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Frank, Ellinor

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PO Box 117
221 00 Lund
+46 46-222 00 00

Microbial Source Tracking for Bathing Water Quality Assessment: *E. coli* and Bacterial Communities in Coastal Environment

ELLINOR M. FRANK

FACULTY OF ENGINEERING | LUND UNIVERSITY



Microbial Source Tracking for Bathing Water Quality Assessment: *E. coli* and Bacterial Communities in Coastal Environment

Ellinor M. Frank



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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Engineering at Lund University to be publicly defended on October 24, 2025 at 10.15 in room V:C, V-building, Klas Anshelms väg 14, Lund.

Faculty opponent
Professor David Werner

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Title and subtitle Microbial Source Tracking for Bathing Water Quality Assessment: <i>E. coli</i> and Bacterial Communities in Coastal Environment			
Abstract Microbial contamination in coastal environments and groundwater is explored within this thesis, focusing on fecal contamination from anthropogenic sources and microbial community dynamics. The spread and persistence of microbial contaminants in complex aquatic environments with multiple intermittent and point sources of contamination, was studied in order to lay a groundwork for developing more robust monitoring framework of water quality. A combination of molecular methods, chemical analyses, and classical microbiological techniques was utilized to study several different sites. In the first study, remediation strategies performed in a site contaminated with chlorinated solvents were evaluated. Chemical monitoring, compound-specific isotope analysis, and 16S rRNA gene sequencing were performed to assess remediation effectiveness and microbial community shifts during biotic and abiotic degradation of contaminants. Subsequent studies were performed in an urban coastal environment, where sediment and water microbial communities were investigated with a particular focus on fecal indicator bacteria (<i>Escherichia coli</i> and intestinal enterococci) and other sewage-associated taxa. Results indicate that sediments can act as reservoirs for <i>E. coli</i>, and that fecal indicator bacteria monitoring could benefit from introduction of additional bacterial groups as sewage indicator bacteria. Phenotypic and genotypic characterization of sediment-associated <i>E. coli</i> further revealed adaptations that may facilitate survival in marine sediments, including virulence factors, biofilm formation, and halotolerance. In a longitudinal study of sediment microbial communities, it was found that treated effluent from a wastewater treatment plant was a significant source of both <i>E. coli</i> and sewage-associated taxa, while combined sewer overflows caused a more localized and transient effect on the microbial community. Additionally, the presence of sewage-associated taxa in sediments distant from known discharge points suggested contribution from intermittent sources, such as stormwater. Analysis of microbial communities in urban bathing water revealed that the microbial communities are influenced by environmental parameters such as temperature and rainfall, and that sewage- and gut-associated bacterial groups have complex spatial and temporal distribution. While the fecal and sewage indicator bacteria could be connected to environmental parameters and anthropogenic influences to some degree, spatial sensitivity was very high. <i>E. coli</i> was found to be an incomplete indicator of anthropogenic impact on coastal waters. Overall, this thesis highlights the importance of combining microbial community analysis, source tracking, and environmental data to achieve a more nuanced understanding of microbial contamination in aquatic environments. The results contribute to the development of more effective monitoring and management strategies for protecting water quality and public health in urban coastal areas.			
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Ellinor M. Frank



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*A dancer dies twice — once when they stop dancing, and this
first death is the more painful.*

Martha Graham

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Slutligen, tack till du lurviga och roliga varelse, min älskade hund Siri.

Abstract

Microbial contamination in coastal environments and groundwater is explored within this thesis, focusing on fecal contamination from anthropogenic sources and microbial community dynamics. The spread and persistence of microbial contaminants in complex aquatic environments with multiple intermittent and point sources of contamination, was studied in order to lay a groundwork for developing more robust monitoring framework of water quality.

A combination of molecular methods, chemical analyses, and classical microbiological techniques was utilized to study several different sites. In the first study, remediation strategies performed in a site contaminated with chlorinated solvents were evaluated. Chemical monitoring, compound-specific isotope analysis, and 16S rRNA gene sequencing were performed to assess remediation effectiveness and microbial community shifts during biotic and abiotic degradation of contaminants. Subsequent studies were performed in an urban coastal environment, where sediment and water microbial communities were investigated with a particular focus on fecal indicator bacteria (*Escherichia coli* and intestinal enterococci) and other sewage-associated taxa. Results indicate that sediments can act as reservoirs for *E. coli*, and that fecal indicator bacteria monitoring could benefit from introduction of additional bacterial groups as sewage indicator bacteria. Phenotypic and genotypic characterization of sediment-associated *E. coli* further revealed adaptations that may facilitate survival in marine sediments, including virulence factors, biofilm formation, and halotolerance. In a longitudinal study of sediment microbial communities, it was found that treated effluent from a wastewater treatment plant was a significant source of both *E. coli* and sewage-associated taxa, while combined sewer overflows caused a more localized and transient effect on the microbial community. Additionally, the presence of sewage-associated taxa in sediments distant from known discharge points suggested contribution from intermittent sources, such as stormwater. Analysis of microbial communities in urban bathing water revealed that the microbial communities are influenced by environmental parameters such as temperature and rainfall, and that sewage- and gut-associated bacterial groups have complex spatial and temporal distribution. While the fecal and sewage indicator bacteria could be connected to environmental parameters and anthropogenic influences to some degree, spatial sensitivity was very high. *E. coli* was found to be an incomplete indicator of anthropogenic impact on coastal waters.

Overall, this thesis highlights the importance of combining microbial community analysis, source tracking, and environmental data to achieve a more nuanced understanding of microbial contamination in aquatic environments. The results contribute to the development of more effective monitoring and management strategies for protecting water quality and public health in urban coastal areas.

Popular science summary in Swedish

När man en varm sommardag rör sig ner till stranden för ett svalkande dopp är det sista man vill se en stor varningsskylt vid bryggan; "VARNING, höga bakteriehalter, badning avrådes". Höga halter av fekala bakterier i badvatten är ingen nytt och kan möjligen ses som ett resultat av ökad populationstäthet. Mer människor, mer avlopp, mer bakterie-förorening? Var bakterierna verkligen kommer ifrån är ett utav målen i detta forsknings-projekt. När man vet med signifikans var de till största del kommer ifrån, först då kan man ta första steget mot att hitta en lösning på problemet. Att leta efter fekal kontaminering i badvatten är viktigt då man kan bli rejält sjuk av många av dessa bakterier, men även en mildare släng av magsjuka är inget man vill åka på.

Det finns massor av olika bakterier som enbart finns i mage och tarm hos människor och boskap, och som inte finns i sjö- och havsvatten, som skulle kunna fungera som indikatorer för kontaminering av den anledningen. Dock så är inte alla bakterier lika lätta att få att överleva i ett labb, och därför är det väldigt svårt att räkna deras antal. En viss art av bakterie som används som indikator för fekal förorening är *Escherichia coli* (*E. coli*) och den är tacksam att odla i labb. *E. coli* tillsammans med intestinala enterokocker används i nuläget som fekala indikatorer i badvatten överallt i hela Sverige och även internationellt. Den nuvarande labb-metodiken för att räkna dessa har en bristfällig faktor dock; det tar tre dagar från provtagning till resultat. Provtagning görs i regel en gång i veckan och detta gör att badarna aldrig har ett precist värde på hur badvattenkvaliteten ser ut "just nu". Vattnet rör sig, resultatet kommer fördröjt, det är inte konstigt att det finns en viss misstro till badvatten-värdena som invånarna har att gå på.

Alla vattendrag är komplexa på sina egna vis, och Öresund är inget undantag. Flertalet bäckar och floder rinner ut i Öresund både på Sveriges och på Danmarks sida, och såklart finns många vattenreningsverk för att ta hand om avlopp ifrån de miljontals människor som bor här. De flesta reningsverken har inte processer som specifikt ska döda bakterier då fokus ligger på att avlägsna kemiska ämnen och läkemedel. Detta kan vara en orsak till att *E. coli* finns i vårt badvatten. En annan källa till fekala bakterier är dagvatten, som är det vattnet som hamnar på våra gator och i urbana vattendrag. Fåglar och andra djur gör direkt förorening på dessa ytor och vatten, och detta vatten blir i flesta fall aldrig renat. I kombinerade avloppssystem rinner dagvatten och avlopp till samma slutdestination (reningsverket), så i dessa system renas dagvatten till en viss grad. Nackdelen med kombinerade system är att när för mycket regn flödar till reningsverket på en gång tvingas reningsverken släppa ut denna orenade kombinerade vattenmassan direkt ut till havet utan rening för att inte överbelasta reningsverket. Vid dessa händelser är det inte förvånande att höjda halter av *E. coli* upptäcks.

Alla celler innehåller DNA; en lång tråd av kod som innehåller all information cellen behöver för att fungera, men även information om vem cellen är. Med hjälp av DNA ifrån badvatten kan man alltså få en bild över vilka bakterier som finns i vattnet. Med prover tagna över en lång sträcka av kusten kan man använda denna information för att källspåra var den troligaste punkten för huvudsaklig kontaminering är. Genom att även undersöka sedimenten på havets botten hoppas man se om, och till vilken grad, sedimenten fungerar som en reservoar för bakterierna att överleva och bo i. För *E. coli* är ju som sagt en bakterie som lever i våra tarmar och föredrar 37 grader varmt. Botten av Öresund är kallt, bara några plusgrader, hur lyckas de överleva där? Tyvärr är bakterier väldigt tåliga, och kan gå in i en slags dvala vid behov. I kombination med deras korta generationstid kan de snabbt anpassa sig till en ny miljö. Ett känt ordspråk är "dilution is the solution to pollution", vilket på svenska innebär att om man spär ut kontaminering tillräckligt mycket så löser det problemen. Men forskare börjar på riktigt ifrågasätta detta, särskilt gällande bakterier då de är så skickliga på att överleva. Och med tanke på de ständiga problem vi ser med *E. coli*-förorening i våra badvatten, kan vi inte längre blint säga att det blir utspäddt nog. Visst, Öresund är ingen dricksvatten-källa, men om man tittar på det stora hela så är det ändå samma vatten som rör sig runt i vårt kretslopp. Och om vi vill ha "lyxen" att kunna bada i sjöar och hav i vår urbaniserade värld så måste vi skydda vårt vatten, både salt som sött.

List of Publications

Appended papers

Paper I: Biochemical changes associated with two different in situ remediation strategies for tetrachloroethene

Authors: Sofia Åkesson, Ellinor M. Frank, Charlotte Sparrenbom, Zixuan Zhang, Henry Holmstrand, Catherine J. Paul

Status: Manuscript for *Science of The Total Environment*

Paper II: Marine sediments are identified as an environmental reservoir for *Escherichia coli*: comparing signature-based and novel amplicon sequencing approaches for microbial source tracking

Authors: Ellinor M. Frank, Jon Ahlinder, Therese Jephson, Kenneth M. Persson, Elisabet Lindberg, Catherine J. Paul

Status: Published in *Science of The Total Environment*

DOI: 10.1016/j.scitotenv.2023.167865

Paper III: *Escherichia coli* in urban marine sediments: interpreting virulence, biofilm formation, halotolerance, and antibiotic resistance to infer contamination or naturalization

Authors: Isabel K. Erb, Carolina Suarez, Ellinor M. Frank, Johan Bengtsson-Palme, Elisabet Lindberg, Catherine J. Paul

Status: Published in *FEMS Microbes*

DOI: 10.1093/femsmc/xtae024

Paper IV: Microbial Contamination in Urban Marine Sediments: Source Identification Using Microbial Community Analysis and Fecal Indicator Bacteria

Authors: Ellinor M. Frank, Carolina Suarez, Isabel K. Erb, Therese Jephson, Elisabet Lindberg, Catherine J. Paul

Status: Published in *MDPI Microorganisms*

DOI: 10.3390/microorganisms13050983

Paper V: Spatiotemporal analysis of coastal bathing water: bacterial communities and viable *E. coli* and intestinal enterococci

Authors: Ellinor M. Frank, Carolina Suarez, Moa Jinbäck, Therese Jephson, Catherine J. Paul

Status: Manuscript for *Environmental Sciences Europe*.

Author's contribution to appended papers

Paper I: Formal analysis, Data curation, Software, Visualization, Writing – review & editing.

Paper II: Methodology, Formal analysis, Data curation, Software, Visualization, Writing – original draft.

Paper III: Investigation, Formal analysis, Visualization, Writing – review & editing.

Paper IV: Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Software, Visualization, Writing – original draft.

Paper V: Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Software, Visualization, Writing – original draft.

Abbreviations and technical terms

ANI = Average Nucleotide Identity

ANOVA = Analysis of variance

ARG = Antibiotic resistance gene

ASV = Amplicon sequence variant

Bp = Base pair

BSA = Bovine Serum Albumin

Ca. = Candidatus

CARD = Comprehensive Antibiotic Resistance Database

CCA = Chromogenic Coliform Agar

CFU = Colony-forming units

cis-DCE = *cis*-1,2-Dichloroethene

CSO = Combined sewer overflow

CST = Curated source tracking

DNA = Deoxyribonucleic acid

DO = Dissolved oxygen

DOC = Dissolved organic carbon

EU = European Union

EUCAST = European Committee on Antimicrobial Susceptibility Testing

ExPEC = Extraintestinal *E. coli*

FIB = Fecal indicator bacteria

GAO = Glycogen-accumulating organism

GTDB = Genome Taxonomy Database

HSD = Honestly Significant Difference

InPEC = Intestinal *E. coli*

ISO = International Organization for Standardization

LB = Lysogeny broth

MiDAS = Microbial Database for Activated Sludge
 MIP = Membrane Interface Probe
 MLST = Multilocus sequence typing
 MPN = Most probable number
 MST = Microbial source tracking
 NCBI = National Center for Biotechnology Information
 NMDS = Non-metric multidimensional scaling
 OTU = Operational taxonomic unit
 PCA = Principal component analysis
 PCE = Tetrachloroethene
 PCoA = Principal coordinates analysis
 PERMANOVA = Permutational analysis of variance
 Phylogroups = Phylogenetic groups
 qPCR = Quantitative polymerase chain reaction
 RDA = Redundancy analysis
 RDP = Ribosomal Database Project
 RGI = Resistance Gene Identifier
 rRNA = Ribosomal ribonucleic acid

SB = Slanetz and Bartley
 SGT = Sewage and gut taxa
 spp. = Species pluralis, Latin for "multiple species"
 ST = Sequence type
 TCE = Trichloroethene
trans-DCE = *trans*-1,2-Dichloroethene
 UPEC = Uropathogenic *E. coli*
 UTI = Urinary tract infection
 UV = Ultraviolet
 UWWTD = Urban Wastewater Treatment Directive
 VBNC = Viable but nonculturable
 VC = Vinyl chloride
 WFD = Water Framework Directive
 WHO = World Health Organization
 W-UPEC = Wastewater-associated uropathogenic *E. coli*
 WWO = Wastewater outlet
 WWTP = Wastewater treatment plant
 ZVI = Zero-valent iron

Introduction

Urban coastal bathing water quality deterioration is a worldwide problem (Globevnik et al., 2020; World Health Organization, 2021). Aging infrastructure as well as expansion of urbanized areas is often seen as a factor in contamination with municipal wastewater (Mance et al., 2021). Additionally, other anthropogenic factors negatively affect surface water quality such as agricultural practices, mining, factories, landfills, cemeteries and traffic (Akhtar et al., 2021). With increasing population sizes these contamination sources grow, and coastal ecosystems experience heightened bacterial concentrations. Hence, the connection and interchanges between different media (e.g., sediment, sand and water) are also valuable to study (Weiskerger et al., 2019).

Environmental factors, such as precipitation, are also affecting bathing water quality. Rainfall impact on drainage and diversion systems has been seen to have a negative effect on microbiological quality of bathing water (Ordulj et al., 2022). Short duration intense rainfall is the type of rainfall that is most connected to high concentrations of fecal coliforms, and since this type of rain difficult to predict with accuracy, forecasting bacterial loads in bathing water is a challenge (Krupska et al., 2024).

Number of people visiting a beach has been shown to be positively connected to concentration of fecal indicator bacteria (FIB) in multiple studies (Torres-Bejarano et al., 2018; Toubiana et al., 2021). Other factors such as beach type (sand versus gravel), sun exposure and wave height are also influencing fecal bacteria concentrations in bathing water (Aragónés et al., 2016).

In the city of Helsingborg, Sweden, there has been issues with bathing water quality for many years, as measured by quantification of the FIB *Escherichia coli* and *Enterococcus* spp. (Svensson, 2016, 2019; Ferm and Sonander, 2025). While some beaches are more affected than others, the overall irregularities in occurrence and locations of extreme bacterial concentrations led to the municipality starting a collaboration with Lund University in search of answers and possible solutions (Sweden Water Research, 2025).

Research objectives & aims

The complex dynamics of microbial contamination in coastal environments and groundwater is studied in this thesis work. A particular focus lies on fecal pollution, microbial community composition, and persistence and transport of microbial contaminants. Utilizing both classical microbiological and molecular methodologies, in combination with advanced bioinformatic approaches, improved understanding of contamination spread and microbial reservoirs.

To investigate the sources, persistence, and dynamics of microbial contamination in coastal environments and groundwater is the aim of this thesis. Utilizing molecular and culture-based approaches, the thesis work aims to improve the understanding of how urban marine sediments may act as microbial reservoirs, how environmental factors influence fecal pollution in recreational coastal waters, and how microbial communities in groundwater respond to remediation strategies.

The thesis consists of five studies, each addressing a distinct aspect of the overall aim:

- **Paper I** aimed to evaluate two different in situ remediation strategies for groundwater contaminated with chlorinated solvents, by applying a combination of molecular and chemical analyses, with the goal of determining the effectiveness of the remediation attempts.
- **Paper II** focused on testing the hypothesis that coastal sediments near wastewater effluent points can act as reservoirs for viable *Escherichia coli*, and evaluated the spread of sewage- and fecal-associated bacteria in the sediments to estimate the wastewater treatment plant's (WWTP) influence.
- **Paper III** explored the genomes of *E. coli* isolates from urban marine sediments to investigate their virulence, antibiotic resistance potential, biofilm formation capability, and halotolerance. This deepens the understanding of their persistence in sediments, as well as the potential health risk these isolates may pose.
- **Paper IV** aimed to characterize the temporal dynamics of coastal sediment microbial communities over a three-year period, to evaluate how community shifts are connected to environmental parameters and potential contamination sources (treated wastewater, combined sewer overflows (CSOs), stormwater).
- **Paper V** sought to assess bathing water quality at multiple recreational beaches using multiple years of FIB data, meteorological records, and molecular analyses. The study aimed to untangle effects of environmental parameters and intermittent- and point-sources of fecal contamination, to

better understand transport and patterns of fecal contamination in coastal bathing waters.

These studies combined provide deeper understanding of microbial contamination in coastal environments, highlighting the connection between environmental conditions, microbial reservoirs, and fecal pollution sources. The findings contribute to the development of improved monitoring and management strategies for safeguarding water quality and public health in urbanized aquatic environments.

Studying sediments over several years and in combination with a bathing water study is a tailored and novel approach. These studies have led to an increased knowledge of microbial changes over a large span of time in an area affected by multiple anthropogenic and environmental influences. Combining this approach with a detailed look at the area's *E. coli* strains' genomic and phenotypic diversity makes this Helsingborg study quite unique. Further, these are the first academic articles regarding Helsingborg's bathing water and sediments, in the distinctive environmental location that is the Öresund strait.

Background

Urban bathing water

Mitigation of bad bathing water quality is complex, but not impossible, and different cases providing diverse solutions have generated a positive effect on the microbial quality of bathing water. Construction of a new WWTP, moving pastures away from close proximity to upstream river, and alteration of stormwater and CSO systems to hinder direct contamination of bathing site, helped improve the bathing water quality in Lacuisine, Belgium. In Ardmore, Ireland, an additional treatment step was implemented in the nearest WWTP during summer months, which significantly lowered FIB concentrations down to safe levels. In Blackpool, United Kingdom, in an area where stormwater and CSO were suspected as large sources of bacterial pollution at the coast, large storage tanks were constructed to temporarily hold stormwater, as well as a new wastewater treatment facility to handle a larger population and the stormwater. This resulted in beaches previously being classified as 'poor' quality, successively reached 'good' and 'excellent' quality (Globevnik et al., 2020). It is clear that improvement of bathing water quality is a task that demands collaboration between both research and governing authorities (Wuijts et al., 2022).

The European Environment Agency recommended additional treatment of wastewater, such as disinfection, chlorination and ozonation, in order to lower fecal bacteria concentrations at urban beaches in 2016 (European Environment Agency,

2016). Recently, the revised Urban Waste Water Treatment Directive (UWWTD) went into effect on January 1st 2025, and it demands monitoring and stricter removal of micropollutants (including *Escherichia coli* and *Enterococcus* spp.) in sewage wastewater with full compliance by 2030. This means that treatment plants located in sensitive areas (such as sites with close proximity to bathing areas) will have to comply with demands that the treated wastewater must have a *E. coli* concentration of < 10 colony-forming units (CFU) per 100 mL (European Union, 2024). Due to the staggeringly high concentrations of *E. coli* in incoming sewage to treatment plants, even with high removal efficiencies around 91.8-96.5 % with mechanical-biological treatment (no disinfection) (Raboni et al., 2016), it does not result in low enough concentrations to meet the new demands on treated wastewater. The revised UWWTD have also added stricter regulations regarding CSOs (which includes that CSOs must be eliminated or significantly reduced in sensitive areas by 2030) and introduced directives for stormwater management, including monitoring and implementation of stormwater retention infrastructure. Stormwater discharges into sensitive areas will have same acceptable threshold levels as is for European Union (EU) bathing water sites (with full compliance requirement by 2030) (European Union, 2024).

Improvement of the monitoring of bathing water quality does not only provide more reliable information about bathing water safety, it can also aid in determining the major sources that is causing contamination for areas where this is a challenging task (for example international waters). The EU-project WATERCARE proposed a system including forecasting the presence of pollutants in seawater (Penna et al., 2021). While forecasting of bathing water quality based on machine learning algorithms is not 100 % accurate, it does add safety (early warnings) and value (awareness and decision-making) for citizens (Krupska et al., 2024).

Conventional methods for bathing water quality determination

Coliforms have been recognized as an indicator for fecal contamination in water since late 1800s (Leclerc et al., 2001). The group name ‘coliforms’ consists of lactose fermenting aerobic bacteria, specifically *Escherichia coli*, *Klebsiella*, *Citrobacter* and *Enterobacter* spp., and was suggested in 1937 (Breed and Norton, 1937). During the 1960s, the term ‘fecal coliforms’ was introduced to subset the coliforms that are a more accurate representation of fecal pollution. These are the coliforms that are more thermotolerant (ability to grow at 44-46 degrees), and it includes *E. coli*, *Klebsiella* spp. and some *Citrobacter* spp. (Dufour, 2021).

In 1976 the European Union released Bathing Water Directive (76/160/EEC), and in 1984 World Health Organization (WHO) published their recreational water quality guidelines for coastal and fresh water. Both these frameworks emphasized fecal coliforms as the microbial indicator to use for bathing water safety (European Union, 1976; World Health Organization, 1984). In 2006, a new Bathing Water

Directive (2006/7/EC) was published, and the fecal indicator of choice was changed to *E. coli* and intestinal enterococci (European Union, 2006), which is very much in line with the revised guidelines that WHO published in 2003 (World Health Organization, 2003). In the latest revision released by European Union in 2014 (European Union, 2014), the reporting requirements got stricter, requiring results from testing to be publicly available on online platforms, and other bacterial pollutants were introduced as a risk (such as blue-green algae (cyanobacteria)) in alignment with the Water Framework Directive (WFD) (European Union, 2000). Similarly, in the latest revised guidelines from WHO in 2021, microbial indicators such as *Bacteroides* spp. and *Vibrio* spp. are discussed as emerging concerns. Guidelines regarding increased monitoring of cyanobacteria are introduced, as well as recommendations for utilizing real-time monitoring and forecasting of bacteria. Other aspects that are important when evaluating bathing water safety are surface waste such as oil and debris, eutrophication, pathogens (specific bacteria, protozoa and viruses) and various chemical compounds (pesticides, persistent organic pollutants and heavy metals) (World Health Organization, 2021).

Quantification methodologies recommended by the EU Bathing Water Directive include ISO 9308-1 (International Organization for Standardization) and ISO 9308-3 for *E. coli*, and ISO 7899-1 and ISO 7899-2 for intestinal enterococci (European Union, 2006). ISO 9308-1 uses membrane filtration with subsequent incubation on selective media (Chromogenic Coliform Agar (CCA)), and results are in CFU per volume of water. ISO 9308-3 instead utilizes a most probable number (MPN) method, either Multiple-Tube fermentation or an enzyme-specific assay (target enzyme: β -Glucuronidase) that allows quantification by color changes and fluorescence from fluorogenic substrates (ISO, 1998, 2014). The recommended methodologies for quantifying intestinal enterococci are very similar to the ones for *E. coli*, but different agar is used (Slanetz and Bartley (SB) Agar) in the membrane filtration workflow, and the MPN method only includes an enzyme-specific assay (target enzyme: β -Glucosidase) with fluorescence detection (ISO, 2000a, 2000b).

The term FIB is widely used, and while it often is used when talking exclusively about *E. coli* and intestinal enterococci in bathing water settings, the term is broader than that. In addition to fecal coliforms, there are other bacteria also considered FIB, such as: *Clostridium perfringens* (Bezirtzoglou et al., 1994), *Bacteroides* spp. (Fiksdal et al., 1985), *Lactobacillus* spp. (Marti et al., 2010), *Streptococcus* spp. (Sinton et al., 1993), *Campylobacter* spp. (Hänninen et al., 2003), and *Salmonella* spp. (Lemarchand and Lebaron, 2003). Moving forward in this thesis however, the term FIB will be used to describe the most widely used FIB today: *E. coli* and intestinal enterococci.

In recent years, the need for culture independent methods, as well as alternative indicators of fecal pollution, has increased. For example, the use of human enteric

viruses (Teixeira et al., 2020), host-specific DNA-based markers (Li et al., 2019), and antibiotic resistance genes (ARGs) (Chen et al., 2023). This is due to the inconsistency between FIB concentrations and health risks (Lemarchand and Lebaron, 2003; McKee and Cruz, 2021), naturalization of FIB in environmental water, sand and sediments (Devane et al., 2020), and for FIB's inherent non source specificity.

Microbial source tracking

Introduction of Microbial source tracking

Microbial source tracking (MST) refers to methods used to identify the sources of fecal contamination in the environment (Bernhard and Field, 2000; Harwood et al., 2014). While cultivation and quantification of FIB can be considered a culture dependent branch of MST, MST generally refers to methods that allow for source distinguishing (e.g., human, agricultural, wildlife or other sources) (Field and Samadpour, 2007). A significant advancement in MST was the development of SourceTracker, a Bayesian model that uses high-throughput sequencing data to estimate the relative contributions of potential contamination sources (Knights et al., 2011). This tool has become widely used for its ability to handle complex microbial community data and provide probabilistic estimates of contamination sources in environmental samples. MST does not have to be limited to fecal contamination tracking, the concept of tracking the source of microorganisms can be utilized in other contexts (Staley et al., 2012). Oil and hydrocarbon contamination can be tracked by identifying oil-degrading bacteria (Krolicka et al., 2019), harmful algal blooms can be tracked with cyanobacteria (Scherer et al., 2017), soil contamination by industrial sources can be tracked by heavy metal-resistant bacteria (Jarosławiecka and Piotrowska-Seget, 2022). To evaluate the effect and efficacy of bioremediation of contaminated soil and groundwater, the bacteria that metabolize the contaminating substance and its metabolites should be analyzed over space and time (Andreoni and Gianfreda, 2007; Fischer et al., 2021).

MST technologies

The rise of new methodologies that quantify DNA-markers of source specific origin have extended the MST field with a culture independent branch (Fulke et al., 2025). Within this field there are multiple different approaches that can be used, most widely used being quantitative polymerase chain reaction (qPCR), shotgun metagenomics, signature-based MST (using predefined fecal reference sequence libraries), and bacterial community composition analysis (Demeter et al., 2023). With qPCR technology, known genetic markers such as *BacH* (Human specific) and *Rum2Bac* (Ruminant specific) are quantified with high sensitivity and specificity, which enables uncovering of the source of the fecal contamination (Boehm et al.,

2013). Metagenomics is a wide term, but for usage in MST purposes, shotgun metagenomics is a useful approach as it allows for sequencing all DNA present in a sample, including plasmids (Bengtsson-Palme et al., 2014). Another similar approach is to do whole genome sequencing of isolated strains, either with short-read sequencing (e.g., with Illumina) (Ma et al., 2022) or long-read sequencing (e.g., with Nanopore) (Finton et al., 2020). Sequencing of whole genomes generates incredibly information rich data, and in addition to containing enough information to determine an organism's classification to species-level and even with strain-level differentiation, host specific genes and functional genes can be identified which aids the source tracking purpose (Sekse et al., 2017). Examples of functional genes of interest in this context can be ARGs, virulence factors, and biofilm formation genes. Bacterial community composition analysis utilizes sequencing of the bacterial (and archaeal) specific 16S rRNA gene, aiming to produce a dataset representing the bacterial community in a sample. While the sequencing reads produced are generally accurate, the composition of the community cannot be considered a true biological representation (Fouhy et al., 2016). The produced DNA strands can be taxonomically assigned down to genus level when specific variable regions are sequenced (such as V1-V2, V3-V4), however species resolution can be achieved if the whole gene is sequenced (Johnson et al., 2019). Genus resolution is often sufficient for MST applications (Song and Unno, 2024), and other factors such as cost-efficiency, taxonomic assignment accuracy, and rare taxa (which contaminating bacteria usually is) sensitivity make the variable regions 16S rRNA gene approach a solid choice (Abellan-Schneyder et al., 2021). Earlier mentioned benefits of metagenomic sequencing make that approach more suitable when knowledge about highly diverse species and their functionality is needed. Furthermore, by combining cultivation methods (using species selective agar) with metagenomics technology, the source tracking reaches new realms of information. Not only is it confirmed that the bacteria were alive at the time of sampling, whole genome sequencing yields far more specific information about the organism than gene marker analysis or bacterial community analysis.

Fecal source tracking in coastal waters

Coastal waters are widely studied in fecal source tracking contexts at recreational bathing sites, but also at sites used for shellfish harvesting and aquafarming purposes (Paruch and Paruch, 2022). Sources that are relevant to investigate in urban coastal waters in terms of fecal contamination are municipal sewage treatment plants (Zimmer-Faust et al., 2021), boat traffic (Koboević et al., 2021), agriculture (Walters et al., 2011), livestock industry (Ahmed et al., 2010), wild animals (Araújo et al., 2014), domestic animals (Tarek et al., 2023), stormwater discharges (Hart et al., 2020), and direct shedding by bathers at bathing sites (Li et al., 2021). While these point and non-point sources contribute to fecal contamination the water, it has been shown that meteorological parameters such as rainfall (Henry et al., 2016),

temperature (Penna et al., 2023), solar radiation (Aragonés et al., 2016), wind (O'Mullan et al., 2017), and tidal patterns (Halliday et al., 2015) also have significant influence. As each studied site is unique, every site benefits from a case study to determine the major contributors and what can be done for mitigation. To pinpoint point sources of fecal contamination in environmental waters, multiple samples are collected over the area to observe how the studied contaminant concentration fluctuates. Collecting samples over time (multiple occasions) aids accuracy as it can reveal variability, which might allow even intermittent sources to be revealed.

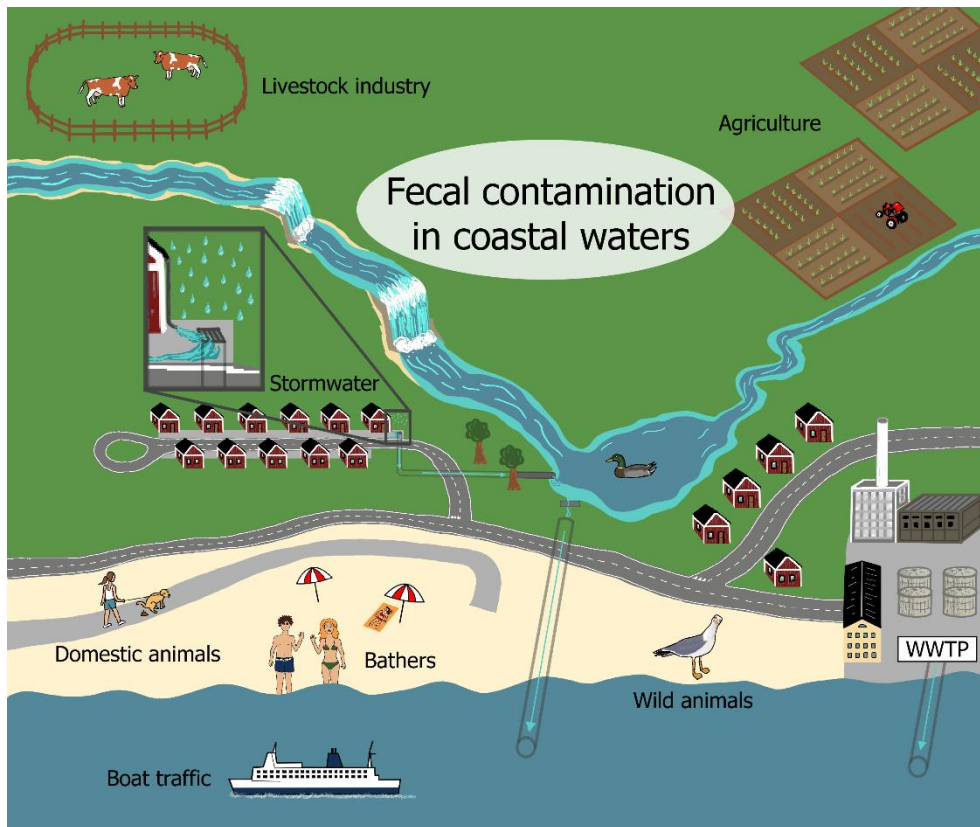


Figure 1. Potential sources of fecal contamination in coastal waters; municipal sewage treatment plants (Zimmer-Faust et al., 2021), boat traffic (Koboević et al., 2021), agriculture (Walters et al., 2011), livestock industry (Ahmed et al., 2010), wild animals (Araújo et al., 2014), domestic animals (Tarek et al., 2023), stormwater discharges (Hart et al., 2020), and direct shedding by bathers at bathing sites (Li et al., 2021).

Fecal source tracking in sediments

The different environment that sediments provide, compared to water, allows for better survival rates for fecal bacteria due to protection from ultraviolet (UV) radiation and environmental stresses (Korajkic et al., 2019). Because of this, sediments can be considered a secondary contamination source, when sediments near point sources allow for very high concentration of fecal bacteria to survive. Then during resuspension events, these bacteria might reach the bathing water (Huang et al., 2015).

Similar to sediments, fine grain sand at bathing sites also act as reservoirs for fecal bacteria (Henry et al., 2016). The submerged sand at bathing sites experience, in addition to the natural physical processes causing resuspension, increased resuspension due to human disturbance (Whitman et al., 2014). Resuspension is amplified during storm events (higher waves, increased turbidity) (Pachepsky and Shelton, 2011) and tidal events (Halliday et al., 2015), resulting in increased exchange of fecal bacteria from sand to water.

Challenges in fecal source tracking studies

Studies of environmental phenomena have many intrinsic challenges, such as sample representation concerns, temporal and spatial complexity, interconnectedness of variables, and cross-scale effects. Concrete examples are particle association and particle sinking (which influences transport and exposure to environmental stress), differential persistence and growth, exchange between sediment-water-air (particularly seen with high turbidity) (O'Mullan et al., 2017). Aerosol formation can transport the bacteria on to land, and beach sand have been found to function as a reservoir for FIB (Bonilla et al., 2007). On a larger spatial scale, studies have shown that gulls transport FIB from landfills and wastewater facilities to recreational beaches (Nelson et al., 2008; Alm et al., 2018). Avian genetic markers have been linked to higher intestinal enterococci and *E. coli* concentrations in beach water (Shrestha et al., 2020).

Additionally, as previously mentioned, a bacterial community dataset generated by 16S rRNA gene sequencing does not represent the true composition, and it includes sequences from both living and dead cells. Not distinguishing between live and dead cells is a problem for genetic marker approaches as well, and further, the degradation of genetic material in the environment is not linear, making the quantifications less accurate (Ahmed et al., 2016).

The gut microbiota

The human gut harbors trillions of microbes (Bäckhed et al., 2005), with the dominant phyla being Bacillota (65%), Bacteroidota (16%), Actinomycetota (9%), and Pseudomonadota (5%) (Belizário and Faintuch, 2018). The microbiota varies

along the gastrointestinal tract, with lower abundance in the stomach due to acidic conditions, higher abundance in the small intestine, and the most microbiota rich and diverse region being the colon with predominantly anaerobic bacteria (Martinez-Guryn et al., 2019). The primary function is fermentation of dietary fibres and complex polysaccharides (Williams et al., 2017), synthesizing vitamins (Munteanu and Schwartz, 2024), and digesting proteins, fats and polyphenols (Loo et al., 2020; Vernocchi et al., 2020). Many factors influence the composition, such as diet, age, lifestyle and genetics (Odamaki et al., 2016; Parizadeh and Arrieta, 2023).

Bacillota (older name *Firmicutes*)

Intestinal enterococci is a group of species within the genus *Enterococcus* (family Enterococcaceae), all found in the gastrointestinal tract of humans and animals, such as *E. faecalis*, *E. faecium*, *E. hirae* and *E. avium*. There are multiple health risks associated with intestinal enterococci, such as urinary tract infections (UTIs), bacteremia, infective endocarditis, and wound and soft tissue infections (Arias and Murray, 2012; Esmail et al., 2019). Enterococci infections are a large problem in hospital settings, particularly *E. faecium* is found to be particularly hard to treat due to its multidrug-resistance (Miller et al., 2014).

Intestinal enterococci are halotolerant and can thus survive in marine environments (Byappanahalli et al., 2012). By attaching to suspended particles or sediments, their survival is aided by protection from UV radiation and better nutrient availability (Kay et al., 2005). *Enterococcus* species have also been found to survive through wastewater treatment (da Silva et al., 2006).

Trichococcus is a genus within the family Carnobacteriaceae. Well studied environments in which *Trichococcus* is found with high abundance are sewer pipes (McLellan and Roguet, 2019), influent wastewater (Dottorini et al., 2021), activated sludge (Nierychlo et al., 2020), anaerobic digesters (Jiang et al., 2021), and treated wastewater (Kristensen et al., 2020). *Trichococcus* exhibit both psychrotolerance and halotolerance (Strepis et al., 2020), and is thus found in higher abundance in treatment plants in cold and temperate climates compared to warm and tropical (Dueholm et al., 2022). *Trichococcus* has been found to survive wastewater treatment, even with UV disinfection, and to be resistant to multiple antibiotics (Zhang et al., 2019).

Lachnospiraceae and Ruminococcaceae are two families whose presence in human gut and sewage are well established (McLellan et al., 2013; Newton et al., 2015), however Ruminococcaceae's name has recently been altered to **Oscillospiraceae** (Tindall, 2019). Lachnospiraceae maintains gut barrier integrity and is a vital part of a healthy gut, and studies have shown that shifts in Lachnospiraceae abundance is linked to gut dysbiosis. Some important genera within Lachnospiraceae in term of gut health are *Roseburia*, *Anaerostipes* and *Coproccoccus* (Vacca et al., 2020).

Similarly to Lachnospiraceae, Oscillospiraceae presence in the gut play a crucial role in fermentation of dietary fibres and butyrate production (which supports gut epithelial health) (Leth et al., 2023). Increased abundance of genera *Oscillospira*, *Faecalibacterium* and *Butyricicoccus* are beneficial for health, and have been found to be negatively correlated with obesity and inflammatory bowel disease (Eeckhaut et al., 2013; Miquel et al., 2013; Konikoff and Gophna, 2016; Zeng et al., 2019).

Romboutsia, a genus within the same class as Lachnospiraceae and Oscillospiraceae, have similar role in the human gut (e.g., production of short-chain fatty acids from dietary fibers). *Romboutsia* can ferment amino acids into ammonia, contributing to nitrogen cycling in the gut (Gerritsen et al., 2017). Similarly to Lachnospiraceae and Oscillospiraceae, *Romboutsia* presence is connected to gut health, and higher abundance has been linked to gut dysbiosis and obesity (Zeng et al., 2019; Therdtatha et al., 2021). Due to *Romboutsia*'s fecal origins, it is often found in wastewater treatment plants and in bioaerosols produced in the treatment process (Liu et al., 2023).

Bacteroidota (older name *Bacteroidetes*)

Bacteroides is one of the most abundant group of bacteria in the human intestines, and its metabolism focuses on fermentation of complex polysaccharides into sugars and short-chain fatty acids (Cheng et al., 2022). *Bacteroides* contributes to maintaining gut homeostasis by maintenance of the mucosal gut barrier, and prevention of inflammation by modulating immune responses (Telesford et al., 2015). While *Bacteroides* is a commensal gut bacteria, pathogenicity has been seen expressed in *Bacteroides fragilis* (Yekani et al., 2020).

Bacteroides is widely used in MST due to its host specificity in fecal markers. The use of qPCR markers including *BoBac* (bovine) (Layton et al., 2006), *HF183* (human) (Ahmed et al., 2016), and *BacCan-UCD* (dog) (Kildare et al., 2007), have demonstrated excellent host-sensitivity and host-specificity. *Bacteroides* is an excellent marker also due to it not being able to reproduce in the aquatic environment (Fiksdal et al., 1985), and its higher die-off rates than fecal coliforms and *Enterococcus* (Ballesté and Blanch, 2010).

Prevotella is a genus of bacteria that contains some species that live in the human gut, and some species that live in the oral cavity (Tett et al., 2021). In the gut, *Prevotella* metabolizes polysaccharides into short-chain fatty acids, and thus help maintain a healthy gut barrier and reduce inflammation (Chen et al., 2017).

Actinomycetota (older name *Actinobacteria*)

Ca. ***Microthrix*** is a problematic bacterium in wastewater treatment plants (specifically activated sludge processes (sludge bulking and foaming)) due to their filamentous nature (Rossetti et al., 2005). It grows best at lower temperatures (10-15 °C), and is thus mainly an issue in temperate climate zones (Nierychlo et al.,

2021). It has a hydrophobic cell surface, and metabolizes hydrophobic substances such as long-chain fatty acids. Thus, *Microthrix* plays a part in lipid degradation in wastewater treatment (Nielsen et al., 2002).

Pseudomonadota (older name *Proteobacteria*)

Escherichia coli is a species in the family Enterobacteriaceae and is often the most abundant facultative anaerobe in the intestines of mammals (Welch, 2006). There are several different strains of *E. coli* that are pathogenic by having acquired virulence genes. These can be grouped into two categories: extraintestinal *E. coli* (ExPEC) and intestinal *E. coli* (inPEC) (Kaper et al., 2004). Within ExPEC there are strains causing UTIs (uropathogenic *E. coli* (UPEC)) and bacteremia (Flores-Mireles et al., 2015). For inPEC, there are many different strain types, most causing diarrhea (Liu, 2015).

Despite its enteric origins, *E. coli* has been found to survive in marine environments for as long as three years (as long as the experiment lasted) (Byrd and Colwell, 1993). *E. coli* that can survive and reproduce in new environments are called naturalized (Devane et al., 2020), and *E. coli* has been found to survive in multiple environments such as water (Berthe et al., 2013), beach sand (Rumball et al., 2021), and sediments (Sciarrillo et al., 2020). *E. coli* in marine waters have been found to have longer survival and growth when in contact with sediments (organic matter) (Gerba and McLeod, 1976; Craig et al., 2004). This is in line with *E. coli* having higher survival rates in water when being attached to particles, rather than being planktonic (Brettar and Höfle, 1992). Thus, *E. coli*'s ability to form biofilm is closely linked to their survival in aquatic environments (Berthe et al., 2013). Furthermore, *E. coli*'s survival through wastewater treatment is established (Raboni et al., 2016), which poses a risk since these can be resistant to antibiotics (Aslan et al., 2018) and be pathogenic (Zhi et al., 2020). Antibiotic resistance of bacteria in the environment is often tested by quantification of ARGs. While that does have value, it is important to note that a bacterium carrying ARGs does not automatically make it resistant to corresponding antibiotics, as the genes may not be expressed. Thus, to confirm if a bacterium is resistant, one can perform phenotypic antibiotic susceptibility testing (e.g., disk diffusion test) (Maunsell et al., 2021).

Ca. ***Competibacter*** is a genus within the class Gammaproteobacteria, and is commonly known as a glycogen-accumulating organism (GAO) in wastewater treatment plants. It is mostly associated with the enhanced biological phosphorus removal process, and because it competes with phosphate-accumulating organisms for the same carbon sources (e.g., acetate and propionate), it is important to hinder GAO populations from dominating to ensure efficient phosphorus removal (McIlroy et al., 2014).

Acinetobacter, also a genus within Gammaproteobacteria, has a more widespread habitat than *Competibacter*. *Acinetobacter* is a dominant presence in sewage

influent and sewer pipes upstream of the treatment plant (Vandewalle et al., 2012; McLellan and Roguet, 2019), however it is a very low abundant genus in the human gut (Adewoyin and Okoh, 2018). *Acinetobacter* is a good indicator for risk for human health, as there are many species within this genus that are pathogenic, for example *A. baumannii*, *A. lwoffii*, *A. pittii*, *A. nosocomialis*, *A. haemolyticus* and *A. ursingii*. These species are associated with hospital-acquired infections causing a wide array of diseases (e.g., pneumonia, bacteremia, UTIs, wound infections, and meningitis), and many are multidrug-resistant (Ku et al., 2000; Visca et al., 2011; Chiu et al., 2015; Carvalheira et al., 2021; Gheorghe-Barbu et al., 2024). While a few species (*A. johnsonii*, *A. lwoffii* and *A. radioresistens*) have also been found in natural waters and sediments (Adewoyin and Okoh, 2018), their relevance in source tracking remains because the abundances found in natural environments have been very low, abundances in the environment have been increasing with closer distance to wastewater discharges (Yang et al., 2020), and the fact that the genus contains many pathogenic species.

Arcobacter, a genus within Arcobacteraceae, is an emerging pathogen found in many aquatic environments, such as coastal surface water (Fera et al., 2004; Carney et al., 2020), sewage sludge (Stampi et al., 1999), influent sewage and treated wastewater (Kristensen et al., 2020), sewer pipes (McLellan and Roguet, 2019), and stormwater (Carson et al., 2024). *Arcobacter* species have been found to survive wastewater treatment processes (Webb et al., 2016). The most clinically significant species, *Arcobacter butzleri*, causes diarrhea, vomiting, and enteritis in humans (Vandenberg et al., 2004) and diarrhea, mastitis and abortion in livestock (Ramees et al., 2017). Despite that *Arcobacter* can be found in natural waters, its strong association with sewage and fecal contamination, as well as its pathogenicity, makes it highly relevant to surveillance in coastal waters.

Aliarcobacter was proposed as a new genus diverging from *Arcobacter* by Pérez-Cataluña et al. (2018). This work was validated (Oren and Garrity, 2020), but later studies rejected the proposed genus due to inaccuracies in their phenotypic distinction between the newly suggested genus and *Arcobacter* (On et al., 2021). In this thesis work, when *Aliarcobacter* is mentioned it is assumed as a synonym to *Arcobacter* (On et al., 2021).

Bioremediation of groundwater

Bioremediation is an environmentally friendly and cost-effective process that utilizes living organisms, primarily microorganisms, to degrade or detoxify pollutants from contaminated environments, such as soil, groundwater, and sediments (Vidali, 2001). This natural attenuation process relies on the metabolic activities of bacteria, fungi, or plants to break down harmful substances into less toxic or non-toxic forms.

There are two primary strategies to enhance bioremediation effectiveness: bioaugmentation and biostimulation. Bioaugmentation involves the introduction of specific microbial strains or consortia with the ability to degrade targeted pollutants, which is particularly useful when native microbial communities lack the metabolic capacity for complete degradation (Gentry et al., 2004). On the other hand, biostimulation focuses on optimizing the environmental conditions (e.g., adding nutrients, oxygen, or electron donors) to stimulate the activity of indigenous microbial populations capable of breaking down contaminants (Tyagi et al., 2011).

The choice of bacterial groups for bioremediation depends on the type of contaminant present in the environment. For instance, when dealing with groundwater contaminated by chlorinated solvents such as tetrachloroethene (PCE) and trichloroethene (TCE), bacteria like *Dehalococcoides mccartyi*, *Desulfitobacterium* spp., *Geobacter*, and *Methanobacterium* are commonly utilized. These bacteria degrade chlorinated compounds through a process called anaerobic organohalide respiration, where chlorine atoms are replaced by hydrogen, thereby detoxifying the pollutants (Dolinová et al., 2017).

Materials and methods

Field investigation

Alingsås

A location contaminated with chlorinated solvents, found in Alingsås, Sweden, is studied in Paper I. The effect of bioremediation attempts are evaluated, which were performed to tackle a spill of approximately 200 L of PCE at a dry-cleaning facility in the 1960s (Cedhagen, 2002; Haglund and Larsson, 2010). Studying the geology of the area revealed that the bedrock is dipping towards the northwest, and this is also the direction of the groundwater flow (Branzén et al., 2016; SGU, n.d.). The spill of PCE occurred where well B2 is located, and the extent of the estimated plume stretches from the contamination site out towards the northwest (red dashed line) (Figure 2). The highest PCE concentrations were detected in along the A-A' and B-B' dashed turquoise lines, according to a previous study utilizing Geoprobe® Membrane Interface Probe (MIP) (Figure 2) (Blazarini and Van Herreweghe, 2017; Davidsson et al., 2020).

Bioremediation was carried out on multiple occasions, and is visualized with colored dots (green, purple and orange) in figure 2. The first event was in 2012 (green dots, called area C), when a biostimulation effort was made utilizing Newman Zone® (electron donors), molasses and zero-valent iron (ZVI). In 2014, the same spots were injected with KB-1® (microbial culture, inter alia *Dehalococcoides*) (SiREM, ON, Canada) (Branzén et al., 2016). Within the present study, further remediation attempts were made, by injecting ZVI, CAT 100™ (granular activated carbon, ZVI and starch), Trap & Treat® bacteria concentrate, and Provect CH4® Methane Inhibitor in area A (orange dots) in 2017. In area B (purple dots) Provectus ERD-CH4™ Olé Ego (electron donors) and microbial consortium SDC-9™ (dehalogenating bacteria, inter alia *Dehalococcoides mccartyi*, *Dehalogenimonas* spp., *Desulfovibrio* spp. and *Desulfitobacterium* spp.) (RNAS Remediation Products, MN, USA) were injected at the same occasion.

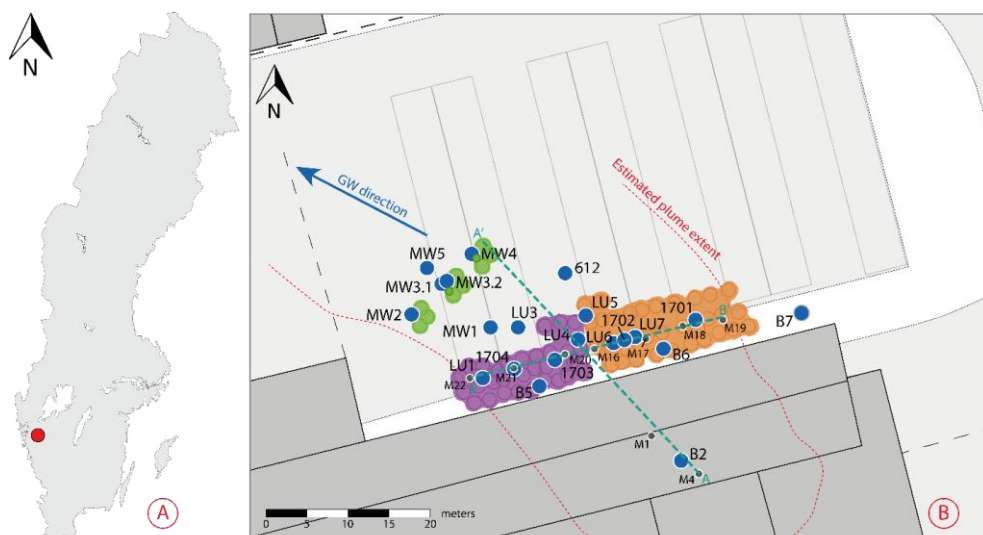


Figure 2. A: The studied site in Alingsås, Sweden is marked with a red dot. B: Map of the contaminated site. Blue dots are the monitoring wells from present study, and the smaller black dots are from the previous study utilizing MIP. Background dots in orange indicate area A, purple indicate area B, and green indicate area C. (Figure is modified from figure 1 in Paper I.)

The first groundwater sampling was performed in October 2017, prior to the remediation efforts of that year (Paper I). Sampling was then conducted in October of 2018 and 2019 to study the effect of the remediation. Before each sampling, each well was pre-purged until field parameters gave stable readings (water temperature, oxidation-reduction potential, pH, electrical conductivity, total dissolved solids, and salinity). The groundwater was collected using an Eijkelpap peristaltic pump.

Helsingborg

In Helsingborg, a city in southern Sweden, the focus of Paper II-V is the bathing water quality of its coastal beaches. The central beaches, being positioned in an urbanized area, are influenced by multiple potential point sources of microbial contamination (e.g., WWTPs, stormwater drainage, CSOs). The parameters of interest were the traditional *E. coli* (Paper II-V) and intestinal enterococci (Paper V), and alternative fecal and sewage indicator bacteria such as Lachnospiraceae (Paper IV and V) and *Trichococcus* (Paper II, IV, V). To investigate the effects from both point and intermittent sources, sediments (Paper II-IV) and bathing water (Paper V) were collected during three- and two-year periods, respectively.

Sediment sampling was conducted outside Helsingborg in the strait of Öresund during six occasions (once every six months during 2019-2021) (Paper II-IV). Sediment cores were extracted using a core sampler, and the top 1 cm layer was

collected into sterile tubes for analysis. Between 15-17 samples were collected on each occasion, at varying depths (between 4-28 meters) along an approximately 4 km stretch (north to south), between approximately 300-700 meters offshore (Figure 3). Samples were stored on ice until arrival at the lab.

Bathing water sampling was conducted during the summer months of 2020 and 2021 (Paper V). Samples were collected once a week (Mondays) at the six central recreational beaches (Figure 3), and every other week for the rural recreational beaches (three located north of the central beaches, five located south). Water was collected in sterilized bottles 30 cm below the surface, where the water was 1 meter deep (thus at slightly different distance offshore for each beach). Samples were kept on ice until arrival at the lab.

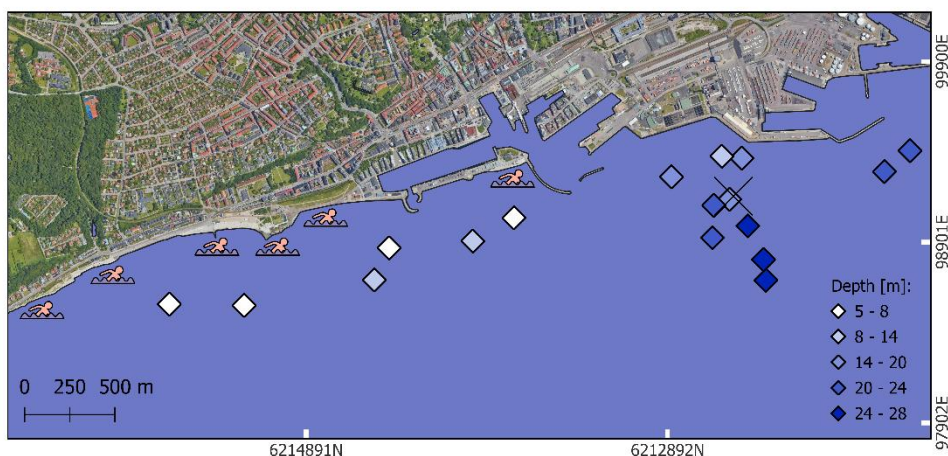


Figure 3. Displays the six central beaches in Helsingborg, marked with a symbol of a human bathing in waves. Squares with colors ranging from white to dark blue show the sediment sampling locations performed in August 2021. The colors indicate at which depths the sediments were, with details displayed in the bottom right corner legend. X marks the spot for the city's treated wastewater effluent point. (Figure is modified from figure 1 in Paper III.)

For signature-based source tracking, a library of 16S rRNA gene (V4 region) communities from feces from seven different species, and influent sewage was constructed by Hägglund et al. (2018). Raw water from drinking water plants were also sequenced to be incorporated as background signal. In order to extend this fecal source tracking library to include a signature for treated sewage, treated sewage samples were collected from 12 municipal WWTPs (Paper II).

Laboratory work

Groundwater was analyzed for concentrations of PCE and its metabolites (TCE, *cis*-1,2-Dichloroethene (*cis*-DCE), *trans*-1,2-Dichloroethene (*trans*-DCE), vinyl chloride (VC), and ethene), sulfate, dissolved oxygen (DO), dissolved organic carbon (DOC), ammonium, phosphorus, methane, and iron. For microbial analysis, the groundwater was filtered through 0.22 μm filters until clogging, and the filter papers were used in DNA extraction (FastDNA Spin Kit for Soil (MP Biomedicals, CA, USA)), in order to do sequencing of the 16S rRNA gene (V3-V4 region) utilizing a Illumina MiSeq system with 2×300 base pairs (bp) paired-end reads (Illumina, CA, USA) (Paper I).

Sediment samples were homogenized and prepared for *E. coli* quantification, as well as replicates being put in cryotubes in a $-80\text{ }^{\circ}\text{C}$ freezer until DNA extraction (Paper II-IV). For *E. coli* quantification, sediments were rocked with MilliQ-water to extract the bacteria from the sediments into the liquid. After rocking, tubes were allowed to settle, and liquid could thereafter be collected into a new tube. For Colilert-18 using Quanti-Tray/2000 (IDEXX, ME, USA), this liquid was diluted in a dilution series, and total coliforms and *E. coli* was subsequently quantified.

After quantification with Quanti-Tray/2000, liquid from wells positive for *E. coli* were extracted and streaked on HICrome Agar B plates (Sigma-Aldrich, MA, USA) (Paper III). After incubation, blue colonies were streaked again, and subsequently grown in lysogeny broth (LB). These colonies were used in DNA extraction (GeneJet Genomic DNA Purification Kit (Thermo Fisher Scientific, MA, USA)) and sequencing (NextSeq 550 (Illumina, CA, USA), 2×150 bp paired end), disk diffusion assay (cefotaxime, meropenem, ciprofloxacin, tetracycline, and gentamicin), crystal violet biofilm growth assay, and growth rate comparison between LB media and LB media with NaCl.

DNA extraction and sequencing of sediments were conducted at two different occasions (Paper II and IV). During both occasions, DNA extraction was performed using FastDNA Spin Kit for Soil (MP Biomedicals, CA, USA). In Paper II, DNA amplification was done with primers 343F/806R (V3-V4 region of the 16S rRNA gene) (Herlemann et al., 2011), and sequencing using an Illumina MiSeq system with 2×300 bp paired-end reads (Illumina, CA, USA). In Paper IV, primers 341F/806R (V3-V4 region of the 16S rRNA gene) (Klindworth et al., 2013) were amplified for sequencing using an Illumina NovaSeq system with 2×250 bp paired-end reads (Illumina, CA, USA).

Water samples were filtered through 0.2 μm filters, and the filter papers were subsequently used in DNA extraction (FastDNA Spin Kit for Soil (MP Biomedicals, CA, USA)) (Paper V). Primers 341F/806R (V3-V4 region of the 16S rRNA gene) (Klindworth et al., 2013) were amplified for sequencing using an Illumina NovaSeq system with 2×250 bp paired-end reads (Illumina, CA, USA).

DNA extraction of treated sewage was done using SoilMaster DNA Extraction Kit (Epicentre Biotechnologies, WI, United States) (Paper II). DNA amplification was done with primers 515F/806R (V4 region of the 16S rRNA gene) (Caporaso et al., 2010). Sequencing was performed using an Illumina MiSeq system with 2×300 bp paired-end reads (Illumina, CA, USA). The reads generated were incorporated into the existing library established by Hägglund et al. (2018) as part of the treated sewage dataset.

Bioinformatic analysis

The majority of bioinformatic analyses were performed in the RStudio environment using the R programming language. Throughout the duration of this thesis work, multiple versions of RStudio were used, beginning with the 2018 release of RStudio 1.2 and concluding with version 2023.9.1.494 (Posit Team, 2023). Similarly, various versions of R were utilized, ranging from version 4.1.0 to 4.4.1 (R Core Team, 2023).

Filtering and trimming of reads, sample inference, dereplication, and merging forward reads with reverse reads, were all conducted utilizing the DADA2 package (Callahan et al., 2016) (Paper I, II (sediments), IV, V). Whether primers were present or not depended on sequencing instance, and removal of primers was only necessary for Paper I. Removal of primers is necessary as they are sequencing artefacts, and would interfere with assignment of taxonomy. After preparation of sequences through the DADA2 pipeline, the reads now classified as amplicon sequence variants (ASVs). To ensure that only correct ASVs were included in the dataset, ASVs with base pair lengths of less than 350 bp and more than 500 bp were removed, based on the length of the V3-V4 region (Rausch et al., 2019).

The treated sewage sequences were processed using QIIME (Caporaso et al., 2010) for demultiplexing, filtering, and trimming with cutadapt (Martin, 2011), and forward and reverse reads were merged using FLASH (Magoc and Salzberg, 2011). Reads were clustered together into operational taxonomic units (OTUs) based on 97 % sequence identity using UCLUST (Edgar, 2010). For MST analysis, the SourceTracker tool was utilized (Knights et al., 2011) in order to create a treated sewage library (Paper II).

For the whole genome sequencing study (Paper III), reads were assembled (SKESA (Souvorov et al., 2018)), contamination removed (CheckM (Parks et al., 2015), ConFindr (Low et al., 2019)), and taxonomic assignment using Genome Taxonomy Database (GTDB) was performed. Phylogenetic groups (phylogroups) were assigned using ClermonTyping (Beghain et al., 2018) to classify *E. coli* into established evolutionary lineages. Multilocus sequence typing (MLST) was performed with the MLST webtool (Larsen et al., 2012) following the Achtman

scheme (Wirth et al., 2006) to determine the sequence types (STs) of *E. coli*, providing insight into genetic variation and potential epidemiological relationships. To assess genomic similarity, Average Nucleotide Identity (ANI) was calculated using fastANI (Jain et al., 2018), which quantifies genetic relatedness between *E. coli* genomes. ANI values were then converted into dissimilarities to facilitate clustering. To examine the previous occurrence of STs, the MLST query tool of EnteroBase (Zhou et al., 2020) was used with the Achtman MLST scheme. This step allowed for the comparison of identified STs with those in public databases, providing information on their geographic distribution and epidemiological relevance. Different individual sources were categorized into main source groups to facilitate comparative analyses.

Genes associated with virulence in *E. coli* were identified using VirulenceFinder (Joensen et al., 2014; Tetzschner et al., 2020), with the 2022-02-12 database version. A BLAST search was conducted against the assembled genomes to determine the presence of known virulence factors. This step was performed to assess the potential pathogenicity of the isolates. To identify genes potentially linked to antibiotic resistance, the Resistance Gene Identifier (RGI) was used with the Comprehensive Antibiotic Resistance Database (CARD) v3.2.6 as a reference (Alcock et al., 2019, 2023). This analysis aimed to detect known resistance genes within the assembled genomes, providing insights into their antimicrobial resistance profiles.

Genes associated with biofilm production and halotolerance were annotated in the assembled genomes using eggNOG-mapper (Cantalapiedra et al., 2021). The annotation was performed with DIAMOND (Buchfink et al., 2021), utilizing the eggNOG 5.0 database (Huerta-Cepas et al., 2019) and Prodigal (Hyatt et al., 2010). This step was conducted to assess potential adaptations related to environmental persistence and stress tolerance.

For taxonomic assignment of ASVs in 16S rRNA gene studies (Paper I, II, IV, V), there are several different publicly available databases that are appropriate to use in academic research. There is the RefSeq-RDP database, which is combining the NCBI RefSeq database with the Ribosomal Database Project (RDP) classifier, and it provides broad coverage of microbial taxa from many environments. The NCBI Refseq is curated and manually reviewed, and is integrated with new species continuously (DADA2 formatted version 1 (Alishum, 2019) used in Paper I). GreenGenes is a database which is well-structured and standardized, however it has not been updated since 2013 (version 13.8 (McDonald et al., 2012) used in Paper II, for treated sewage samples). Usage of a database curated for a specific environment can sometimes be beneficial, for example when studying WWTPs. MiDAS (Microbial Database for Activated Sludge) is such a database, and is curated by experts in the field, providing a database optimized for wastewater and activated sludge (version 4.8.1 (Dueholm et al., 2022) used in Paper II, sediments). The Genome Taxonomy Database (GTDB) is a database using whole-genome phylogenetics rather than 16S classification. This database, just like NCBI, is

frequently updated and actively maintained to ensure that the latest revision of taxonomic groupings is reflected (version 07-RS207 (Parks et al., 2022) used in Paper III, and DADA2 formatted version 4.3 (Alishum, 2022) used in Paper IV and V).

After taxonomic assignment, the package decontam (Davis et al., 2018) was used to remove contaminating sequences (Paper IV and V). Following this, the R-package phyloseq was utilized for its convenience in data handling, statistical analyses, and compatibility with other R-packages such as DESeq2, vegan and ggplot2 (McMurdie and Holmes, 2013) (Paper I, II, IV, V). The DESeq2 package was used for differential abundance analysis, allowing for identification of differentially expressed taxa between two groups (e.g., different areas, or before and after treatment) using a negative binomial distribution (Love et al., 2014) (Paper I). The analysis can also test whether there is a linear relationship between microbial abundance and continuous variable (e.g., *E. coli* concentration) (Paper II). Additionally, to identify microbial signatures associated with environmental factors, penalized regression models were applied using the coda4microbiome package (Calle, 2023) (Paper IV). The R-package vegan was used for multivariate analyses, including alpha and beta diversity measurements, ordination methods, and other ecological statistics (Oksanen et al., 2022) (Paper I, II, IV, V).

There are multiple different analyses that can be utilized in ordination analysis, for example non-metric multidimensional scaling (NMDS) (Kruskal, 1964) (Paper I and II), principal coordinate analysis (PCoA) (Gower, 1966) (Paper IV), principal component analysis (PCA) (Pearson, 1901) (Paper I and V), and redundancy analysis (RDA) (Rao, 1964) (Paper III). Plotting results from this type of analysis helps with interpreting high-dimensional data (e.g., ASV-tables with 50000+ columns) by reducing it into a few interpretable dimensions. In NMDS, one must first transform the ASV-table with a distance matrix (also called dissimilarity matrix). The most commonly used in microbial studies are the Jaccard index (Jaccard, 1901) and the Bray-Curtis dissimilarity (Bray and Curtis, 1957). By plotting the two dimensions that represent the largest differences between the samples (based on the rank-order of distances), one can get a better understanding of the overall patterns in community composition, how samples cluster based on similarity, and which environmental or biological factors may be driving these differences. Like NMDS, PCoA begins with a distance matrix. However, instead of using iterative optimization, it applies eigenvalue decomposition to identify new coordinate axes that best preserve pairwise distances. Unlike NMDS, PCoA maintains the actual distances between samples, allowing each axis to have a defined percentage of the total variance it explains. A downside with PCoA is that it is a linear method, and does not handle non-linear patterns as well as NMDS. PCA also uses eigenvalue decomposition like PCoA, however PCA is not dependent on the use of distance matrices, rather it uses raw data directly. Because of this, inclusion of environmental parameters is possible within the same analysis, and can

help identify patterns in microbial abundance and environmental variation directly. However, by using linear regression, environmental variables can be fitted onto an ordination (e.g., NMDS or PCoA), allowing for the visualization of how environmental gradients correlate with community variation (Legendre and Legendre, 2012) (Paper II). RDA is similar to PCA; however, instead of finding axes that maximize overall variance, it first creates axes based on environmental parameters (explanatory variables). The microbial data (response variables) is then aligned with these axes, showing how taxa/species/strain abundances vary in relation to the environmental factors. This was used to explore how different phylogroups of *E. coli* vary in their gene content, particularly genes linked to biofilm formation and halotolerance, and how these genetic differences correlate with phenotypic traits such as growth rate and biofilm formation in the presence and absence of NaCl. By applying RDA, the extent to which these genes are associated with specific phylogroups and how they influence bacterial adaptation under different environmental conditions was examined.

For a different approach to microbial source tracking (rather than signature-based MST and whole genome sequencing), a literature review-based source tracking methodology was developed (Paper II, IV, V). Microbial community studies (16S rRNA gene) of fecal matter, gastrointestinal tract, sewage pipes, inside WWTPs, and effluent (treated) wastewater were investigated, and a comprehensive list of all genus and species found in these studies was created. Each taxon in the list was further investigated to evaluate whether the taxon is exclusively found in the gut and sewage, or if it is also found in other environments. If a taxon had also been detected in other environments (e.g., natural waters and pristine sediments), it was removed from the list. This curated library was imported into R, where a script was created to extract all ASVs with matching taxonomic assignments. By adding the abundances of the chosen ASVs together for each sample, the varying influence of gut and sewage over the study area could be evaluated using various statistical tests. In Papers V, the library was split into two groups (gut and sewage separate) to enhance the resolution of the analysis, making it possible to better distinguish between alternative sources of gut taxa in the environment.

In another approach, when investigating the interconnectedness between sediment and water microbial communities, the core community of the sediments was first established using the package microbiome (Lahti and Shetty, 2019) (Paper IV). Evaluation of the presence of the sediment core community in the bathing water was done to inspect if resuspension of sediment could be the cause of increased *E. coli* concentrations (Paper V). Each ASV from the bathing water dataset was compared with the core community ASVs from Paper IV to identify exact DNA sequence matches, using the package stringr (Wickham, 2023).

Additional statistical tests conducted throughout this thesis work include a range of correlation analyses and group comparison tests. To assess monotonic relationships between variables, Spearman's rank correlation test was applied (Papers I, II, IV,

V), while Pearson's correlation was used for detecting linear relationships between continuous variables (Papers IV and V). Group differences were evaluated using the Kruskal-Wallis test by ranks, a non-parametric method suitable for comparing three or more groups, followed by Dunn's post-hoc test to identify specific pairwise differences (Paper I). For comparisons between two related groups, the Wilcoxon signed-rank test was employed (Papers III and IV), while differences among multiple groups were analysed using one-way analysis of variance (ANOVA), with Tukey's Honestly Significant Difference (HSD) test for post-hoc pairwise comparisons (Paper III). To assess differences in microbial community composition across groups, Permutational Multivariate Analysis of Variance (PERMANOVA) was conducted, with p-values adjusted using the Benjamini-Hochberg correction to control for false discovery rates (Paper IV). Diversity within microbial communities was evaluated using the Shannon and inverse-Simpson indices (Paper II), which measure species richness and evenness (Shannon, 1948; Simpson, 1949).

Results

Groundwater

Groundwater was studied to evaluate the effects of bioremediation strategies in a site contaminated with PCE, as part of the thesis work conducted for Paper I. Effects and efficiency of the bioremediation were studied by quantifying chemical compounds over time that are related to the degradation of PCE to ethene, as well as investigating if change in concentration of contaminants and metabolites could be reflected in shifts in the microbial communities.

Chemical analysis

In the study area, increased iron concentration in area A was confirmed after injection of ZVI. Concentrations had started to decline by the end of the study. Sulfate concentrations were stable in the Source zone, but were decreasing in area A, B and C after injections. DO was decreasing in all areas after injection, with exception of three wells, and were within anoxic conditions (< 0.5 mg/L) (US EPA, 1998; AFCEE, 2004) during the whole study period. DOC concentration was high enough to supply electrons (> 5 mg/L) (US EPA, 1998; Swedish Geotechnical Society, 2011) in area A and B after injections, with the exception of one well in area A. In area C, concentrations were generally low (< 3.1 mg/L). An outlier was well LU1 (area B) with a very high concentration of > 400 mg/L. Ammonium concentration was highest in area C, followed by area A, then area B, and lastly the Source zone had very low concentrations. Amount of phosphorus was measured by quantifying phosphate, and it was noticeably higher in area B compared to all other areas. It increased after injections, and then decreased with time. Methane was only found to be produced in three wells in area A prior to injections (however area C was not tested for methane during this time). After injections, methane was detected and increasing in all samples. Concentration was generally higher and with more rapid increase in area A.

Concentrations of PCE, TCE, *cis*-DCE, *trans*-DCE, VC, and ethene, was likewise measured before and after injections. PCE was in high enough concentration in the Source zone to be in free phase (not fully dissolved in the water) throughout the study period, except for the last sampling occasion where the concentration was considerably lower. Area C was the area with second highest concentrations of PCE

(after Source zone), and there free phase also occurred in two wells. Third highest concentrations had area A, and for all areas the PCE concentrations fluctuated over time, but ended up having lower concentration by the end of the study period compared to before the injections. TCE was detected in the Source zone on all occasions and retained static concentration over the study period. Area A had consistently low concentration, while area B showed a decrease, and area C was inconsistent. *Cis*-DCE was found in low concentration in the Source zone. In area A, B and C, production of *cis*-DCE was observed after the injections, increasing the concentrations until the end of the study period. For *trans*-DCE, only very low concentrations were detected in area A, B and C, which happened after injections. VC was only detected in area C before the injections, and after injections it could be measured in area A, B and C. Similar to *cis*-DCE, concentrations kept rising and had the highest value at the end of the study period. Ethene was not produced in the Source zone, but was consistently increasing over time in the other three areas.

Microbial analysis

DNA sequencing of the 16S rRNA gene (V3-V4 region) resulted in a dataset of 6466 ASVs, which represented 596 different taxonomic groups. Utilizing PCA to investigate the overall differences in microbial composition between areas before injections (year 2017), area A and B are clustering together and overlapping, while area C is further away. After the injections (year 2018), all three areas are clustering together but with no overlapping, and the PCA plot remains looking very similar in 2019.

Comparing specific taxonomic groups' change in abundance before and after injection was done to get an understanding of what taxa might be involved in the PCE degradation process. In area A, Actinomycetota, Bacteroidia, and Gammaproteobacteria were decreasing in relative abundance after injections, while Deltaproteobacteria, *Thermodesulfovibrio*, Methanobacteria, and Parcubacteria were increasing. In area B, same taxa showed a decrease in relative abundance, and Methanobacteria, Methanomicrobia, and Anaerolineae were increasing. Due to area C having received a bioaugmentation treatment in 2014 that area A and B had not, it is reasonable that this area was differing in microbial composition. In this area, there was a notable increase in Methanomicrobia and Methanobacteria after injections. The relative abundances of the known genera that were injected were summed together, this group including *Dehalococcoides* (Dehalococcoidia), *Dehalogenimonas* (Dehalococcoidia), *Desulfovibrio* (Deltaproteobacteria), and *Desulfitobacterium* (Clostridia). Spearman's rank correlation test was used to see if the relative abundance of these taxa could be correlated to PCE or *cis*-DCE concentrations, but no significant correlations could be found for any of the years. Kruskal-Wallis test determined that there was significant difference in median of PCE and *cis*-DCE concentrations between areas (p -value = 0.03125 and p = 0.0006,

respectively). DESeq2 was utilized to investigate which ASVs could be linked to higher concentration of *cis*-DCE, and this yielded eight ASVs. Six out of these could be taxonomically assigned down to genus level, which were *Clostridium*, *Parcubacteria*, *Syntrophomonas*, and *Methanobacterium*. As injections and treatments varied between areas, DESeq2 was also applied to compare pairwise between the areas, including only samples taken after injections were made. Comparing area B and C, area C had a significantly higher presence of *Rhodoferrax*, *Cellulosilyticum*, *Ancalomicrobium*, *Dechloromonas* and *Paludibacter*, and area B had a significantly higher presence of *Geobacter* and *Mycobacterium*. Comparing area A and C, *Dehalobacter* and *Methylocystis* had significantly higher presence in area A. Another DESeq2 was run, finding taxa that were correlating to the injected bacteria's cumulative relative abundances (*Dehalococcoides*, *Dehalogenimonas*, *Desulfovibrio*, and *Desulfitobacterium*) during 2018 and 2019. With this analysis, *Acidovorax*, *Magnetosprillum*, *Methylovirgula*, *Sporichthya* and *Dehalobacter* were found to have a positive relationship to the injected bacteria in area B, while *Dehalococcoides* and *Pseudomonas* had a negative relationship.

Bathing water quality in urban coastal beaches

For bathing water quality investigation in natural waters, a broad approach is necessary. Looking at both sediments (Paper II-IV) and water (Paper V) in the area of interest, both with microbial community analysis (16S rRNA gene sequencing) and viable *E. coli* and intestinal enterococci quantification analysis, was performed in this thesis work.

***Escherichia coli* in sediments**

Escherichia coli in the sediments along the coast of Helsingborg, Sweden were studied during three years time (Paper II-IV). Concentration of viable *E. coli* show temporal and spatial variation in the three years (six occasions) that were tested. The area has several points where introduced fecal bacteria to the natural microbial community might come from.

During the first round of sampling on 2019-03-19, sediments were sampled at 16 locations (Paper II). Eight locations were in close proximity to the treated wastewater effluent (≤ 440 meters) and eight locations were further away ($1230 \leq x \leq 3340$ meters). The locations further away were located in close proximity to recreational beaches, which was planned with the aim that microbial communities and *E. coli* in the sediments could help understand whether beaches with poor bathing water quality could be linked to bacteria in nearby sediments. *E. coli* concentrations in the sediments had a distinct distribution, with measurable

quantities only detected in sediments close to the wastewater outlet (WWO), with distance ≤ 440 meters. The close proximity samples ranged between 20 MPN/100 mL to 3097 MPN/100 mL on this sampling occasion.

In the longitudinal dataset (2019-2021), the spread of *E. coli* varied greatly, as most locations had high *E. coli* at least once during the three year period, but no clear patterns in the spread were evident (Paper IV). With a mean of 148 MPN/100 mL and a median of 24 MPN/100 mL, one trend that could be seen was that the highest measured concentration during five out of six occasions were located with close proximity to WWO (distance ≤ 440 meters). Since the Colilert-18 procedure was based on resuspension of sediments in MilliQ-water, this may have caused bacterial cell lysis due to osmotic stress. Because of this, the *E. coli* concentrations reported in both Paper II and IV are likely conservative estimates.

During the last sampling occasion (2021-08-24), *E. coli* was quantifiable in 15 out of 17 locations. Solution containing viable *E. coli* from the 15 positive samples were cultivated, which yielded 41 isolates that were subsequently used in whole genome sequencing analysis (Paper III). Despite using *E. coli* selective agar, only 37 out of the 41 genomes of the isolates were classified as *E. coli*. Out of these 37 genomes, nine were assigned to phylogroup A, 16 were assigned to phylogroup B1, nine were assigned to phylogroup B2, two were assigned to phylogroup D, and one was assigned to phylogroup E. The 37 isolates were classified as 30 different sequence types. The sequence types could be linked to different sources using EnteroBase, which gave hits in human, wild animals, domestic animals, avian, and environment databases. The human associated sequence types contained two strains that are ExPEC strains.

Within the genomes of the *E. coli* strains, 66 genes were found (with > 90 % identity) that are linked to virulence. Virulence genes were detected within all five phylogroups, but B2 had significantly higher virulence gene count compared to the other two large phylogroups (A and B1) (ANOVA). Genes detected within the B2 phylogroup were vacuolating autotransporter toxin (*vat*), fimbrial-like protein (*yfcV*), siderophore yersiniabactin receptor (*fyuA*), outer membrane hemin receptor (*chuA*), iron transport protein (*sitA*), increased serum survival lipoprotein (*Iss*), and outer membrane usher P fimbriae (*papC*). In the B1 phylogroup, a gene for long polar fimbriae (*lpfA*) was detected.

Furthermore, genes responsible for antibiotic resistance was detected in all genomes, which included resistance to cefotaxime (cephalosporin), meropenem (carbapenem), ciprofloxacin (fluoroquinolone), tetracycline, and gentamicin (aminoglycoside). When isolates were tested for resistance using disk diffusion assay, only one isolate (N119_F1) was resistant (to ciprofloxacin and tetracycline). This result was reflected in the genome, being the only isolate containing *tet(B)* and *tetR* (tetracycline resistance genes) and specific variants of *gyrA* and *parC* (fluoroquinolone resistance genes) (Nguyen et al., 2014; Redgrave et al., 2014).

Using *de novo* assembly, it was found that the genes for tetracycline resistance likely were located on a plasmid.

The isolates' ability to form biofilm was investigated through both cultivation and genomic analysis. Isolates grown in LB media and LB with 3.5 % NaCl, were analysed using crystal violet biofilm growth assay, and biofilm formation was significantly lower in the NaCl group (Wilcoxon signed rank test, p -value < 0.0001). Two isolates had increased biofilm formation with NaCl (W497_F1 and S1008_F3). In pure LB media, nine isolates showed no growth of biofilm, and in LB with NaCl, 27 isolates showed no growth of biofilm. Using ANOVA and Tukey's HSD test, a significant difference between phylogroups was only detected between A and B1 in the pure LB group (phylogroup A forming less than B1) (ANOVA, p -value = 0.035. Tukey's HSD test, p -value = 0.022). ANOVA in the NaCl group did not generate a significant result (p -value = 0.29). In terms of growth rate, generation time was significantly shorter for phylogroup B1 compared to B2 in the NaCl group (ANOVA, p -value = 0.021. Tukey's HSD test, p -value = 0.019).

Fecal source tracking in sediments

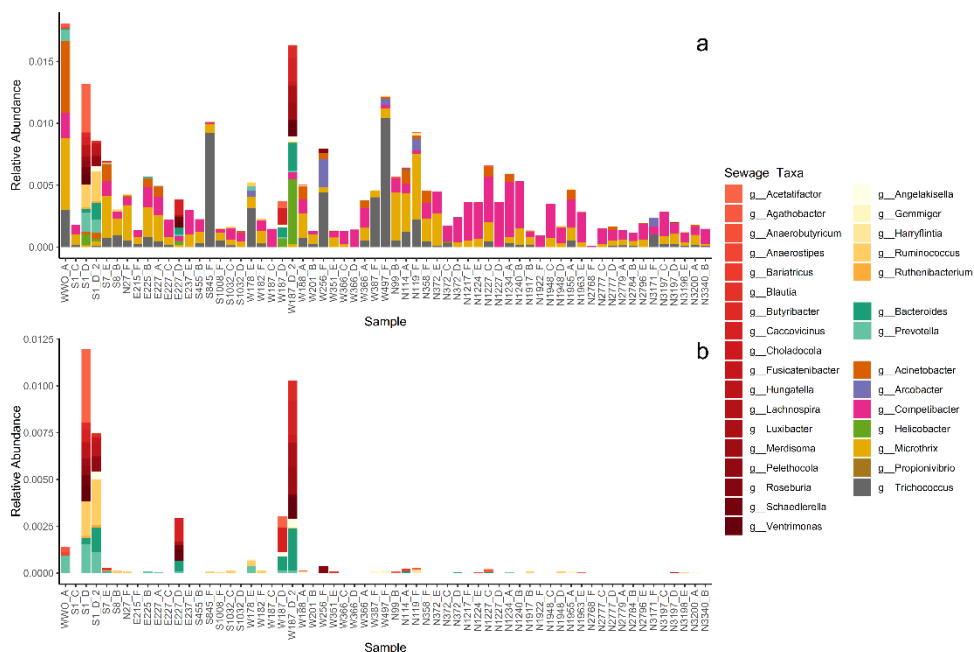
The first fecal source tracking approach utilized was signature-based MST. For Paper II, a new treated sewage library was created by sequencing eleven samples of treated sewage, and added to the existing library of various sources published in Hägglund et al. (2018). The north samples were set as background signal in the MST analysis, and a treated sewage signal could be detected in samples N110, WWO, and S260.

It was desired to try another approach of source tracking to validate the already applied method, and also to explore a new way of quantifying the influence of sewage and gut bacteria in the sediments (Paper II). Initially, thirteen genera were selected to represent the library called "Sewage and gut taxa" (SGT). These thirteen genera were selected thorough literature research, to include taxa found within sewage and the gastronomical tract, and that are not found in the natural environment. All taxa in the sediments were looked through for matches in the library using a custom script. When a match was identified, the taxa's abundances were added to a new data frame. After all sediment taxa have been looked through, the total abundances of all taxa in the data frame represents the sewage and gut influence. This method was named curated source tracking (CST).

In Paper II, SGT were detected in all samples except the three northmost ones. With Spearman's rank correlation test it was confirmed that a significant negative correlation can be made between SGT and distance to WWO (p -value = 0.0001; $\rho = -0.820$). Similarly, a significant positive correlation could be made between SGT and *E. coli* concentration (p -value = 0.0010; $\rho = 0.740$).

In Paper IV, the CST library was expanded to contain thirty-eight genera. Similarly to Paper II, the SGT were highest in close proximity to the WWO, which would be the expected result (Figure 4a). Correlation results were not as strong as when looking at March 2019 alone. While the Spearman's rank correlation still yielded significant p-value for both parameters (p-value < 0.05), the correlation coefficients revealed that the distance parameter had a stronger correlation to SGT than *E. coli* concentration had (−0.46 for distance, −0.40 for *E. coli*). When looking at seasonality, no significant difference between spring and summer was found.

When investigating genera within Lachnospiraceae, Oscillospiraceae, and Bacteroidaceae within the CST library, it could be seen that these families' distribution in the sediments differed from the total thirty-eight genera's distribution (Figure 4b). Singling out these families resulted in spikes in abundances during one specific occasion, which happened to be the date that a large CSO occurred in the area. Considering that when sampling occurred several days after a CSO during another sampling occasion, and no spike of sewage and gut taxa occurred then, it can be assumed that effects from CSO are short living.



To try an additional approach to fecal source tracking, penalized linear regression analysis was applied to find taxa within the sediment dataset whose abundances could be linked to the SGT abundances. Taxa that could be linked positively to the SGT were the genera *Methanotrix*, *Sulfurovum*, *Lamprocystis*, *Microthrix*, and *Aliarcobacter*.

Microbial communities in sediments

Non-metric multidimensional scaling (NMDS) was used to construct a plot visualizing differences in the ASV composition of the March 2019 sediment dataset (stress = 0.043), using Bray-Curtis dissimilarity. Multiple regression analysis was applied, and the environmental variables that significantly could be linked to the beta diversity of ASV composition were depth and distance to WWO (p-value = 0.001 for both, r^2 -values 0.9194 and 0.8336, respectively). No significance was found for *E. coli* concentration (p-value = 0.142) (Paper II).

To test how taxa within the dataset varies along an environmental parameter, differential abundance analysis was applied. In the sediments dataset it was of interest to investigate the distance and *E. coli* concentration parameters closer. Some taxa that were found connected to proximity to WWO were *Hydrogenophaga*, *Mesorhizobium*, *Trichococcus*, *Methyloceanibacter* and *Desulfobulbus*. For *E. coli*, taxa that were having similar presence pattern were *Bacteroides*, *Hydrogenophaga* and *Trichococcus* (Paper II).

To investigate the natural community of the longitudinal sediments (Paper IV), a core members analysis was run, to determine which ASVs that are present in > 90 % of the samples. This resulted in 260 ASVs and they represented on average 38 ± 4.5 % (mean $\pm$ standard deviation) of the abundance in each sample. Some interesting taxa found within the core community were *Sulfurovum* (Campylobacterales), S5133MH16 (Desulfobacterales), *Ilumatobacter* A (Acidimicrobiia), *Nitrosopumilus* (Thermoproteota) and *Parahaliea* (Haliaceae).

For the longitudinal sediment dataset (Paper IV), another type of beta diversity visualization was applied different to what was utilized in Paper II. Here, Jaccard index was used instead of Bray-Curtis dissimilarity, to focus solely on the presence or absence of taxa rather than their relative abundances. PCoA confirmed that the variance in the dataset could in part be explained by the depth (p-value = 0.001), distance to WWO (p-value = 0.001), and cardinal direction in relation to WWO (p-value = 0.035). The cardinal direction groups that were significantly similar were East-WWO and South-West.

Bathing water analysis

Two types of data were collected in regard to bathing water for this thesis work (Paper V). 77 samples of bathing water were collected during summers of 2020 and 2021, and after DNA sequencing and quality filtering of reads it resulted in 56957 ASVs and 2442 taxa. Data containing information about routinely measured bathing water quality in Helsingborg were collected from a national database (Havs- och vattenmyndigheten, 2024), selecting samples collected in the span of 1996-06-04 to 2023-08-29. This 28-year dataset (samples collected between May and September), contained information regarding location, date, *E. coli* concentration, intestinal enterococci concentration, and water temperature. When removing samples with values above detection limit (*E. coli* and intestinal enterococci), it resulted in a dataset of 2774 samples, and values below detection limit was set to 0. To include meteorological data into this dataset, datasets containing rainfall (mm/24 hours), air temperature, wind speed, and wind direction were collected from SMHI (SMHI, 2024). Merging the SMHI-data with the “Havs och vatten”-data resulted in missing values for a few dates, and after removal of these, the remaining dataset contained 2547 samples. This dataset is now referred to as the longitudinal dataset.

The *E. coli* concentrations in the longitudinal dataset had a mean of 120 ± 489 (mean $\pm$ standard deviation) and a median of 10 MPN/100mL. Intestinal enterococci concentration had a mean of 24 ± 78 and a median of 0 CFU/100mL. Using Fligner-Killeen test, it was determined that the variance of both types of bacteria was not equal in between beaches (p-value < 0.05). The highest mean and standard deviation of *E. coli* was found at B04 (240 ± 823 MPN/100mL), but the highest mean and standard deviation of intestinal enterococci was found at B02 (45 ± 130 CFU/100mL). Second highest mean was found at B02 and B06 respectively.

To evaluate the connections between bacterial concentrations and environmental parameters, linear correlations were made. The bacterial concentrations were converted to $\log(\text{concentration} + 1)$, day number was added as a parameter to see if sampling time had an effect (while disregarding what year), and wind direction was removed as a parameter as this is not a linear value. With Spearman's rank correlation, *E. coli* was positively correlated to intestinal enterococci, day number, water temperature, rainfall, and windspeed (p-value < 0.05). Intestinal enterococci was positively correlated to *E. coli*, day number, rainfall, and windspeed, and was negatively correlated to air temperature (p-value < 0.05). The same result was found for *E. coli* using Pearson correlation, while for intestinal enterococci the result differed. Intestinal enterococci was still positively correlated to *E. coli*, day number, rainfall, and windspeed, but the negative correlation with air temperature was not significant. The strongest correlation in terms of correlation coefficient was found between *E. coli* and intestinal enterococci (Spearman: 0.535, Pearson: 0.558). The second strongest correlations for both *E. coli* and intestinal enterococci were wind

speed (*E. coli*: Spearman: 0.193, Pearson: 0.189, Intestinal enterococci: Spearman: 0.183, Pearson: 0.186).

Looking closer at the DNA data, principal coordinates analysis (PCoA) was applied to see differences in the microbial communities in between beaches. Bray-Curtis dissimilarity was calculated from the ASV-table (transformed from absolute abundances to relative abundances), and PCoA resulted in the first two axes representing 26.25 % of the variability. Despite the relatively low cumulative variance, the beach samples are heavily overlapping, indicating that the samples have similar ASV composition to some degree. Due to all samples being bathing water from the same water body, it is reasonable that many bacterial species will be the same throughout the area and over time. However, since the analysis was performed on ASV level, and not taxa level, it is also reasonable that a large degree of variation in the dataset cannot be described by the first two principal coordinates.

Before analysing what environmental parameters have the largest impact on the microbial community structure, the dispersion between the groups within each parameter need to be tested, as dispersion being even is an assumption of PERMANOVA. The function `permutest` from `vegan` was used for this, with pairwise post-hoc testing with Tukey's HSD test. For beach, `permutest` gave a significant result, which indicates that the dispersion is not even between groups. However, the results from Tukey's HSD test found that no pairs of beaches had significantly different dispersion. Because of this, PERMANOVA can still be used, but it is important to keep in mind that the differences found in PERMANOVA may be due to differences between groups, but could also be due to dispersion differences. When running `permutest` for the year-parameter, it also returned a significant result. Tukey's HSD test was also significant, indicating that the dispersion is significantly different between the two years. Because of this, year was not included in the PERMANOVA. For week, the `permutest` was not significant, indicating even dispersion between groups, thus fulfilling the assumption of PERMANOVA. Permutation test for homogeneity gave a significant result for $\log(E. coli \text{ concentration} + 1)$, and Tukey's HSD test yielded non-significant result for 86.5 % of the tested pairs. Due to the large fraction of non-significant pairs in Tukey's HSD test, *E. coli* was still included in PERMANOVA.

PERMANOVA was run to test if the variance found in the Bray-Curtis dissimilarity matrix could be tied to the environmental variables of the dataset. The following order was used; beach, week, *E. coli* and sequence depth. PERMANOVA yielded significant p-values for beach and week (p-value = 0.001), with explained variance in the microbial community composition of 18.8 % and 8.22 % respectively. Due to the high richness of the dataset (56957 unique ASVs), it is reasonable that the *E. coli*-parameter did not yield a significant result, both due to *E. coli* quantification having a large margin of error, and due to *E. coli* being a contaminating bacteria in the bathing water.

CST was utilized on the bathing water dataset (Paper V), using the same genera that were used in the sediment analysis (Paper IV), with the exception of *Arcobacter* which was removed. Out of the 37 genera in the library, 21 genera were detected in the bathing water. In the analysis for this study, a clearer division was made between sewage prevalent bacteria and animal gut prevalent bacteria. Sewage taxa detected were *Competibacter*, *Microthrix*, *Propionivibrio*, *Trichococcus*, and *Acinetobacter*. Gut taxa detected were *Gemmiger*, *Ruminococcus_F*, *Ruthenibacterium*, *Bacteroides*, *Bacteroides_G*, *Prevotella*, *Helicobacter_C*, *Helicobacter_D*, *Acetatifactor*, *Anaerobutyricum*, *Blautia_A*, *Butyribacter*, *Caccovicinus*, *Peletothocola*, *Lachnospira*, and *Roseburia*. Mean relative abundance of sewage taxa and gut taxa was $0.446 * 10^{-3} \pm 0.772 * 10^{-3}$ and $0.321 * 10^{-3} \pm 0.444 * 10^{-3}$ respectively (n = 77). Highest relative abundance of sewage taxa was found at B03 and highest relative abundance of gut taxa was found at B04. Over the whole sampling period, B02, B03 and B04 had the highest cumulative relative abundance of sewage and gut taxa.

In order to evaluate how environmental parameters could be linked to sewage and gut taxa, the ASV dataset and the longitudinal dataset was merged. Exact matches were found for 64 samples. Principal component analysis (PCA) was conducted including all samples (n = 64), and also for beach B04 (n = 19) and B06 (n = 19) separately (other beaches had too few samples). For PCA, $\log(E. coli \text{ concentration} + 1)$, $\log(\text{intestinal enterococci concentration} + 1)$, water temperature (°C), rain (mm rain the day before sampling), air temperature (°C), windspeed (m/s) and relative abundance of sewage-taxa and gut-taxa (separately), was included as environmental parameters. The PCA for all beaches yielded a cumulative proportion of 46.1 % for PC1 and PC2. For B04 and B06, this number was 54.6 % and 48.3 % respectively. The fact that the cumulative proportion increased when testing individual beaches indicates that the parameters are affecting the beaches differently. At B04, *E. coli* and enterococci are closely linked, and are found connected to rainfall and wind speed. At B06, *E. coli* and enterococci were also linked, but not as strongly as at B04. *E. coli* and enterococci were connected to sewage and gut taxa to some degree, and gut taxa was found to be connected to rainfall.

Discussion

Groundwater contaminated with PCE

Results strongly suggest that ZVI and bioremediation strategies enhanced the degradation of PCE to *cis*-DCE and further (Paper I). The chemical concentrations indicate that the degradation process started faster in area A compared to B. The difference between these two areas could be due to area A having a higher starting concentration, and that it was injected at an earlier time. Due to areas having different starting conditions, and the areas' close proximity to each other, the effectiveness of the two different remediation strategies is challenging to fully evaluate separately.

In area C a remediation attempt was performed prior to the start of this study, and effects of it could still be detected three years later (at the start of this study), in terms of iron concentration and microbial groups. A delay in the response to the bioremediation could be seen in area C compared to area A and B, matching what is known about the hydraulic conductivity of the site.

While PCE concentrations were consistently high in the Source zone, except for the last sampling occasion, the bacterial groups injected in area B were seen to also increase in the Source zone. This could be due to the higher concentration of nutrients of choice in that area causing the bacteria to proliferate and migrate. PCE concentrations over the area indicated transport of PCE from the Source zone to area A and B, and further away to area C, as was hypothesized. Fluctuating concentrations of PCE were likely due to the bioremediation injections' and the ZVI's varying distribution speed and concentrations over the area.

An interesting result was found in area A, where the degradation of PCE all the way to ethene was observed in all wells except one: the well closest to the Source zone (named B6). In this well, PCE concentrations were consistently high, and its metabolite concentrations were low. Iron concentration and DOC were also very low, despite being injected at this very spot. The difference of this well could also be seen in the microbial community, and this well's community did not change as much over time as the other wells in area A did. The genus *Pseudonocardia* was found in this well, but was not found in the other wells of area A.

Area B also had an outlier well, named LU1, which had much higher concentrations of iron, DOC, methane, magnesium, sodium, calcium, and potassium than all other

wells in area B. Due to it being these specific compounds having such high concentrations, it would seem that this well is affected more by the injection done in area A, rather than the injection done closely by in area B. Despite LU1 not being positioned adjacent to area A, a hydrogeological connection between these sites, due to fractures in bedrock or sediments, could be the cause of this effect (Christiansen, 2010; Christiansen et al., 2012). While the chemical composition resembles area A rather than area B, the microbial community in this well was similar to other wells in area B. All wells in area B showed an effect from the injection in area B, and the likely transport of injected compounds and bacteria between areas might also in part explain the fluctuations that were seen in PCE metabolites, DO, and methane concentrations throughout the study site.

Overall, the degradation process was found to be more effective in area A compared to area B. Area C also showed evidence of degradation, however since this area is positioned lower than other areas, the increase in metabolites may be due to them being transported from area A and B. This effect would also be magnified by the fact that the metabolites get higher mobility throughout the degradation process (Pankow and Cherry, 1996). The consortium of bacteria added in area A was called Trap & Treat®, but what bacterial groups this contains is not openly published by the company that manufactures it. In area A, the classes Methanomicrobia and Methanobacter were not present in 2017, but were found in high relative abundance in 2018, which could indicate that these were added by Trap & Treat. Likewise, the class Clostridia showed a large increase between these two years as well.

The four known genera of bacteria that were injected in area B as a consortium (*Dehalococcoides*, *Dehalogenimonas*, *Desulfovibrio*, and *Desulfitobacterium*) were detected in very low abundances throughout the areas, and could not be correlated with PCE- or *cis*-DCE concentrations. While concentration of said bacteria within the injected consortium is unknown, a higher presence of these bacteria was expected. However, introducing new bacterial groups into an already rich and established microbiome, it is also expected that competition for nutrients is high and that the slow spatial distribution of a highly concentrated consortium will likely result in die off of bacteria. The fact that these taxa could not be correlated with PCE and *cis*-DCE could also be indicating that these are not the only bacterial groups that are responsible for the degradation. Furthermore, just the presence of PCE and metabolites may not be all that the bacteria require to metabolize the contaminants. For example, *Dehalococcoides mccartyi*, one of the known species in the consortium, can metabolize TCE all the way to ethene, but only if vitamin B12 is available (Yan et al., 2021). Whether vitamin B12 was available in the groundwater at the site is unknown, and thus the effectiveness of added *D. mccartyi* is also unknown.

Another bacterial group of interest, a class of bacteria called Clostridia, became more abundant in all areas after injections. This class contains *Desulfitobacterium* and *Dehalobacter*, and may contain many more genera also capable of degradation

of PCE and its metabolites. Additionally, correlations not being significant could also be due to relative abundances of certain DNA sequences being compared to quantified concentrations of chemical compounds, which are very different methodologies with varying precision and accuracy. There could also be naturally occurring microorganisms in the groundwater that are capable of degradation of PCE and its metabolites, that were stimulated by the bioremediation process, but might be completely unknown species. Since the large spill of PCE occurred in the 1960s, it is plausible that naturally occurring species capable of the degradation have been selected for during these past five decades.

All in all, evidence was found that all different remediation strategies had positive effects on the site. The measured concentrations of PCE, *cis*-DCE and VC indicated that degradation did occur, however making a distinction of which strategy was most effective was not possible. As the degradation could be of both abiotic and biotic nature, it further complicates distinction. Further, due to complexities such as varying initial concentrations in the areas, and uneven transport of PCE from the Source zone into area A and B, distinction between strategies could not be uncovered with the set sampling scheme. Furthermore, nutrient contents of the groundwater are unknown, so if conditions were met for the microorganisms to metabolize the contaminants are unknown. Whether the microbial groups (both natural community and the injected bacteria) present in the water were contributing to the degradation of PCE cannot be fully proven, but there are indications that they did.

Sediments

Viable *E. coli* quantified in Paper II and IV were detected with varying concentrations over the ≈ 4 km long study area, but with distinct increases in close proximity to a treated wastewater effluent. The initial study (Paper II) examined the microbial community and viable *E. coli* during one sampling occasion, and in order to make stronger conclusions from the site, five more sampling occasions were performed. *E. coli* was quantified in the same manner for following field investigations, and sediments from all six occasions were then sequenced to get a complete dataset stretching over the three years (Paper IV). 16S rRNA gene sequencing of the V3-V4 region was performed to analyse the microbial communities' spatial and temporal variations, and large focus was put on fecal source tracking using a variety of techniques. Established methods, such as signature-based source tracking (Paper II), differential abundance analysis (Paper II), and penalized linear regression (Paper IV), as well as developing a new method of source tracking called CST (Paper II and IV).

E. coli in sediments have been studied in multiple parts of the world, and culturable *E. coli* can be found in both marine and freshwater environments (Craig et al., 2001; Beversdorf et al., 2007; Ishii et al., 2007; Walk et al., 2007; Mackowiak et al., 2018). While it is not their native environment, *E. coli*'s ability to survive in water environments is largely documented (Ishii and Sadowsky, 2008; van Elsas et al., 2011), and their survival success is aided by their short generation time and ability to share beneficial genes through horizontal gene transfer (Hasegawa et al., 2018; Pérez-Etayo et al., 2020). Not only is *E. coli* one of the most well studied bacteria in the world (Edberg et al., 2000), it is of large interest in environmental studies due to their status as a FIB (Paruch and Mæhlum, 2012). *E. coli* has been shown to survive better in sand than in water (Hartz et al., 2008), with a preference for finer-grained sediments such as sand over coarser materials like gravel (Craig et al., 2001; Pachepsky and Shelton, 2011; Perkins et al., 2014; Aragonés et al., 2016). While containing important results, it is important to not draw generalized conclusions of *E. coli*'s survival and behaviour based on single studies. Studies are conducted on different continents, and environmental factors and anthropogenic influences will vary greatly from site to site. Furthermore, due to *E. coli*'s large genetic diversity (Selander and Levin, 1980; Rousset et al., 2021), generalizations should be made with caution (Hassard et al., 2016).

The treated effluent of the nearby WWTP was hypothesized to be a major point source of fecal contamination in the area. This due to its close proximity to the recreational beaches, and due to it not utilizing a disinfection step such as chlorination (US EPA, 1999a), ozone (US EPA, 1999b) or UV-treatment (US EPA, 1999c; NSVA, 2020). The effluent location is located in the deeper zone, and thus effects of sampling depths, distance to the effluent point, and salinity are entangled. The treated effluent likely contains planktonic microorganisms as well as microorganisms attached to particles. As attachment to particles offers higher survival chance for *E. coli* (Vogeleer et al., 2014), it is likely that this occurs. Since particle size and weight will affect the settling velocity of the particle in the receiving waters, studying the particle size distribution of the treated effluent would aid in evaluating how far particles carrying *E. coli* potentially could travel. *E. coli* have been found to associate to smaller sewage particles ($\leq 12 \mu\text{m}$) to a larger degree than to larger particles (Walters et al., 2013), which could indicate that *E. coli* might be able to be transported in the water column for some distance. However, due to consistently high concentrations in the samples collected closest to the effluent, this points to *E. coli* sinking fast to the bottom to a large degree from the consistent source.

The first source tracking methodology utilized was signature-based source tracking, and it indicated wastewater influence in three samples (N110, WWO, S260), all which also had detectable levels of viable *E. coli*. This method was only applied on the March 2019 dataset (Paper II). As the source tracking library was sequenced using another region of the 16S gene (V4, rather than V3-V4), as well as different

bioinformatic pipelines for sequence reads handling, this analysis' results may be an underestimation. Further, when performing DNA extraction on sediment samples, rather than water samples, different types of inhibiting substances (such as organic compounds) are likely to be co-extracted, despite the addition of Bovine Serum Albumin (BSA). This will affect the next step, DNA amplification, where the varying composition of inhibitors will affect the amplification in different ways, potentially causing mismatches between the resulting datasets (Sidstedt et al., 2020). The source tracking library for treated wastewater used in the signature-based source tracking method was sampled in another part of the country, and thus there may be local differences in wastewater composition (including the microbiome) that negatively affects this source tracking method's accuracy. Further, the north samples (distance > 440 meters from WWO) were set as background signal in the MST analysis (Paper II), but Paper IV revealed that sewage influence were present in these samples consistently, albeit at a lower abundance than in the south samples. Thus, this may have caused the MST analysis to underestimate the sewage signal throughout the dataset in Paper II.

The CST approach utilized in Paper II, IV and V identified taxonomic groups from a manually made library of sewage- and gut-associated bacteria in the studied coastal environment. In March 2019 (Paper II), eight out the 16 sediment samples contained taxa from this library ($n = 13$). In the longer study (Paper IV), every single sample contained taxa from the expanded library ($n = 38$). A reason why the results differ, particular for the same samples of March 2019 that were sequenced twice (once per paper) and had different results, could be due to sample heterogeneity, varying sequencing protocols, and the library being expanded. Both studies showed that a negative correlation exist between the distance to the WWO and the relative abundance of SGT, validating that despite different sequencing runs the results say the same. Similarly, in both studies there were a strong connection between *E. coli* concentration and SGT abundance. In the longitudinal dataset it was seen that when grouped based on SGT abundance (high vs low), *E. coli* only had a significant correlation in the high group. This strengthens the hypothesis that while *E. coli* is added to the environment by treated sewage, it is not the only source of *E. coli*.

That *E. coli* is originating from other sources was investigated particularly in the northernmost sediments (≥ 3.2 km north), as influence from the WWTP decreased at ≈ 1.9 km north of the WWO according to the CST analysis. In Paper III, strains from phylogroup B2 was found in these sediments, which could indicate extraintestinal pathogenic *E. coli* (Denamur et al., 2021). If the theory that the WWTP's effect does not reach that far north is true, that would mean that another source is contaminating this site. Sources such as stormwater (Marsalek and Rochfort, 2004; Hart et al, 2020), boat traffic (Koboević et al., 2021), direct fecal contamination by wild animals (Araújo et al., 2014), domestic animals (Tarek et al., 2023) and humans (Li et al., 2021), and influence from other further away WWTPs could all be factors, and due to the inherent intermittence of these sources, it is

difficult to prove or disprove their effects on the bacterial contents with current datasets. Furthermore, due to *E. coli*'s ability to survive and become naturalized, the *E. coli* isolated from bathing water and sediments may not be due to recent contamination (Devane et al., 2020).

To get a better understanding of the possible impact from intermittent sources, looking into specific genera of bacteria might reveal more clues. The challenge is that many groups of bacteria inhabit more than one environment. *Arcobacter*, for example, is a bacteria largely abundant in both influent and effluent sewage (Kristensen et al., 2020), but they are also found in stormwater (Carney et al., 2020; Carson et al., 2024). Stormwater is a particularly tricky source to evaluate the impact of, as its bacterial content varies largely both spatially and temporally (Fraser and Preheim, 2021). One of the most abundant sewage bacteria in the sediments was *Trichococcus*, which is a strong sewage indicator (Kristensen et al., 2020) (Paper II and IV). In several sediment samples where *Trichococcus* had a large presence there was a clearly larger abundance of *Arcobacter* compared to other samples. This would indicate that the *Arcobacter* in these cases did originate from sewage.

An intermittent source that is important to investigate, and to be able to separate from the effect of the treated effluent, is CSOs. The bacterial composition of a CSO water mass is expected to vary, containing bacteria from human fecal matter, influent sewage, and stormwater, and containing lower abundances of bacteria enriched in WWTP reactors. To investigate the effect of CSOs, certain bacterial groups were selected: Lachnospiraceae, Oscillospiraceae, and Bacteroidaceae (McLellan et al., 2013; Newton et al., 2015). Since there was much stronger presence of these bacterial groups during one specific sampling occasion, it suggests that it could have been caused by a CSO event. The four affected locations were located south of the CSO outlet, which matches the general southward direction of the water under the halocline (Jephson, 2012), and the fact that there had very recently been a CSO event. Although CSO volumes are very low in comparison to the treated sewage volumes at this site, it cannot be disregarded due to the high concentration of bacteria it contains. CSOs have been shown to elevate FIB levels and sewage related bacteria such as *Acinetobacter*, *Arcobacter*, and *Trichococcus* in the receiving water body in multiple studies (Newton et al., 2013; Al Aukidy and Verlicchi, 2017). In part due to the nature of CSOs, heavy rain is a parameter often positively connected to FIB levels (Newton et al., 2013; Henry et al., 2016).

In addition to the CST approach, differential abundance analysis was utilized in Paper II. It was found that *E. coli* concentration was significantly connected to *Trichococcus*, *Hydrogenophaga*, and *Bacteroides*, which are sewage- and gut-associated bacteria (Kämpfer et al., 2005; Newton et al., 2015; Kristensen et al., 2020). This further strengthens the hypothesis that *E. coli* to a large degree originates from the WWTP. Another method utilized was penalized linear regression (Paper IV), but here to detect taxa in the dataset that could be connected to the sewage- and gut-bacteria relative abundance. *Microthrix* was among the

detected, which makes sense as it is part of the CST library. *Methanothrix* was also a reasonable find, as it is prevalent in anaerobic digestors in wastewater treatment (Sato et al., 2007). An interesting find was the sulfur-metabolizing bacteria *Sulfurovum* (Mino et al., 2014), *Lamprocystis* (Frigaard and Dahl, 2009), and *Desulfosarcina* (Watanabe et al., 2017), as their presence in sediments with higher impact from sewage might benefit from the addition of sulfur and sulfate in the effluent from the treatment plant. One taxon detected, *Aliarcobacter*, is of concern due to it being a human pathogen with good survivability in water environments (Chieffi et al., 2020). As it has been found present in sewage and CSO discharges in previous studies (Kristensen et al., 2020; Venâncio et al., 2022; Zan et al., 2023), it does highlight the importance of studying the impact of wastewater and other anthropogenic contamination in our environment.

Both Paper II and IV strongly suggest that the treated effluent from the local WWTP is affecting the microbial communities of the sediments. Sediments furthest to the north indicated that other sources may be involved, and on one occasion there seemed to be influence in some southward sediments from a recent CSO (Paper IV). While significant correlations could be made between studied parameters in the dataset, there were challenges regarding pinpointing intermittent sources due to multiple factors. The sampling interval of six month makes it difficult to fully investigate the effects of short term events such as CSOs, stormwater discharge, and sporadic contamination. The results from the Colilert-18 method for quantifying viable *E. coli* are compared to relative abundances derived from 16S rRNA amplicon sequencing, which is a method that does not differentiate between viable and dead cells. Thus, there are concerns regarding the comparability of the data produced by these two methods. Further, duplicate sequencing samples generated from the same sediment samples showed high variation in taxonomic composition, indicating that the spatial variation in the sediments is very high. Due to many taxonomic groups being found in multiple environments, a qPCR-based approach utilizing source specific markers could have aided in determining certain sources, such as for dogs (Rothenheber and Jones, 2018), seagulls (Goodwin et al., 2016) and humans (Li et al., 2021). While great care was taken to only include sewage- and gut-associated bacteria in the CST library, full knowledge of every genera of bacteria does not exist. While a bacterium's presence is often studied in the WWTP context, the same species can inhabit other, not considered, environments. Even the well studied fecal indicator bacteria *E. coli* have strains that are naturalized in the aquatic environment (Devane et al., 2020). Due to high spatial variability of sediments (Tolu et al., 2017), sample representability could be questioned. Sediment mixing resurfaces lower layers of sediments through natural processes, which adds complexities when comparing top-layer sediments over a time series.

Escherichia coli in sediments

Survival and growth of *E. coli* in the environment have been shown in multiple studies (Luo et al., 2011; van Elsas et al., 2011; Rumball et al., 2021). While some *E. coli* are naturalized in the aquatic environment, WWTPs have been shown to be a significant contributor of *E. coli* (Anastasi et al., 2012; Zieliński et al., 2021). To get a better understanding of the *E. coli* present in our study area, whole genome sequencing of collected isolates was conducted (Paper III).

Among the *E. coli* isolates analyzed, one was assigned to phylogroup E, which includes both commensal strains and the highly pathogenic serotype O157:H7 (Clermont et al., 2021). The majority of isolates (15 out of 37) belonged to phylogroup B1. This phylogroup has been frequently associated with animal sources (Higgins et al., 2007; Carlos et al., 2010; Johnson et al., 2017), but it is also commonly observed in aquatic environments (Berthe et al., 2013; Touchon et al., 2020; Rumball et al., 2021), suggesting that B1 isolates in the Öresund sediments may originate from animal sources or could be naturally adapted to the marine environment.

Six isolates collected near the WWO were classified as phylogroup B2, a group known to include several extraintestinal pathogenic *E. coli* (ExPEC) strains (Johnson and Russo, 2002; Denamur et al., 2021). ExPEC are known to persist through wastewater treatment processes (Raboni et al., 2016; Zhi et al., 2020; Yu et al., 2022) and often belong to phylogroups B2 and D (Picard et al., 1999; Johnson and Russo, 2002). More specifically, wastewater-associated uropathogenic *E. coli* (W-UPEC) have been shown to cluster primarily within phylogroups B1, B2, and D, with B2 being the most prevalent (Zhi et al., 2020). These findings suggest that some of the *E. coli* isolates, particularly those found near the WWTP, may be pathogenic and likely originated from the WWTP. In contrast, others likely entered the environment via alternative routes, including CSOs, stormwater runoff, or local animal populations (Wright et al., 2009; McCarthy et al., 2017; McGinnis et al., 2022).

The diversity observed in MLST further supports multiple sources of *E. coli* in the sediments. A recent study from the Salish Sea showed that higher ST diversity was associated with areas impacted by wastewater discharges (Grunwald et al., 2022). Approximately 20 % of the STs identified in this study were frequently reported in humans, including ST10, ST73, ST131, and ST127. Notably, ST73 and ST131 are well-established ExPEC lineages (Nicolas-Chanoine et al., 2014; Riley, 2014), and both ST131 and ST127 have been previously linked to wastewater environments (Finn et al., 2020; Zhi et al., 2020). ST131 has also been recovered from marine sediments (Vignaroli et al., 2013) and is associated with virulence factors linked to complicated UTIs and treatment failure (Can et al., 2015). Other isolates such as ST8972 (S1008_F1) and ST214 (N1922_F2) were more rarely reported, with the

former previously found only in surface soil (Dusek et al., 2018) and the latter in a single human isolate (GenBank: GCA_900490405.1). Additionally, STs 10, 162, 362, and 2144 have been found in aquatic animals such as fish and seals (Grunwald et al., 2022), suggesting a broad ecological distribution.

Virulence gene profiles differed across phylogroups, with B2 isolates exhibiting the highest abundance of virulence genes, consistent with prior observations (Picard et al., 1999). Genes of concern included *vat*, *yfcV*, *fyuA*, *chuA*, *sitA*, *iss*, and *papC*, all of which are associated with ExPEC virulence and uropathogenic potential (Spurbeck et al., 2012; Sarowska et al., 2019). These genes were more frequently detected in isolates from sediments near the WWO, supporting the hypothesis that proximity to the WWTP contributes to the presence of potentially pathogenic strains. Furthermore, the prevalence of virulence genes has been shown to increase after wastewater treatment (Osińska et al., 2020), reinforcing the WWTP as a likely source.

Although only one isolate showed phenotypic antibiotic resistance according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines, several others harboured ARGs, some likely carried on plasmids (e.g., N119_F1 and N119_F3), which raises concerns about horizontal gene transfer in sediments (Wang et al., 2021). Aquatic sediments have previously been recognized as reservoirs for ARGs (Luo et al., 2010; Wu et al., 2021), and the Öresund sediments may function similarly.

An environmental factor of interest in the marine environment is salinity. Survival of *E. coli* in saline water has been studied and confirmed (Byrd and Colwell, 1993), thus the consistent *E. coli* presence detected at certain sites in this thesis work could be an indication of survival and possible proliferation. However, another established fact regarding *E. coli* is that it is prone to enter a viable but nonculturable (VBNC) state when salinity is high (Roth et al., 1988). *E. coli* isolated from this study area were able to grow under saline conditions resembling the study site's during laboratory experiments, hence it is evident that some *E. coli* are more halotolerant than others (Paper III). An added complexity to the study area is that a halocline exists, positioned at 10-15 meters depth separating the shallow water (salinity of 10-20 (PSS78)) and the deeper water (salinity of ≈ 30 (PSS78)) (Jephson, 2012; SMHI, 2025). Thus, the sediments extracted in close proximity to the WWTP are all below the halocline, whereas the samples further north with closer proximity to the recreational beaches are all above it. The greater halotolerance observed in phylogroup B1 compared to B2 may reflect the ecological adaptation of B1 to naturalized environments, whereas B2 is more commonly linked to human and animal sources (Clermont et al., 2013; Martak et al., 2020).

The ability to form biofilm was also studied in Paper III, as presence of biofilm aid the survival of *E. coli* (Hassard et al., 2016; Decho and Gutierrez, 2017). Phylogroup B1 isolates exhibited greater biofilm formation capacity compared to other

phylogroups, in line with earlier studies (Olowe et al., 2019). Interestingly, the *lpfA* gene, which encodes a major subunit of long polar fimbriae, was observed in 14 isolates, all within phylogroup B1. This gene is commonly found in intestinal pathogenic strains, including O157:H7 (Zhou et al., 2019), and is associated with enhanced adhesion and biofilm formation (Ross et al., 2015; Madoshi et al., 2016; Zhou et al., 2021). Its presence may provide a survival advantage in marine sediments, potentially explaining the high abundance of B1 isolates. Biofilm formation enhances bacterial survival in aquatic sediments (Dang and Lovell, 2016; Flemming and Wuertz, 2019), further supporting the ecological success of B1 strains in these environments. Interestingly, within ST155, isolate N2036_F1 did not produce biofilm, while the closely related W182_F3 (ANI > 99.9 %) exhibited low biofilm production in LB medium. Isolates from phylogroup A generally showed lower biofilm formation, consistent with prior findings (Martínez et al., 2006), likely due to the absence of key biofilm-related genes. However, two phylogroup A isolates, S1008_F3 (ST10) and W497_F1 (ST1443), showed increased biofilm formation when grown in LB supplemented with 3.5 % NaCl, suggesting a role for environmental conditions in modulating this trait. These findings highlight the need for a multifaceted approach to source tracking in marine environments, going beyond enumeration of *E. coli* to include phylogenetic, genotypic, and functional assessments.

Bathing water

The variance of *E. coli* concentration distribution between the beaches was not equal, suggesting that some beaches may have less consistent levels of *E. coli* than others. The beach with both highest mean and variance of *E. coli* concentration was B04, which could be an indication that this beach is experiencing more disturbance and contamination.

This beach, B04, is according to the city one of their most problematic beaches. This view stems from the often occurring peaks of FIB concentrations in the water. These FIB concentrations were utilized in this study to get a better understanding of their patterns and to see if source tracking was possible by combining that data with the DNA data gathered. By analyzing FIB concentrations together with environmental parameters at this beach, *E. coli* and intestinal enterococci is the only combination that yields a positive significant correlation, meaning that when *E. coli* levels are high intestinal enterococci often is too. When adding the results from the DNA data, and looking for connections in between parameters using PCA, it revealed that the FIBs are both linked to increased rainfall and wind speed. When it comes to the sewage- and gut-taxa, these display different patterns than the FIB, which could indicate that the FIB concentrations at this beach are not caused by the treated

wastewater effluent, CSOs, or direct human shedding to a large degree. Rather, it would seem that due to the connection to rainfall and wind speed, the FIB might be increased due to a local stormwater discharge point and possibly due to sediment resuspension of FIB, that could be happening to a larger degree during stormy weather.

Beach B06 is located further south than B04, and is closer to both CSO outlet and the treated wastewater outlet. In this location, the correlation between the two FIBs were not as strong as it was at B04, which could mean that they do not originate from the same source as often, or that the transport of the bacteria is more complex (Amorim et al., 2014). At this beach, PCA revealed that both FIB are connected to both sewage taxa and gut taxa to some degree, but *E. coli* stronger so than intestinal enterococci. Gut taxa showed an increase with increased rainfall, which could be an indication of CSO influence. From all these observations in the results, it is clear that the sources contaminating this beach are not uniform or consistent. Likely, the sources causing increase of FIB are multiple, sporadic and complex. There is difficulty in pinpointing a major source of the contamination, just as it is difficult to exclude any potential sources, both anthropogenic and environmental. As this beach is one of the most popular beaches in the city, this beach is likely the most affected by human interactions, both through direct shedding in the water, and by disturbance of sediments (Pachepsky and Shelton, 2011; Byappanahalli et al., 2012; Aragonés et al., 2016). Whether this effect is significant or not is not possible to say with the collected data.

The gut taxa detected in the bathing water suggests several different sources. *Anaerobutyricum*, *Bacteroides*, *Blautia*, *Butyribacter*, *Gemmiger*, *Helicobacter*, *Lachnospira*, *Prevotella*, *Roseburia*, *Ruminococcus*, and *Ruthenibacterium* have all been found in human fecal matter (Qin et al., 2010; The Human Microbiome Project Consortium, 2012; Yatsunenkov et al., 2012; McLellan et al., 2013; Newton et al., 2015; Ahmed et al., 2016; Shkoporov et al., 2016; Shetty et al., 2018; Tett et al., 2021; Zou et al., 2021; Ke et al., 2022). Other taxa detected, *Acetatifactor*, *Caccovicinus*, and *Peletthocola*, are associated with bird feces (Gilroy et al., 2021). Since the microbial communities in feces can overlap between different mammalian species, and even with those of birds, the taxa detected may originate from additional sources beyond those identified here (Häggglund et al., 2018; Boukerb et al., 2021). *Bacteroides*, for example, is also found in dog and bovine feces (Layton et al., 2006; Kildare et al., 2007).

Looking at all beaches together, matching the microbial community data with the *E. coli*- and environmental-data, PERMANOVA was utilized to uncover more information. The global PERMANOVA revealed that beach and week were the only two parameters that significantly influenced the microbial community. Beach having a significant effect is reasonable, as large differences in FIB concentrations

are commonly detected between adjacent beaches. PERMANOVA confirmed however, that these differences do not only apply to FIB, but can be seen in the whole microbial community as well. The second strongest parameter, week, can be due to several reasons such as temperature changes (in both air and water), rainfall occurrence, and human activity (which also commonly is linked to both temperature and rainfall). In this area over the course of the summer season, there are variations in these factors. Warmer temperatures during July during both studied years could have contributed to these significant changes in the microbial community, and as higher temperatures can enhance bacterial growth and survival (Fuhrman et al., 2015). During these weeks there were also less rainfall (compared to the whole dataset), which can also be connected to lower runoff-related contamination which might be reflected in the microbial community (Shrestha et al., 2020). Further, peak summer weeks are associated with higher beach attendance and increased human shedding of bacteria into the water, with potential to alter microbial composition and FIB levels (Graczyk et al., 2010; Toubiana et al., 2021). The results from the global PERMANOVA truly shows that the spatiotemporal variations at these beaches are significant and undeniable. It also shows that there is an influence on the microbial community from environmental factors.

Utilizing multiple analyses that included both FIB concentrations and sewage- and gut-taxa abundances, made it evident that these do not always correlate or co-occur. This raises important questions about current water quality monitoring standards. Similar to the results from Paper II and IV, the results from Paper V also reveals that the standard FIB utilized for bathing water quality assessment does not always align with what can be seen in terms of sewage and fecal contamination. The results from these papers are in line with many other studies supporting the need of expanding routine bathing water quality assessment with new methods, such as molecular microbial source tracking (Boehm et al., 2013; World Health Organization, 2021). To better the accuracy of water quality assessment could in turn lead to identification of contamination sources, and to better assess the risk to human health when in contact with the water.

Conclusions and Future work

Investigation of sources of microbial contamination in coastal environments led to the treated sewage being a significant source of *E. coli* and other sewage and fecal associated bacteria. This was possible due to the treated wastewater being added to the area continuously, and could therefore be detected despite six months interval between sediment samplings. Intermittent sources, such as stormwater and CSOs could be indicated at certain locations and time, but due to time resolution of sampling, the data was not strong enough to make any firm conclusions. Likewise, in the bathing water study, both spatial and temporal variations of both FIB concentrations and microbial community composition were high, indicating that potentially both sampling interval and sampling distances were too large for it to be possible to determine intermittent and unknown sources. Sources such as boat traffic, wild animals, domestic animals, stormwater, and human shedding at beaches are all parameters that can influence bathing water quality, but to determine their impact was not possible solely on the water and sediment data collected.

When it comes to environmental factors influencing fecal pollution in bathing water, both intestinal enterococci and *E. coli* were positively correlated to day number, rainfall, and windspeed. Even though differences in between beaches were high when it comes to what factors could be linked to the sewage- and gut-associated taxa, the mentioned environmental factors and FIB concentrations could be significantly correlated when looking at all samples together. However, in order to truly establish what are causing the peaks in FIB concentrations in the bathing water, one would benefit of looking closer into each potential source, such as measure FIB concentrations in stormwater ponds and retention basins, runoff, and any treated effluent that will enter the receiving water body. Further, one could perform a qPCR study utilizing source specific fecal markers such as *DF475* (dogs), *HF183* (humans), and *Gull2* (gulls) (Rothenheber and Jones, 2018). Additionally, one could evaluate the impact of bathers by counting number of people visiting a selected beach, and measure FIB concentrations during morning and afternoon for several weeks. Similarly, one can count the number of wild and domestic animals in the vicinity.

Investigating the persistence of *E. coli* in coastal environments was done by testing halotolerance, biofilm formation capacity, and antibiotic resistance. Strains belonging to different phylogroups showed varying results, implying both genetic and phenotypic diversity of *E. coli* of the area. Persistence (halotolerance and biofilm formation capacity) was higher in the source ambiguous (can be both from animals and environmental) phylogroup B1, which was found throughout the sampling area (except south of the WWO).

Investigating the sediments of the area was performed to get a better understanding of whether it could be possible that resuspension of viable *E. coli* was the cause of

high *E. coli* levels at the beaches. The sediment studies showed that live *E. coli* was present during all sampling occasions, and results primarily suggest the treated wastewater effluent as a source, with CSOs having a short-lasting effect. Resuspension may be the cause for some *E. coli* at the beaches, as *E. coli* was present in sediments across the whole area. However, resuspension likely causes only a fraction of the *E. coli* at the beaches. Moreover, it is of higher interest to investigate what is causing the high numbers of live *E. coli* in the sediments, as the sources contaminating the sediments are likely also contaminating the bathing water.

Investigation of how the microbial communities in groundwater respond to remediation strategies revealed only minor changes in the microbial community. While certain groups of bacteria did slightly increase post remediation, such as Clostridia, no significant correlations between bacterial groups of interest and PCE and its metabolites existed. As both abiotic and biotic degradation of these substances can occur, it could not be proven or statistically supported that the microbial groups (both natural community and injected bacteria) were the major degraders of PCE. There were, however, indications that some bacteria were part of the degradation process.

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