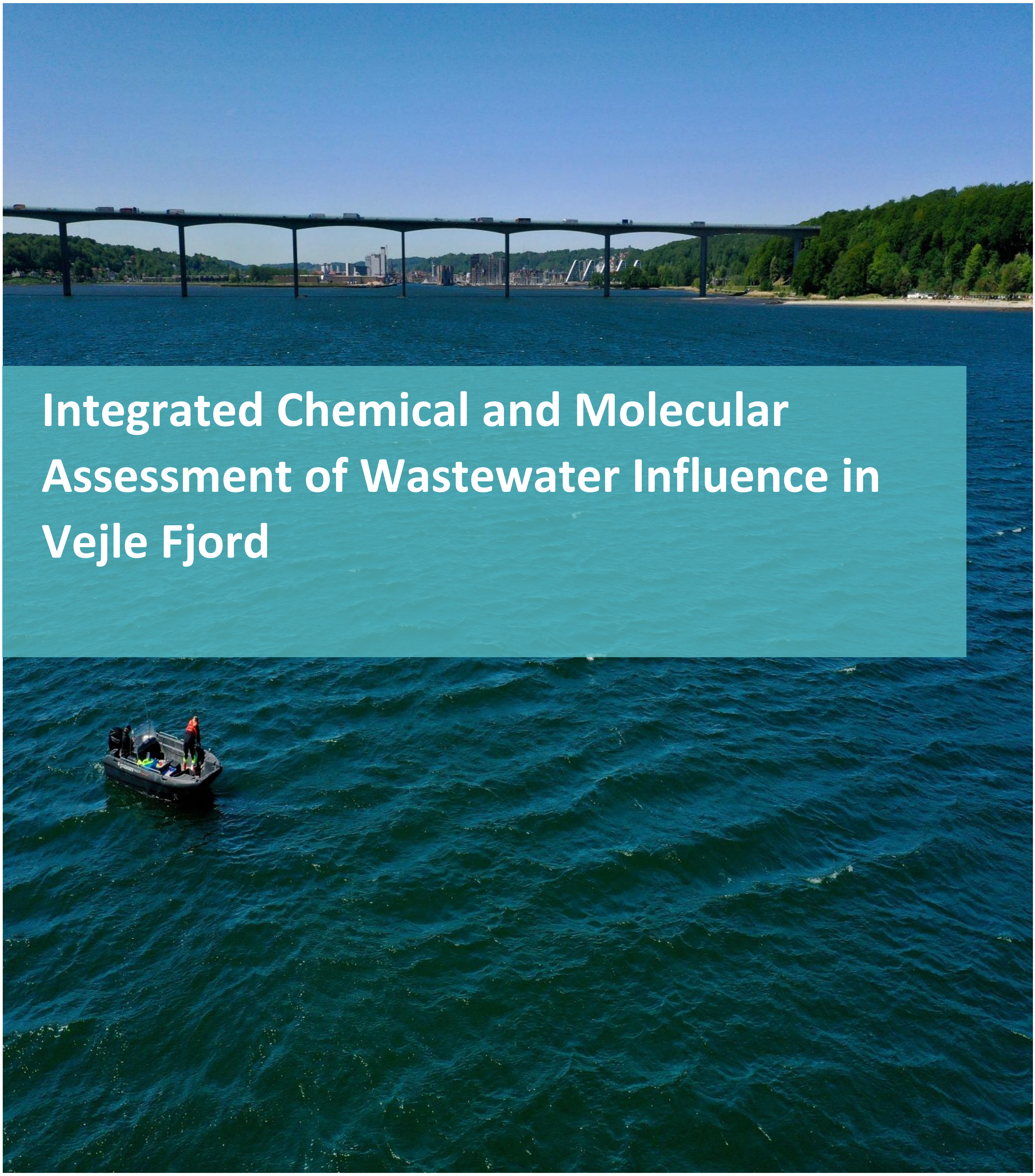
Integrated Chemical and Molecular Assessment of Wastewater Influence in Vejle Fjord



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[eDNA, sediment, wastewater, chemical]

Summary

IVL Swedish Environmental Research Institute thanks Bæredygtigt Landbrug for the opportunity to carry out this assignment. The study was specifically designed to determine whether microorganisms and pharmaceutical residues indicative of wastewater treatment plant (WWTP) influence could be detected in sediments from Vejle Fjord. No targeted analyses were conducted for contaminants or microbial signals related to agriculture, natural processes, or other diffuse sources.

Vejle Fjord was chosen because it represents a typical Danish fjord with a clear gradient from urban areas in the inner basin to more marine conditions toward the Kattegat. Sediment samples were collected at 19 stations along the fjord and two additional sites in Vejle River. Analyses included 16S rRNA metabarcoding and shotgun metagenomics, complemented by targeted chemical analysis of pharmaceuticals in the same sediment material.

The results revealed clear signatures of wastewater-related microorganisms and pharmaceutical residues in the inner fjord, particularly near the discharge point. Several bacterial taxa characteristic of activated sludge processes—such as *Candidatus Accumulibacter*, *Tetrasphaera*, and *Nitrospira*—were enriched in these areas, while their abundance declined toward the outer fjord. Elevated concentrations of antidepressants and antibiotics were found in the same region. Together, these consistent molecular and chemical patterns demonstrate that microbial and pharmaceutical indicators can effectively trace wastewater influence in fjord sediments, even at low contamination levels.

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1 Introduction

Local Wastewater Treatment Plants

Vejle Spildevand operates several wastewater treatment plants across the municipality, of which Vejle Centralrenseanlæg is the largest and most directly connected to Vejle Fjord. The plant employs mechanical, biological, nitrification, denitrification and chemical treatment (MBNDK) processes and in 2023 treated approximately 99,768 m^3 of municipal wastewater. The annual load corresponded to around 74 tons of nitrogen, 2.7 tons of phosphorus and 33.9 tons of biodegradable organic matter (BOD_5). Other plants, including Haraldskær and Brejning Centralrenseanlæg, contribute to total discharges within the municipality, but the Vejle Central plant remains the single largest point source affecting the fjord. The Haraldskær wastewater treatment plant, located upstream along Vejle Å, primarily serves the western part of the catchment. It discharges treated effluent into the river system, which ultimately flows into the inner fjord. Although smaller in capacity than the central plant, Haraldskær may still influence the chemical and biological conditions of sediments in the upper estuarine zone, especially during periods of high flow or combined runoff.

In its 2023 overflow report, Vejle Spildevand documented a substantial reduction in overflow volume from its main outfall—decreasing from approximately 213,000 m^3 in 2022 to 47,000 m^3 in 2023, despite exceptionally high rainfall that year (Vejle Spildevand, 2023). In 2022, this outlet represented nearly 30 % of the total overflow volume in the municipality, but in 2023 its share fell to 4 %. The reduction is attributed to the commissioning of a new treatment line in 2022, which doubled the hydraulic capacity from 2,500 $m^3\ h^{-1}$ to 5,000 $m^3\ h^{-1}$. This technical upgrade, together with improved process control and buffer capacity, has clearly enhanced the hydraulic and environmental performance of the facility. Nevertheless, residents along the southern shoreline near Ibæk Strandvej have reported local sludge deposits suspected to originate from the wastewater system—an issue currently under investigation.

Hydrodynamics and Circulation of Vejle Fjord

Vejle Fjord, located on the east coast of Jutland, is a 22 km long semi-enclosed estuary characterized by steep bathymetric slopes and limited shallow areas. The fjord receives freshwater inflow mainly from the Vejle Å river and opens into the

Kattegat. Its circulation follows a typical estuarine pattern, with brackish surface water flowing seaward and saline bottom water entering from the Kattegat. This two-layer flow is primarily driven by wind forcing, tidal exchange and density gradients between fresh and saline waters.

Wind-induced currents often cause an outflow along the southern shore and an inflow along the northern side. Because the fjord deepens rapidly from the coast, water exchange between its inner and outer sections is limited, resulting in long water residence times and strong spatial gradients in salinity, oxygen and nutrient concentrations (Christensen et al., 1999). These circulation dynamics are critical for interpreting patterns of contaminant transport, sedimentation, and ecological gradients in the fjord.

Recent simulations by the University of Southern Denmark (Lees et al., 2022) provide a quantitative context for nutrient loading and circulation patterns within the fjord. The model results indicate a pronounced eutrophication gradient extending from the inner to the outer fjord, driven mainly by diffuse inputs from agricultural and natural catchment runoff. Point sources such as municipal wastewater and aquaculture facilities contributed smaller, yet measurable fractions of total nitrogen and phosphorus. The study also highlighted that hydrodynamic retention times are longest in the inner fjord, where limited water exchange amplifies nutrient effects and supports elevated planktonic production. These findings align with the conceptual framework of the present study, where molecular and chemical indicators were expected to show similar spatial gradients reflecting cumulative anthropogenic pressure rather than a single dominant source.

Environmental Status and Anthropogenic Pressures on Vejle Fjord

Vejle Fjord has long been regarded as one of Denmark’s most heavily affected coastal ecosystems. It has suffered from chronic eutrophication, loss of eelgrass (*Zostera marina*) and blue mussel (*Mytilus edulis*) beds, and frequent oxygen depletion (Miljøstyrelsen, 2023). The fjord is currently classified as having poor ecological status and not good chemical status under the Danish national monitoring programme (NOVANA).

The SUND Vejle Fjord project, led by the University of Southern Denmark, has modelled water retention times, nutrient reduction effects, eelgrass restoration and overflow events to guide local management (SDU, 2022). These studies highlight the fjord’s limited self-purification capacity and high sensitivity to both diffuse and point-source inputs.

In late summer 2023, the fjord was described as being near ecological collapse after an extensive oxygen depletion event that caused mass mortality among benthic fauna and fish (Miljøstyrelsen, 2023). Such hypoxic episodes have become more frequent under conditions of high nutrient loading and rising summer temperatures.

Pharmaceutical Residues as Emerging Contaminants

Pharmaceutical residues are an increasingly recognized environmental concern due to their persistence, bioactivity and potential for accumulation in aquatic environments. Wastewater treatment plants are major entry points of pharmaceuticals to surface waters (aus der Beek et al., 2016). Pharmaceutical substances such as antibiotics, analgesics, and psychopharmaceuticals are frequently detected in aquatic and coastal sediments worldwide (Gaw et al., 2014; Maghsodian et al., 2022). Even at low concentrations, these compounds can alter microbial community composition and potentially affect ecosystem functioning (Świacka et al., 2023). Chronic exposure and bioaccumulation have also been shown to propagate through aquatic food webs, highlighting the risk of long-term ecological effects (Xie et al., 2017).

Most conventional wastewater treatment systems are not designed to remove these microcontaminants, allowing many active compounds to pass through and reach the receiving waters (Jelić et al., 2012). Once in the aquatic environment, pharmaceuticals can exist dissolved in the water column or adsorbed to particles that settle to sediments, making sediments both a sink and long-term reservoir for these compounds (aus der Beek et al., 2016).

Even at low concentrations, certain pharmaceuticals can disrupt aquatic life. Synthetic oestrogens from contraceptives may cause feminisation in fish (Kidd et al., 2007), antibiotics can promote antibiotic-resistant bacteria (Martinez, 2009; Bengtsson-Palme & Larsson, 2016), and nonsteroidal anti-inflammatory drugs (NSAIDs) can impair fish organ function and behaviour (Corcoran et al., 2010).

Microorganisms as Indicators of Environmental Impact

Microorganisms play a central role in nutrient cycling and ecosystem functioning and are sensitive indicators of anthropogenic impact. Sediment microbial communities respond strongly to nutrient and organic inputs from both agriculture and wastewater (Beattie et al., 2020). Antibiotic resistance genes (ARGs) have been detected in sediments downstream from treatment plants, often spread via horizontal gene transfer between bacteria (Marti et al., 2013).

Thus, metagenomic and metabarcoding analyses of microbial assemblages can reveal changes in community composition, ecosystem function and resistance patterns, providing a basis for ecological risk assessment (Rizzo et al., 2013). Combining chemical analyses of pharmaceuticals with molecular profiling of microbial communities enables a dual perspective on contamination: the presence of pollutants and the biological responses they trigger.

Sediment Analysis as an Environmental Assessment Tool

In coastal ecosystems such as fjords, sediments act both as a sink and an archive of environmental contamination (Förstner & Wittmann, 2012). Contaminants attached to fine particles accumulate over time, providing an integrated record of historical and ongoing pollution inputs. Sediment analysis is therefore a robust method for evaluating spatial and temporal gradients of contamination.

In Vejle Fjord, sediment monitoring is particularly relevant because it can reveal gradients of contamination extending from the inner fjord—where the main wastewater outlet is located—toward less impacted outer zones. In addition to the central plant in Vejle, the Haraldskær wastewater treatment plant, located further upstream along Vejle Å, also contributes to discharges within the catchment. The two sampling stations in Vejle Å were therefore included to assess whether wastewater-related indicator taxa and chemical tracers can be detected further upstream in the river system.

Pharmaceutical residues are of special concern due to their persistence and biological activity. Many compounds degrade slowly under the low-oxygen and low-light conditions typical of fjord sediments (Patel et al., 2019). The presence of such residues provides clear evidence of wastewater influence and can serve as a chemical tracer of anthropogenic pressure.

The HELCOM (2021) report on the Baltic Sea region identifies municipal wastewater treatment plants as the dominant source of pharmaceuticals, estimating that several thousand tonnes are discharged annually, with less than half efficiently removed during treatment. Anti-inflammatory drugs (e.g. diclofenac, ibuprofen), cardiovascular agents and CNS-active pharmaceuticals such as carbamazepine were among the most frequently detected compounds in sediments and surface waters. Danish NOVANA monitoring has reported similar compound classes in national surveys, underscoring the relevance of analysing these in Vejle Fjord.

Rationale for the Present Study

In fjord ecosystems, sediments function both as sinks and archives of environmental contamination, integrating signals from multiple sources over time (Förstner & Wittmann, 2012). Because contaminants attached to fine particles accumulate gradually, surface sediments provide a robust indicator of both recent and historical pollution inputs. In Vejle Fjord, where a wastewater outlet is located in the inner basin, sediment monitoring offers a powerful means to trace gradients of contamination extending outward toward less impacted zones and upstream reference areas in Vejle Å (SDU, 2022).

Pharmaceutical residues are of particular concern due to their persistence and biological activity. Many compounds degrade slowly under the low-oxygen and low-light conditions typical of fjord sediments (Patel et al., 2019). The presence of such residues thus provides clear evidence of wastewater influence and can serve as a chemical tracer of anthropogenic pressure. The HELCOM (2021) report on the Baltic Sea region identifies municipal wastewater treatment plants as the dominant source of pharmaceuticals, releasing several thousand tonnes annually, with less than half efficiently removed during treatment. Danish NOVANA monitoring has reported similar compound classes, including anti-inflammatory drugs (e.g., diclofenac, ibuprofen), cardiovascular agents, and CNS-active pharmaceuticals such as carbamazepine, underscoring the relevance of analysing these compounds in Vejle Fjord.

To capture both chemical and biological aspects of such pressures, surface sediment samples (0–5 cm) were collected in May 2025 from Vejle Fjord and Vejle Å, representing the most recently deposited material. The aim was to obtain a snapshot of pharmaceutical occurrence and microbial community composition in areas influenced by wastewater discharges and agricultural runoff. By combining pharmaceutical residue analysis with metagenomic profiling of microbial communities, this study provides an integrated, sensitive, and scalable framework for assessing anthropogenic impacts. The results establish a baseline for evaluating spatial gradients of contamination and for guiding future monitoring and restoration efforts aimed at improving the ecological resilience of Vejle Fjord.

2 Methodology

Fieldwork

In mid-May 2025, sampling was carried out in Vejle Fjord and its surrounding area (fig 1), for detailed map of the inner fjord and Vejle Å see supplementary maps S1 and S2. Sediment ampling was conducted from a boat using a Van Veen grab sampler, except for sampling point 1 (taken from a bridge) in the middle of the river, just downstream Vejle Centralrenseanlæg. Sampling points 20 and 21 were collected from the shoreline using the same grab sampler. Samples for DNA-related work were collected in triplicates (n=63) and preserved in PowerProtect DNA/RNA stabilization reagent by QIAGEN, which penetrates cells and halts biological activity to maintain microbial community profiles and nucleic acid integrity during transport and storage.

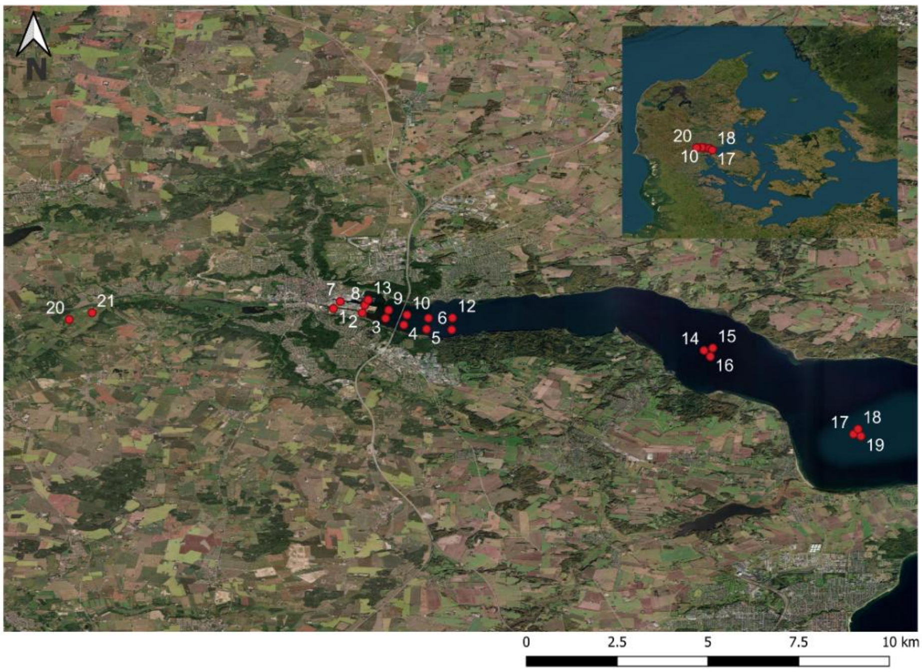

Figure 1. Overview map of Vejle Fjord sampling stations. Map showing the 21 sediment sampling stations (Vejle 1–21) along the fjord transect. The locations of Vejle Å (stations 20–21), the main wastewater treatment plant discharge point, and the inner, mid, and outer fjord zones are indicated. Background map: © Geodatastyrelsen, Denmark.

DNA extraction

DNA was extracted using the MagAttract® PowerSoil® Pro DNA kit on a KingFisher Flex instrument. Negative controls were included, and

ZymoBIOMICS™ Microbial Community Standard (D6300) was used as a positive control following the during the DNA extraction. DNA concentration was measured using an Implen Nanophotometer N60.

Metabarkodning and PCR-amplification

To study the bacterial communities, a metabarcoding approach based on the 16S rRNA gene was used. This gene is a well-conserved genetic marker found in both bacteria and archaea. It contains both conserved and hypervariable regions, which makes it particularly useful for distinguishing between different species. In this study, the V3–V4 region was selected due to its suitability for broad taxonomic coverage, enabling identification at a relatively detailed level—from genus to species. The primer pair 341F and 805R (Table 1) was used to amplify this region. Metabarcoding was performed using a two-step PCR protocol. In the first step, DNA was amplified with 16S primers equipped with Illumina adapters (TruSeq-style SP1/2 sites) at the 5′ end (underlined nucleotides in Table 1).

Table 1. Primer sequences used for PCR amplification of the V3–V4 region of the 16S rRNA gene.

Namn	Sekvens (5’-3’)
341F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTACGGGNGGCWGCAG
805R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGACTACHVGGGTATCTAATCC

DNA amplification was performed using a MiniAmp™ thermal cycler and ZymoBIOMICS™ Microbial Community Standard (D6300) as the positive control. Each 20 µl reaction contained 10 µl of 2X Phusion™ Plus Green PCR Master Mix, 0.5 µM of each primer (341F and 805R; Table 4), 0.5 mg/ml BSA, 4 µl of template DNA, and nuclease-free water to a final volume of 20 µl.

The thermal cycling program consisted of an initial denaturation at 98 °C for 30 s, followed by 30 cycles of 98 °C for 10 s, 57 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 2 min and a hold step at 6 °C (∞).

The purified PCR products were sent to BMKgene for sequencing. At BMKgene, the amplified PCR products underwent quality control to assess their suitability for sequencing based on purity and concentration. All samples passed quality control and were prepared for sequencing using the Illumina sequencing adapters incorporated during the first PCR (Table 1 above) and dual-index primers for multiplexing. All samples were approved for sequencing and were sequenced on the Illumina NovaSeq 6000 platform using paired-end 250 bp reads (PE-250).

Metagenomic sequencing

DNA extracts from sediment samples were pooled (10 µl from each) from the same triplicate subsamples that were used for metabarcoding analysis, in order to obtain a representative composite for each sampling station. In addition, a positive control consisting of a ZymoBIOMICS™ Microbial Community Standard (Zymo Research, USA) was included to verify sequencing performance and taxonomic accuracy.

Prior to shipment, all DNA extracts were quantified using a Qubit 4 fluorometer (Thermo Fisher Scientific, USA) and assessed for purity based on A260/280 and A260/230 ratios using a NanoDrop spectrophotometer. Only extracts with sufficient concentration (>10 ng/µL) and high purity (A260/280 ≈ 1.8–2.0) were selected for sequencing.

Samples were stored at −20 °C until dispatch and transported to BMKgene who performed an additional quality control assessment using agarose gel electrophoresis and quantification. Libraries were prepared using the Illumina TruSeq DNA PCR-Free Library Preparation Kit according to the manufacturer's protocol. Sequencing was carried out on an Illumina NovaSeq 6000 platform (paired-end 150 bp mode), generating high-quality metagenomic reads suitable for downstream taxonomic and functional profiling.

Bioinformatic analysis metabarcoding

Amplicon sequencing of sediment and control samples from Vejle Fjord was analyzed using the nf-core/ampliseq pipeline (v2.14.0; Straub et al., 2020) implemented in Nextflow. Raw Illumina paired-end reads were quality-checked with FastQC (Andrews, 2010) and summarized using MultiQC (Ewels et al., 2016). Primer sequences were removed with Cutadapt (Martin, 2011). Subsequent denoising and quality filtering were performed with DADA2 (Callahan et al., 2016), which included error correction, trimming, and chimera removal. Taxonomic assignment was carried out in DADA2 using the SILVA v138.2 prokaryotic SSU reference database (Quast et al., 2013).

Bioinformatic analysis metagenomics

Shotgun metagenomic sequence data from Vejle Fjord were processed using the standardized and reproducible workflow nf-core/taxprofiler (v2.0.1). The analysis was executed with Nextflow (v23.10.1) on a local high-performance computing system under a Linux environment. All runs were containerized with Singularity to ensure full version control and reproducibility.

Paired-end metagenomic reads (*.fastq.gz) were provided as input, organized in a Nextflow samplesheet. Taxonomic classification was performed using the Kraken2 module within nf-core/taxprofiler, employing a locally built reference database (k2_standard_2025_08) based on NCBI RefSeq, encompassing bacteria, archaea, viruses, and eukaryotes. Kraken2 assigns taxonomic identities to reads using a k-mer–based matching approach, and the resulting profiles were exported as .kraken2.report.txt files containing relative abundances for each detected taxon.

To refine species-level abundance estimates, Bracken (v2.9) was applied to all Kraken2 output files. Bracken re-estimates abundances based on the probabilistic distribution of k-mers in the reference database, improving quantitative accuracy at lower taxonomic ranks. Species- and genus-level abundance tables from both Kraken2 and Bracken classifications were processed using custom Python scripts that matched detected taxa against a predefined list of wastewater-related indicator organisms. The scripts applied minimum thresholds (≥50 reads and ≥0.01% relative abundance) and automatically assigned each sample to one of five spatial zones along the Vejle Fjord transect. The output included the number and relative abundance of indicator taxa per sample and category, mean values per zone, and the ten most abundant indicators within each zone, providing a consistent basis for comparing WWTP-associated microbial signatures across the fjord gradient.

Method for Identifying Microbial Indicators of Wastewater Impact

To identify microbial indicators of wastewater treatment plant (WWTP) influence, a reference list was compiled based on previous studies describing bacterial groups typically associated with wastewater, treatment processes, pathogens, and antibiotic resistance genes in nutrient-enriched recipient environments. The foundation was primarily drawn from Chu et al. (2018) and Beattie et al. (2020), complemented by studies focusing on activated sludge microbiology, resistance dissemination, and the occurrence of opportunistic pathogens within and downstream of WWTPs.

The indicator list was constructed by extracting taxonomic names of bacterial genera and species reported in the literature as being linked to four main categories of influence: WWTP_process, Recipient_indicator, Pathogen, and ARG (see Supplementary Table 3). These categories represent distinct microbial components of the wastewater-associated footprint and served as the analytical framework for interpreting both metagenomic and metabarcoding data.

The WWTP_process category includes bacterial taxa typically present in biological treatment stages of WWTPs, indicating direct influence from process water or leakage of activated sludge into the recipient. Key examples include *Candidatus Accumulibacter, Tetrasphaera, Nitrosomonas, Nitrospira, Thauera, Paracoccus*, and *Zoogloea*, all of which play essential roles in nitrification, denitrification, phosphorus uptake, and biofilm formation in activated sludge (Coskuner & Curtis, 2002; Johnston et al., 2019). Detection of these taxa outside the treatment process is interpreted as evidence of process-related influence.

The Recipient_indicator category includes taxa not directly tied to the treatment process but known to increase in abundance in nutrient- and organic-rich sediments and water bodies downstream of point sources. Typical examples are *Arcobacter, Acinetobacter, Bacteroides, Prevotella, Clostridium, Fusobacterium*, and *Enterococcus*, which frequently occur in fecally or urban-impacted waters (Fisher et al., 2014; Kristensen et al., 2020; Ghaju Shrestha et al., 2019; Beattie et al., 2020). Their presence thus serves as a broader ecological indicator of wastewater impact in receiving environments.

The Pathogen category comprises potentially pathogenic or opportunistic bacteria commonly found in municipal or hospital wastewater. This group includes *Aeromonas, Klebsiella, Pseudomonas aeruginosa, Stenotrophomonas maltophilia, Legionella pneumophila*, and *Mycobacterium avium*, all of which have been reported to survive treatment processes and persist in effluents or sludge (Męcik et al., 2024; Eze et al., 2021). These taxa act as specific warning indicators associated with health-related risks.

Finally, the ARG (antibiotic resistance genes) category encompasses bacterial taxa frequently carrying resistance determinants and thereby functioning as indicators of antibiotic resistance dissemination from urban and clinical point sources. Representative examples include *Acinetobacter baumannii, Klebsiella pneumoniae, Escherichia coli ST131, Enterococcus faecium*, and *Enterococcus faecalis*, all of which are recurrently detected in wastewater, sludge, and downstream water environments (Marutescu et al., 2023; Murphy et al., 2021; Park et al., 2024; Radisic et al., 2024).

The list was taxonomically harmonized against the SILVA v138.1 reference database to ensure consistency with the classification used in the 16S rRNA metabarcoding analysis.

For the metagenomic dataset, taxonomic profiles were generated with Kraken2 and adjusted with Bracken, with the latter used for downstream analyses due to its higher accuracy in species-level abundance estimation. For 16S rRNA metabarcoding, ASV-level data produced through the nf-core/ampliseq pipeline (DADA2 denoising; SILVA v138.1 classification) were used.

All identified taxa from metabarcoding and metagenomic analysis were compared to the indicator list, and matches were counted per sample and per zone (T1–6 inner, T7–13 middle, T14–19 outer, T20–21 river inlet). For each dataset, the number of indicator detections was calculated, and overlaps between metagenomic and metabarcoding indicators were quantified. Only indicators occurring in at least two independent samples per zone were retained for zone-specific comparisons. Results were visualized as "Top 10 indicators per zone" plots for both Kraken2 and Bracken and as comparative summaries across methods.

Pharmaceuticals

Sediment samples were extracted using liquid–liquid extraction followed by solid-phase extraction (SPE) on HLB cartridges. The analysis was performed by HPLC–MS/MS at IVL's laboratory in Stockholm. The following isotope-labeled internal standards were used for quantification: Carbamazepine-$^{13}C^{15}N$, Diclofenac-$^{13}C_6$, Hydrochlorothiazide-$^{13}C_6$, Atenolol-d_7, Metoprolol-d_7, Ibuprofen-d_3, Ciprofloxacin-d_8, Fluconazole-d_4, Sulfamethoxazole-$^{13}C_6$, Clarithromycin-N-methyl-d_3, and Trimethoprim-d_3.

The resulting concentration data provided the foundation for subsequent occurrence scoring and index calculations. For each analyte and sampling station, three distinct outcomes were obtained following instrumental quantification: (i) quantified concentrations above the limit of quantification (LOQ), (ii) detections below LOQ, and (iii) non-detects (ND). To ensure consistency across compounds and stations, these categories were standardized according to the conventions applied in the pharmaceutical assessment. Detailed descriptions of the chemical analyses are provided in the IVL report Pharmaceuticals, 25–0149, Vejle Fjord (IVL Swedish Environmental Research Institute). The processed dataset was subsequently organized into an analyte-by-station matrix, in which human pharmaceuticals and technical substances were classified and treated separately to maintain interpretative clarity. The subsequent data preprocessing and scoring procedures are described below.

For each station (s), the Occurrence Score (OccScore) was computed as the sum of per-analyte weights across all human pharmaceuticals. The per-analyte weight ($w_{(i,s)}$) was set to 1.0 when the concentration exceeded the limit of quantification (LOQ), 0.5 when the analyte was detected but below LOQ, and 0 when it was not detected (ND). In other words, the OccScore for a given station equals the total number of human pharmaceuticals quantified (> LOQ) plus half the number detected < LOQ, with non-detects contributing zero (see Pharmaceutical Report, Appendix X for LOQs and detection frequencies).

Σ(6&4)-methylbenzotriazole (MeBT) was treated separately because it is a technical corrosion inhibitor and hydrological plume tracer rather than a human medicine. The MeBT component for station (s) was defined as follows: if MeBT was quantified (> LOQ), the component equaled the MeBT concentration divided by 10; if MeBT was detected < LOQ, the component equaled 0.5; and if MeBT was not detected, the component equaled 0. Plain benzotriazole was not included in the pharmaceutical sum.

The Human Pharmaceutical Index (HPI) for each station was then obtained by adding the OccScore and the MeBT component, such that HPI = OccScore + MeBT component. Sensitivity analyses using alternative weighting and normalization schemes were performed to assess the robustness of the index.

3 Results

3.1 Metabarcoding

The sequencing and bioinformatics workflow was successfully completed using the nf-core/ampliseq pipeline (v2.14.0) under Singularity, including quality control, primer trimming, DADA2 denoising, taxonomic classification, and read distribution assessment. All samples passed the initial quality filters, and DADA2 inferred a high number of unique Amplicon Sequence Variants (ASVs), indicating excellent sequence diversity and low noise, while chimera removal was efficient with less than five percent of reads flagged as chimeric. The Barrnap module confirmed the presence of expected rRNA gene content, verifying successful amplification of the targeted region and absence of off-target or contaminant sequences. Sequencing depth was consistent across replicates, as shown by the stacked read distribution chart, ensuring comparability between samples. Taxonomic classification using SILVA v138.1 revealed a broad representation of bacterial taxa dominated by Proteobacteria, Bacteroidota, and Actinobacteriota, with most reads classified to genus level, indicating high database coverage and reliable sequence data. Overall, the sequencing and analysis demonstrate high data quality and methodological robustness, providing a solid foundation for downstream analyses, including indicator species evaluation.

The sequencing data showed consistently high read retention and processing efficiency across all 63 Vejle Fjord samples (see Supplementary Table 1). On average, 98.9 % of raw reads passed initial Cutadapt filtering, confirming excellent sequencing quality and effective primer removal. Subsequent DADA2 processing maintained similarly high efficiency, with approximately 90–99 % of reads retained after filtering, denoising, merging, and chimera removal. The total dataset comprised 4.78 million raw reads, of which 4.73 million passed filters and 1.29 million high-quality merged, non-chimeric reads were ultimately retained as Amplicon Sequence Variants (ASVs). These reads represented 178,644 unique Amplicon Sequence Variants (ASVs) across all samples. Individual samples exhibited minor variation, with most retaining over 90 % of sequences, except a few lower-yield samples (e.g., Vejle20–21, Vejle19) that showed higher read loss (20–30 %) during merging and taxonomic filtering, likely reflecting variation in input biomass or amplicon quality. Overall, data integrity and sequence recovery were excellent, confirming high-quality input DNA, and efficient amplification and processing. The final dataset is robust and well-suited for downstream analyses of the Vejle Fjord system.

The 16S rRNA metabarcoding analysis yielded a total of 178,644 ASVs across all samples, corresponding to 2.46 million sequence counts. The majority of ASVs were assigned to Bacteria (98.5 %), while 1.4 % were classified as Archaea and < 0.2 % as Eukaryota. Taxonomic classification reached 90.4 % of ASVs at the phylum level, 70.2 % at the order level, and 26.4 % at the genus level. A total of 9 % of ASVs (≈ 16,000) were successfully classified to species level, providing fine-scale resolution of sediment microbial communities.

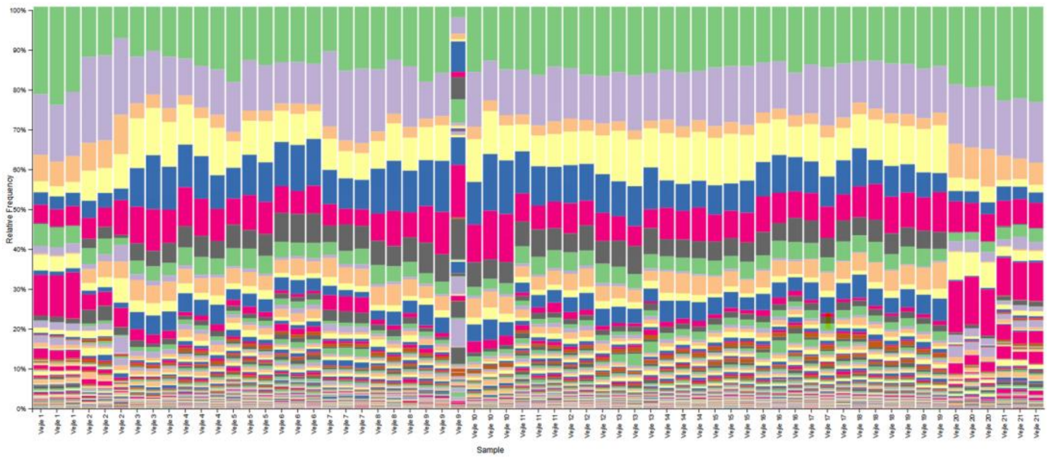

Figure 2. Relative abundance of bacterial taxa in sediment samples from Vejle Fjord based on 16S rRNA metabarcoding. Each bar represents one sediment sample (Vejle 1–21), and colors indicate the relative frequency of taxa at the family or genus level. Three technical replicates were analyzed per station, allowing evaluation of reproducibility across samples.

The overall bacterial community composition is shown in Figure 2. Replicates generally displayed high consistency, demonstrating good reproducibility of DNA extraction and sequencing. A few replicates deviated slightly, likely reflecting small-scale spatial variability in sediment composition or variation in DNA quality. One replicate (Vejle 9-3) deviated more clearly from the other two replicates at the same station. This discrepancy may be due to local micro-scale heterogeneity in the sediment or minor technical variation during extraction or amplification. Given the overall consistency across other stations, this replicate was retained but interpreted with caution.

Samples from the fjord mouth (Vejle 1) and upstream river sites (Vejle 20–21) exhibited distinct community profiles compared with the more marine stations, indicating a freshwater influence in these locations. This pattern likely reflects the gradual transition from marine to brackish and freshwater conditions along the fjord–river continuum.

3.2 Metagenomic results

A total of 22 metagenomic sediment samples were successfully sequenced by BMKgene using the Illumina NovaSeq 6000 platform. The sequencing output ranged from 21.8 to 38.6 million paired-end reads per sample, corresponding to 6.5–11.6 Gb of high-quality data (see Supplementary Table 2). Across all samples, base quality scores were good, with Q20 = 100 % and Q30 values between 92.3 %

and 95.1 %, indicating high sequencing accuracy and minimal error rates. The GC content varied from 48.5 % to 60.0 %, reflecting the natural diversity of microbial and eukaryotic DNA present in the sediment metagenomes. In total, the sequencing campaign produced approximately 567 million read pairs (≈ 170 Gb) of raw data.

The taxonomic profiles generated with nf-core/taxprofiler revealed a clear pattern of microbial indicators associated with wastewater influence and antibiotic resistance genes (ARGs) in the inner parts of Vejle Fjord, with a gradual decrease in occurrence toward the outer recipient area. Classification using Kraken2 followed by adjustment with Bracken showed similar overall trends, but the Bracken results were used as the primary basis for quantitative comparisons, as they provide more reliable species-level abundance estimates.

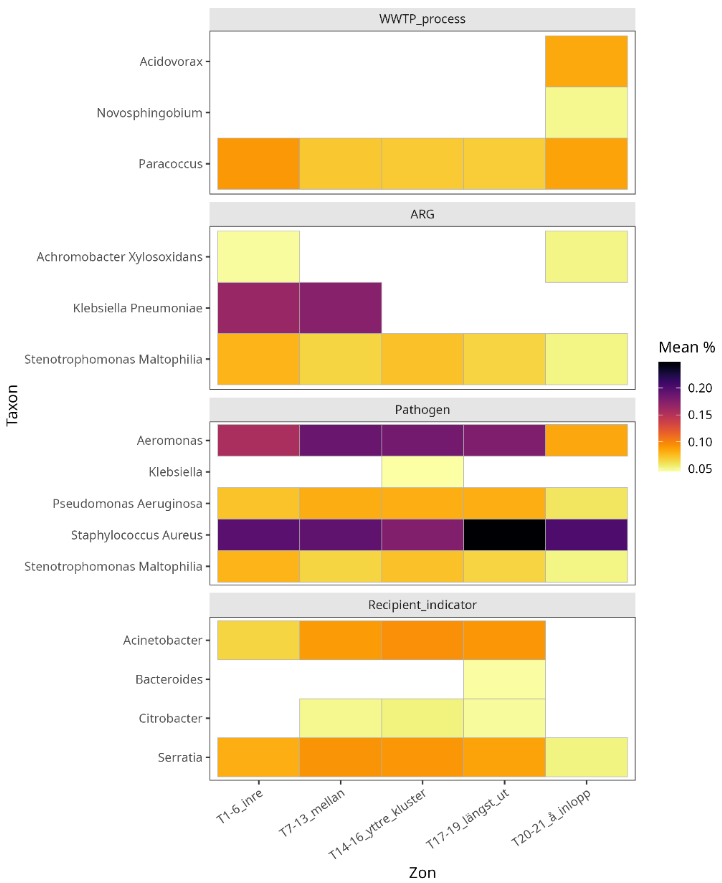

Figure 3. Top indicators per zone – Kraken2

The zonal summary table (table x) presents the most abundant taxa that matched the predefined WWTP- and recipient-indicator lists. This overview shows that taxa associated with wastewater treatment processes (*Paracoccus*, *Thauera*, *Acidovorax*, *Zoogloea*) dominate in the inner fjord zones (1–6) and gradually decrease toward the outer and river zones, where recipient-related indicators (*Arcobacter*, *Acinetobacter*, *Aeromonas*, *Enterococcus*) become more frequent.

Tabell 2. Top indicator taxa per zone based on Bracken results. Relative abundance (%) is averaged per zone. Only the ten most frequent indicator taxa across all samples are shown.

Zone	Dominant indicator taxa (Top 10 overall)	Indicator category	Mean relative abundance (%)	Interpretation
1–6 (Inner fjord)	Paracoccus, Acidovorax, Nitrosomonas, Thauera, Zoogloea, Tetrasphaera, Defluviicoccus, Dechloromonas, Candidatus Accumulibacter, Propionivibrio	WWTP_process	1.0–2.0	Strong WWTP signal; dominated by typical nitrifiers and denitrifiers linked to activated sludge processes.
7–13 (Mid fjord)	Paracoccus, Rhodocyclus, Thauera, Zoogloea, Nitrospira, Acidovorax	WWTP_process	0.5–1.0	Transitional zone; reduced abundance of WWTP-associated taxa.
14–16 (Outer fjord)	Nitrospira, Candidatus Amarolinea, Propionivibrio, Candidatus Microthrix	WWTP_process	0.2–0.6	Low abundance of residual process-related taxa; background community dominates.
17–19 (Outer fjord)	Arcobacter, Acinetobacter, Aeromonas, Pseudomonas, Shewanella	Recipient_indicator	0.2–0.6	Shift toward general recipient indicators and marine heterotrophs.
20–21 (River inflow)	Arcobacter, Acinetobacter, Aeromonas, Enterococcus, Vibrio	Recipient_indicator	0.4–0.8	Strong freshwater influence; dominance of recipient and fecal indicators.

The accompanying heatmap visualizes the same spatial gradient for additional indicator categories—antibiotic-resistance (ARG), pathogens, and recipient indicators—showing how the relative abundance of these groups varies between zones.

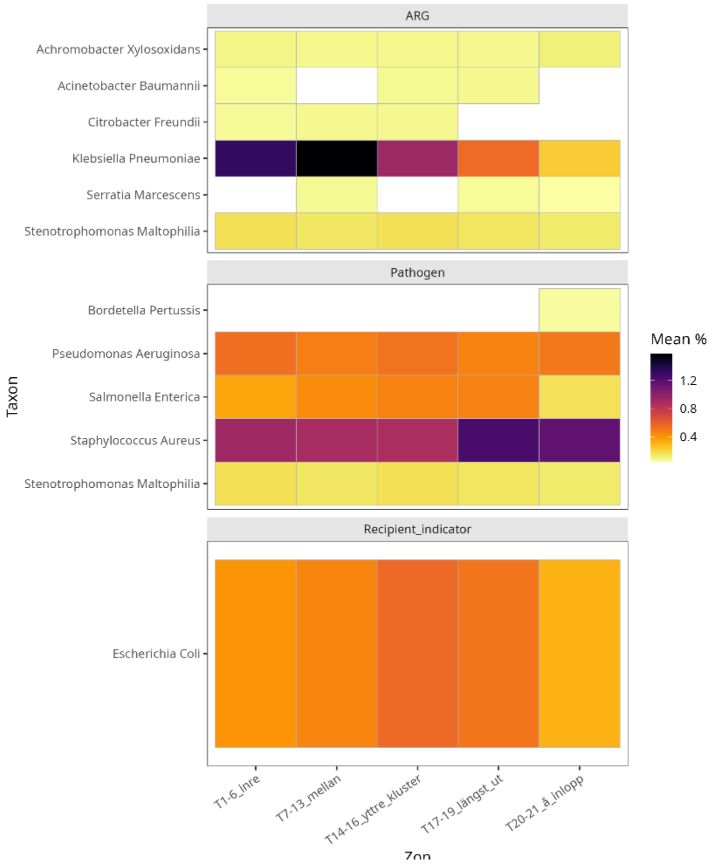

Figure 4. Top indicators per zone – Bracken

Together, these results provide a consistent picture of indicator distributions, where inner fjord zones are characterized by wastewater- and pathogen-related taxa, while outer and upstream zones are increasingly dominated by recipient and freshwater-associated indicators.

Taxa associated with wastewater treatment processes, such as *Paracoccus* and *Acidovorax*, were mainly detected in the inner zones closest to the discharge point (1–6_inre and 20–21_å_inlopp). Their abundance declined sharply further out in the fjord, indicating a distinct gradient of wastewater-related influence.

ARG-associated taxa, including *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia*, and *Achromobacter xylosoxidans*, showed elevated abundances in the inner zones (1–6 and 14–16), with *K. pneumoniae* representing the highest relative proportion (>1% of reads per sample). The occurrence decreased toward the outer recipient (17–19), suggesting that antibiotic resistance markers are dispersed from the discharge point but diluted as they spread through the fjord.

Pathogenic bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Stenotrophomonas maltophilia* dominated several zones, especially near the discharge point and in the inner fjord. *S. aureus* and *P. aeruginosa* exhibited the highest abundances among all indicator groups, reaching up to 1.2% of total reads per sample in the Bracken results. These findings indicate that these species are robust indicators of microbial impact from wastewater.

Taxa classified as recipient indicators, including *Escherichia coli*, *Bacteroides*, and *Acinetobacter*, were found across all zones but reached their highest relative abundances in the inner fjord. *E. coli* displayed consistently elevated values across all zones, indicating background influence rather than distinct point-source contamination.

Comparison between Kraken2 and Bracken

Kraken2 and Bracken revealed broadly similar gradients and dominant taxa, but Kraken2 tended to overestimate taxa with low sequence uniqueness (e.g., Klebsiella spp.). The Bracken correction reduced these effects, providing clearer separation between indicator groups. Zone-wise summaries showed that Bracken yielded a higher total proportion of reads classified as indicators, particularly for ARGs and pathogens (2–2.5% compared to <1% for Kraken2). The combined results show that microbial indicator species linked to both antibiotic resistance and pathogenicity are concentrated in the inner fjord areas, especially near the wastewater discharge. The results from bracken results show that the average proportion of reads assigned to the ARG and pathogen groups ranged between 1–2 % in the inner zones (1–6 and 7–13) and gradually decreased toward the outer fjord zones (17–19). Recipient indicators consistently remained below 0.6 %. See Table X for a zone-wise summary.

Tabell 3. Zone-wise summary of indicator groups (Bracken results).
Mean values represent the relative proportion of reads (%) and the number of identified taxa per indicator group and fjord zone.

Zon	ARG – mean %	ARG – taxa (n)	Pathogen – mean %	Pathogen – taxa (n)	Recipient – mean %
1–6	1.86	62	2.13	57	0.4
7–13	2.12	75	2.1	78	0.45
14–16	1.55	34	2.2	34	0.56
17–19	1.12	36	2.49	37	0.51
20–21	0.6	16	2.05	13	0.31

The Bracken results present mean values for three indicator groups — antibiotic-resistant bacteria (ARG), pathogenic indicator taxa, and recipient indicators — calculated per zone in Vejle Fjord. The values represent the average proportion of reads (%) and the number of identified taxa in each category. The highest mean values for both ARGs and pathogenic indicators were observed in the inner fjord zones (1–6 and 7–13), indicating a spatial gradient consistent with influence from WWTP effluent, superimposed on natural and diffuse background variation. Recipient indicators showed lower and more stable values throughout the fjord. This indicates that both resistant and potentially pathogenic bacteria are transported into the recipient environment but gradually diluted and decrease in abundance toward the open fjord.

Ecological and environmental interpretation

The results reveal clear microbial signatures consistent with the influence of wastewater treatment plant effluents. The dominant indicator taxa in the inner fjord zones — such as *Paracoccus*, *Acidovorax*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* — are well-known inhabitants of treatment plant environments and thus serve as strong markers of this type of impact. At the same time, organisms with broader ecological distributions were also detected, suggesting that the fjord is affected by a combination of sources, including natural microbial communities and diffuse runoff from surrounding catchments.

The observed gradient, with higher abundances of these indicators in the inner fjord and progressively lower levels further out, supports the interpretation that wastewater influence can be detected and spatially delineated using metagenomic methods. However, the results should be interpreted considering the fjord’s complex hydrology and the cumulative impacts from multiple sectors. This study demonstrates that environmental DNA and metagenomics can be used to objectively distinguish and quantify the influence of point sources, while still accounting for contributions from other anthropogenic or natural processes.

3.3 Pharmaceutical Residues

Overall Results and Categorization

The detection matrix for 32 pharmaceuticals including the corrosion inhibitor Σ(6&4)-methylbenzotriazole (MeBT) is presented in Figure 5. Each compound is categorized as not detected (ND), detected below the limit of quantification (<LOQ), or quantified above LOQ (>LOQ).

Σ(6&4)-MeBT was detected at all 21 stations and quantified above LOQ at most sites, confirming its suitability as a robust tracer of urban and wastewater-derived inputs. Several pharmaceuticals, including azithromycin, citalopram, metoprolol, and oxazepam, were also detected—primarily in sediments from the inner and mid-fjord stations—while fewer compounds were found in the outer fjord and river sites.

This spatial pattern indicates a gradient consistent with wastewater influence originating from the inner fjord and diminishing toward less impacted zones.

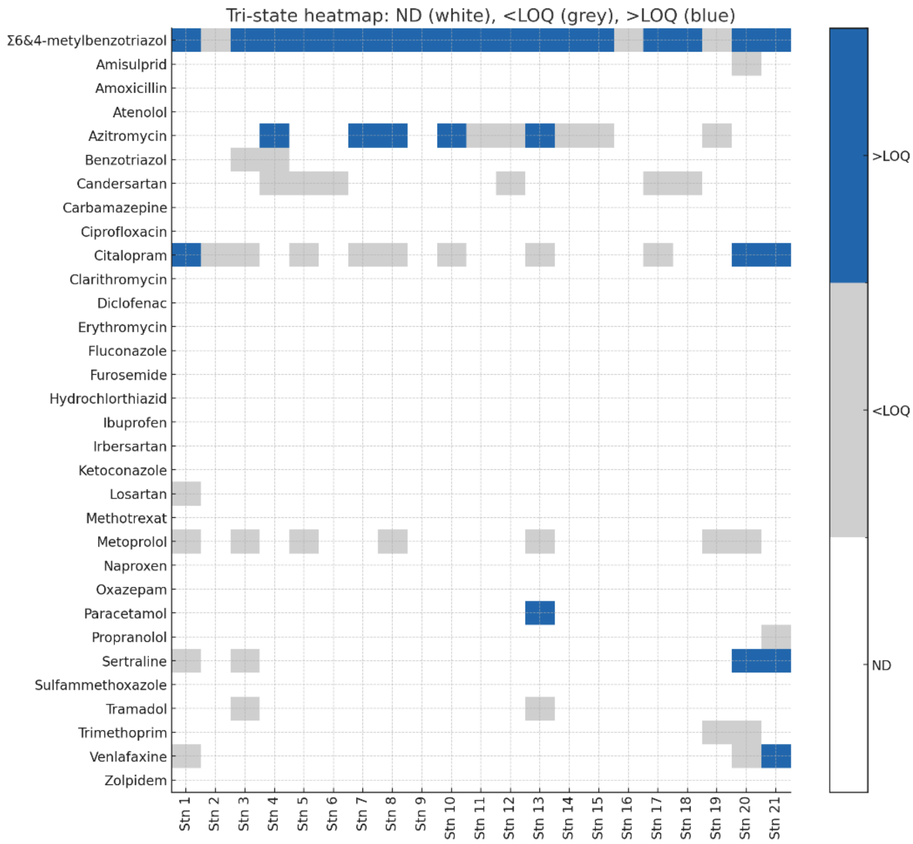

Figure 5. Tri-state heatmap showing detection frequencies of Σ(6&4)-methylbenzotriazole and selected pharmaceuticals across 21 sediment stations in Vejle Fjord. White = not detected (ND), grey = <LOQ (below limit of quantification), blue = >LOQ (quantified).

Human Pharmaceuticals – Occurrence, Levels, and Interpretation

In addition to MeBT, several human pharmaceuticals were detected, collectively forming a distinct urban contamination signature. SSRI/SNRI antidepressants appeared both as quantified concentrations and as <LOQ detections. Citalopram was quantified near the outlet (Stn 1 ≈ 4) and especially in the river stations (Stn 20

≈ 5.9; Stn 21 ≈ 21), demonstrating clear human influence even upstream of the inner fjord. Sertraline (Stn 20 ≈ 3.4; Stn 21 ≈ 8.5) and Venlafaxine (Stn 21 ≈ 10) followed similar patterns. The co-occurrence of multiple antidepressants is typical of municipal wastewater inputs and indicates continuous rather than episodic emissions. Azithromycin occurred at quantifiable levels at several stations (e.g., Stn 4 ≈ 5; Stn 10 ≈ 4; Stn 13 ≈ 9.3) and as <LOQ detections elsewhere; together with Trimethoprim (mainly <LOQ), this supports a predominantly human rather than veterinary origin. Paracetamol showed a distinct peak in the marina (Stn 13 ≈ 24), suggesting recent human input and/or limited degradation under sheltered conditions. Cardiovascular drugs such as Metoprolol, Losartan, and Candesartan were mainly <LOQ, but their widespread occurrence contributed to the overall urban chemical profile.

Collectively, these findings reinforce the interpretation of a WWTP-dominated signal, while typical agrochemical markers were absent from the analytical panel. The composite Human Pharmaceutical Index (HPI) summarizing the number of quantified compounds per station is shown in Figure 6.

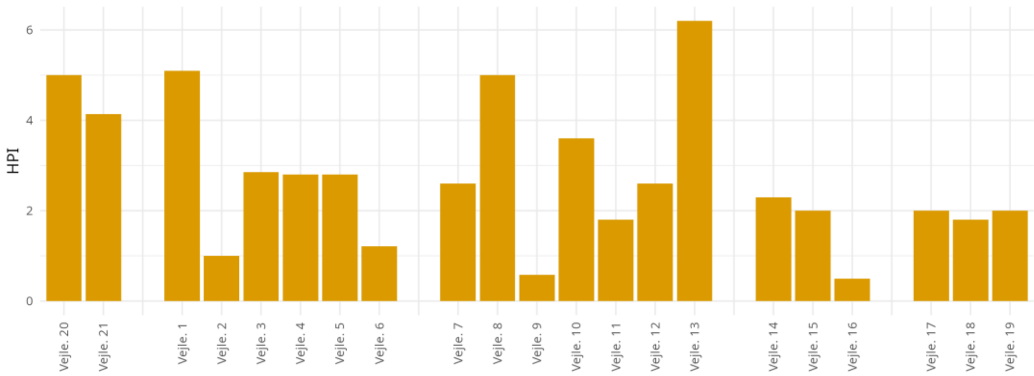

Figure 6. Human Pharmaceutical Index (HPI) per sediment station in Vejle Fjord. The index represents the count of pharmaceuticals quantified above the limit of quantification (>LOQ) at each station.

Local Patterns and Source Indications

The plume from the main WWTP is evident in the inner fjord but shows hydrodynamic complexity. Within the Stn 1–6 sector, MeBT concentrations remained consistently elevated and several human pharmaceuticals were detected, yet the concentrations did not decline strictly with distance from the outlet. This suggests that mixing, local circulation patterns, and retention influence the observed distribution as much as simple spatial geometry. Further out, this is reflected at Stn 8, where MeBT reached approximately 30 despite the distance from the discharge point—indicating residual impact and/or retention zones. Stn 10 also maintained a high level (MeBT ~21), and in the marina (Stn 13) the pattern of accumulation became pronounced: high MeBT (~27) coincided with Paracetamol (~24) and Azithromycin (~9.3), typical for a sheltered environment where chemicals can accumulate and recirculate between water and surface sediment. In Vejle Å (Stn 20–21), several SSRI/SNRI compounds occurred at quantified levels (Citalopram 5.9/21; Sertraline 3.4/8.5; Venlafaxine 9.8), indicating that urban influence is already strong upstream of the inner fjord and that the river acts as a transport pathway for human pharmaceuticals downstream. The stations where MeBT was only detected <LOQ (Stn 2, 16, 19) suggest areas with lower primary impact or stronger dilution at the time of sampling. Overall, the spatial distribution—reinforced by the HPI ranking, with Stn 13 highest followed by Stn 1/8/20—indicates that the observed patterns are consistent with influence from WWTP discharges; however, additional inputs from urban runoff and other sources cannot be ruled out.

4 Discussion / Analysis

Indicator taxa and source interpretation

To contextualize the quantitative findings presented in Table 1, a qualitative assessment was conducted of the most abundant microbial indicator taxa observed via Bracken-adjusted metagenomic profiling. These taxa fall broadly into two categories: (i) genera strongly associated with activated-sludge and wastewater-treatment processes, and (ii) genera indicative of human or faecal/zoonotic origin. The interpretation is based primarily on Bracken results; Kraken2 is referenced only as supporting context.

Among process/activated-sludge associated genera, *Paracoccus* and *Acidovorax* were consistently detected and are well documented in treatment-plant microbial cores. For example, *Paracoccus limosus* was isolated from activated sludge in a municipal sewage-treatment plant (Lee & Lee, 2013). These genera thus serve as credible indicators of WWTP-derived biomass in sediments and water columns, especially in inner-fjord zones. Acinetobacter is also frequently enriched in activated-sludge systems, sometimes linked to antibiotic-resistance gene propagation (Kisková et al., 2023). Its elevated relative abundance in the inner zones supports the interpretation of a WWTP-related microbial signal.

The second group of taxa comprises genera such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, which are frequently associated with wastewater of human/clinical origin. For *S. aureus*, for example, sewage-plant studies have shown prevalence of methicillin-resistant isolates downstream of treatment plants (Amirsoleimani et al., 2019; Oladipo et al., 2023). Their presence in the sediment/water profile further strengthens a human/WWTP component in the microbial footprint.

Genera such as *Escherichia coli* and *Salmonella enterica* remain important as faecal or zoonotic indicators, but are less source-specific: they may derive from municipal sewage, stormwater runoff or agricultural runoff. For example, studies show *Salmonella enterica* detection in surface waters associated with livestock runoff (Chung et al., 2023). Thus, their occurrence in the fjord must be interpreted more cautiously regarding WWTP-specific influence.

The relative abundances observed here — typically in the range of a few tenths of a percent up to ~1–2 % per taxon — are reasonably comparable with values observed in full-scale activated-sludge community studies. For example, a large survey of Danish municipal activated-sludge plants found that core species accounted for large fractions of reads, but individual genera often contributed much less (Vestergaard et al., 2024). The spatial gradient observed in Table 1 — higher indicator abundances in inner fjord zones (closest to discharge) and reduced levels in outer zones — is consistent with a dilution and mixing scenario of treated effluent into the fjord.

In summary, the detected indicator taxa principally reflect microbial communities derived from wastewater-treatment processes, thereby supporting the study objective of identifying a WWTP-specific signature. At the same time, the occurrence of general faecal/zoonotic genera underscores that other pathways (including agricultural/land-based runoff) cannot be excluded. Overall, the results indicate that wastewater effluent exerts a measurable influence on the microbial composition in the inner fjord zones, while additional diffuse inputs from other sources may also contribute to the observed patterns.

Occurrence Patterns of Pharmaceuticals and Related Contaminants

In total, 13 different compounds were detected in the sediment samples from Vejle. Among these, Σ(6&4)-methylbenzotriazole was dominant (detected in all 21 samples, 18 above LOQ), followed by citalopram (14/21), azithromycin (11/21), metoprolol and candesartan (7/21 each), sertraline (6/21), and venlafaxine (5/21).

Other substances were found more sporadically (trimethoprim 3/21; benzotriazole, tramadol 2/21; paracetamol, losartan, propranolol, amisulpride 1/21). For several compounds, a large proportion of detections were below the LOQ, indicating widespread but low-level presence in the sediments. This detection profile is consistent with earlier findings from the Baltic Sea region, where CNS drugs (SSRI/SNRI), cardiovascular agents (beta-blockers, ARBs), and analgesics/anti-inflammatory drugs have been the most frequently reported in aquatic environments, particularly near municipal WWTP discharges (HELCOM, 2021).

The compound detected in all samples was Σ(6&4)-methylbenzotriazole, with concentrations above LOQ in 18 of 21 sediment samples. This compound, used as a corrosion inhibitor in industrial products, is not a pharmaceutical per se but is well known for its persistence and frequent occurrence in urban and industrial recipient waters. The related compound benzotriazole was also detected, but only in two samples and below LOQ, possibly due to lower usage or differing transport behavior. Among the pharmaceuticals, the antibiotic azithromycin was one of the most frequently detected (11/21 samples, 6 >LOQ). Azithromycin is a macrolide antibiotic known for its environmental stability and contribution to antimicrobial resistance development. Similarly, the antidepressant citalopram was found in 14 samples, though only 4 above LOQ. These compounds are both widely used in households and possess physicochemical properties that promote long-term accumulation in sediments. Other antidepressants, such as sertraline (6/21, 2 >LOQ) and venlafaxine (5/21, 2 >LOQ), occurred less frequently but still confirm that psychopharmaceuticals are common in the environment, even at trace levels. They have been shown to affect fish behavior and reproduction at environmentally relevant concentrations. Analgesics like tramadol and paracetamol were found in fewer samples (2 and 1, respectively), consistent with their relatively rapid degradation in aquatic environments. Beta-blockers and antihypertensives such as metoprolol (7/21), candesartan (7/21), losartan (1/21), and propranolol (1/21) generally occurred below LOQ, which may reflect low sediment affinity or degradation in the water column prior to sedimentation. The antibiotic trimethoprim—commonly detected in surface waters—was only found in 3 samples at very low levels (<LOQ), despite its known persistence.

Interactions Between Chemical Properties and Environmental Behavior

The sediment occurrence patterns closely reflect known environmental characteristics such as low degradability, high usage frequency, and physicochemical properties favoring particle adsorption. Compounds that are water-soluble yet resistant to degradation (e.g., azithromycin, citalopram) are more frequently detected than those that are more biodegradable or less widely used. The fact that many detections are below LOQ but still measurable highlights the importance of analytical sensitivity and suggests that even low concentrations should be considered when assessing environmental risk—particularly for substances with additive or synergistic effects.

Comparisons with regional studies show that the Vejle results resemble the typical wastewater-related pattern observed in aquatic sediments. The high detection frequency of Σ(6&4)-methylbenzotriazole—a corrosion inhibitor of urban origin—together with several pharmaceuticals commonly associated with municipal effluents (e.g., SSRI/SNRI antidepressants, beta-blockers, ARBs, and antibiotics) suggests that wastewater inputs likely represents an important source component in the inner fjord. At the same time, the high proportion of <LOQ findings aligns with previous observations that pharmaceuticals in sediments are often present at low concentrations yet can still be ecologically relevant (HELCOM, 2021). Experimental studies in the region have shown, for example, that citalopram can alter fish behavior at ≤0.15 µg/L (Brodin et al., 2013; Kellner et al., 2015), and that compounds such as propranolol, diclofenac, and ibuprofen can affect blue mussel byssus production, energy balance (Scope for Growth), and algal photosynthesis at low levels (Ericson et al., 2010; Eriksson Wiklund et al., 2011; Kumblad et al., 2015; Oskarsson et al., 2012, 2014). This indicates that even the sub-LOQ concentrations found in Vejle sediments may contribute to long-term ecological effects, particularly in combination. Altogether, the pharmaceutical profile of Vejle sediments not only aligns with regional patterns but also suggests potential risks of chronic ecological impacts.

Integration of molecular and chemical indicators

The integration of molecular and chemical evidence provides a coherent picture of wastewater influence in Vejle Fjord. Both the metagenomic and metabarcoding datasets were analysed against a predefined list of wastewater-related indicator taxa representing three functional categories: (i) WWTP process indicators (e.g., Paracoccus, Tetrasphaera, Nitrospira), (ii) antibiotic resistance-associated taxa (ARG indicators), and (iii) recipient indicators reflecting environmental response.

In the metagenomic data, the relative abundance and number of indicator taxa were highest in the inner fjord zones, particularly near the discharge area, and decreased gradually toward outer fjord zones. This trend coincided with elevated concentrations of pharmaceuticals such as antidepressants and antibiotics detected in sediment samples, confirming the presence of an urban-wastewater signature.

The 16S metabarcoding dataset was evaluated separately using the same indicator list to allow direct comparison between methods. Several WWTP process and ARG-associated taxa were detected exclusively in the inner fjord samples, while recipient indicators were more evenly distributed or occurred at low relative abundance throughout the fjord. The consistency between the two molecular approaches strengthens the interpretation that these taxa represent reliable indicators of wastewater influence in fjord sediments.

When compared to the chemical results, the molecular indicators—particularly *Tetrasphaera*, *Nitrospira*, *Paracoccus*, and *Acidovorax*—aligned with zones showing the highest cumulative pharmaceutical loads. Together, these molecular and chemical signals delineate a clear spatial gradient consistent with hydrodynamic and nutrient modelling of Vejle Fjord (Lees et al., 2022). The integrated evidence suggests that inner-fjord sediments act as a local sink for wastewater-derived microorganisms and compounds, while outer-fjord zones reflect background or diffuse inputs.

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Appendices

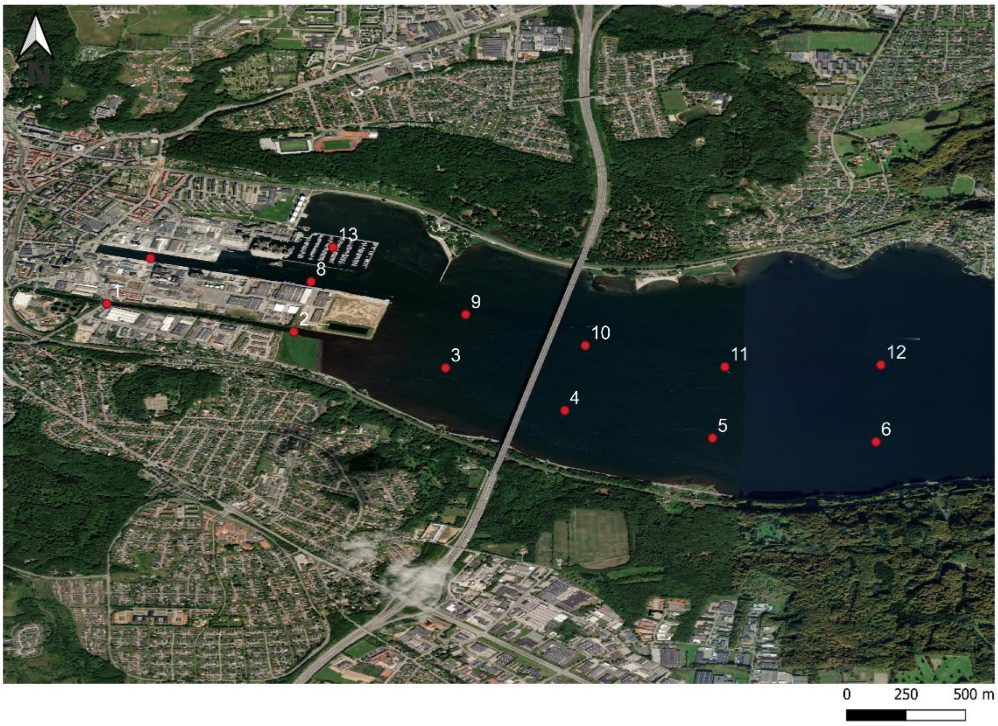

Figure S1. Zonal grouping of sampling stations in Vejle Fjord. Map illustrating the four spatial zones used in the analyses: Inner fjord (stations 1–6), Mid fjord (7–13). Background map: © Geodatastyrelsen, Denmark.

Figure S2. Sampling stations of Vejle Å (stations 20–21). Background map: © Geodatastyrelsen, Denmark.

Supplementary Table S1. Quality control and read-processing summary for representative sediment samples from Vejle Fjord (nf-core/ampliseq v2.14.0). The table summarizes the main processing steps from raw reads to final taxonomically classified sequences of samples (BaVejle1–63). Columns show the number of read pairs at each stage of the workflow: total reads processed by Cutadapt, reads passing primer and quality filters, and reads retained after DADA2 filtering, denoising, merging, and chimera removal. "Input tax filter" refers to the number of reads subjected to taxonomic classification, while "filtered tax filter" shows the number of successfully classified reads. "Retained percent" indicates the proportion of reads retained after all quality control steps, and "lost percent" represents reads removed during filtering and chimera detection. Overall, data quality and read retention were high (>90 % in most samples), confirming efficient sequencing and robust bioinformatic processing.

sample	cutadapt total processed	cutadapt passing filters	cutadapt passing filters percent	DADA2 input	filtered	denoisedF	denoisedR	merged	nonchim	input tax filter	filtered tax filter	lost	retained percent	lost percent
BaVejle1	93,452	92,321	98.8%	92321	85161	64063	68440	26203	23899	23899	21681	2218	90.7	9.3
BaVejle2	81,165	79,589	98.1%	79589	69907	52446	56285	21794	19548	19548	17450	2098	89.3	10.7
BaVejle3	74,992	74,021	98.7%	74021	68467	51384	55102	21785	19842	19842	17789	2053	89.7	10.3
BaVejle4	76,954	76,320	99.2%	76320	69020	54105	57549	25256	22838	22838	17197	5641	75.3	24.7
BaVejle5	56,826	55,986	98.5%	55986	50915	38462	40997	17144	15995	15995	13072	2923	81.7	18.3
BaVejle6	95,065	94,145	99.0%	94145	87018	67519	71794	28193	25302	25302	25105	197	99.2	0.8
BaVejle7	64,773	64,081	98.9%	64081	58944	46370	48047	19430	17511	17511	17378	133	99.2	0.8
BaVejle8	67,923	67,237	99.0%	67237	62372	49312	50862	19856	17652	17652	17259	393	97.8	2.2
BaVejle9	64,539	63,916	99.0%	63916	57881	45426	47068	18939	17038	17038	16528	510	97.0	3.0
BaVejle10	77,993	77,287	99.1%	77287	70869	55623	57363	22665	20344	20344	19584	760	96.3	3.7
BaVejle11	65,760	65,116	99.0%	65116	58597	45400	46807	18430	16628	16628	15591	1037	93.8	6.2
BaVejle12	57,694	57,061	98.9%	57061	50984	39128	41119	15617	14307	14307	14184	123	99.1	0.9
BaVejle13	88,503	87,524	98.9%	87524	78976	61000	63316	24254	22260	22260	20868	1392	93.7	6.3
BaVejle14	52,669	52,004	98.7%	52004	47390	36087	37619	14900	13784	13784	13135	649	95.3	4.7
BaVejle15	85,463	84,346	98.7%	84346	76050	59320	61362	23932	21492	21492	20204	1288	94.0	6.0
BaVejle16	74,965	74,176	98.9%	74176	67835	53382	54770	22518	20819	20819	20433	386	98.1	1.9
BaVejle17	72,179	71,624	99.2%	71624	65380	51989	53725	22868	21037	21037	20659	378	98.2	1.8
BaVejle18	92,675	91,522	98.8%	91522	80206	63859	66098	26144	23763	23763	23389	374	98.4	1.6
BaVejle19	100,220	99,413	99.2%	99413	90311	73849	76339	36605	28337	28337	21856	6481	77.1	22.9
BaVejle20	130,325	128,922	98.9%	128922	109602	90595	93290	45743	34806	34806	24944	9862	71.7	28.3
BaVejle21	53,229	52,579	98.8%	52579	47500	37754	39351	18980	15081	15081	10168	4913	67.4	32.6
BaVejle22	63,106	62,511	99.1%	62511	56850	44338	45853	17738	16011	16011	14622	1389	91.3	8.7
BaVejle23	67,843	67,248	99.1%	67248	60714	47858	49429	19329	17338	17338	16785	553	96.8	3.2
BaVejle24	65,148	64,398	98.8%	64398	59719	46713	48890	20124	18060	18060	16848	1212	93.3	6.7
BaVejle25	92,862	91,673	98.7%	91673	84048	69619	71304	31903	27657	27657	26910	747	97.3	2.7
BaVejle26	83,925	83,146	99.1%	83146	76168	62185	63857	26775	23148	23148	21940	1208	94.8	5.2
BaVejle27	111,293	110,291	99.1%	110291	99071	80860	84479	42567	36500	36500	36157	343	99.1	0.9
BaVejle28	69,756	68,995	98.9%	68995	62469	51016	53001	23293	19897	19897	17599	2298	88.5	11.5
BaVejle29	66,273	65,687	99.1%	65687	59489	48427	50088	21352	18729	18729	17464	1265	93.2	6.8
BaVejle30	103,727	102,764	99.1%	102764	93498	77199	78958	34099	28196	28196	25406	2790	90.1	9.9
BaVejle31	80,135	79,351	99.0%	79351	71346	55627	57708	22272	20513	20513	19944	569	97.2	2.8
BaVejle32	63,000	62,389	99.0%	62389	56709	43624	45342	17611	16447	16447	15692	755	95.4	4.6
BaVejle33	98,481	97,350	98.9%	97350	87719	69104	70953	28934	26401	26401	24955	1446	94.5	5.5
BaVejle34	91,757	90,797	99.0%	90797	82417	65081	67329	27454	24722	24722	23839	883	96.4	3.6
BaVejle35	65,993	65,425	99.1%	65425	59967	46684	48090	19735	18009	18009	17145	864	95.2	4.8
BaVejle36	52,910	52,146	98.6%	52146	47743	36482	37805	14122	12974	12974	12328	646	95.0	5.0
BaVejle37	52,999	52,570	99.2%	52570	47738	37212	38395	14647	13134	13134	12858	276	97.9	2.1
BaVejle38	50,680	50,241	99.1%	50241	45438	35511	36943	14409	12961	12961	12787	174	98.7	1.3
BaVejle39	68,245	67,576	99.0%	67576	61331	49261	50614	20922	18915	18915	18802	113	99.4	0.6
BaVejle40	59,385	58,612	98.7%	58612	52894	41294	42795	17755	16364	16364	15884	480	97.1	2.9
BaVejle41	89,097	88,122	98.9%	88122	76322	60059	62205	24992	22962	22962	21759	1203	94.8	5.2
BaVejle42	65,560	64,797	98.8%	64797	59126	46105	47873	19594	17949	17949	17122	827	95.4	4.6
BaVejle43	83,094	82,169	98.9%	82169	74595	58564	61036	24313	21705	21705	20561	1144	94.7	5.3
BaVejle44	67,043	66,479	99.2%	66479	60504	47386	49455	21126	19393	19393	18375	1018	94.8	5.2
BaVejle45	78,252	77,506	99.0%	77506	70391	54639	57160	22240	19849	19849	18806	1043	94.7	5.3
BaVejle46	73,218	72,577	99.1%	72577	65497	51887	54004	24054	21827	21827	19182	2645	87.9	12.1
BaVejle47	71,139	70,543	99.2%	70543	64812	50439	52525	21095	19290	19290	17771	1519	92.1	7.9
BaVejle48	86,617	85,548	98.8%	85548	78477	62037	64257	27658	25450	25450	23489	1961	92.3	7.7
BaVejle49	77,888	77,225	99.1%	77225	71065	55990	57907	23883	21467	21467	20776	691	96.8	3.2
BaVejle50	90,400	89,443	98.9%	89443	83098	66384	68015	29241	25900	25900	23794	2106	91.9	8.1
BaVejle51	66,457	65,849	99.1%	65849	59575	46057	47597	18867	17406	17406	16712	694	96.0	4.0
BaVejle52	88,201	87,177	98.8%	87177	76490	60871	62803	25740	23041	23041	22698	343	98.5	1.5
BaVejle53	56,329	55,632	98.8%	55632	50634	38229	39905	15515	14414	14414	13899	515	96.4	3.6
BaVejle54	91,118	90,303	99.1%	90303	78991	62320	64457	25821	23113	23113	22633	480	97.9	2.1
BaVejle55	76,034	75,427	99.2%	75427	68564	53897	55959	23037	20918	20918	20247	671	96.8	3.2
BaVejle56	70,360	69,345	98.6%	69345	63479	49566	51797	20964	19113	19113	18602	511	97.3	2.7
BaVejle57	101,452	100,342	98.9%	100342	92252	74882	77409	34743	30347	30347	29316	1031	96.6	3.4
BaVejle58	71,207	70,259	98.7%	70259	64136	49133	52703	20828	18951	18951	18563	388	98.0	2.0
BaVejle59	61,878	61,246	99.0%	61246	56204	42495	45710	17774	16410	16410	15891	519	96.8	3.2
BaVejle60	50,563	49,964	98.8%	49964	45267	33864	36690	14128	13105	13105	13046	59	99.5	0.5
BaVejle61	84,625	83,746	99.0%	83746	75667	58317	61790	24714	22660	22660	21266	1394	93.8	6.2
BaVejle62	74,119	73,419	99.1%	73419	66736	50802	54477	21687	19567	19567	17774	1793	90.8	9.2
BaVejle63	69,726	69,030	99.0%	69030	63097	48103	51415	20304	18702	18702	17338	1364	92.7	7.3

Supplementary Table S2. Sequencing output and quality metrics for metagenomic libraries from Vejle Fjord. The table summarizes raw read statistics and sequencing quality for 22 metagenomic sediment samples (Vejlemg1–22) generated on the Illumina NovaSeq 6000 platform (paired-end 150 bp mode). Columns show the total number of read pairs, total yield in gigabases (Gb), and the proportion of bases with Phred quality scores above 20 (Q20) and 30 (Q30), respectively. GC (%) indicates the overall guanine-cytosine content per sample, reflecting the diversity of microbial and eukaryotic DNA present in the sediments. All libraries exhibited excellent sequencing quality, with Q20 = 100 % and Q30 values between 92 % and 95 %, confirming high data integrity suitable for downstream taxonomic and functional analyses.

Sample ID	Reads (pairs)	Yield (Gb)	Q20 (%)	Q30 (%)	GC (%)
Vejlemg1	22304465	6.683377369	100	92.38	59.31
Vejlemg2	23393777	7.008491058	100	93.38	55.39
Vejlemg3	29180892	8.745954016	100	94.05	54.72
Vejlemg4	28946747	8.674894994	100	94.44	54.46
Vejlemg5	26681671	7.997727472	100	94.35	53.52
Vejlemg6	22636369	6.785153765	100	94.33	53.55
Vejlemg7	21870940	6.555224707	100	94.83	51.19
Vejlemg8	22158983	6.641665532	100	94.54	53.65
Vejlemg9	23849941	7.148843457	100	94.05	54.46
Vejlemg10	28558956	8.560087442	100	94.13	54.55
Vejlemg11	23678435	7.097179152	100	94.66	53.12
Vejlemg12	38617208	11.57452438	100	94.24	54.38
Vejlemg13	24115415	7.228564323	100	94.31	53.59
Vejlemg14	22716253	6.809193432	100	94.25	54.05
Vejlemg15	27928705	8.371147633	100	94.67	53.08
Vejlemg16	24046659	7.207355545	100	93.56	53.67
Vejlemg17	24609205	7.376088902	100	94.36	53.06
Vejlemg18	24877417	7.45672646	100	94.35	53.36
Vejlemg19	22996199	6.89198172	100	94.06	53.46
Vejlemg20	33545316	10.05158125	100	92.29	60.03
Vejlemg21	27456195	8.227805445	100	92.6	59.27
Vejlemg22	22604588	6.776484011	100	95.06	48.51

Supplementary Table S3. List of indicator taxa used for classification of wastewater-related influence in Vejle Fjord and Vejle Å sediments. Taxa are grouped into four categories representing (i) wastewater treatment plant process indicators, (ii) general recipient indicators, (iii) human pathogens, and (iv) antibiotic resistance gene (ARG)–associated taxa.

category	taxon	category	taxon
WWTP_process	candidatus accumulibacter	Pathogen	aeromonas
WWTP_process	tetrasphaera	Pathogen	klebsiella
WWTP_process	dechloromonas	Pathogen	pseudomonas aeruginosa
WWTP_process	defluviicoccus	Pathogen	stenotrophomonas maltophilia

WWTP_process	candidatus competibacter	Pathogen	legionella pneumophila
WWTP_process	nitrosomonas	Pathogen	mycobacterium avium
WWTP_process	nitrospira	Pathogen	yersinia enterocolitica
WWTP_process	parcubacteria	Pathogen	salmonella enterica
WWTP_process	saprospiraceae	Pathogen	shigella
WWTP_process	thauera	Pathogen	vibrio cholerae
WWTP_process	zoogloea	Pathogen	vibrio vulnificus
WWTP_process	paracoccus	Pathogen	vibrio parahaemolyticus
WWTP_process	rhodocyclus	Pathogen	listeria monocytogenes
WWTP_process	propionivibrio	Pathogen	streptococcus pneumoniae
WWTP_process	hyphomicrobium	Pathogen	streptococcus pyogenes
WWTP_process	novosphingobium	Pathogen	staphylococcus aureus
WWTP_process	acidovorax	Pathogen	helicobacter pylori
WWTP_process	pseudoxanthomonas	Pathogen	chlamydia trachomatis
WWTP_process	candidatus amarolinea	Pathogen	neisseria meningitidis
WWTP_process	candidatus microthrix	Pathogen	bordetella pertussis
WWTP_process	candidatus vilivitalis	Pathogen	clostridioides difficile
WWTP_process	candidatus poribacteria	Pathogen	burkholderia cepacia complex
Recipient_indicator	arcobacter	ARG	acinetobacter baumannii
Recipient_indicator	acinetobacter	ARG	klebsiella pneumoniae
Recipient_indicator	bacteroides	ARG	escherichia coli st131
Recipient_indicator	prevotella	ARG	proteus mirabilis
Recipient_indicator	clostridium	ARG	providencia stuartii
Recipient_indicator	fusobacterium	ARG	morganella morganii
Recipient_indicator	enterococcus	ARG	citrobacter freundii
Recipient_indicator	escherichia coli	ARG	bacteroides fragilis
Recipient_indicator	citrobacter	ARG	prevotella intermedia
Recipient_indicator	serratia	ARG	staphylococcus epidermidis
Recipient_indicator	moraxella	ARG	enterococcus faecalis
Recipient_indicator	kingella	ARG	enterococcus faecium
Recipient_indicator	campylobacter	ARG	corynebacterium striatum
Recipient_indicator	leptotrichia	ARG	serratia marcescens
Recipient_indicator	treponema	ARG	stenotrophomonas maltophilia
Recipient_indicator	veillonella	ARG	achromobacter xylosoxidans
Recipient_indicator	bifidobacterium	ARG	ralstonia pickettii
Recipient_indicator	lactobacillus		
Recipient_indicator	rikenellaceae		
Recipient_indicator	parabacteroides		

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This report has been reviewed and approved in accordance with IVL's audit and approval management system.