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From Promise to Practice: Advancing Bacterial Biomonitoring of Salmon Aquaculture

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Dissertation

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PREFACE

Over the past decade, advances in molecular biology have transformed our ability to study environmental microbial communities. High-throughput sequencing has revealed the remarkable sensitivity of bacteria to ecological disturbance and established microbial communities as promising indicators of ecosystem health. Despite this promise, however, bacterial metabarcoding has remained largely within the domain of research. Routine environmental monitoring continues to rely predominantly on conventional approaches, reflecting the need for greater confidence in the robustness, reproducibility and practical applicability of molecular methods before they can be widely adopted for regulatory assessment.

This dissertation was motivated by a simple question: "*what is required to translate the promise of bacterial biomonitoring into routine environmental practice?"* Rather than focusing solely on the discovery of bacterial indicators, this work addresses the broader scientific requirements necessary for implementation. These include understanding methodological variability, distinguishing ecological change from natural variability, identifying reproducible bioindicators, and exploring practical approaches for their application in routine monitoring. Together, these components form the foundation needed to move bacterial biomonitoring from proof-of-concept towards practical environmental assessment.

To address this objective, the dissertation is organized into four interconnected chapters, each representing a key step in the transition from scientific promise to practical application. Chapter I evaluates the analytical robustness of bacterial metabarcoding by investigating methodological sources of variation and sampling-related variability. Chapter II examines the ecological robustness of bacterial communities by assessing temporal variability and their response to organic enrichment under realistic farming conditions. Building upon this, Chapter III identifies reproducible bacterial bioindicators using complementary analytical approaches validated across independent datasets. Finally, Chapter IV explores the practical implementation of these findings through the development and validation of targeted digital PCR assays for selected bacterial indicators.

Together, these chapters follow a common trajectory: from establishing confidence in bacterial metabarcoding to demonstrating its practical application for environmental assessment. It is this progression that inspired the title of this dissertation, "*From Promise to Practice: Advancing Bacterial Biomonitoring of Salmon Aquaculture."*

General Introduction

1.1 Aquaculture expansion and environmental pressure

The growing global population and increasing demand for high-quality protein place considerable pressure on existing food production systems. Capture fisheries have largely reached their maximum sustainable yield, limiting their capacity to meet future demand (Garcia and Rosenberg, 2010). As a result, aquaculture has emerged as a critical component of the global food supply, providing an increasingly large proportion of aquatic protein consumed worldwide (FAO, 2024; Mair et al., 2023). This rapid expansion has positioned aquaculture as a central component of the so-called "*blue economy*", aligned with the United Nations' Sustainable Development Goals (SDGs) (United Nations, 2015), and framed as a sustainable solution to future food provision (Simpson, 2011; Garlock et al., 2020). Among aquaculture sectors, Atlantic salmon farming represents one of the most economically significant and technologically advanced industries (FAO, 2024), particularly in regions such as Northern Europe. This growth has been driven by increasing market demand, advances in farming technology, and the high economic value of salmon products (Boyd et al., 2022).

Despite its economic importance, intensive salmon aquaculture exerts well-documented pressures on surrounding marine ecosystems, particularly on benthic habitats (Pearson and Black, 2001; Buschmann et al., 2006; Taranger et al., 2015; Tett et al., 2018; Quiñones et al., 2019). The primary driver of these impacts is the deposition of organic material in the form of uneaten feed pellets and fish faeces beneath production cages (Carroll et al., 2003; Keeley et al., 2013; Bannister et al., 2016; Quimpo et al., 2020). These inputs settle rapidly to the seafloor, leading to elevated organic carbon concentrations in the sediment and creating pronounced spatial gradients of enrichment extending outward from the cages (Mayor and Solan, 2011; Keeley et al., 2012; Cranford et al., 2022; Vopel et al., 2026). The accumulation of organic matter stimulates intense microbial decomposition within the sediment (Hou et al., 2022).

As microbial degradation proceeds, oxygen consumption at the sediment-water interface frequently results in hypoxic or anoxic conditions (Gray et al., 2002). Under these conditions, sediment biogeochemistry shifts towards anaerobic pathways, including sulfate reduction and methanogenesis, altering nutrient cycling and redox conditions within the sediment (Holmer et al., 2005; Kalantzi and Karakassis, 2006; Keeley et al., 2013). These biogeochemical changes fundamentally modify the physicochemical environment of the

sediment and create conditions that favor microbial taxa adapted to oxygen-depleted, organically enriched habitats (Ape et al., 2019).

These environmental changes have profound consequences for benthic communities (Pearson and Rosenberg, 1978). Macrofaunal assemblages typically shift towards dominance by opportunistic and pollution-tolerant species, while microbial communities respond rapidly to changes in redox conditions and organic matter availability (Borja et al., 2000; Bae et al., 2017; Shi et al., 2021). In highly enriched sediments, anaerobic and sulfur-cycling bacteria frequently become dominant, reflecting the altered biogeochemical state of the environment (Aranda et al., 2015; Zhang et al., 2020).

Consequently, sediments beneath and surrounding aquaculture installations represent highly dynamic systems in which organic loading, sediment biogeochemistry, and benthic community composition are closely interconnected (Choi et al., 2018). The pronounced spatial structure of these ecological changes highlights the importance of reliable approaches for assessing environmental condition beneath and surrounding salmon farms. A conceptual overview of the environmental gradient associated with organic enrichment beneath Atlantic salmon aquaculture installations is presented in Figure 1.

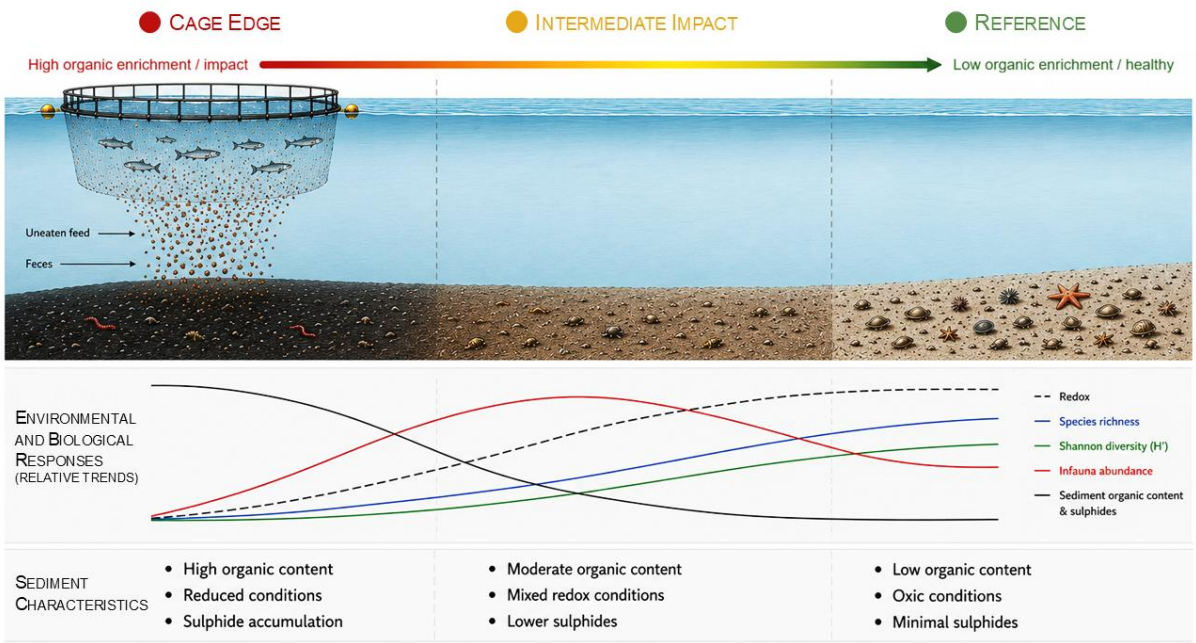


Figure 1. Conceptual schematic illustrating the environmental gradient associated with organic enrichment beneath Atlantic salmon aquaculture cages and the corresponding benthic ecological responses. *Elevated deposition of uneaten feed and feces near cages promotes organic matter accumulation, sulphide production, and reduced sediment conditions, resulting in altered benthic community structure and reduced ecological quality. The figure summarizes the generalized ecological patterns and sediment characteristics commonly associated with benthic responses to salmon aquaculture. Created by the author with AI-assisted illustration tools, inspired by Keeley et al. (2014).*

1.2 Current approaches for benthic impact assessment in aquaculture

The ecological consequences associated with organic enrichment beneath salmon farms extend beyond localized shifts in sediment chemistry and benthic community composition. Because benthic ecosystems underpin many key ecosystem functions, disturbances to these habitats can have far-reaching implications for marine ecosystem functioning. Benthic communities contribute to nutrient regeneration, organic matter degradation, carbon cycling, and overall ecosystem stability (Haese, 2002; Huettel et al., 2014). Consequently, severe organic enrichment can compromise essential ecosystem processes, alter trophic interactions, and reduce benthic biodiversity and resilience (Hale et al., 2016; Xu et al., 2022).

Because these impacts develop along pronounced spatial gradients and persist over time, accurate assessment of benthic condition has become central to sustainable aquaculture management (Wang et al., 2025). Although physicochemical variables such as sulphide

concentration, redox potential and sediment organic content provide valuable information on the intensity of organic loading, they primarily characterize the environmental conditions that drive ecological change. In contrast, benthic biological communities integrate the cumulative effects of multiple stressors over ecologically relevant timescales, providing a direct measure of ecosystem response and ecological integrity (Holt and Miller, 2011; Parmar et al., 2016). Effective management, however, depends on a prerequisite: the ability to assess environmental impact accurately and consistently (Caro-Gonzalez et al., 2021). To that end, regulatory frameworks have been established globally to monitor and manage the environmental consequences of aquaculture. In marine environments, this includes legislative instruments such as the European Water Framework Directive (WFD, 2000), which establishes standards for assessing and maintaining ecological status, as well as comparable national regulatory frameworks in other regions, such as the New Zealand Coastal Policy Statement (2010) and Scotland's Strategic Environmental Assessment framework (Thompson et al., 2010).

In the context of salmon aquaculture, environmental monitoring is implemented throughout different stages of farm development and operation (Wilson et al., 2009). Prior to the establishment or expansion of aquaculture installations, Environmental Impact Assessments (EIAs) are conducted to evaluate the potential ecological consequences of proposed activities (Phillips et al., 2009). Once farms become operational, routine compliance monitoring is required to assess ongoing benthic environmental condition and ensure adherence to regulatory standards (Keeley et al., 2014).

Because the ecological impacts of organic enrichment are most strongly expressed within seabed sediments beneath and surrounding production cages, benthic communities have become central components of aquaculture monitoring frameworks (Papageorgiou et al., 2009; Sanz-Lázaro and Marín, 2011). Among these, benthic macrofaunal assemblages have long been used as biological indicators of environmental disturbance due to their sensitivity to changes in oxygen availability, organic enrichment, and sediment geochemistry (Borja et al., 2000, 2003, 2009; Muxika et al., 2005). Predictable shifts in macrofaunal community structure, including reductions in biodiversity and increased dominance of opportunistic and pollution-tolerant taxa, provide the basis for assessing ecological condition beneath salmon farms.

Over the past decades, numerous benthic indicators and biotic indices have been developed and evaluated for the assessment of aquaculture-derived environmental impacts (Borja and Dauer, 2008). These approaches include indices based on trophic structure, species

sensitivity, diversity, abundance, biomass, and community composition (Edgar et al., 2005; Aguado-Giménez et al., 2007). Among the most widely applied are the Infaunal Trophic Index (ITI) (Word, 1979) and AZTI's Marine Biotic Index (AMBI) (Borja et al., 2000), both of which have been successfully used to assess benthic impacts associated with marine aquaculture across different environmental settings (Maurer et al., 1999; Carvalho et al., 2006; Bouchet and Sauriau, 2008).

Consequently, macrofaunal analyses form the foundation of many regulatory monitoring programs in marine aquaculture, including those implemented by the Scottish Environment Protection Agency (SEPA, 2023). In Scotland, ecological condition beneath salmon farms is commonly assessed using the Infaunal Quality Index (IQI), a multimetric benthic index developed within the framework of the European WFD (Phillips et al., 2014). IQI integrates multiple components of macrofaunal community structure, including taxonomic composition, diversity, abundance patterns, and species-specific sensitivity to organic enrichment, the latter commonly represented through the incorporation of AMBI (Culhane et al., 2014). By combining these ecological metrics into a standardized assessment framework, IQI enables classification of benthic ecological status along gradients of anthropogenic disturbance and currently forms a central component of regulatory compliance monitoring in Scottish marine aquaculture (Phillips et al., 2014). A simplified overview of benthic community responses and associated ecological status changes, as well as IQI boundaries along gradients of organic enrichment is presented in Figure 2.

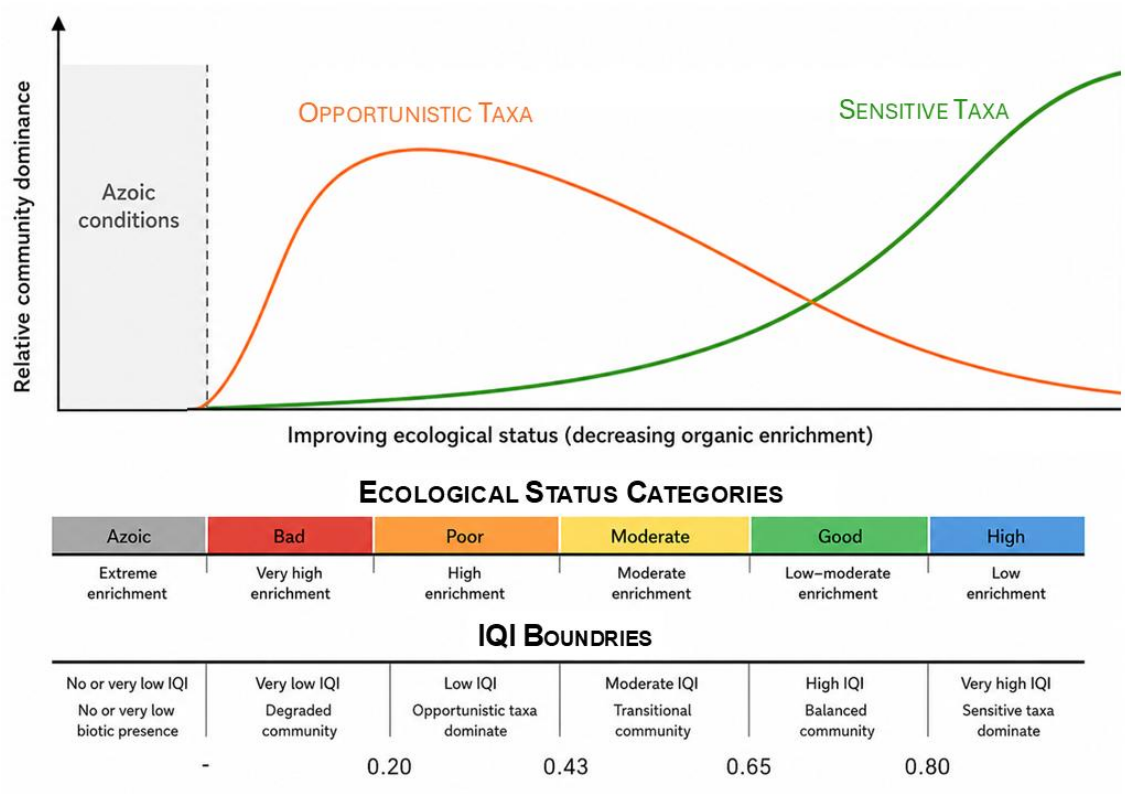


Figure 2. Conceptual representation of simplified benthic community responses and ecological status transitions along gradients of increasing organic enrichment beneath salmon aquaculture installations. *Increasing organic loading is associated with declining dominance of sensitive taxa and increasing prevalence of opportunistic and pollution-tolerant taxa, ultimately resulting in severely degraded or azoic conditions under extreme enrichment. Adapted conceptually from Pearson and Rosenberg (1978), Borja et al. (2000), and Phillips et al. (2014).*

Although macrofaunal indices provide ecologically meaningful assessments of benthic condition and remain central to regulatory compliance monitoring, their implementation is associated with several practical and methodological limitations. Traditional benthic assessments rely heavily on labor-intensive sample processing and taxonomic identification, requiring substantial expertise, time, and financial resources (Goodwin et al., 2017). Because accurate species-level identification is pivotal for these indices, taxonomic misidentifications can directly influence ecological quality assessments (Martinez-Crego et al., 2010), particularly when different developmental or life stages of organisms are difficult to distinguish morphologically (Darling and Mahon, 2011). Consequently, reliable assessment requires highly trained taxonomic specialists (Schander and Willassen, 2005), while sample processing and identification workflows may take weeks or even months before ecological quality status can be determined (Aylagas et al., 2014). These constraints can limit monitoring scalability

and restrict the capacity for timely and effective environmental management (Hajibabaei et al., 2011; Lejzerowicz et al., 2015; Danovaro et al., 2016), highlighting the need for complementary approaches capable of providing rapid, scalable, and reliable assessment of benthic environmental condition.

1.3 Environmental DNA and molecular approaches to biomonitoring

The limitations associated with traditional morphology-based monitoring approaches have driven increasing interest in molecular methods capable of transforming environmental assessment in marine ecosystems. The emergence of environmental DNA (eDNA) technologies has fundamentally reshaped the way ecological systems can be observed and assessed (Shokralla et al., 2012). eDNA refers to genetic material released into the environment by organisms through cells, tissues, secretions, or other biological material that can be recovered directly from environmental samples such as water or sediment (Taberlet et al., 2012). Because eDNA can be collected without isolating or visually identifying individual organisms, it provides a non-invasive means of characterizing biological communities and assessing biodiversity.

The analytical potential of eDNA has been realized through advances in high-throughput sequencing technologies, which enable the simultaneous analysis of large and complex biological communities from environmental samples (Taberlet et al., 2018). Improvements in sequencing throughput, read quality, and cost efficiency have substantially increased the capacity to characterize biodiversity and community composition across diverse ecosystems (Baird and Hajibabaei, 2012; Taberlet et al., 2012), thereby providing access to biological information at resolutions that were previously unattainable using conventional methods (Baird et al., 2012; Valentini et al., 2016).

A key application of high-throughput sequencing in environmental research is metabarcoding, in which taxonomically informative genetic marker regions are amplified from eDNA and sequenced simultaneously from complex biological samples (Taberlet et al., 2012; Deiner et al., 2017). The resulting sequence data are clustered into amplicon sequence variants (ASVs), providing high-resolution information on community composition without the need for individual organism isolation or morphological identification (Callahan et al., 2017; Taberlet et al., 2018). By enabling comprehensive characterization of biological communities

from a single environmental sample, metabarcoding has become one of the most widely adopted molecular approaches for biodiversity assessment and biomonitoring.

Early applications of metabarcoding in environmental assessment primarily focused on evaluating the capacity of molecular approaches to reproduce taxonomic inventories generated through conventional morphology-based identification of bioindicator communities (e.g., Hajibabaei et al., 2012; Carew et al., 2013). As sequencing technologies and analytical frameworks advanced, it became increasingly evident that molecular data could provide ecological information beyond simple taxonomic inventories. Rather than merely reproducing morphology-based species lists, bacterial community composition itself could be used to infer ecological condition by identifying consistent responses to environmental gradients and anthropogenic disturbance (e.g., Pawlowski et al., 2014; Pascault et al., 2014). This transition transformed metabarcoding from a tool for taxonomic identification into a promising approach for aquatic environmental assessment (Stat et al., 2017; Aylagas et al., 2018; Pawlowski et al., 2021; Oladi et al., 2022).

The potential of eDNA metabarcoding for environmental assessment has stimulated growing interest in its application to aquaculture biomonitoring (Figure 3). Numerous studies have demonstrated the ability of metabarcoding approaches to characterize biological responses to organic enrichment beneath fish farms, using a range of indicator groups including macrofauna, meiofauna, protists, and microbial communities (Pochon et al., 2015; Pawlowski et al., 2014, 2016; Stoeck et al., 2018; He et al., 2021; Nguyen et al., 2026). These studies have shown that molecular data can effectively discriminate between impact levels and capture ecological gradients associated with aquaculture-derived disturbance. Among the potential indicator groups, sediment bacterial communities have attracted considerable attention due to their close association with sediment biogeochemical processes and rapid response to organic enrichment (Nogales et al., 2011; Dowle et al., 2015; Aylagas et al., 2017; Stoeck et al., 2018).

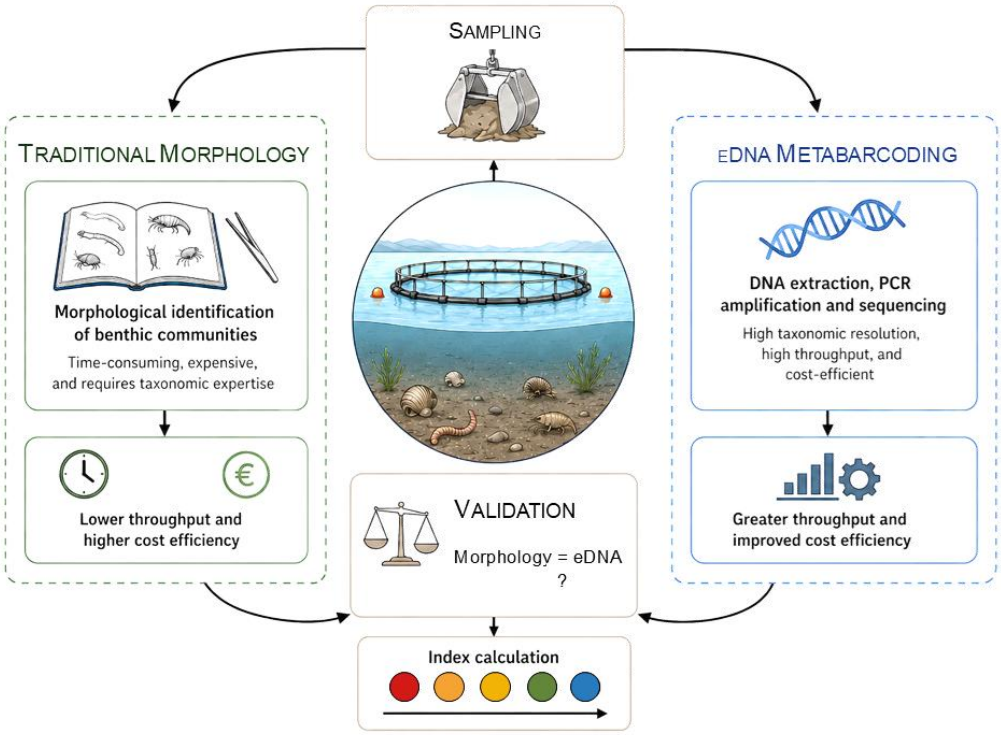


Figure 3. Overview of the principal approaches used in the environmental impact assessment of salmon farms. *Routine compliance biomonitoring has traditionally relied on morphological identification of benthic communities, which is time-consuming and expensive, and demands taxonomic expertise (left panel). In contrast, eDNA metabarcoding offers higher taxonomic resolution, greater throughput, and improved cost efficiency, substantially enhancing the capacity to characterize biodiversity and community composition for environmental impact assessment (right panel). However, before molecular approaches can be adopted as routine regulatory monitoring tools, their ecological assessments must be rigorously validated against established morphology-based methods to demonstrate that they provide comparable and reliable measures of environmental status. Created by the author with AI-assisted illustration tools with inspiration from Pawlowski et al. (2018).*

Altogether, the aforementioned studies established bacterial metabarcoding as a promising alternative for benthic environmental assessment. However, demonstrating ecological sensitivity alone does not ensure that molecular approaches are sufficiently robust, reproducible, or practical for routine biomonitoring. Addressing these requirements represents the next stage in the development of bacterial biomonitoring.

1.4 Bacterial communities as indicators of environmental change

Microbial communities are fundamental components of sediment ecosystems, driving many of the biogeochemical processes that underpin ecosystem functioning (Lim et al., 2011; Madsen, 2011; Liu et al., 2021). Often described as the "*hidden backbone*" of ecosystems, microorganisms regulate nutrient cycling, support food-web structure, and contribute to ecosystem stability and resilience (Jousset et al., 2017; Sagova-Mareckova et al., 2021). Through their involvement in organic matter degradation, nutrient regeneration, carbon cycling, and sulfur transformations, sediment microbes play a central role in maintaining ecosystem functioning and the delivery of ecosystem services (Falkowski et al., 2008; Bodelier, 2011; Miki et al., 2014). Because these processes are tightly coupled to local physicochemical conditions, microbial communities respond rapidly to changes in organic matter availability, oxygen concentration, redox state, and nutrient fluxes, often preceding observable responses in higher trophic levels (Bisset et al., 2007; Reimers et al., 2013). This close coupling between microbial assemblages and sediment conditions makes them particularly attractive candidates for environmental assessment and biomonitoring (Paerl et al., 2006; Kawahara et al., 2009; Nogales et al., 2011).

Among sediment microorganisms, bacteria are particularly attractive for environmental assessment because they dominate many of the biogeochemical processes associated with organic matter degradation and nutrient cycling (Falkowski et al., 2008; Dowle et al., 2015). Their short generation times and rapid metabolic responses enable bacterial communities to respond quickly to changes in environmental conditions, making them sensitive indicators of ecosystem disturbance (Smith et al., 2015; Aylagas et al., 2017).

In aquaculture environments, these microbial communities in particular have attracted increasing attention as potential indicators of organic enrichment because of their rapid responsiveness to environmental change and their pivotal involvement in sediment biogeochemical processes (Bissett et al., 2006; Kawahara et al., 2009; Dowle et al., 2015; Fodelianakis et al., 2015). As bacteria directly mediate many of the biogeochemical transformations affected by organic loading, changes in sediment conditions are often rapidly reflected in their community composition. Numerous studies have consequently documented consistent shifts in bacterial assemblages along aquaculture-derived enrichment gradients, highlighting their potential as sensitive indicators of environmental disturbance (McCaig et al.,

1999; Bissett et al., 2006; Keeley et al., 2018; Stoeck et al., 2018; Cordier, 2020; Frühe et al., 2021; Wilding et al., 2023).

Although bacteria possessed these ecological characteristics all along, their use in biomonitoring remained limited until advances in high-throughput sequencing and metabarcoding enabled comprehensive characterization of bacterial communities directly from environmental samples (Sogin et al., 2006; Taberlet et al., 2012). These advances revealed that bacterial communities respond predictably to organic enrichment associated with fish farming activities, exhibiting shifts in diversity, composition, and functional structure that often parallel environmental gradients identified through conventional benthic monitoring approaches (Dowle et al., 2015; Keeley et al., 2018; Verhoeven et al., 2018; Stoeck et al., 2018; Frühe et al., 2021; Wilding et al., 2023; Liu et al., 2024). Although these studies clearly demonstrate that bacterial communities respond predictably to organic enrichment, the development of routine biomonitoring tools requires the identification of indicators that respond consistently across different methodological approaches, environmental conditions, and temporal scales (Mathieu et al., 2020).

A variety of analytical approaches have been applied to bacterial metabarcoding datasets to infer environmental conditions and identify candidate microbial indicators in aquaculture-impacted sediments. These include approaches aimed at ecological quality prediction, such as quantile regression spline (QRS) modelling and supervised machine-learning algorithms, as well as methods designed to identify enrichment-responsive taxa, including indicator value analysis (Verhoeven et al., 2018; Keeley et al., 2018; Leontidou et al., 2023; Johansen et al., 2024; Rubel et al., 2025; Wyness et al., 2026). Together, these approaches have shown that bacterial community data can be used both to approximate established macrofaunal indices and ecological quality classes, and to identify specific ASVs that respond consistently to organic enrichment gradients.

While these advances have substantially improved the capacity to identify candidate microbial indicators, translating observed community responses into robust and broadly applicable biomonitoring tools remains a significant challenge (Cordier et al., 2021). Although many bacterial taxa exhibit consistent responses to organic enrichment, observed community patterns may reflect not only environmental disturbance, but also methodological artefacts and natural ecological variability. Consequently, a clearer understanding of the different sources of

variation underlying bacterial metabarcoding data is required before microbial indicators can be reliably integrated into routine monitoring frameworks.

1.5 Requirements for routine bacterial biomonitoring tools

As bacterial metabarcoding continues to mature as a tool for environmental assessment, the central challenge is no longer simply demonstrating that microbial communities respond to anthropogenic disturbance. Numerous studies have shown that bacterial assemblages exhibit predictable shifts along environmental gradients and possess considerable potential for ecological assessment (e.g., Dowle et al., 2015; Keeley et al., 2018; Stoeck et al., 2018; Frühe et al., 2021; Liu et al., 2024). The successful implementation of these observations into routine biomonitoring, however, requires considerably more than responsive microbial communities alone. Reliable biomonitoring depends on the development of methods that are analytically robust, ecologically interpretable, reproducible across studies, and ultimately suitable for practical implementation within regulatory monitoring programmes. More generally, effective environmental monitoring relies on maximizing the ratio between impact-related ecological signal and unrelated methodological or biological noise, thereby improving the reliability and efficiency of ecological assessment (Wilding et al., 2023).

Meeting these requirements is challenging because bacterial assemblages are influenced by multiple sources of variation that can operate simultaneously across spatial and temporal scales (Sadeghi et al., 2021; Tian et al., 2022). Laboratory methodologies may introduce technical biases (Goldberg et al., 2016; Dully et al., 2021a, b), while natural ecological dynamics can generate substantial variability independent of anthropogenic disturbance (Böer et al., 2009). In parallel, organic enrichment associated with aquaculture activities drives additional shifts in community composition through alterations in sediment biogeochemistry and ecosystem functioning. Together, these processes complicate the interpretation of microbial community patterns in environmental monitoring programmes. Each of these sources of variation must therefore be considered when developing bacterial biomonitoring approaches capable of producing reliable and reproducible assessments.

Among these, methodological variation represents the most immediate challenge because it originates from the analytical workflow itself and can influence observed community patterns independently of environmental conditions. The generation of metabarcoding data involves multiple analytical steps, including sample collection, preservation, DNA extraction,

Polymerase Chain Reaction (PCR) amplification, sequencing, and bioinformatic processing, each of which has the potential to introduce technical biases (Hiergeist et al., 2016; Boers et al., 2019; Ashley-Wheeler et al., 2026). Consequently, numerous studies have investigated the influence of sampling strategies, sample preservation, DNA extraction procedures, sequencing depth, PCR amplification protocols, and bioinformatic processing on microbial community characterization (Pearman et al., 2020; Dully et al., 2021a, b; Hestetun et al., 2021; Ashley-Wheeler et al., 2026). Collectively, these studies demonstrate that variability may be introduced throughout the metabarcoding workflow, emphasizing the importance of standardized methodologies capable of generating reproducible ecological assessments.

A second requirement for routine biomonitoring is that bacterial indicators remain reliable despite natural ecological variability. Environmental indicators must also remain reliable despite the natural ecological variability that characterizes marine ecosystems (Mathieu et al., 2020). Bacterial assemblages are shaped by a complex interplay of biotic and abiotic factors, including temperature, nutrient availability, primary production, hydrodynamic conditions, and seasonal cycles (Delille, 1995; Findlay and Watling, 1998). Such natural ecological variability can result in pronounced temporal changes in community structure, even in the absence of anthropogenic disturbance. Seasonal variation has received particular attention in microbial ecology because temperature-driven changes in metabolic activity and resource availability can influence both the composition and functioning of sediment bacterial communities (Verhoeven et al., 2018; Quero et al., 2020; Johansen et al., 2024). For bacterial biomonitoring to be integrated into existing regulatory programmes, these natural dynamics must not obscure the ecological signal associated with aquaculture-derived organic enrichment.

The ultimate objective of bacterial biomonitoring is the identification of microbial indicators that respond consistently to environmental disturbance while remaining robust to both methodological and natural ecological variability (Wilding et al., 2023). Organic enrichment beneath salmon farms associated with aquaculture activities also induces pronounced shifts in microbial assemblages (Holmer et al., 2005; Kawahara et al., 2009; Tamminen et al., 2011; Frühe et al., 2021; Johansen et al., 2024). The organic enrichment gradients generated beneath fish farms alter sediment biogeochemistry and create strong selective pressures that restructure bacterial communities, leading to predictable changes in community composition along impact gradients (Bissett et al., 2007; Keeley et al., 2018; Frühe et al., 2021). Unlike many forms of natural variability, these responses are often directional, reflecting progressive changes in environmental conditions associated with increasing organic

loading and oxygen depletion (Verhoeven et al., 2018). As a result, numerous studies have reported consistent increases in enrichment-associated taxa and corresponding declines in microorganisms characteristic of well-oxygenated reference sediments across geographically distinct aquaculture systems (Dowle et al., 2015; Keeley et al., 2018; Stoeck et al., 2018; Frühe et al., 2021; Johansen et al., 2024). The reproducibility of these patterns has formed the basis for the development of bacterial indicators intended to infer environmental conditions and ecological quality status in aquaculture monitoring programs (Keeley et al., 2018; Stoeck et al., 2018; Cordier, 2020; Frühe et al., 2021). However, confidence in these indicators depends not only on their ecological responsiveness, but also on their reproducibility across analytical approaches, datasets, and environmental contexts.

Ultimately, the transition from promising molecular techniques to routine biomonitoring depends on building scientific confidence at every stage of the workflow. Such confidence arises not from demonstrating microbial responses alone, but from showing that those responses remain robust to methodological variation, resilient to natural ecological variability, reproducible across analytical approaches and datasets, and translatable into practical monitoring tools (Mathieu et al., 2020). **Together, these four requirements form the conceptual framework of this dissertation and provide the foundation for the research presented in the following chapters.**

Identifying robust bacterial indicators represents an important milestone, but practical biomonitoring ultimately requires tools capable of translating these indicators into rapid, standardized assessments. A recent study has combined metabarcoding with complementary approaches such as shotgun metagenomics to validate enrichment-responsive taxa, increasing confidence in their robustness, transferability, and suitability for subsequent development into targeted molecular assays (Rubel et al., 2025). A logical next step is the translation of sequencing-derived indicators into quantitative decision-support frameworks capable of directly measuring changes in indicator abundance across environmental gradients. Such an approach would complement community-level metabarcoding by enabling the targeted quantification of selected indicator taxa. Digital PCR (dPCR) is particularly well suited for this purpose because it enables highly sensitive and absolute quantification of target DNA molecules without reliance on standard curves (Quan et al., 2018; Salipante and Jerome, 2020), providing a promising route for translating sequencing-derived microbial indicators into practical monitoring tools. The integration of validated bacterial indicators with targeted molecular quantification represents an important step towards the development of rapid, cost-

effective decision-support tools capable of complementing routine environmental assessment in aquaculture.

1.6 Digital PCR and quantitative molecular biomonitoring

Digital PCR (dPCR), also known as the third generation of PCR technology after the conventional end-point PCR and real-time quantitative PCR (qPCR), is an emerging DNA detection method that provides absolute quantification of target DNA (Quan et al., 2018). It is based on the partitioning of a PCR reaction into thousands of individual microreactions, in which target DNA molecules are randomly distributed (Figure 4B; Mao et al., 2019). During PCR amplification, a fluorescent dye or sequence-specific fluorescent probe generates a signal in partitions where the target sequence is successfully amplified, whereas partitions lacking the target remain non-fluorescent (Figure 4C). Following amplification, each partition is therefore classified as either positive or negative based on its fluorescence intensity, and the number of target molecules in the original sample is estimated using Poisson statistics (Figure 4D & E; Poisson, 1837; Tiwari et al., 2022). These characteristics offer several advantages for environmental biomonitoring, including absolute quantification without reliance on standard curves, high sensitivity for low-abundance targets, and improved robustness to PCR inhibitors present in complex environmental matrices (Figure 4F; Hindson et al., 2011; Nathan et al., 2014; Doi et al., 2015).

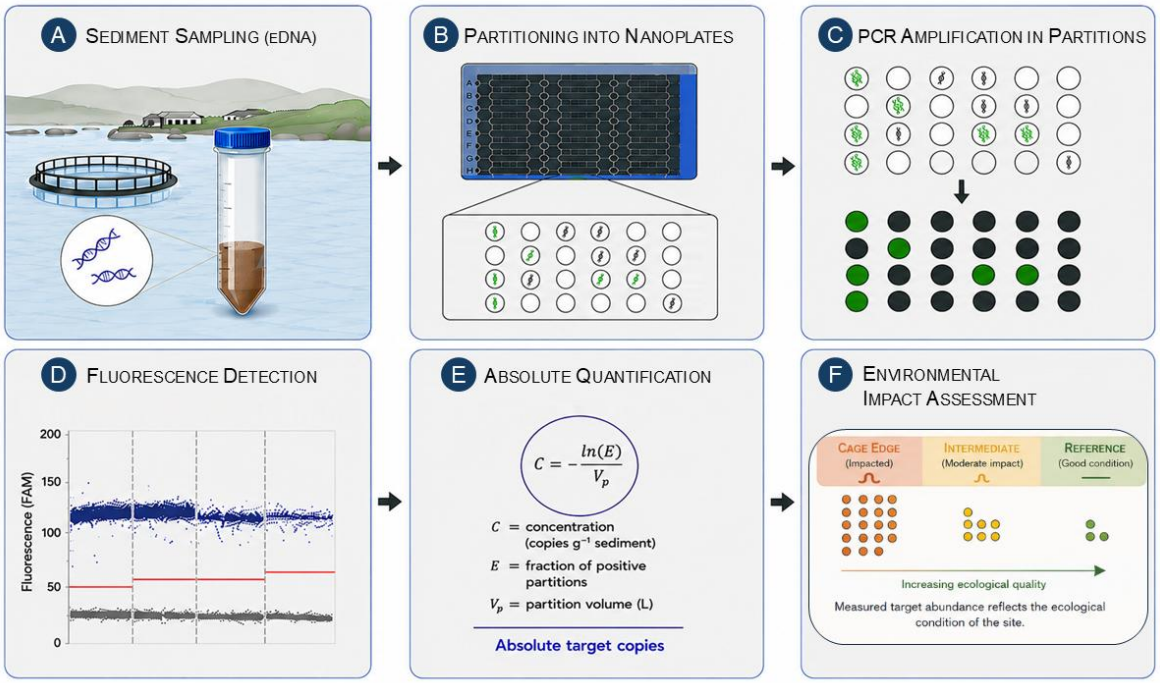


Figure 4. Conceptual workflow illustrating the application of dPCR for eDNA-based biomonitoring of salmon aquaculture impacts. *Surface sediment samples are collected from salmon farm sites and subjected to eDNA extraction (A). DNA extracts are partitioned into thousands of individual nanowells on a nanoplate, resulting in the random distribution of target DNA molecules (green) and non-target DNA molecules (black) among partitions (B). PCR amplification occurs independently within each partition, with only those partitions containing the target sequence generating a positive amplification signal (green) following PCR (C). Fluorescence signals are used to classify partitions as positive or negative for the target sequence (D). The proportion of positive partitions is then converted to absolute target abundance using Poisson statistics, enabling direct quantification of target DNA copies (E). Absolute abundances of environmental indicator targets can subsequently be interpreted within the context of benthic ecological status, where changes in the target copy numbers may reflect different levels of organic enrichment and environmental impact from salmon aquaculture activities (F). The conceptual example shown in panel F illustrates a high-impact indicator whose abundance increases along the enrichment gradient. Created by the author with AI-assisted illustration tools.*

The ability to accurately quantify specific DNA targets has led to the increasing application of dPCR in environmental sciences. Environmental DNA studies have successfully employed dPCR for the detection and quantification of invasive species, endangered organisms, pathogens, microbial source-tracking markers, and antimicrobial resistance genes across a wide range of aquatic environments (Blaya et al., 2015; Doi et al., 2015; Baker et al., 2018; Schweiss et al., 2020; Shea et al., 2020; Harringer and Alfreider, 2021; Condachou et al., 2024). More recently, dPCR has been increasingly applied to environmental surveillance

and water-quality monitoring, where accurate quantification of low-abundance molecular targets is often required (Coertze and Bezuidenhout, 2018; Vezzulli et al., 2021; Mirabile et al., 2024). These applications demonstrate the capacity of dPCR to provide sensitive and reproducible measurements of molecular targets across a wide range of environmental matrices.

While dPCR has become increasingly established for the quantification of microbial pathogens, source-tracking markers, and antimicrobial resistance genes in environmental samples (e.g., Netzer et al., 2021; Tiwari et al., 2022), its application to microbial indicators of environmental condition and ecological quality assessment remains comparatively unexplored. Most microbial biomonitoring approaches in aquaculture continue to rely on community-level metabarcoding data and relative abundance patterns. Consequently, the potential for targeted dPCR assays to quantify enrichment-responsive bacterial indicators and support environmental assessment has received little attention.

1.7 Aims and objectives

The overarching aim of this thesis was to evaluate the potential of sediment bacterial communities as biomonitoring tools for assessing environmental impacts associated with salmon aquaculture and to advance their application towards routine environmental monitoring. Developing reliable bacterial biomonitoring tools requires more than demonstrating microbial responses to organic enrichment. Robust microbial indicators must be analytically robust to methodological variation, resilient to natural ecological variability, reproducible across datasets and analytical approaches, and amenable to practical molecular implementation. Achieving these requirements demands an understanding of how technical, ecological, and impact-related processes shape bacterial community composition and influence the identification of reliable bacterial indicators. This thesis therefore combined methodological evaluation, ecological validation, consensus indicator selection, and quantitative assay development to establish the scientific foundations for identifying and validating bacterial indicators suitable for routine molecular biomonitoring. To achieve this aim, the following objectives were addressed:

i. To evaluate the influence of methodological sources of variation, including PCR amplification protocols and sampling-related variability, on bacterial metabarcoding-based environmental assessment, and to determine whether these sources of variation

limit the robustness of bacterial biomonitoring relative to conventional macrofaunal approaches, which is addressed in Chapter I, "*Methodological Sources of Variation in Bacterial Metabarcoding-Based Biomonitoring*."

ii. To determine whether natural temporal variability alters bacterial community responses to aquaculture-derived organic enrichment, thereby evaluating the ecological robustness of bacterial indicators over time, which is addressed in Chapter II, "*Disentangling Natural and Impact-Induced Dynamics in Sediment Bacterial Communities*."

iii. To identify bacterial bioindicators that exhibit consistent and reproducible responses to organic enrichment across complementary analytical approaches and independent datasets, which is addressed in Chapter III, "*Identification of Robust Bacterial Indicators for Aquaculture Biomonitoring*."

iv. To evaluate the feasibility of translating sequencing-derived bacterial bioindicators into targeted quantitative assays using digital PCR, thereby advancing their application in routine molecular biomonitoring, which is addressed in Chapter IV, "*Quantitative Validation of Bacterial Bioindicators Using Digital PCR*."

Chapters I, II, and IV have been published as:

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Altogether, these objectives define a pathway from the promise of bacterial biomonitoring towards its practical implementation. This dissertation approaches this transition as a bridge that must be built progressively: from establishing methodological and ecological robustness, through identifying reproducible bacterial indicators, to evaluating their translation into targeted quantitative assays. Figure 5 illustrates this framework and the four research questions that form the foundation of the bridge towards routine bacterial biomonitoring.

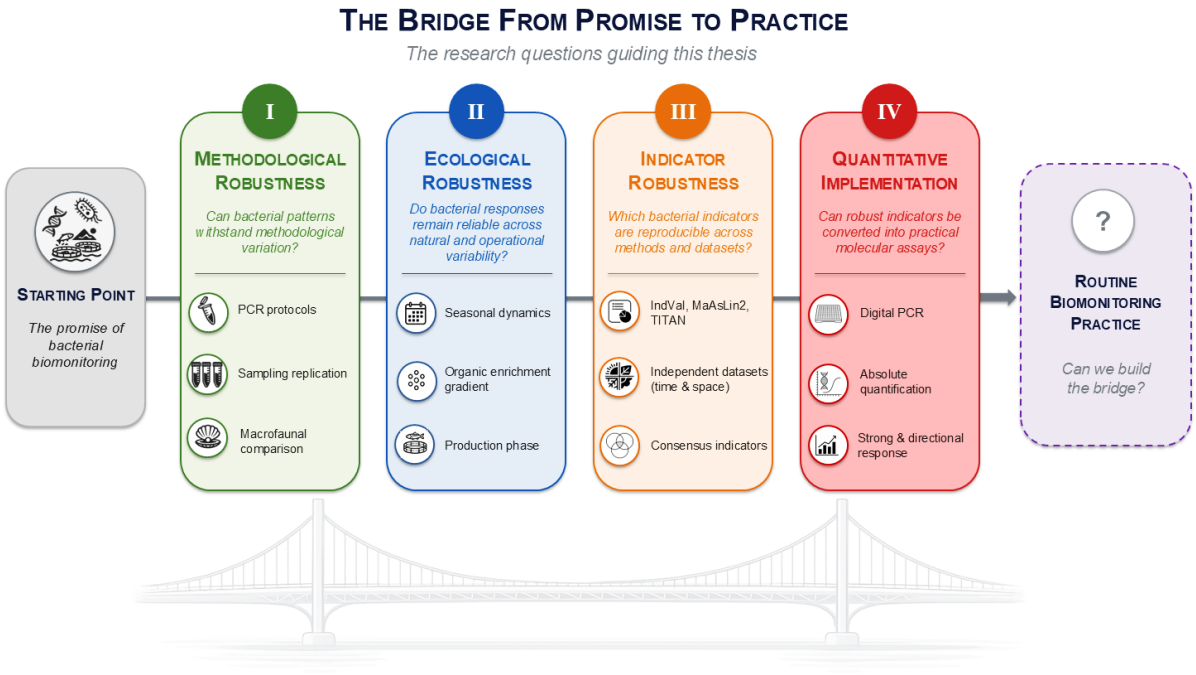


Figure 5. Conceptual framework and progression of the thesis. *The four chapters address complementary requirements for advancing bacterial biomonitoring of salmon aquaculture towards practice, progressing from methodological and ecological robustness to the identification of reproducible bacterial indicators and their translation into targeted quantitative tools.*

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CHAPTER I

METHODOLOGICAL SOURCES OF VARIATION IN BACTERIAL METABARCODING-BASED BIOMONITORING

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2.1 Summary

2.1.1 Background

The application of bacterial metabarcoding to environmental monitoring has expanded rapidly over the last decade, with numerous studies demonstrating strong responses of sediment bacterial communities to organic enrichment associated with salmon aquaculture (Dowle et al., 2015; Keeley et al., 2018; Stoeck et al., 2018; Cordier, 2020; Frühe et al., 2021; Wilding et al., 2023). Despite this progress, the successful implementation of bacterial community analyses into routine compliance monitoring requires more than the identification of ecological patterns. It is equally important to establish that observed community differences reflect genuine environmental variation rather than methodological artefacts (Alberdi et al., 2018; Bruce et al., 2021).

Methodological sources of variation may arise at multiple stages of the metabarcoding workflow, including sample collection, laboratory processing, PCR amplification, sequencing, and bioinformatic analysis (Alberdi et al., 2018; Bruce et al., 2021; Dully et al., 2021a). Consequently, considerable effort has been devoted to standardizing eDNA workflows and evaluating the effects of methodological decisions such as sample preservation (Dully et al., 2021b; Ashley-Wheeler et al., 2026), DNA extraction (Hestetun et al., 2021b; Ruan et al., 2022; Pawlowski et al., 2022), sequencing depth (Dully et al., 2021c), and analytical reproducibility (Dully et al., 2021a). Despite these advances, PCR amplification remains one of the most widely recognized sources of technical variation in metabarcoding workflows, and previous studies have demonstrated that methodological choices during PCR amplification and downstream sequencing may influence estimates of community composition (Kennedy, 2014; Boers et al., 2019). However, it remains unclear whether such technical variation is sufficiently large to affect ecological interpretations and biomonitoring conclusions, particularly in the context of environmental assessments conducted across different laboratories and monitoring providers. If methodological sources of variation substantially affect estimates of community composition, they may compromise the reliability, reproducibility, and comparability of biomonitoring outcomes.

In addition to laboratory-derived sources of variation, reliable biomonitoring requires an understanding of sampling-related variability. Environmental samples are inherently heterogeneous, and differences among replicate samples may arise from both sampling error and natural small-scale spatial variability (McBean and Rovers, 1985). In conventional

macrofaunal monitoring, such variability is accounted for through the collection of multiple sediment grab replicates from each sampling location (Pochon et al., 2020; Lejzerowicz et al., 2015). Similar patchiness has also been documented for benthic microbial communities, highlighting the importance of replicate sampling in eDNA-based monitoring programmes (Hestetun et al., 2021a, b; Beentjes et al., 2019; Dully et al., 2021a; Wilding et al., 2023). However, the magnitude and sources of sampling-related variability in bacterial metabarcoding datasets, and the extent to which this variability influences ecological interpretations, remain insufficiently characterized. Furthermore, for bacterial eDNA approaches to serve as viable alternatives to conventional monitoring methods, the magnitude of sampling-related background noise must be understood and should not substantially exceed that observed in established macrofaunal assessments (Wilding et al., 2023). Quantifying this variability is therefore essential for evaluating the practical suitability of bacterial metabarcoding for routine compliance monitoring.

Accordingly, this chapter evaluates two key methodological sources of variation in bacterial metabarcoding-based biomonitoring. First, the influence of alternative PCR amplification protocols on bacterial community assessments was examined. Second, sampling-related variability was quantified and compared directly with the background noise observed in conventional macrofaunal monitoring. Taken together, these analyses provide a critical assessment of the robustness and reproducibility of bacterial metabarcoding and represent an important step towards its implementation in routine environmental monitoring of salmon aquaculture impacts.

2.1.2 Methods

Sediment samples were collected in March 2022 from two Atlantic salmon farms located on the west coast of Scotland (CRE and DUN), representing contrasting production states at the time of sampling (Wilding et al., 2012). At each farm, sampling was conducted along an organic enrichment gradient comprising the cage edge (CE), intermediate (Allowable Zone of Effect; AZE), and reference (REF) stations. Three replicate Van Veen grab samples were collected at each station. From every grab, three independent sediment subsamples were collected for eDNA-based bacterial metabarcoding, and the rest was retained for conventional macrofaunal analysis (SEPA, 2023). A schematic overview of the sampling design and replication structure is provided in Figure 6.

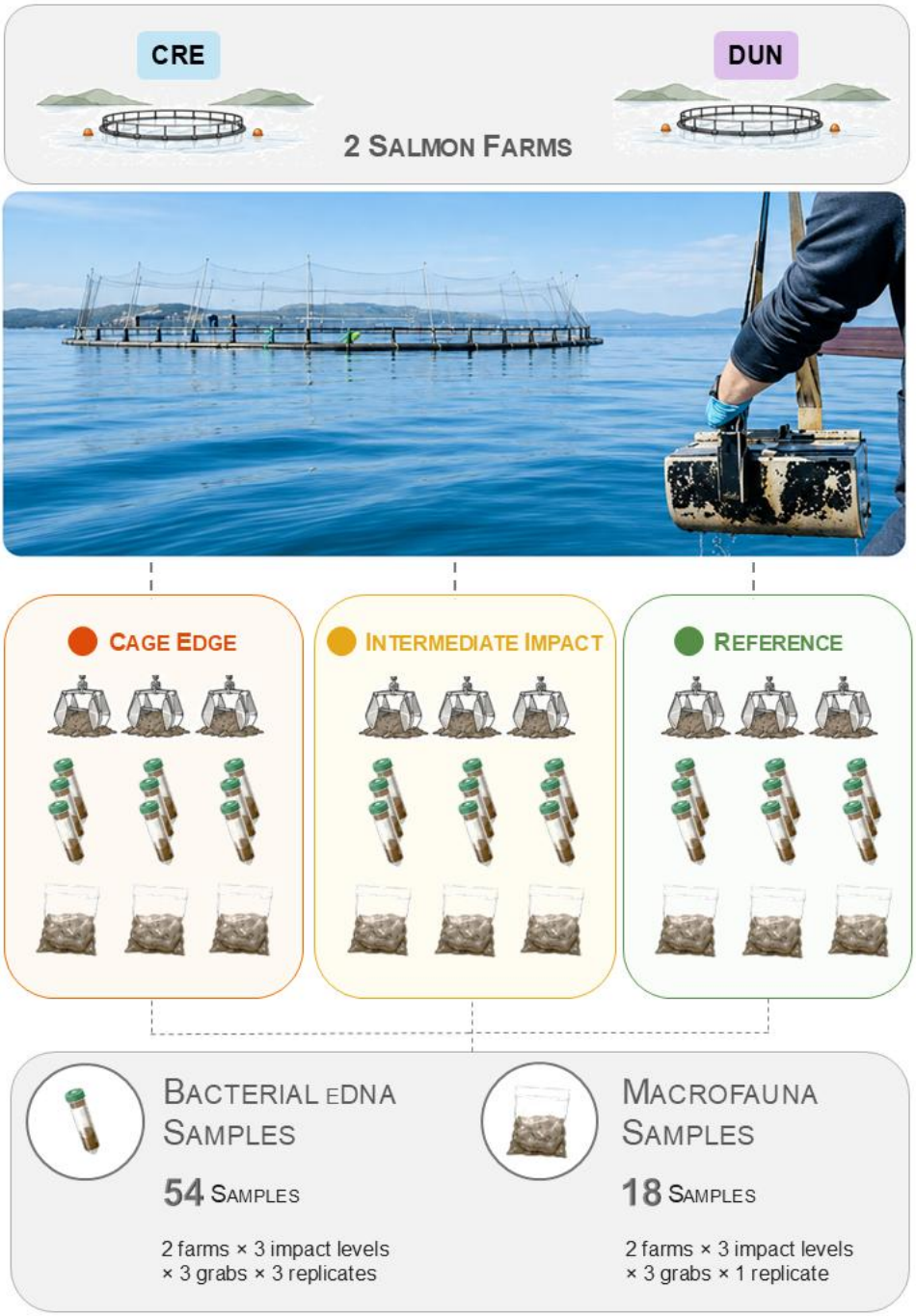


Figure 6. Overview of the sampling design. *Sediment samples were collected from two salmon farms (CRE and DUN) across three impact categories representing an organic enrichment gradient: cage edge, intermediate impact, and reference stations. Three replicate grab samples were obtained at each station, with each grab subsequently processed for both macrofaunal analysis and bacterial eDNA metabarcoding. This resulted in a total of 54 eDNA samples (2 farms × 3 stations × 3 grabs × 3 subsamples) and 18 macrofauna samples (2 farms × 3 stations × 3 grabs). The design enabled direct comparison of sampling-related variability between conventional and molecular biomonitoring approaches.*

Following collection, sediment samples designated for bacterial metabarcoding were frozen at -20°C within a few hours of sampling and stored until DNA extraction. The remaining sediment for macrofaunal analysis was sieved through a 1 mm mesh and the retained fauna was preserved in 4% borax-buffered formaldehyde. Macrofaunal specimens were subsequently identified to the lowest practical taxonomic level by a certified taxonomic laboratory.

DNA was extracted from sediment samples using the DNeasy PowerSoil Pro Kit (Qiagen), and bacterial communities were characterized by targeting the V3-V4 16S rRNA gene. To assess the influence of PCR methodology on biomonitoring outcomes, aliquots of the same DNA extracts were amplified using two commonly applied first-stage PCR protocols that differed in DNA polymerase (Roche KAPA HiFi HotStart and NEBs Phusion® High-Fidelity) and amplification conditions (e.g., protocol 1; Wilding et al., 2023 vs. protocol 2; Dully et al., 2021b), while all subsequent library preparation and sequencing steps were standardized. Amplicon libraries were sequenced on an Illumina MiSeq platform (2 × 300 bp).

Raw sequence data were processed using a standardized bioinformatic pipeline, and clustered into Amplicon Sequence Variants (ASVs) using DADA2 (Callahan et al., 2016). Taxonomic assignments were performed against the SILVA reference database (Quast et al., 2013), and only bacterial ASVs were retained for downstream analyses. The resulted data were normalized and analysed using univariate and multivariate statistical approaches implemented in R v.4.3.1 (R Core Team, 2022). PCR protocol performance was evaluated by comparing ASV recovery, alpha diversity, and bacterial community composition, after which the most appropriate protocol was selected for subsequent analyses. Sampling-related variability was assessed by comparing bacterial and macrofaunal communities from the same sampling locations using Bray-Curtis dissimilarities, ordination, and multivariate dispersion analyses. Finally, bacterial community variation was partitioned into within-grab and between-grab components to quantify the relative contribution of different levels of sampling replication.

2.1.3 Results and Discussion

The two PCR protocols generated substantially different numbers of ASVs despite using identical DNA extracts and primer pairs. Protocol 1 recovered considerably more ASVs than Protocol 2 at both farms. Although a large proportion of ASVs were unique to each protocol, 5,148 and 5,189 ASVs were shared between protocols at the CRE and DUN farms, respectively. Despite differences in ASV recovery, both protocols produced highly similar

taxonomic profiles dominated by Proteobacteria and Desulfobacterota at both farms (Figure 7A). No major taxonomic groups were exclusively associated with either protocol among the dominant phyla. Likewise, alpha- and beta-diversity analyses demonstrated that both protocols consistently distinguished among impact categories and revealed comparable community patterns (Figures 7B & C). The lower ASV richness recovered by Protocol 2 may reflect differences in polymerase performance, amplification efficiency, and error-correction mechanisms between the two PCR chemistries (Nagai et al., 2022). Both polymerases are designed for high-fidelity amplification, yet previous studies have shown that different enzymes can produce substantial differences in amplicon yield and the number of recovered sequence variants (Damaso et al., 2021). Nevertheless, the observed differences highlight the importance of protocol standardization to ensure comparability among studies, laboratories, and monitoring providers.

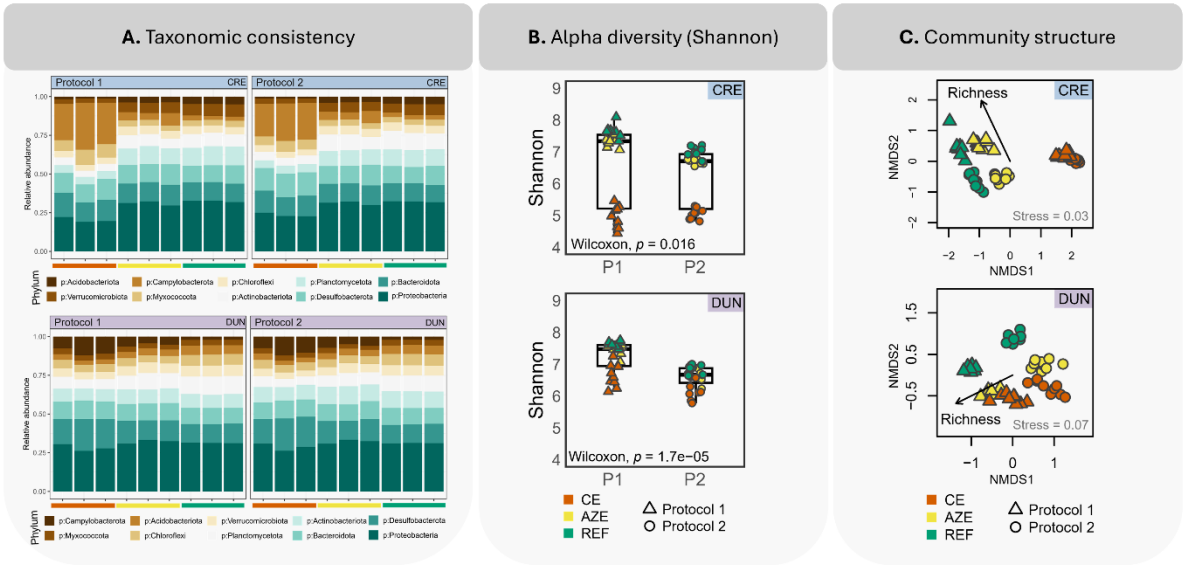


Figure 7. Comparison of bacterial community profiles generated using two PCR amplification protocols at two salmon farms (CRE and DUN). *(A) Relative abundance of the ten most abundant bacterial phyla recovered by Protocol 1 and Protocol 2 across the organic enrichment gradient. Despite differences in amplicon sequence variant (ASV) recovery, both protocols produced highly similar taxonomic profiles and consistently identified the dominant bacterial phyla. (B) Shannon diversity indices obtained using each protocol. Significant differences in alpha diversity estimates were detected between protocols at both farms using the Wilcoxon test, indicating that protocol choice influenced quantitative diversity estimates. (C) Non-metric multidimensional scaling (NMDS) ordinations based on Bray–Curtis dissimilarities showing bacterial community structure. Samples clustered primarily according to impact category (CE = cage edge, AZE = allowable zone of effect, REF = reference) rather than PCR protocol.*

A central objective of this chapter was to determine whether sampling-related variability in bacterial metabarcoding exceeds that observed in conventional macrofaunal monitoring. Despite fundamental differences between molecular and morphological approaches, bacterial and macrofaunal communities exhibited highly comparable responses to aquaculture-derived organic enrichment. Community composition differed consistently among impact categories, with environmental gradients explaining approximately 75% of the observed variation in both datasets, while sampling-related variability remained comparatively low. These findings are consistent with previous studies reporting strong concordance between benthic microbial and macrofaunal community responses to aquaculture impacts, supporting the use of bacterial communities as reliable bioindicators of environmental quality (Stoeck et al., 2018; Keeley et al., 2018). Importantly, the magnitude of background noise in bacterial eDNA samples was similar to that observed in conventional macrofaunal assessments, indicating that the molecular approach does not introduce additional uncertainty relative to the current regulatory standard. Furthermore, variability among replicate samples collected within the same sediment grab was generally of a similar order of magnitude to variability among replicate grabs collected from the same sampling location. This suggests that small-scale spatial heterogeneity occurs at multiple sampling scales but remains considerably weaker than the ecological signal associated with organic enrichment (Hestetun et al., 2021). Consequently, the results support a practical sampling strategy consisting of three replicate grabs per station with a single eDNA subsample from each grab, thereby minimizing sampling-induced uncertainty while maintaining compatibility with existing compliance monitoring frameworks.

2.1.4 Contribution to the Thesis

This chapter disentangled methodological from ecological sources of variation by demonstrating that PCR protocol choice can substantially influence bacterial diversity estimates, whereas sampling-related variability in bacterial eDNA was comparable to that observed in conventional macrofaunal monitoring. Together, these findings show that, although methodological variation is not negligible, it can be minimized through standardized laboratory workflows and consistent sampling strategies. This provides the methodological foundation for the subsequent chapters, enabling natural temporal dynamics and impact-induced changes in sediment bacterial communities to be interpreted with confidence that the observed ecological patterns are not primarily driven by technical artefacts.

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2.2 Publication

Towards a Standardized Operating Procedure for eDNA-Based Biomonitoring in Coastal Marine Salmon Aquaculture

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2.2.1 Abstract

The marine aquaculture industry and regulators are in the process of implementing environmental DNA (eDNA) metabarcoding of microbial communities for compliance monitoring. This requires standardization of sampling, laboratory, and data analysis protocols. Towards this goal, we in this study completed two further milestones using samples collected from two Scottish salmon farms: (i) We tested the effect of using two different PCR protocols (i.e., different DNA polymerases, master mixes, and annealing temperatures), which are frequently being used in eDNA biomonitoring of aquaculture installations, for the amplification of the taxonomic marker gene (V3-V4 hypervariable region of the bacterial 16S rRNA gene). (ii) We quantified sampling background noise obtained from eDNA samples and statistically compared results with the sampling bias observed in macrofaunal samples from the same source sediments. We detected differences in bacterial community structures resulting from the performance of different PCR protocols, profoundly influencing the interpretation of biomonitoring results. Furthermore, we found that sampling-induced errors for eDNA samples were similar to errors for macrofaunal samples collected according to compliance monitoring protocol (~25% variability in both cases). Finally, we showed that within-grab variances of microbial community structures were in the same order of magnitude (less than 10× difference in all cases) as the one obtained from replicate grabs collected from the same locale (impact category). Based on our findings, we suggest using a consistent PCR protocol for biomonitoring efforts to improve the comparability of results, especially when different service providers are conducting the biomonitoring. We propose a sampling scheme to be considered in eDNA biomonitoring that includes taking three replicate grabs at each locale, with one replicate sample from each grab. This minimizes sampling-induced errors and makes upcoming

eDNA-based monitoring results comparable with previous compliance monitoring results obtained from macrofaunal data.

Keywords: eDNA metabarcoding, Salmon aquaculture, Biomonitoring, Variability, Reproducibility.

2.2.2 Introduction

Marine aquaculture is one of the fastest growing food-production industries worldwide to satisfy the increasing global demand for seafood, micronutrients and protein (FAO, 2022). With wild fish stocks declining due to overfishing and environmental changes, aquaculture has become crucial, supplying over half of the world's fish consumed by humans (Mair et al., 2023). Among the marine finfish, Atlantic salmon (*Salmo salar*) dominates aquaculture production by far (FAO, 2022). However, the growth of the industry has come with a myriad of environmental challenges, including but not limited to, habitat destruction, pollution, pathogen proliferation and disease outbreaks, as a result of the accumulation of organic material on top of the sediment in the vicinity of aquaculture installations (reviewed by Pearson and Black, 2001; Quiñones et al., 2019). Therefore, sustainable salmon farming practices, along with continuous research and biomonitoring efforts, are essential to maximizing the industry's benefits while minimizing its negative environmental effects (Martinez-Porchas and Martinez-Cordova, 2012). Accordingly, most salmon farming corporates are applying Sustainable Development Goals (SDGs), issued by the United Nations (United Nations, 2015) into their “blue economy” practice. This includes, for example, salmon farms in Scotland, which are bound to ensure a positive contribution to sustainable development according to license conditions issued by the Scottish Environment Protection Agency (SEPA). This requires effective biomonitoring and environmental impact assessment. The current compliance monitoring protocol in place is the assessment of changes in the benthic macrofaunal communities (assemblage diversity and indicator species abundance) using morphological identifications (microscopy). This is expensive, demands taxonomic expertise, and above all, takes more time to produce results than is needed for active management (Hajibabaei et al., 2011). In addition, this approach cannot easily be scaled up to meet increasing sample numbers in updated compliance monitoring regulations (SEPA, 2023).

Therefore, the common goal of industry and regulators is the implementation of environmental (e)DNA-based biomonitoring into compliance monitoring regulations. This

innovative molecular technique involves the simultaneous sequencing of short DNA fragments exclusive to certain taxonomic groups in environmental samples such as water, sediment, etc. (Taberlet et al., 2012). There are numerous research studies that provide crucial guidance for eDNA users to adopt best practices, enhance the effectiveness of marine biodiversity monitoring, and identify critical knowledge gaps that need to be addressed for advancing the field (e.g., Miya, 2022; Shea et al., 2023; Takahashi et al., 2023). In this context, the use of benthic bacterial communities as biomarkers for monitoring marine benthic environments is well-supported by scientific research, highlighting their effectiveness in assessing environmental quality (Dowle et al., 2015; Keeley et al., 2018; Stoeck et al., 2018a; Laroche et al., 2018; Xie et al., 2018; Cordier, 2020; Lee et al., 2020; Aylagas et al., 2021; Frühe et al., 2021; Garlapati et al., 2021; Oladi et al., 2022; Wilding et al., 2023). The implementation of eDNA-based biomonitoring using microbial communities into official regulations requires a standardized operating protocol (SOP) considering sampling, laboratory procedures, and data analyses. A SOP allows for robust and reproducible results, in particular, if biomonitoring is being conducted by different commercial providers. Some challenges were addressed in previous studies, which include, for example, the effects of different sediment sample preservation methods (Dully et al., 2021a), DNA extraction methods (Hestetun et al., 2021a; Pearman et al., 2020), and the minimum required sequence data depth (Dully et al., 2021b).

However, the effects of other known critical steps in sampling and laboratory procedures on the interpretation of eDNA-based biomonitoring results using benthic bacterial communities have remained unexplored. This includes (a) the possible technical biases raised by using different PCR protocols for the amplification of the target gene (V3-V4 region of the hypervariable bacterial small subunit ribosomal RNA gene, 16S rRNA); And b) the bias that could possibly come from sampling error (e.g., much less source material compared to that in morphological approaches). One of the main sources of technical biases in metabarcoding studies is PCR amplification, which can be influenced by the annealing step (Piñol et al., 2015) and the extension step (mostly influenced by the type of DNA polymerase). Several previous studies have demonstrated that the enzyme used for 16S rRNA PCR-amplification may influence sequencing quality and bias as well as measures of alpha- and beta-diversity (Nichols et al., 2018; Pereira et al., 2018; Nagai et al., 2022). However, it is unknown to what extent this may influence interpretations of results in the context of biomonitoring of marine aquaculture installations. Currently, there is a lack of standardization in eDNA-based biomonitoring

protocols, including but not limited to PCR analysis. This broader issue affects the establishment of universally accepted guidelines for various aspects of eDNA workflows.

Likewise, understanding the variability between sampling replicates, which includes both sampling errors as well as genuine natural variability (i.e., independent of the impact-related change; also referred to as 'background noise'; see Wilding et al., 2023) is crucial to be considered in compliance biomonitoring (McBean and Rovers, 1985). Spatial heterogeneity of macrofaunal communities at meter-scale is well known and accounted for in compliance biomonitoring regulations by collecting 3-6 sediment grab replicates from the same sampling locality (e.g., SEPA, 2023; Pochon et al., 2020; NS, 2009; Lejzerowicz et al., 2015). Several previous studies demonstrated the necessity of collecting replicate samples also for eDNA-based sampling of benthic marine microbial communities to account for patchiness (Hestetun et al., 2021a, b; Laroche et al., 2017; Stoeck et al., 2018a; Beentjes et al., 2019; Dully et al., 2021c; Wilding et al., 2023). However, the relative sources of variance in replicate sampling (e.g., within sediment grab replicates and between grab replicates of the same locality) in eDNA-based biomonitoring using bacterial communities and the degree to which these variances influence result interpretations are scarcely explored (Wilding et al., 2023). In addition, to make previous compliance biomonitoring results based on macrofaunal communities comparable with upcoming eDNA-based results using bacterial communities, the background noise obtained from eDNA sampling should not noteworthily exceed that of macrofaunal sampling.

To contribute two further milestones towards a standardized operating protocol for biomonitoring in the salmon aquaculture industry we first: (i) tested two PCR protocols which are frequently being used in eDNA biomonitoring of aquaculture installations. Each of these protocols employed a different DNA polymerase, master mixes, and annealing temperatures. (ii) Then, using field site data from two salmon farms in Scotland, we performed rigorous statistical testing to compare sampling background noise obtained from eDNA samples and macrofaunal samples of the same source sediments.

2.2.3 Material and methods

Study area and sampling

This study was conducted at two marine coastal Atlantic salmon (*Salmo salar*) aquaculture installation sites on the western coast of Scotland near Oban operated by Scottish Sea Farms Ltd (Equitable House, London). Detailed descriptions of both farms, CRE and DUN, including arrangements of cage arrays are available in Wilding et al. (2012). In brief, both farms have been in use for salmon production for at least 20 years, and, therefore, have most likely reached equilibrium conditions in terms of environmental impact status. Both farms are situated in typical fjordic sea lochs with the CRE site being centrally located within the loch, approximately 400 m from both shores with a depth varying between 20 and 33 m. In contrast, the DUN site is more exposed, positioned over a relatively flat seabed at a depth of 40 m (>5 km to the nearest land to the south and west). Typical sediment characteristics at both farming sites include a mean sediment particle size ranging between 30 and 50 µm with a slightly coarser sediment at the CRE site. Accordingly, loss of ignition at 500 °C is typically lower at the CRE site (ca. 4%) compared to the DUN site (ca. 9%). The dry solid flux is typically slightly higher at CRE compared to DUN. Sampling occurred in March 2022. At the time of sampling, the CRE farm was close to the production peak, while the DUN farm was fallow since August 2021. Sediment was collected along a gradient of organic enrichment resulting from deposits of feed and fish feces on the seabed, including the outer cage edge (CE), an intermediate impact zone (Allowable Zone of Effect, AZE) and a reference site further away from aquaculture activities (REF). At each site, three replicate Van Veen grab (0.1 m^2 surface area) sediment samples were collected. Three scoops of the top 10 mm of the sediment from each side of one grab (six scoops in total per grab) were combined into a sterile plastic tube. From each grab, three such replicates, each containing six scoops of surface sediment, were collected for DNA extractions. The grab itself was thoroughly rinsed after collecting each sample and before taking the next sample. In addition, a new sterile scoop was used to collect each sample. All sediment samples were subsequently frozen at -20°C within a few hours of collection until further processing. This sampling scheme resulted in a total of 54 (2 farms × 3 sampling sites per farm × 3 grabs per site × 3 replicates per grab) samples for DNA extractions. The macrofauna was obtained from Van Veen grabs after subsampling for DNA analyses. Therefore, the remaining sediment was washed through a 1 mm sieve, and the residue was fixed in 4% borax-buffered formaldehyde prior to macrobenthic sorting and counting. The sieve-retained fauna was identified to the lowest taxonomic level possible under the National

Marine Biological Quality Control Scheme (NMBAQCS) by Ocean Ecology Limited, Oban, UK. This resulted in 18 macrofauna samples (2 farms × 3 sampling sites per farm × 3 grabs per site).

Molecular analyses

We stuck to the "clean laboratory" protocol throughout all stages of laboratory procedures. This means that all procedures were done in a laboratory dedicated to eDNA molecular work, equipped with all necessary tools for DNA extraction and library preparation. All surfaces were sterilized, and all lab consumables were autoclaved prior to any experiment. Total DNA was extracted from sediment samples using the Qiagen DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions with a slight modification as described in Oladi et al. (2022). The concentration and quality of extracted DNA were measured with a NanoDrop 2000 spectrophotometer (Peqlab, Erlangen, Germany). As metabarcodes, we targeted the V3-V4 hypervariable regions of the bacterial 16S rRNA gene using the primer pair Bakt341F (5′-CCTACGGGNGGCWGCAG-3′) and Bakt805R (5′-GACTACHVGGGTATCTAATCC-3′) (Herlemann et al., 2011).

We used the two-step polymerase chain reaction (PCR) approach that was originally developed for microbiome studies based on the 16S Metagenomic Sequencing Library Preparation Protocol by Illumina (www.illumina.com). This approach includes a first-stage PCR, at which the targeted gene region is amplified with Illumina overhang adaptors attached to the PCR primers mentioned above. This is then followed by the second-stage PCR that appends sample-specific sequences (IDs) to amplicons. To assess the effects of the first-stage PCR protocol for compliance monitoring protocol standardization, we subjected aliquots of the same genomic DNA extracts to two different commonly used first PCR stage PCR protocols, both of which employ the same PCR primer set as described above.

For the first PCR protocol (protocol 1, e.g., Wilding et al., 2023), the reaction mix contained ~ 20 ng of template DNA, 12.5 µL of 2x KAPA HiFi HotStart ReadyMix and 0.2 µM of each primer, adjusted to a final volume of 25 µL using PCR-grade water. PCR cycling conditions were as follows: 95 °C (3 min), followed by 25 cycles of 95 °C (30 s), 55 °C (30 s), and 72 °C (30 s), followed by a final extension at 72 °C (5 min). The second PCR protocol (protocol 2, e.g., Dully et al., 2021a; Oladi et al., 2022), included ~ 20 ng of template DNA, 1 unit of NEB's Phusion® HighFidelity DNA polymerase, 1× Phusion® HF Buffer, 0.5 µM/L of each primer, and 200 µM dNTPs in a final volume of 50 µL. The thermal cycling profile

consisted of an initial 30 s denaturation step at 98 °C, 27 cycles of 10 s at 98 °C, 30 s at 60 °C, and 30 s at 72 °C, followed by a final 5 min step at 72 °C. For all PCR, both negative (using nuclease-free water instead of template DNA) and positive (mock samples) controls were included to ensure the accuracy and reliability of the results. All PCR amplifications were performed in triplicate for each sample (technical replicates). All three technical PCR replicates per sample were pooled into an equal volume for each protocol and purified using Qiagen's MinElute PCR purification kit. The second-stage PCR was carried out by the sequencing company (StarSeq Co., Mainz, Germany), which was identical for all samples resulting from the two above-described first-stage PCR protocols. At this stage, the P5 and P7 adaptors, as well as index sequences, were bound to complementary sequences on the Illumina flow cell. Thereafter, sequencing was done on a MiSeq platform (Illumina) with the NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB) by StarSeq Co. (2 × 300 bp paired-end). The raw sequences are publicly available at the Sequence Read Archive under the BioProject ID PRJNA1078566, accession numbers SAMN40006391 - SAMN40006444.

Bioinformatics

FastQC quality control tool (Andrews, 2010) was used for the first evaluation of the quality of the raw sequences based on an assigned quality score across all bases. Removal of primer and adapter sequences was performed using cutadapt v.2.11.0 (Martin, 2011) with the default parameters. Quality filtering and trimming (truncLen = c(230, 225), maxEE(F & R) = 2) and formation of merged sequences (minOverlap = 20), as well as clustering them into Amplicon Sequence Variants (ASVs) were performed using the Divisive Amplicon Denoising Algorithm (DADA2) (Callahan et al., 2016) pipeline in R v.4.1.1 (R core Team, 2022). The truncation length for R1 and R2 reads was decided by choosing the sequence position with a Phred score of ≥30 (Q3) for at least 51% of all reads in a dataset (=base call accuracy 99.9%) (Ewing et al., 1998). The paired-end sequences were merged (mergePairs function) using a minimum overlap of 20 bp and an allowed mismatch of two bases. Chimeras were removed using the uchime_denovo function of vsearch (Rognes et al., 2016) with default parameters. Taxonomic assignment of ASVs was performed using the sintax function from vsearch against the SILVA v.138 reference database (Quast et al., 2013) based on the last common ancestor (LCA) approach. For downstream analyses, we selected all ASVs that were assigned to the domain Bacteria, and then mitochondrial and Chloroplast 16S sequences and ASVs with only one sequence read (singletons) were discarded.

Statistical analyses

All statistical analyses were conducted in the R environment (v.4.3.1), with some calculations being done using Microsoft Excel formulas. Sampling saturation depth for V3-V4 amplicon datasets was assessed using the rarecurve function of the R package vegan v.2.6-4 (Oksanen et al., 2020).

To compare the results obtained from the two PCR protocols, we first normalized each sample to 5,000 reads using the rarefy_even_depth function of the R package phyloseq v.1.44.0 (McMurdie and Holmes, 2013). This normalization accounted for uneven sample sizes (Supplementary Table S1) and the number of 5,000 was chosen because previous studies identified this number as sufficient for the characterization of benthic bacterial communities at marine aquaculture installations (Dully et al., 2021b).

Venn diagrams were used to visualize the number of shared and unique ASVs when comparing the rarefied results of the two different PCR protocols (venn.diagram function of the VennDiagram R package; Chen and Boutros, 2011). The alpha diversity measures i.e. ASV richness, Shannon diversity and evenness were calculated from the rarefied amplicon datasets with the vegan package of R using the specnumber and diversity functions and then checked for normality distribution using the Shapiro-Wilk test (function shapiro.test of the R package stats v.4.3.1). As all alpha diversity measures were not normally distributed ($p < 0.05$), the Wilcoxon test (Wilcoxon, 1945), as a robust alternative to parametric tests, was used to test for significant differences between the alpha diversity measures obtained from the two PCR protocols (function wilcox.test of the R package stats). Finally, we compared the rarefied amplicon datasets from both PCR protocols in terms of their taxonomic structures. Therefore, all ASVs obtained from each individual sample of PCR protocol 1 were merged into one sample and the same was applied to all ASVs from PCR protocol 2. For the 50 most abundant ASVs of each of the two datasets, we then generated pie-donut charts using the PieDonut function of the webr R package v.0.1.5.

All following analyses were conducted using the amplicon data obtained from PCR protocol 1 (for reasons see Results and Discussion sections). It should be mentioned that after plotting the sampling coordinates of the DUN farm site (Fig. S1), we noticed a shift in CE grab 2 replicates, which were taken at closer proximity to the AZE than to the CE, due to the vessel drifting during sample collection. Accordingly, we changed the sample design by grouping CE

grab 2 with the other three AZE grabs for further analyses (i.e., 2 grabs for CE, 4 grabs for AZE, and 3 grabs for REF). This was done for both macrofauna and bacterial datasets.

To assess how the sampling error (noise) obtained from the eDNA samples (bacterial communities) compares to the noise obtained from macrofaunal samples, we first pooled the within-grab replicates into one sample. For example, for grab number 1 taken at CRE cage edge sample, we had three within-grab replicates, which were pooled into one sample to make the CRE cage edge grab 1 sample. The same was performed for the other two grabs taken at CRE cage edge. This scheme was applied to all collected eDNA samples (i.e., for CRE AZE and REF as well as for DUN CE, AZE and REF). Thus, the eDNA dataset for this statistical comparison with macrofauna consisted of the same sample design as the macrofaunal samples. From the square-root transformed data matrices, we then calculated Bray-Curtis distances for all eDNA profiles of bacterial communities obtained from PCR protocol 1 and for all macrofaunal communities of the same sampling sites (vegdist function of the vegan R package). The Bray-Curtis dissimilarity was chosen due to its suitability in ecological studies where differences in community composition need to be quantified. We then used the Bray-Curtis dissimilarities to calculate if the distances of replicates to their group centroids (dispersion) were similar for the macrofaunal and for the bacterial eDNA datasets (betadisper function of the vegan R package). We visualized the results in box plots (using ggplot2 v.3.4.3) and ordination diagrams. For the latter, we used a Principal Coordinates Analysis (PCoA, base R) with 95% confidence ellipsoids for sampling sites (cage edge, allowable zone of effect and reference). The non-parametric ANOVA using the adonis2 function implemented in the vegan R package was specifically selected to partition variance between different factors (location and distance from cage edge) without assuming normality, which is particularly important given the nature of our ecological data. This model was chosen to test the interaction between location (farms) and sampling site (CE, AZE, and REF), helping us understand how these factors jointly influence the variance in microbial and macrofaunal communities.

To compare the within-grab replicate variability of bacterial communities with the between grabs replicates variability taken at the same sampling site, we used the Bray-Curtis dissimilarities of the bacterial community structures (using square root-transformed data tables) and calculated the distances of within-grab and between-grab replicates to their group centroids. Group centroids and the distances of each point were extracted from the betadisper output, and within- and between-group distances were calculated using the Euclidean distance

formula ($\sqrt{[(x_2 - x_1)^2 + (y_2 - y_1)^2]}$). Distances to group centroids were then visualized using box plots.

2.2.4 Results

Sequence data overview

The number of sequence reads for all samples from both salmon farms and both PCR protocols that remained after each data cleaning step is provided in Supplementary Table S1. In case of PCR protocol 1, these datasets consisted of 2,994,443 sequences clustering into 30,058 final ASVs (13,441 for the dataset rarefied to 5,000 sequences per sample) for the CRE farm and 3,339,143 sequences clustering into 32,746 final ASVs (15,126 for the dataset rarefied to 5,000 sequences per sample) for the DUN farm. In case of PCR protocol 2, these numbers were: 1,065,722 sequences and 11,562 (8,608) ASVs for CRE and 780,899 sequences clustering into 10,508 (8,452) ASVs for DUN. Sampling saturation profiles showed that all samples were sampled to (near) saturation (Supplementary Figures S2 and S3). In these profiles, it is noteworthy that regardless of the PCR protocol used to generate the datasets, all cage edge (CE) samples from the CRE farm reach their saturation notably sooner than the samples from the Allowable Zone of Effect (AZE) and the Reference (REF). In contrast, this does not apply to the CE samples from the DUN farm. Here, the CE sample saturation curves are nested within the cloud of AZE and REF samples.

Comparisons of results obtained from two different PCR protocols

In case of the CRE farm, 5,148 ASVs were shared between the two PCR protocols, while 8,186 were unique to protocol 1 and 3,449 to protocol 2 (Fig. 1). In case of the DUN farm, these numbers were highly similar: 5,189 ASVs shared, 9,937 unique to PCR protocol 1 and 3,263 to protocol 2.

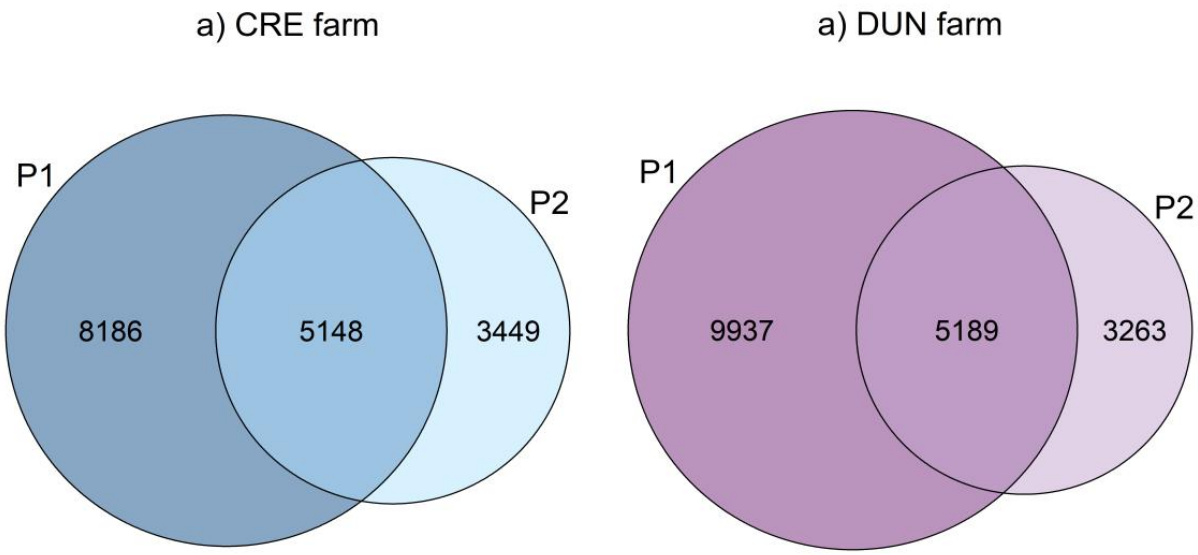


Fig. 1. *Number of unique and shared (overlapping area) ASVs between PCR protocol 1 (P1) and protocol 2 (P2) in both a) CRE and b) DUN farms represented by Venn diagrams.*

Even though the Venn diagrams showed a relatively high proportion of ASVs that were unique to one of the two PCR protocols, the taxonomic inventory (50 most abundant ASVs) obtained from both PCR protocols for the CRE farm (Fig. 2a) and the DUN farm (Fig. 2b) was remarkably similar. To mention a few examples for the CRE farm, Sulfurovaceae were the most abundant accounting for 37% of all ASVs in case of PCR protocol 1 and 32% in case of protocol 2 (Fig. 2a). In terms of abundance, this is then followed by Desulfobulbaceae and Desulfocapsaceae for both protocols. Even the least abundant taxon groups were identical for the two PCR protocols, namely Kiritimatiellaceae and the undefined bacterial group Sva0485 (Fig. 2a). Not a single bacterial taxon group among the 50 most abundant ASVs in both PCR datasets is unique to only one of the two protocols. The same applies to the results obtained for the DUN farm: the most abundant taxon groups (in decreasing order) are Sulfurovaceae, Desulfocapsaceae, and Desulfobulbaceae (Fig. 2b). The two least abundant taxa were Kiritimatiellaceae and the undefined bacterial group Sva0485. Once more, all 23 identified taxon groups at strain or family-level were identical among the two PCR datasets (Fig. 2b).

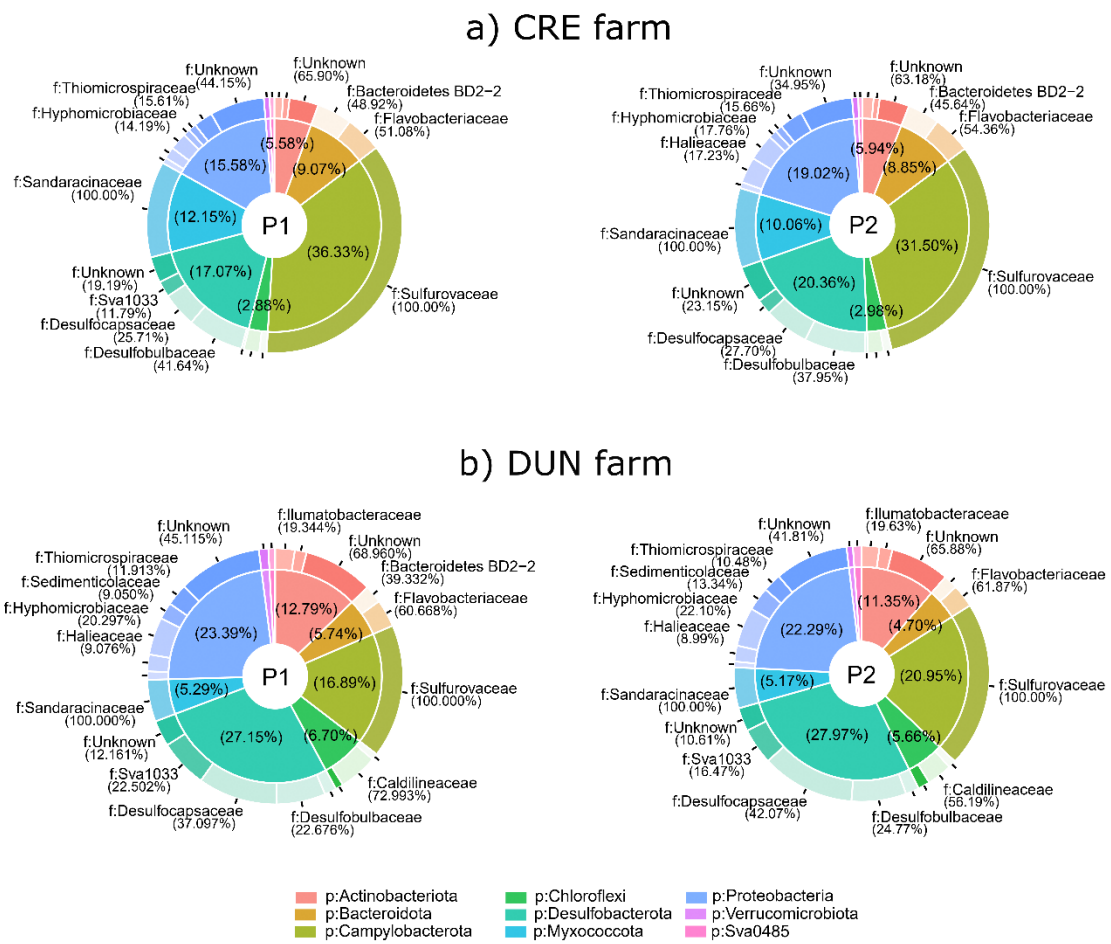


Fig. 2. *Comparison of the taxonomic inventory of the 50 most abundant ASVs generated by two PCR protocols in both a) CRE and b) DUN farms represented by PieDonut charts. Taxa with less than 2% relative abundance are not labeled in the figure. Legend represents the identity of phyla (pies). P1 = protocol 1, P2 = protocol 2.*

ASV richness as well as Shannon diversity were significantly different (Wilcoxon $p < 0.05$) between the two PCR protocols for both farms with notably higher averaged values resulting from protocol 1 samples (Fig. 3). In contrast, evenness was highly similar between PCR protocol 1 and protocol 2 samples at both farms (Wilcoxon $p > 0.05$).

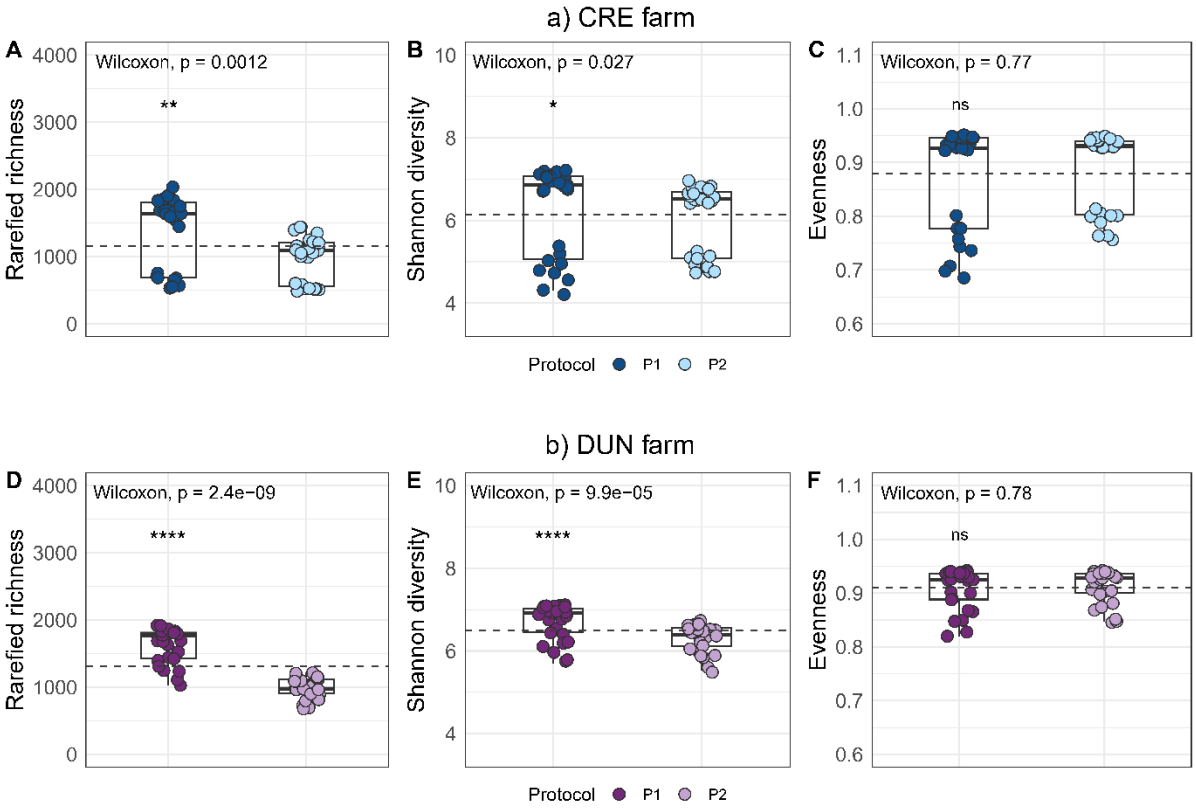


Fig. 3. *Comparison of alpha diversity measures (i.e., A,D= rarefied ASV richness, B,E= Shannon diversity and C,F= Evenness) between PCR protocol 1 and protocol 2 in both a) CRE and b) DUN farms, as well as the corresponding significance test results (Wilcoxon* p-*values). The horizontal dash line represents the base mean. ns= not significant.*

Comparing the variability of DNA replicate samples (bacterial communities) with macrofaunal replicate samples

To assess whether the sampling error (noise) obtained from the eDNA samples (bacterial communities) compares to the noise obtained from macrofaunal samples, we tested whether the variability of grab replicates taken for DNA profiling of benthic bacterial communities is higher, lower, or similar compared to the variability of macrofaunal replicates as taken in compliance biomonitoring of salmon farms. Bray-Curtis (BC) distances for both datasets, macrofaunal and bacterial communities, had highly similar patterns in an ordination analysis (Fig. 4). For both communities at both farms, 95% confidence intervals for sampling sites (CE, AZE, REF) showed no overlap (numerical values of BC distances to their group centroids, i.e. dispersion, are provided as boxplots in Fig. S4).

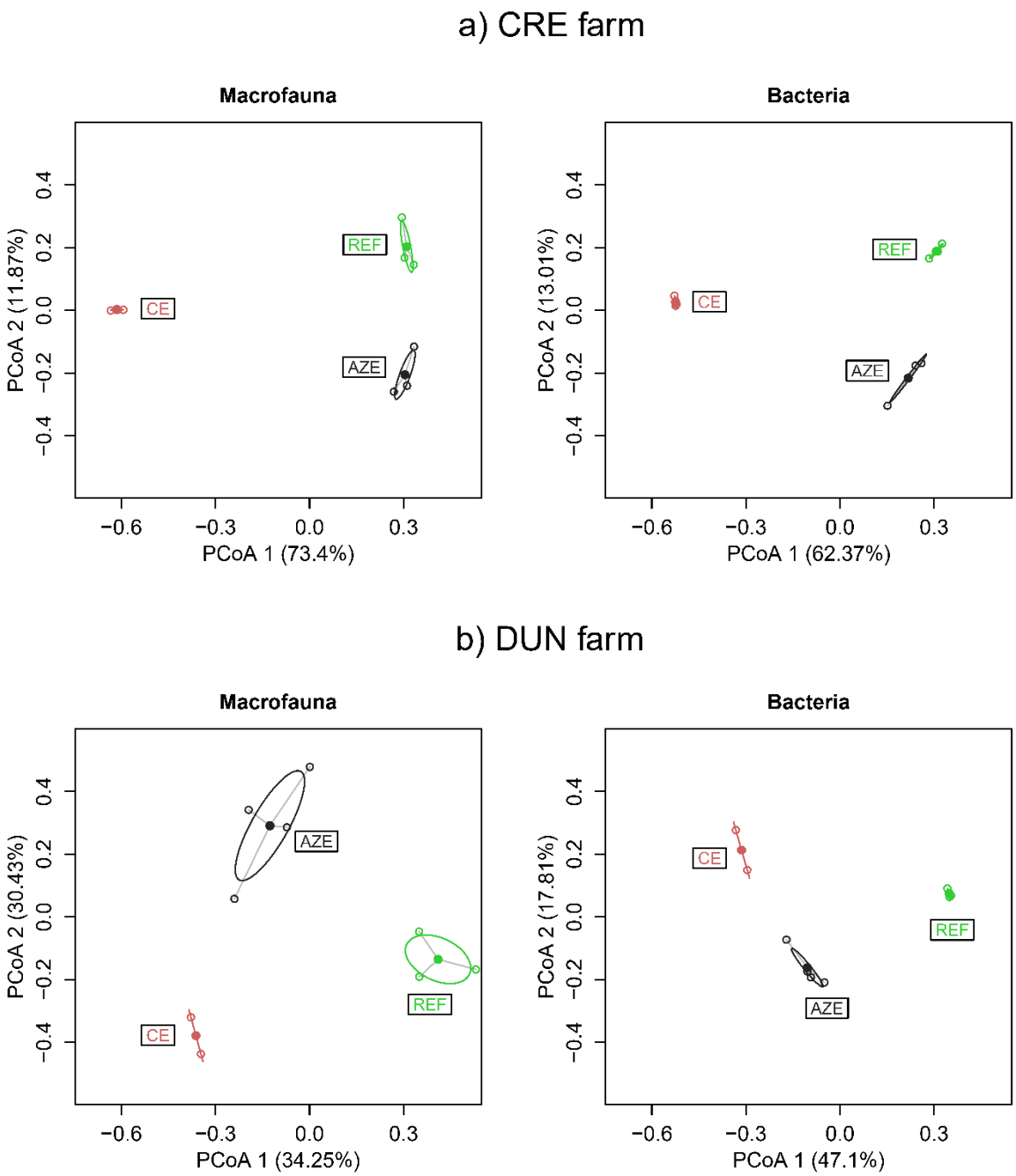


Fig. 4. *Principal Coordinates Analysis (PCoA) derived from Bray-Curtis distances between macrofauna and bacterial samples in both a) CRE and b) DUN farms. Distinct clusters indicate pronounced differences in community structures. Percent of the variations explained by each axis is presented in brackets. Colors correspond to different distances from fish cages i.e. red represents the cage edge, black represents the Allowable Zone of Effect, and green represents the reference site. Please note that group centroids are shown with solid circles.*

A permutational multivariate analysis of variance confirmed that in both cases (bacterial and macrofaunal communities), factors included in the model (i.e., farm, distance, and their interaction) explained ~75% of the variability in the data (Table 1). Intriguingly, distance from fish farming cages accounted for ~37 and 36 % of the variance in bacteria and macrobenthos, respectively, highlighting the highly similar response of these communities to fish farm activities.

Table 1. PERMANOVA results comparing the model performance and explained variations by each factor (farm, distance, and their interaction) between bacterial and macrofaunal communities.

Sample comparison		**Df**	**Sum of Squares**	**R^2**	**F**	**Pr(>F)**
Bacteria	Farm	1	0.846	0.208	10.137	0.001
	Distance	2	1.524	0.374	9.123	0.001
	Farm*Distance	2	0.692	0.17	4.142	0.001
	Residual	12	1.002	0.246		
Macrofauna	Farm	1	1.016	0.171	8.649	0.001
	Distance	2	2.117	0.357	9.008	0.001
	Farm*Distance	2	1.386	0.233	5.895	0.001
	Residual	12	1.41	0.237		

Comparing the within-grab DNA replicate samples with between-grab DNA replicate samples (bacterial communities)

In this part of the study, we compared the magnitude of the sampling error resulting from within a sediment Van Veen grab (three replicate samples taken from within each grab) with the one resulting from two other replicate grabs taken at the same sampling location. While the ideal scenario would involve no differences between within- and between-grab replicates, measuring variations in real scenarios is essential. This would inform strategic decisions on where to focus sampling efforts. Here, we observed a similar order of magnitude (less than ×10 in all cases) in between- and within-grab variations when comparing the data obtained from both farms (Fig. 5). The largest difference between within- and between-grab variability was observed at CRE AZE and DUN CE (×7 more between-grab variability). In case of CRE AZE,

we could link the high between-grab variation to a replicate grab (number 1) which was more distant to the other two replicate grabs of the same site (Fig. S1). Also at the DUN REF site one of the replicate grabs (number 2) was notably more distant to the other two replicate grabs of this site (Fig. S1). Yet, microbial community profiles obtained from all three replicate grabs were highly similar to each other (Fig. 5b). Between-grab variability of microbial community profiles for the CRE REF site were in the same order of magnitude compared to the within-grab variability at the DUN REF site (Fig. 5a & 5b). This indicates relatively similar microbial communities in the larger reference area, whereas closer to the salmon farm site (CE and AZE) microbial community structures vary notably within relatively short distances.

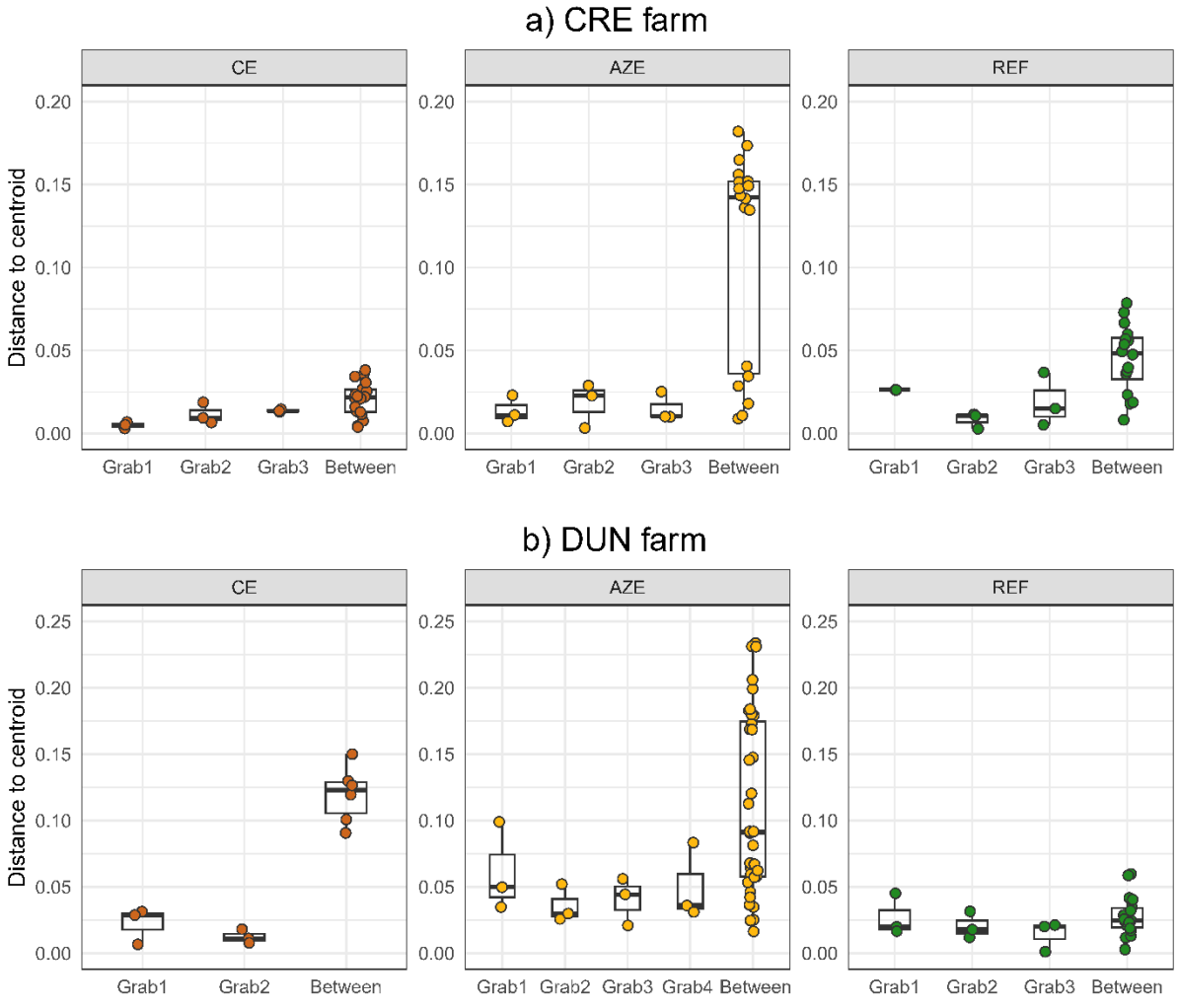


Fig. 5. *Comparison of the magnitude of the sampling noise resulting from within a sediment grab and from between other grabs, based on eDNA metabarcoding of microbial communities, at the same location in both a) CRE and b) DUN farms.*

2.2.5 Discussion

Standardizing eDNA protocols is crucial for ensuring consistency and comparability across different studies and for regulatory applications (Takahashi et al., 2023). Standardization helps in establishing baseline data, facilitating cross-study comparisons, and ensuring that management decisions are based on reliable and comparable data. This study contributes to that effort by evaluating how two distinct PCR protocols influence the accuracy of microbial community detection and by quantitatively comparing the sampling errors between microbial communities and traditional macrofaunal sampling methods, providing crucial insights for improving ecological monitoring practices.

Influence of different PCR protocols on diversity estimates and community composition of bacterial communities

Ideally, different laboratories working on the same sample material in the framework of a compliance biomonitoring should come to the same conclusions regarding environmental quality classifications. It is well known that the choice of the PCR primer set plays a key role in the amplification of microbial community bioindicators (e.g., Fredriksson et al., 2013; Dziurzynski et al., 2022), and, therefore, it is crucial that the same PCR primer set is used in a standardized laboratory protocol for eDNA-based compliance monitoring. Therefore, in most eDNA-based biomonitoring studies of salmon aquaculture installations, the same PCR primer set or slight variations thereof have been used (e.g., Keeley et al., 2018; Stoeck et al., 2018a; Cordier, 2020; Pearman et al., 2020; Aylagas et al., 2021; Frühe et al., 2021; Dully et al., 2021c; Laroche et al., 2021; Wilding et al., 2023). However, the employed protocol for the PCR amplification of benthic microbial communities in these studies differed. Whether (and if so, how) this affects conclusions drawn from analyzed microbial communities in the framework of compliance monitoring was hitherto untested.

One of the notable distinctions between the two protocols was the type of DNA polymerase that was used for amplifications. Both DNA polymerases (Roche KAPA HiFi HotStart and NEBs Phusion® High-Fidelity) used in this study are advertised with a superior proofreading power (due to 3′→ 5′ exonuclease activity), which would lead to very high accuracies during DNA amplification (see manuals provided by the manufacturers of the two enzymes compared in this study). Therefore, we initially predicted congruent results from both protocols, as well as bacterial community structures. However, this was not the case and we

observed significant differences in several diversity measures between the microbial communities analyzed with the two different polymerases.

In agreement with our findings, Damaso et al. (2021) demonstrated in a forensic study that different PCR polymerases generate different amplicon yields. Interestingly, among eleven commercially available enzymes, the highest amplicon yield was obtained with the KAPA HiFi DNA polymerase. Nevertheless, it should be noted that a higher number of amplicons does not necessarily correlate with enhanced accuracy (De Wolfe and Wright, 2023). Amplification success and accuracy rely, among others, on the concentration of salts in PCR buffers (mastermix) that is essential for creating an environment that promotes accurate primer binding, and in turn, reliable results (Singh et al., 2014). The most commonly used salt in PCR is magnesium chloride ($MgCl_2$), which was the salt used in both PCR protocols in this study. However, the final concentration of $MgCl_2$ was higher in KAPA's ReadyMix (2.5 mM) than in Phusion Buffer (1.5 mM). This was also evident in our results (Table S1), where the percentage of non-bacterial ASVs (i.e., Archaea and unassigned ASVs, representing non-specific binding) was higher in protocol 2 (Phusion DNA polymerase) than in protocol 1 (KAPA DNA polymerase).

In addition, it should be pointed out that we did not use a uniform annealing temperature for the applied PCR protocols. This decision was based on the recognition that the performance of DNA polymerases at different temperatures is influenced by several factors, including enzyme fidelity and the thermal stability of the polymerase (Lundberg et al., 1991; Montgomery et al., 2014). Consequently, it is essential to determine the optimal annealing temperature for each set of primers for each DNA polymerase used (using methods such as temperature gradient PCR or Tm calculators), which was precisely followed in our study. According to NEB's Phusion protocol (https://www.neb.com/en/protocols/0001/01/01/pcr-protocol-m0530), the annealing temperatures required for Phusion polymerase are generally higher than those for other polymerases, which justifies the 5 °C higher annealing temperature used for amplifications with Phusion compared to KAPA.

Another source of error in the PCR process is editing errors that occur during DNA polymerase-catalyzed enzymatic copying (Pienaar et al., 2006). Here, the quality of the enzyme used in the PCR seems to play an important role. Nagai et al. (2022) used different polymerases to PCR amplify a eukaryotic mock eukaryotic community. They found that the degree of PCR-generated errors such as chimera, indels, and base substitution differed significantly among

different polymerases. Consequently, this affects measures of diversity for this mock community. This was indeed evident in our DUN dataset, where the percentage of chimeric sequences was almost 4 times more in PCR protocol 2 than in protocol 1 (Table S1). Likewise, Pereira et al. (2018), amplifying different species of the bacterial genus Legionella, showed that an increased error accumulation resulting from a less accurate polymerase artificially inflated species richness compared to the highly accurate KAPA HiFi polymerase. Nichols et al. (2018) compared the potential biases of six commercially available polymerases (among which four were proofreading enzymes) using a combination of mixtures of amplifiable synthetic sequences and real sedimentary DNA extracts. The authors found that polymerase choice can affect both relative abundance estimates and occurrence. As the main source of this bias, the authors identified polymerase preferences for sequences with specific GC contents. According to the manufacturer's (Roche Sequencing and Life Science) information, their KAPA HiFi HotStart ReadyMix has a 100% success rate even at an amplicon GC content reaching up to 85% at a much lower error rate compared to competitor enzymes (https://rochesequencingstore.com/wp-content/uploads/2017/10/Application-Note-High-Fidelity-GC-rich-PCR.pdf, link accessed December 1st 2023).

Based on the results of our study, we recommend to explicitly specify, in a standardized eDNA laboratory protocol for eDNA-based biomonitoring, which polymerase should be used in the PCR to amplify the microbial communities under interrogation. Whilst we only investigated salmon farms, we believe that standardization of polymerase should be part of any biomonitoring SOP. We here tested only two DNA polymerases, which were frequently used in eDNA-based biomonitoring studies of salmon aquaculture installations, and, hence, cannot make any statements regarding the best possible commercially available choice for this specific purpose. Yet, one good option is certainly the KAPA HiFi HotStart ReadyMix by Roche Sequencing and Life Science. This choice is also recommended by Illumina for 16S metagenomic sequencing library preparation for Illumina high-throughput sequencing (www.illumina.com; 16S Metagenomic sequencing library preparation, Illumina: San Diego, CA, USA, 2013).

Assessing eDNA sampling error in marine finfish aquaculture biomonitoring

To reliably interpret results obtained from eDNA-based interrogation of benthic microbial communities in the context of biomonitoring, it is a prerequisite to understand to what extent these results are affected by sampling bias and by spatial heterogeneity (Hestetun

et al., 2021b). As a benchmark for the sampling bias, we here considered results obtained from traditional morphological biomonitoring surveys as part of compliance monitoring protocols. Several previous studies showed a high accordance between beta-diversity patterns of benthic macrofaunal (morphological sampling) and benthic microbial (eDNA) communities obtained from the same samples collected for marine aquaculture biomonitoring (Stoeck et al., 2018a, b; Keeley et al., 2018; Pochon et al., 2015). This corroborates our own findings in this study. Here, we showed in the corresponding multidimensional scaling (PCoA) plots even remarkably stronger segregation of differentially impacted sampling sites in case of the microbial eDNA community profiles compared to macrofaunal communities. Notably, statistical significance testing depicted a clear picture of the similar performance of bacterial and microbenthic communities as a biomonitoring tool for the impact assessment of fish farming. Thus, we can conclude that the small amount of source material that we used for the eDNA profiling achieved the same amount of "signal" compared to the large source sediment volume collected for the interrogation of benthic macrofaunal communities by visual inspections. It is also worth noting that the large amount of sediment for macrofaunal analysis that is needed for capturing as many species as possible can be rather invasive, which would potentially cause disturbance and habitat destruction in the long run (e.g., Beng and Corlett, 2020). Undoubtedly, this is a strong argument that supports the further development of an eDNA-based protocol for compliance monitoring which in the long run could replace the traditional morphological macrofaunal surveys.

Nonetheless, the degree of the random sampling effect to which the eDNA sampling performed compared to the standard benthic macrofaunal sampling was unexpected for two reasons. First, according to ecological theory, a given area may seem like a homogenous habitat for larger organisms (e.g., macrobenthic invertebrates), but constitutes a vast number of different habitats for microbes (Fenchel et al., 2019). Therefore, the expectation would be that the macrofaunal benchmark data should show a lower sampling bias compared to the microbial communities. Second, identification of the macrofaunal inventory is based on the captured community from a relatively large sediment sample (15-20 liters of sediment), whereas the sediment volume from which eDNA is extracted is extremely small to represent the seafloor at the locality of interest.

We explain our finding with the sampling design used to collect sediment for eDNA extraction, which may counteract sampling bias for microbial communities. Collection of six scoops of sediment for each of the three within-grab replicates (amounting to 18 sediment

scoops in total) from different points of the grab, which was based on the Scottish regulator's guidance (https://www.sepa.org.uk/media/594223/seabed-mixing-zone-limit_compliance-assessment-method.pdf), probably led to a reasonable coverage of the overall sediment heterogeneity within cm-scales. As a result, we obtained the nearly full deck of the bacterial diversity present in the sediment surface layer of a Van Veen grab (for support of this assumption, see also the sampling saturation profiles shown in Supplementary Figures S2 and S3). The bacterial diversity we detected within cm-scales corroborated quite well with the bacterial diversity found in meter-scales (which refers to the three grab replicates taken at each locale). This then resulted in a surprisingly small sample bias for eDNA sampling. Even though this had not been demonstrated for 16S rRNA profiling of benthic bacterial communities, Hestetun et al. (2021b) came to the exact same result in an 18S rRNA biomonitoring survey of eukaryote diversity in North Sea sediments. The authors had taken three grab parallels and five separate sediment samples from each grab, for which they obtained the V1-V2 hypervariable region of the eukaryote SSU rRNA gene. Most of these sequence reads were obtained for microbial eukaryotes (unicellular protists). However, their findings were not linked to any explicit ecological driver (Hestetun et al., 2021b).

Nevertheless, we observed greater between-grab variability at AZE compared to CE and REF, particularly at CRE, which was in the production phase at the time of sampling. This observation could be attributed to a more uniform seabed at CE and REF than AZE. We postulate that in the immediate vicinity of the cages, the deposition of uneaten feed and fish feces contributes to the seabed's uniformity. Conversely, in lower-impacted, transient environments such as AZE, biological interactions may supersede the effect of environmental factors (Schafer, 2000), resulting in increased variability among biological replicates (Laroche et al., 2017). This hypothesis is supported by results from the DUN farm, which was in the fallow phase during sampling (i.e., no cages in the sea). As demonstrated in Fig. 5, the between-grab variability at CE is much higher at the DUN farm compared to the CRE farm, highlighting a less uniform seabed at CE and AZE in the fallow farm. Similarly, Dully et al. (2021c) observed such patchiness in macroinvertebrate communities across three AZE stations. They linked their observation to the non-linear organic matter depositions from the fish farm, which influences benthic microbes and macroinvertebrates (Dully et al., 2021c).

While our study provides valuable insights into the conditions at two Scottish fish farms, it is important to note that the findings may not be fully generalizable to other regions or ecological contexts. There are many factors influencing the performance of eDNA

biomonitoring that are site-specific (e.g., variations in environmental conditions, local ecological dynamics, etc.). To balance the need for standardized protocols with the necessity to adapt to site-specific conditions, it is often advocated in the literature to conduct pilot sampling and adjust protocols based on the specific conditions of each site (see Bruce et al., 2021). This approach allows for the initial standardization of core methodologies while incorporating the flexibility to optimize protocols for local conditions (Takahashi et al., 2023). Therefore, expanding the scope of research to include a variety of fish farms in diverse regions will further help to potentially identify additional factors influencing farm conditions as well as the performance of eDNA biomonitoring.

2.2.6 Conclusion

Towards a standardized protocol for biomonitoring in the marine aquaculture industry, this study contributed two further milestones. 1) The choice of PCR protocol for the amplification of the target gene (V3-V4 hypervariable region of the 16S rRNA gene) should be defined in the protocol for compliance monitoring. To make this point, it was sufficient to test only two different PCR protocols which are frequently being used in scientific publications for the amplification of the above-mentioned target gene from marine sediment eDNA. Based on our results, there were significant differences in alpha diversity metrics and the subsequent interpretations, underscoring the importance of employing a consistent PCR protocol in biomonitoring efforts. In this study, the PCR protocol containing Roches KAPA HiFi polymerase performed superior compared to the other protocol. Nevertheless, there may be alternative PCR protocols that have gone untested thus far. Therefore, the final decision about the PCR protocol to be included in the standard operating protocol is left to the regulator. 2) The following sampling design is suggested to be considered for eDNA-based biomonitoring in the marine aquaculture industry: For each locale, three replicate grabs should be taken. It is sufficient to take one sample from each of these grabs, consisting of six scoops of sediment to be collected from different points of the surface sediment in the grab and combined in one sample tube.

Author Contributions

Mahshid Oladi: investigation, formal analysis, data curation, visualization, writing – review and editing. Thomas A. Wilding: conceptualization, sampling, formal analysis, writing – review and editing. Adam Wyness: data interpretation, writing – review and editing. Thorsten Stoeck: conceptualization, sampling, methodology, validation, writing – original draft, supervision, project administration, funding acquisition.

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Conflict of Interest Statement

The authors declare no conflict of interest.

Data availability statement

The data published in this study are available at NCBI BioProject database ID PRJNA1078566, accession numbers SAMN40006391 - SAMN40006444. The R script used to process the data and prepare visualizations is available in the Supplementary Files as Supplementary File 1, and the data tables as Supplementary File 2.

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CHAPTER II

DISENTANGLING NATURAL AND IMPACT-INDUCED DYNAMICS IN SEDIMENT BACTERIAL COMMUNITIES

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3.1 Summary

3.1.1 Background

Having established the methodological robustness of bacterial metabarcoding in Chapter I, the next step towards developing robust bacterial biomonitoring tools is to understand the extent to which natural ecological dynamics influence sediment bacterial communities. Bacterial communities in marine sediments are influenced by both natural environmental fluctuations and anthropogenic disturbance. Seasonal changes in temperature, organic matter availability, and other environmental conditions can alter bacterial community composition (Findlay and Watling, 1998), while organic enrichment from salmon aquaculture promotes predictable shifts towards enrichment-tolerant assemblages (Holmer et al., 2005). Distinguishing natural temporal dynamics from impact-induced responses is therefore essential for identifying bacterial indicators that reliably reflect environmental degradation rather than predictable seasonal variability. By quantifying the relative contribution of natural and impact-induced sources of variation, the robustness of bacterial communities as biomonitoring indicators can be properly assessed.

Anthropogenic organic enrichment associated with salmon aquaculture is not static but varies throughout the production cycle (Verhoeven et al., 2018). As fish biomass increases, uneaten feed and faecal material accumulate on the seabed, progressively altering sediment biogeochemistry through increased organic matter deposition, oxygen depletion, and the development of reducing conditions (Holmer et al., 2005; Keeley et al., 2014). These environmental changes promote predictable shifts in bacterial community composition, favouring anaerobic and sulfide-associated taxa while reducing the abundance of bacterial groups associated with well-oxygenated sediments (Frühe et al., 2021b; Johansen et al., 2024). Following harvest and fallowing, reduced organic loading promotes sediment recovery, allowing bacterial communities to progressively transition towards pre-disturbance conditions (Verhoeven et al., 2018). The magnitude and rate of these changes, however, are likely to depend not only on natural environmental variability but also on management practices, including production intensity, fallowing duration, and site-specific characteristics (Kalantzi and Karakassis, 2006; Cromey et al., 2022).

Although numerous studies have demonstrated clear spatial responses of sediment bacterial communities to aquaculture-derived organic enrichment (Dowle et al., 2015; Keeley et al., 2018; Stoeck et al., 2018; Frühe et al., 2021b; Wilding et al., 2023), considerably less

attention has been given to their temporal dynamics throughout fish production cycles (Verhoeven et al., 2018). As a result, it remains unclear how seasonal environmental variability interacts with progressive organic loading, sediment recovery during fallowing, and changes in farm management to shape bacterial community trajectories over time. Likewise, little is known about whether different positions along the enrichment gradient exhibit similar temporal responses or whether some locations are particularly sensitive to management-related change. Because regulatory monitoring is typically conducted during peak biomass, resolving these uncertainties is essential for determining whether the timing of sampling influences the detection and interpretation of enrichment-related community responses. Addressing these questions is central to determining the temporal robustness of bacterial indicators and their suitability for routine environmental monitoring.

This chapter, therefore, investigates the relative importance of natural temporal variation and aquaculture-derived organic enrichment in structuring sediment bacterial communities over two salmon production cycles. By examining bacterial community dynamics throughout production and fallowing periods, the study evaluates the relative influence of seasonal variability, organic enrichment, and farm management practices on bacterial community trajectories, thereby providing a critical evaluation of the temporal robustness of bacterial communities as indicators of aquaculture-derived environmental disturbance.

3.1.2 Methods

To investigate the relative influence of natural temporal variability and aquaculture-derived organic enrichment on sediment bacterial communities, a field study was conducted at an Atlantic salmon farm on the west coast of Scotland. Sediment samples were collected approximately monthly over two independent production cycles encompassing both production and fallowing periods. Sampling was performed along a well-defined organic enrichment gradient comprising the cage edge (CE), an intermediate impact zone (INT), and a reference site (REF). Notably, the second production cycle followed a longer fallowing period and coincided with a relocation of the fish cages, providing an opportunity to evaluate how differences in farm management influenced bacterial community dynamics.

Bacterial communities were characterized using 16S rRNA gene (V3-V4) metabarcoding following the standardized laboratory workflow established in Chapter I. DNA was extracted from sediment samples, amplified using the optimized PCR protocol identified

previously, and sequenced on an Illumina MiSeq platform. Sequence data were quality filtered, denoised, and clustered into amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016), which were subsequently assigned taxonomic identities using the SILVA reference database (Quast et al., 2013).

Community responses were assessed using complementary multivariate approaches implemented in R v.4.1.1 (R core Team, 2022) to evaluate both compositional patterns and temporal dynamics. Alpha- and beta-diversity analyses, non-metric multidimensional scaling (NMDS), and permutational multivariate analysis of variance (PERMANOVA) were used (using 'vegan' R package; Oksanen et al., 2022) to quantify the relative contributions of organic enrichment, production cycle, seasonal variation, and environmental variables to bacterial community structure. In addition, ecological trajectory analysis was employed (using 'ecotraj' R package; Sturbois et al., 2023) to characterize the magnitude, directionality, and consistency of temporal community change, enabling a dynamic assessment of bacterial assemblage responses to organic loading, sediment recovery during fallowing, and changes in farm management.

3.1.3 Results and Discussion

Bacterial community composition remained strongly structured by the organic enrichment gradient throughout both production cycles. For example, Sulfurovaceae dominated the sediment at both CE and INT (18 and 12% relative abundance, respectively) during both production cycles, while Pirellulaceae was the most abundant family at REF with 12% relative abundance. These patterns are consistent with the ecological characteristics of these taxa. Members of the Sulfurovaceae are sulfur-oxidizing bacteria that are commonly associated with sulfate-rich, organically enriched sediments (Hamilton et al., 2014; Aranda et al., 2015; Pettersen et al., 2022), whereas members of the Pirellulaceae are typically associated with oligotrophic, well-oxygenated environments (Kaboré et al., 2020). The persistence of these dominant taxa across production cycles indicates that organic enrichment remained the primary determinant of bacterial community composition despite temporal environmental variation.

Alpha diversity analyses consistently distinguished the three impact categories, with bacterial diversity declining along the organic enrichment gradient. The lowest diversity was observed at the cage edge (CE), where rarefied ASV richness, Shannon diversity, and Pielou's

evenness reached minimum values of 401, 4.35, and 0.71, respectively, whereas the highest values were recorded at the reference (REF) site (3196, 7.25, and 0.91, respectively). Temporal comparisons revealed no significant differences in alpha diversity between production cycles at either the CE or REF sites. In contrast, the intermediate (INT) site exhibited significantly higher richness, Shannon diversity, and evenness during the second production cycle, suggesting partial recovery of sediment conditions following the extended fallow period (Keeley et al., 2014, 2019; Verhoeven et al., 2018).

These patterns were further supported by multivariate analyses. PERMANOVA identified organic enrichment as the dominant driver of bacterial community composition, explaining substantially more variation than production cycle, temperature, or sampling period (Figure 8). Collectively, these findings demonstrate that seasonal variability exerts only a comparatively minor influence on sediment bacterial assemblages and does not obscure the strong ecological signal associated with aquaculture-derived organic enrichment. This temporal consistency further supports the application of bacterial metabarcoding as a robust tool for routine environmental biomonitoring.

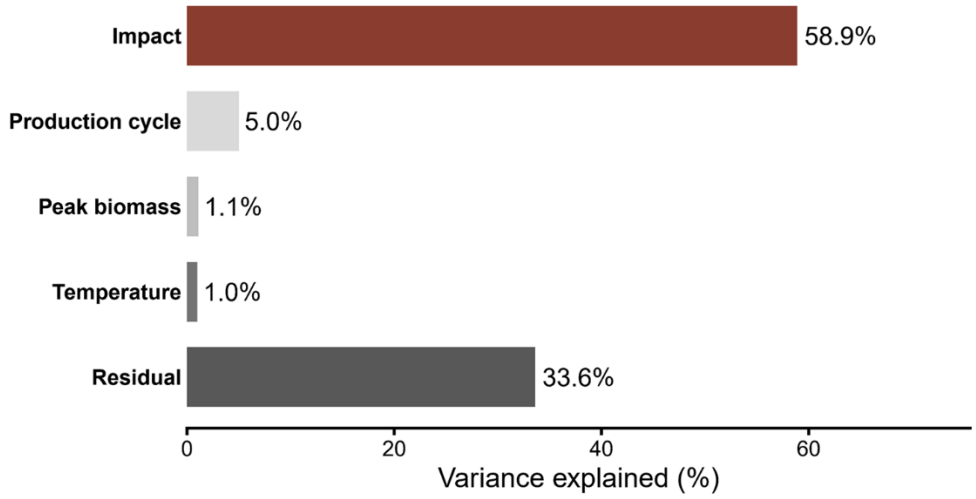


Figure 8. Relative contribution of the explanatory variables included in the PERMANOVA model to the variation in sediment bacterial assemblage composition. *Bars represent the proportion of total variation (R^2) attributed to each predictor and the residual component. The residual component represents variation attributable to factors not included in the model, as well as stochastic and unexplained ecological variation.*

Temporal analyses further demonstrated that bacterial communities responded predictably to changes in production activity and sediment recovery. Ecological trajectory analysis showed that bacterial assemblages at the REF site exhibited the longest trajectories but lacked consistent directional change, indicating that natural environmental variability produced dynamic yet resilient microbial communities rather than progressive shifts in community composition (Keeley et al., 2019). In contrast, communities at CE followed short, constrained trajectories, reflecting reduced temporal variability and convergence towards stable enrichment-tolerant assemblages under chronic organic loading (Allison and Martiny, 2008). The INT site exhibited the greatest temporal responsiveness, with increased bacterial diversity during the second production cycle and stronger directional community change following the extended fallow period. These findings indicate that management interventions, including extended fallowing and cage relocation, can substantially influence bacterial community succession in transitional habitats. Collectively, the results demonstrate that bacterial community trajectories are governed primarily by cumulative organic enrichment and aquaculture management practices, while natural seasonal variability contributes comparatively little to long-term community dynamics. This highlights the potential of bacterial communities as robust indicators for tracking both environmental degradation and ecological recovery.

Differences between the two production cycles further demonstrated that bacterial communities respond not only to organic enrichment but also to aquaculture management practices. The longer fallowing period preceding the second production cycle, together with the relocation of fish cages, resulted in measurable shifts in bacterial community composition, particularly at the intermediate site, where enrichment-tolerant taxa declined and diversity increased. These findings indicate that management interventions can influence the trajectory of bacterial community succession and highlight the importance of considering production history when interpreting temporal ecological change. Collectively, this chapter demonstrates that bacterial community responses are primarily driven by aquaculture-derived organic enrichment while remaining sufficiently resilient to natural temporal variability, reinforcing their suitability as robust indicators for environmental monitoring. A Schematic overview of the main findings of Chapter II is presented in Figure 9.

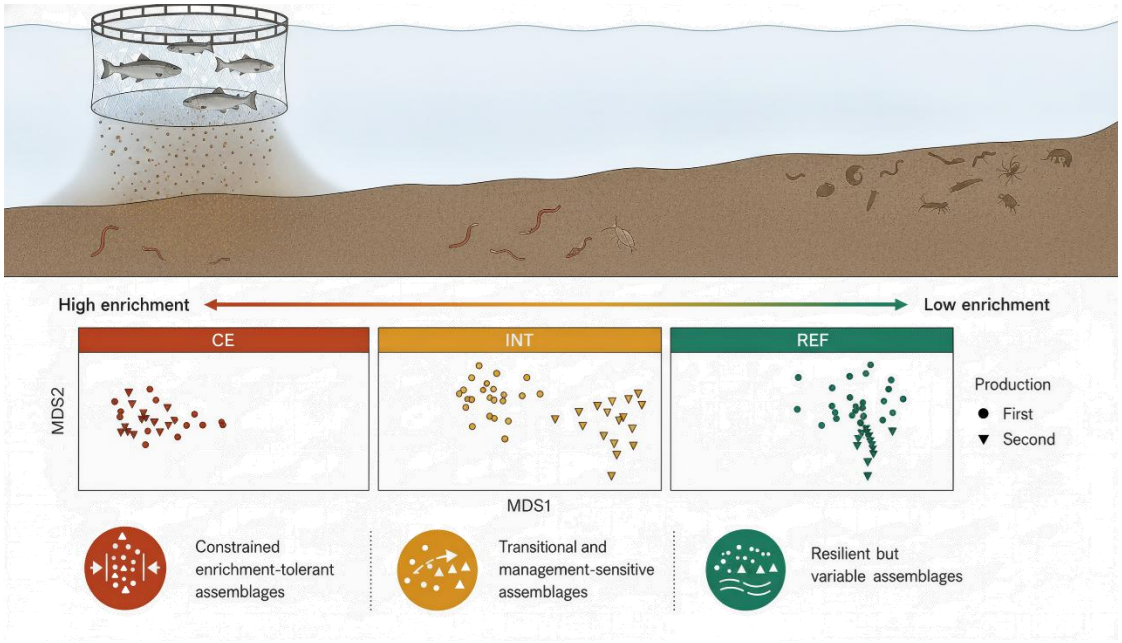


Figure 9. Synthesis of the ecological responses of sediment bacterial communities to aquaculture-derived organic enrichment. *Organic enrichment remained the primary driver of bacterial community composition, overriding seasonal variability throughout two salmon production cycles. Communities beneath the fish cages (CE) converged towards stable enrichment-tolerant assemblages, whereas reference communities (REF) remained resilient while exhibiting natural temporal variability. In contrast, the intermediate impact zone (INT) showed the greatest responsiveness to production history and farm management, reflecting its transitional ecological state. Ordination plots illustrate the separation of bacterial communities between the first and second production cycles at each impact level.*

3.1.4 Contribution to the Thesis

Chapter II demonstrated that sediment bacterial communities are structured primarily by aquaculture-derived organic enrichment, while natural temporal variability contributes comparatively little to overall community composition. Although bacterial assemblages exhibited predictable temporal dynamics throughout the production cycles, these changes did not obscure the ecological signal associated with organic enrichment. Furthermore, bacterial community trajectories responded to farm management practices, with the greatest temporal responsiveness observed at intermediate impact levels, highlighting the importance of considering production history when interpreting ecological change. Together with the methodological foundation established in Chapter I, these findings demonstrate that both technical and natural sources of variation can be effectively accounted for, providing the ecological foundation for the identification and selection of robust bacterial indicators in the following chapter.

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3.2 Publication

eDNA-Based Temporal Trajectories of Bacterial Communities in Salmon Farm Sediments: Disentangling Seasonal Variability from Aquaculture Impacts

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3.2.1 Abstract

Benthic bacterial communities are increasingly recognized as sensitive indicators of aquaculture impacts, yet disentangling the effects of organic enrichment from natural variability remains a challenge, especially in salmon farming. We leveraged 16S-rRNA V3-V4 eDNA metabarcoding and ecological trajectory analysis across an enrichment gradient and two salmon production cycles, testing how cumulative disturbance and management history affect microbial dynamics. Our analyses revealed that nutrient enrichment associated with aquaculture was the dominant factor structuring bacterial communities, while seasonal variability exerted only a modest influence. Diversity metrics consistently distinguished impacted from reference sites, with bacterial richness approximately 3.9× higher and Shannon diversity 1.4× higher at the reference site compared to the cage edge. Multivariate analyses confirmed that organic enrichment explained 59% of the variation in benthic bacterial assemblage composition, surpassing smaller but significant contributions from temperature and production cycle, indicating that seasonal effects do not obscure enrichment-related patterns, including during peak biomass. Trajectory analysis further revealed contrasting temporal dynamics, where reference sites retained variable but resilient assemblages, while cage edge communities stabilized into enrichment-tolerant states with reduced microbial variability. Intermediate sites were most sensitive, shifting from stochastic to directional change in response to altered exposure following farm relocation and longer fallowing. Collectively, we showed that cumulative enrichment not only alters community composition, but also changes how stable and predictable microbial communities are through time. This has critical implications for biomonitoring, as trajectory-based approaches can help build time-aware

bacterial indicators that separate natural variability from disturbance, giving managers clearer signals to guide sustainable aquaculture practices.

Keywords: Sediment microbiome, Salmon farming, Biomonitoring, eDNA metabarcoding, Ecological trajectories, Nutrient enrichment.

3.2.2 Introduction

The rapid evolution of aquaculture, commonly termed the "*blue revolution*", marks its emergence as a vital and highly productive agricultural sector (Costa-Pierce, 2008; Simpson, 2011; Garlock et al., 2020). This expansion is driven by significant increases in fish production, which contributes to global food security and nutrition (Béné et al., 2016), providing a consistent source of high-quality protein (Boyd et al., 2022). A substantial portion of the economic growth of the aquaculture sector has been driven by the expansion of salmon farming (FAO, 2024). Nevertheless, alongside its undeniable socio-economic benefits, aquaculture has been linked to notable adverse environmental effects (Pusceddu et al., 2007), including the accumulation of total organic carbon in sediments, leading to seabed hypoxia (Karakassis et al., 1998; Gray et al., 2002; Hargrave et al., 2008; Dai et al., 2025). These disruptions compromise benthic ecosystem functioning, with consequences for habitat provision and biodiversity maintenance (Mirto et al., 2012; Keeley et al., 2013).

To address the challenges around benthic impacts and promote sustainable aquaculture practices, compliance biomonitoring has become a key management tool. This approach involves the systematic use of biological indicators to evaluate environmental conditions and ensure that aquaculture operations adhere to environmental regulations and standards (Wilding et al., 2018). Effective biomonitoring is therefore critical for detecting and mitigating environmental impacts, safeguarding ecosystem services, and supporting the long-term resilience of aquaculture systems (Martinez-Porchas and Martinez-Cordova, 2012).

Given the pivotal role of the benthic components of the ecosystem in delivering and supporting key ecosystem services (Galparsoro et al., 2014), routine compliance biomonitoring has traditionally relied on morphological identification of a limited set of taxa, mainly meio- and macro-fauna (e.g., Pereira, 1997; Magurran et al., 2010; Mirto et al., 2012; Stojanović et al., 2017; Bouchet et al., 2020). This “Biomonitoring 1.0” approach (Baird and Hajibabaei, 2012) is constrained by high sampling costs, lengthy processing, and delayed data

interpretation (Pawlowski et al., 2018), which hinder timely management responses. Consequently, there is growing interest in alternative approaches that can deliver faster and more comprehensive assessments. High-throughput sequencing (HTS) of environmental samples (Shokralla et al., 2012), termed Biomonitoring 2.0 (Baird and Hajibabaei, 2012), has emerged as a promising tool to meet this need.

Environmental (e)DNA-based methods offer a promising tool for biomonitoring, particularly by enhancing the detectability of a plethora of macro- and micro-organisms from a single sample (Zhang et al., 2020). This allows for capturing a comprehensive picture of the entire microbial community, which is practically impossible through morphological identification alone. This is particularly intriguing, given that microorganisms, especially prokaryotic microbial communities (Archaea and Bacteria), are essential to nutrient recycling and the remineralization of organic matter (Le et al., 2016). In aquaculture settings, bacterial metabolism drives the breakdown of organic deposits and harmful compounds, processes that contribute to oxygen depletion, sulfide accumulation, and sediment acidification, thereby reshaping benthic ecosystems (Kawahara et al., 2008; Reimers et al., 2013; Hornick and Buschmann, 2018). Moreover, bacteria have large population sizes, short generation times, fast growth rates, and respond quickly to various environmental changes (Smith et al., 2015; Aylagas et al., 2017). Consequently, bacteria are increasingly recognized as reliable indicators for monitoring pollution dynamics in various marine ecosystems (Dowle et al., 2015; Caruso et al., 2016; Aylagas et al., 2021; Keeley et al., 2018; Laroche et al., 2018; Stoeck et al., 2018; Cordier, 2020; Frühe et al., 2021a,b; Oladi et al., 2022, 2024; Wilding et al., 2023). Importantly, their responses to aquaculture disturbance have been shown to mirror those of macrofaunal assemblages (Stoeck et al., 2018; Oladi et al., 2024; Wyness et al., 2026), supporting their integration into established ecological quality assessments.

While macrobenthic communities remain the established standard for compliance monitoring, bacterial indicators derived from eDNA metabarcoding offer a powerful and scalable alternative for process-based environmental assessment. By targeting bacterial "indicator species," or more precisely amplicon sequence variants (ASVs), this approach enables high-resolution characterization of bacterial community responses to environmental change, which may improve the sensitivity of environmental monitoring relative to traditional macroinvertebrate indices (Borja et al., 2000, 2009, 2013; Aylagas et al., 2017; Borja, 2018; Frühe et al., 2021a). Their potential can be further explored through various analytical approaches, including supervised machine learning (Frühe et al., 2021a) and dedicated

indicator analyses (Verhoeven et al., 2018; He et al., 2021), and emerging quantitative molecular frameworks targeting specific bacterial indicators (Oladi and Stoeck, 2026), offering a robust complement to established benthic monitoring tools.

Developing a reliable bacterial bioindicator approach requires not only in-depth knowledge of bacterial community structure, but also the underlying ecological processes shaping their dynamics. Bacterial assemblages in aquatic environments are shaped by a complex interplay of natural and anthropogenic factors. Natural drivers, such as seasonal temperature fluctuations, can induce significant changes in microbial assemblage structure (Delille, 1995; Findlay and Watling, 1998). Meanwhile, human-induced disturbances, including nutrient loading from aquaculture or industrial activities, can further alter microbial assemblages, often promoting opportunistic or enrichment-tolerant taxa (Holmer et al., 2005; Frühe et al., 2021b; Johansen et al., 2024; Oladi et al., 2024). These impact-related shifts are critical for determining ecosystem health status, though they can be challenging to disentangle from natural background variability.

Anthropogenic-related stressors tend to accumulate in sediments, particularly once the system's buffering capacity is exceeded (Cranford et al., 2022). This gradual accumulation may drive progressive and directional shifts in microbial assemblage structure, pushing it away from its original state. Conversely, following a reduction in organic input (such as during fallowing), communities may begin to recover, returning toward pre-disturbance conditions (Keeley et al., 2014). Capturing these temporal dynamics is essential for developing bioindicators that reflect the full spectrum of ecological responses, from degradation to potential recovery.

Previous research has demonstrated the potential of benthic bacterial communities as sensitive indicators of environmental change, particularly in response to organic enrichment from marine aquaculture (Dowle et al., 2015; Keeley et al., 2018; Stoeck et al., 2018; Cordier, 2020; Frühe et al., 2021b; Wilding et al., 2023; Oladi et al., 2024). Much of the existing work has focused on spatial comparisons along disturbance gradients, while fewer studies have examined how these communities change temporally under prolonged or cyclical stress regimes such as fish production cycles (Verhoeven et al., 2018; Quero et al., 2020; Johansen et al., 2024). Furthermore, evidence on the influence of seasonal variation on bacterial communities associated with aquaculture remains inconclusive, with studies reporting context-dependent or site-specific effects linked to differences in hydrodynamics, sediment characteristics, seabed topography, and farm management practices (Cromey et al., 2022;

Kalantzi and Karakassis, 2006). Notably, the available studies differ in focus, with some examining communities in the water column (Kyritsi et al., 2023), others in sediments (Wang et al., 2024), and some addressing both compartments (Kolda et al., 2020), complicating direct comparisons and generalizations. As a result, there is still limited consensus on how to integrate bacterial community data into operational ecological quality assessments, particularly in systems where both natural seasonal variability and human-induced pressures co-occur.

To address these critical gaps, we leveraged a well-defined organic enrichment gradient at a salmon farm on the western coast of Scotland, employing eDNA metabarcoding of the 16S rRNA gene (V3-V4 region) over two production cycles. This natural field experiment enabled the simultaneous assessment of temporal and impact-driven shifts in benthic bacterial communities under realistic management regimes. By integrating genetic data collected at different time points with complementary ecological metrics, our study provides novel insights into the interplay between seasonal variability and cumulative anthropogenic disturbance in the response of bacterial communities to aquaculture disturbance.

In addition to identifying key ecological drivers, we evaluated whether the timing of regulatory compliance monitoring, which is typically conducted at peak biomass (when organic loading is highest), could bias the detection of enrichment effects. Seasonal changes in temperature and hydrodynamics may influence sediment oxygenation and organic matter turnover (Grenz et al., 2010; Hicks et al., 2017), yet it remains unclear whether such background variability alters bacterial community signals used for compliance assessments.

We hypothesized that (1) organic enrichment from aquaculture would be the primary driver of bacterial assemblage structure, surpassing seasonal influences, and (2) the timing of sampling (peak vs. non-peak biomass) would not significantly alter the detection of enrichment-related community shifts. If confirmed, this would suggest that monitoring efforts can be confidently aligned with operational schedules without concern for strong seasonal bias, reinforcing the reliability of bacterial assemblage data as a foundation for environmental compliance and adaptive management.

3.2.3 Material and methods

Study site and sampling strategy

We conducted this research on the west coast of Scotland (UK) near Oban, at an Atlantic salmon (*Salmo salar*) aquaculture installation site in Loch Linnhe. The fish farm in this region is located above a moderately flat seabed at an average depth of 40 m (Wilding et al., 2012). The farm, operated by Scottish Sea Farms Ltd (Equitable House, London), has been operating since 1987 and is thus expected to represent equilibrium conditions regarding its impact status. The localized gradient of organic enrichment in the sediment below and adjacent fish cages was confirmed in previous studies (e.g., Wilding et al., 2012, 2023; Oladi et al., 2024). Sampling for this study was performed at various distances from the farm, including the outer cage edge (CE), an intermediate impact zone (INT), and a reference site further away from aquaculture activities (REF).

To evaluate the potential impacts of seasonal variation and organic enrichment on bacterial communities, we collected samples monthly during two separate aquaculture production cycles (Fig. 1a). The first production cycle included in our study started in May 2018, following a five-month fallow period. We collected samples approximately monthly from June 2018 to June 2019, except for November and December 2018 due to poor weather conditions. We also collected an additional sample in November 2019 to represent late autumn, although the farm was in fallow at that time. The farm was active throughout the primary sampling period of this cycle (Fig. 1). The next production cycle included in our study (henceforth the second production cycle) began in May 2022, after a nine-month fallow period. Sampling was conducted from February 2023 to August 2023. It should be noted that the spatial configuration of the farm changed between the two cycles (Fig. 1b & c), with cage positions relocated closer to the intermediate sampling site during the second cycle.

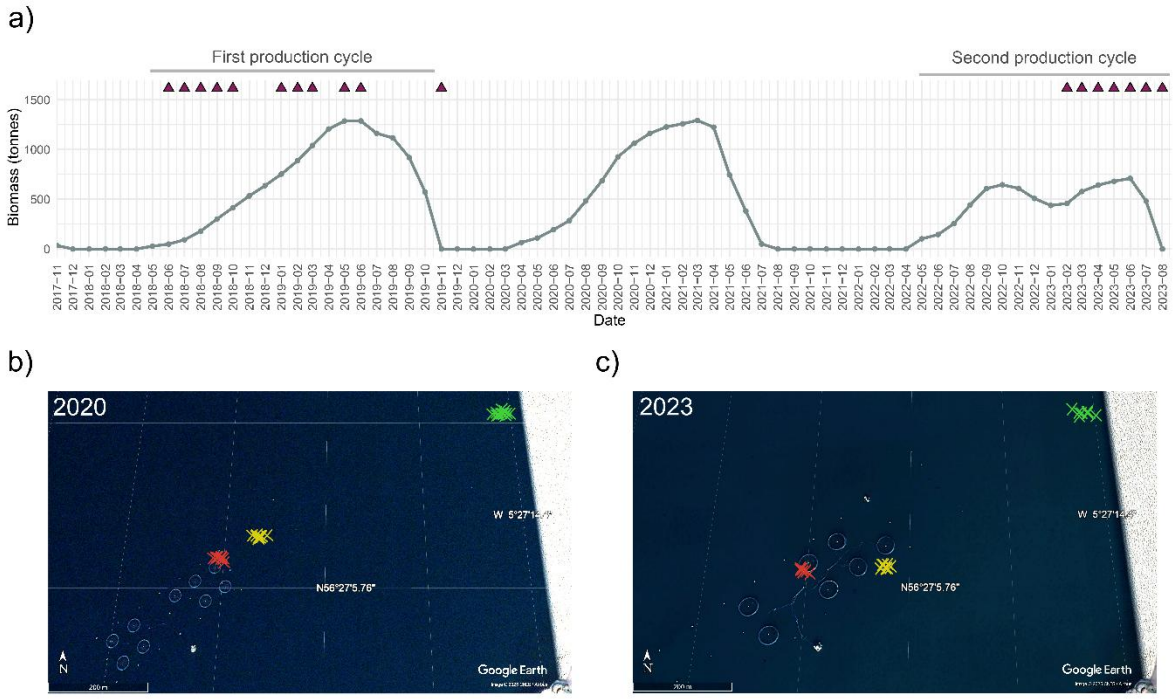


Figure 1. *(a) Time series fish biomass (tonnes) at the studied salmon farm spanning from November 2017 to August 2023. Months of sampling are shown with maroon triangles. Note that the two investigated production cycles were not consecutive; an additional production cycle occurred between them but was not included in the present study. (b, c) Google Earth satellite images from 2020 and 2023, corresponding to the first and second production cycles, respectively, depicting cage locations, with noticeable changes in number, size, and configuration between production cycles. Sampling points are marked as red (cage edge), yellow (intermediate impact zone), and green (reference).*

At each site, we collected three replicate Van Veen grab (0.1 m^2 surface area) sediment samples according to the standardized operating protocol published by the Scottish Environment Protection Agency (SEPA, 2023). From each side of one grab, we combined three scoops (six scoops in total) of the top 10 mm of the sediment into a sterile plastic tube (SEPA, 2023; Wilding et al., 2023; Oladi et al., 2024; Wyness et al., 2026). Following collection, we promptly froze the samples at -20°C until further processing.

DNA extraction, PCR amplification, and 16S rRNA gene sequencing

We used DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) to extract DNA from ~250 mg of each sediment sample. The manufacturer's recommendations were followed, with slight modifications as described in Oladi et al. (2022). The NanoDrop 2000 spectrophotometer (Peqlab, Erlangen, Germany) was used to evaluate both the concentration and quality of the

extracted DNA. A ~400 bp fragment of the V3-V4 hypervariable regions of the bacterial 16S rRNA gene was targeted as metabarcode using the primer pair Bakt341F (5′-CCTACGGGNGGCWGCAG-3′) and Bakt805R (5′-GACTACHVGGGTATCTAATCC-3′) (Herlemann et al., 2011).

As per the recommendation made by Oladi et al. (2024), in order to obtain reliable results, we used the following polymerase chain reaction (PCR) protocol: each sample was amplified in a total volume reaction of 25 µl, containing 12.5 µl of 2X KAPA HiFi HotStart ReadyMix and 0.2 µM of each of the above-mentioned primers and 1 µL of stock DNA. The following PCR program was run in a thermal cycler: initial denaturation was performed at 95 °C for 3 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by final elongation at 72 °C for 5 min. Each PCR run contained positive controls as well as negative controls with no template DNA. All samples were amplified in three independent PCRs (technical replicates), which were pooled into an equal volume after being quality-checked on a 1.5% agarose gel. All amplicons were subsequently purified using Qiagen's MinElute PCR purification kit according to the manufacturer's instructions. PCR products were then sent to StarSeq Co. (Mainz, Germany) for the second-stage PCR (indexing/adapter addition) and sequencing on an Illumina MiSeq platform (2 × 300 bp paired-end), following the provider's in-house protocol. The raw sequences are publicly available at the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under the BioProject ID PRJNA1353086, accession numbers SAMN52926655-SAMN52926804.

Quality filtering, ASV clustering, and taxonomic assignment

Illumina reads were first quality-checked using FastQC (Andrews, 2010) based on an assigned quality score across all bases. Adapters and primers were removed using cutadapt v.2.11.0 (Martin, 2011) with the default settings. Further sequence processing was done using the Divisive Amplicon Denoising Algorithm (DADA2) (Callahan et al., 2016) pipeline in R v.4.1.1 (R core Team, 2022). Briefly, forward reads were truncated to 230 bp and reverse reads to 225 bp, with a maxEE of 2 for both. Reads were dereplicated, denoised, and then merged (minOverlap = 20), which were subsequently screened for chimeric sequences using vsearch (Rognes et al., 2016) with default parameters. The remaining reads were clustered into Amplicon Sequence Variants (ASVs) using DADA2.

Representative sequences for each ASV were taxonomically assigned using vsearch against the SILVA v.138 reference database (Quast et al., 2012) employing the last common

ancestor (LCA) approach. For subsequent analyses, ASVs assigned to the Bacteria domain were retained, while mitochondrial and Chloroplast 16S sequences, as well as ASVs with only one sequence read (singletons), were discarded.

Statistical analysis

All analyses were carried out in the R environment for statistical computing. Graphic visualizations were done using the 'ggplot2' package (Wickham, 2016).

Rarefaction curves were plotted using the R package 'vegan' v.2.6-4 (Oksanen et al., 2022) to check for sufficient sequencing depths. Prior to alpha diversity calculations, differences in sequencing depth among samples (19,384-87,460 reads per sample) were addressed by rarefying samples to the minimum library size (19,384 reads) using the rrarefy function in the 'vegan' package. Rarefaction was applied to standardize sequencing effort across samples prior to comparisons of alpha diversity metrics. Following rarefaction, alpha diversity metrics, including rarefied ASV richness, the Shannon index (H'), and Pielou's evenness, were calculated using the 'vegan' package. Statistical differences in alpha diversity measures among sites and between production cycles were assessed using pairwise Wilcoxon rank-sum tests with Benjamini-Hochberg (BH) correction for multiple comparisons using the 'stat' v.4.3.1 R package (R core Team, 2022). As input data for beta-diversity analyses, the ASV-to-sample matrix of non-rarefied sequence reads was used and the data table was then Hellinger-transformed to reduce the influence of dominant taxa and account for the large number of zero values using the R-package 'funrar' v.1.4.1 (Grenié et al., 2017). Beta diversity was calculated with the Bray-Curtis (BC) index. BC dissimilarity matrix was then used for non-metric multidimensional scaling (NMDS) analyses of the bacterial assemblages using the 'vegan' package. To further visualize temporal trajectories and assess potential cyclicity, we calculated monthly centroids of the ordination coordinates (mean position of biological replicates per month, impact level, and production cycle). These centroids were then sequentially connected in ordination space to illustrate the direction and continuity of community change through time. Environmental vectors were fitted into the ordination space using 'vegan' in R. The two environmental factors were Infaunal Quality Index (IQI) and loss on ignition (LOI). IQI was calculated from macrobenthic communities provided by Ocean Ecology (Oban, UK) using the IQI v2 workbook (Phillips et al., 2014). LOI was measured as mass loss after combustion at 450 °C for 4 h, expressed relative to dry weight (Heiri et al., 2001).

To determine the relative contributions of the impact of nutrient enrichment, different production cycles, and temporal factors to variation in bacterial assemblage structure, we conducted a Permutational Multivariate Analysis of Variance (PERMANOVA). The analysis was performed using the R package 'vegan' on untransformed relative abundance data and BC dissimilarity, as this approach resulted in lower residual variation and better model fit compared to analyses performed on Hellinger-transformed data. Our main objective was to partition the explained variance to identify the main factors driving community change. To further evaluate whether sampling timing or temperature influenced the detection of aquaculture impacts, we extended the model to include scaled temperature (Temp; the 10-year average water temperature of the region) and a categorical factor representing biomonitoring period (PeakBiomass; peak vs. non-peak biomass periods), as well as their interaction. This allowed us to test whether temperature modified assemblage differences between biomonitoring operation phases, thereby assessing potential confounding between seasonality and production activity. The final model included impact level (CE, INT, REF), production cycle, scaled temperature, biomonitoring period, and their interaction (PeakBiomass × Temp).

In addition, we applied ecological trajectory analysis to explore the temporal dynamics of bacterial communities in response to organic enrichment and production activity. This analysis assumes directional (non-cyclical) temporal progression and does not explicitly model periodic or circular patterns such as seasonal loops. This assumption was appropriate given the absence of a clear cyclical structure in ordination space. Using the 'ecotraj' R package (Sturbois et al., 2023), we computed trajectory metrics in the multivariate ordination space derived from non-metric multidimensional scaling (NMDS) of BC dissimilarities. Trajectories were constructed from sequential monthly observations in chronological order within each impact level and production cycle, allowing trajectory metrics to quantify the stepwise temporal movement of communities through ordination space. This approach allowed us not only to detect whether assemblages differed across sites or time points, but also to quantify how consistent and directional those changes were over time. From each trajectory, we extracted summary metrics (including trajectory length, directionality, and internal variance) to characterize the magnitude, orientation, and coherence of community change. To further quantify directional change over time, we used linear models to test how the distance from the starting point in ordination space changed with survey month (slope) and initial differences between sites (initial offset). This allowed us to assess whether community trajectories reflected a gradual accumulation of stress or a tendency toward recovery.

3.2.4 Results

Overview of sequence data

The total number of sequences after bioinformatic processing, filtering, and chimera removal was 14,944,615 reads from 150 eDNA samples (51 samples from CE, 50 samples from INT, and 49 samples from REF). After removing non-target sequences (i.e., non-Bacteria and chloroplastic and mitochondrial sequences) and singletons, 7,762,179 sequences remained, resulting in 48,140 ASVs. Rarefaction curves clearly demonstrated that the sequencing depth was sufficient to achieve a near-complete characterization of bacterial diversity, as evidenced by the plateauing observed in the curves (Supplementary Fig. S1).

Overview of bacterial assemblage structure across sites and production cycles

Overall, the identified ASVs were classified into 69 phyla, 166 classes, 389 orders, and 484 families. Despite this high taxonomic diversity, the relative abundance of the dominant bacterial families remained largely stable over time within each impact category (CE, INT, REF), with only minor temporal fluctuations observed throughout the sampling period (Fig. 2). Major compositional patterns in Fig. 2 are presented for the 10 most relatively abundant families at each site; however, statistical comparisons were performed across all families, with *p*-values corrected for multiple testing (BH procedure).

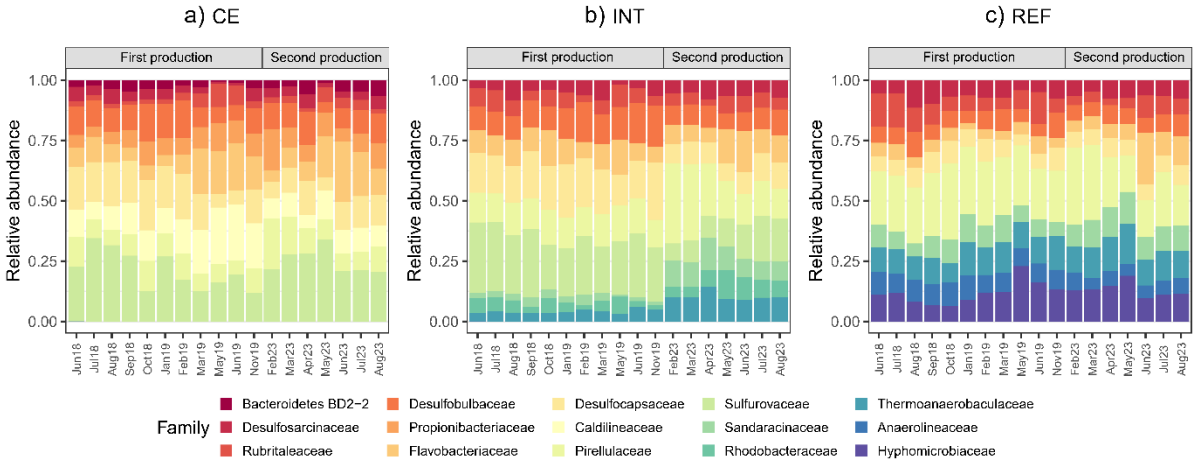


Figure 2. *Stacked bar plots representing the taxonomic composition of the 10 most abundant bacterial families at each impact site over time. Labels on the x-axis show the month and year of sampling. Colors are used to distinguish dominant bacterial families and to facilitate visualization of compositional similarities and differences among impact categories; they do not represent phylogenetic relatedness. a) CE = cage edge. b) INT = intermediate impact zone. c) REF = reference.*

At the CE site, four out of 484 identified bacterial families accounted for approximately 50% of the total relative abundance across all sampling dates. These dominant families included Sulfurovaceae (~18%), Desulfocapsaceae (~11%), Caldilineaceae (~11%), and Flavobacteriaceae (~10%). Additionally, Propionibacteriaceae, Caldilineaceae, and an unclassified Bacteroidetes family affiliated with class BD2-2 were exclusively among the 10 most abundant families at CE, and were not present in the top 10 at the other sites. The overall composition showed minimal changes between the first and second production cycles. While a few families exhibited statistically significant differences in relative abundance, the actual magnitude of change was small. For instance, Caldilineaceae underwent a marginal decline from a median relative abundance of 0.10 in the first production cycle to 0.08 in the second (Wilcoxon rank-sum test, $p_{BH} < 0.05$), and Desulfocapsaceae decreased from 0.13 to 0.10 ($p_{BH} < 0.01$). Although statistically significant, these small relative differences are unlikely to reflect ecologically meaningful shifts.

At the REF site, by contrast, the bacterial assemblage was more evenly distributed across taxa, with lower dominance by individual families. Pirellulaceae was the most abundant family, accounting for approximately 12% of the total relative abundance, while all other dominant families occurred at less than 6% across all samples. Thermoanaerobaculaceae, Hyphomicrobiaceae, and Sandaracinaceae were among the most consistently abundant families throughout the time series, with little variation between production cycles. Notably, Hyphomicrobiaceae and Anaerolineaceae were among the 10 most abundant families exclusively at the REF site and were not detected within the top 10 at CE or INT. Although the overall composition remained stable over time, few families exhibited statistically significant differences between production cycles. However, the changes in relative abundance were generally small and are unlikely to represent meaningful ecological shifts. For example, Anaerolineaceae showed a modest decrease from 0.04 to 0.03 ($p_{BH} < 0.001$).

The INT site shared most of its dominant taxa with both CE and REF, with Rhodobacteraceae being exclusively present among the ten most abundant families at this intermediate location. The most abundant bacterial family in INT, similar to CE, was Sulfurovaceae (~12%), followed by Pirellulaceae (~9%), Desulfocapsaceae (~9%), and Desulfobulbaceae (~7%). Additionally, the INT site was the only one to show notable differences in the relative abundance of several dominant families between the two production cycles. For instance, Sulfurovaceae showed a significant decline, with its median relative abundance decreasing from 0.15 in the first production cycle to 0.06 in the second (Wilcoxon

rank-sum test, $p_{BH} < 0.001$). A similar pattern was observed for Desulfocapsaceae, which declined from a median of 0.12 to 0.05 ($p_{BH} < 0.001$). In contrast, Thermoanaerobaculaceae showed significant increases, with median relative abundances rising from 0.02 in the first production cycle to 0.05 in the second, representing an approximate 2.5-fold increase in both cases ($p_{BH} < 0.001$).

Comparisons of alpha and beta diversity of bacterial assemblages across sites and production cycles

Overall, all alpha diversity measures (rarefied richness, Shannon index, and Pielou's evenness) showed significant pairwise differences among the three impact categories (i.e., CE, INT, and REF; Fig. 3), as determined by pairwise Wilcoxon rank-sum tests with BH correction ($p_{BH} < 0.001$). While minimum values of rarefied bacterial ASV richness, Shannon index, and evenness were obtained from the CE site (401, 4.35 and 0.71, respectively), all maximum values were observed at the REF site (3196, 7.25 and 0.91, respectively). Alpha diversity metrics were additionally compared between production cycles within each site. No significant differences were found between cycles at the CE and REF sites (pairwise Wilcoxon rank-sum test, $p_{BH} > 0.05$). In contrast, the INT site exhibited significantly higher richness, Shannon index, and evenness during the second production cycle compared to the first (Fig. 3; $p_{BH} < 0.001$ for all metrics).

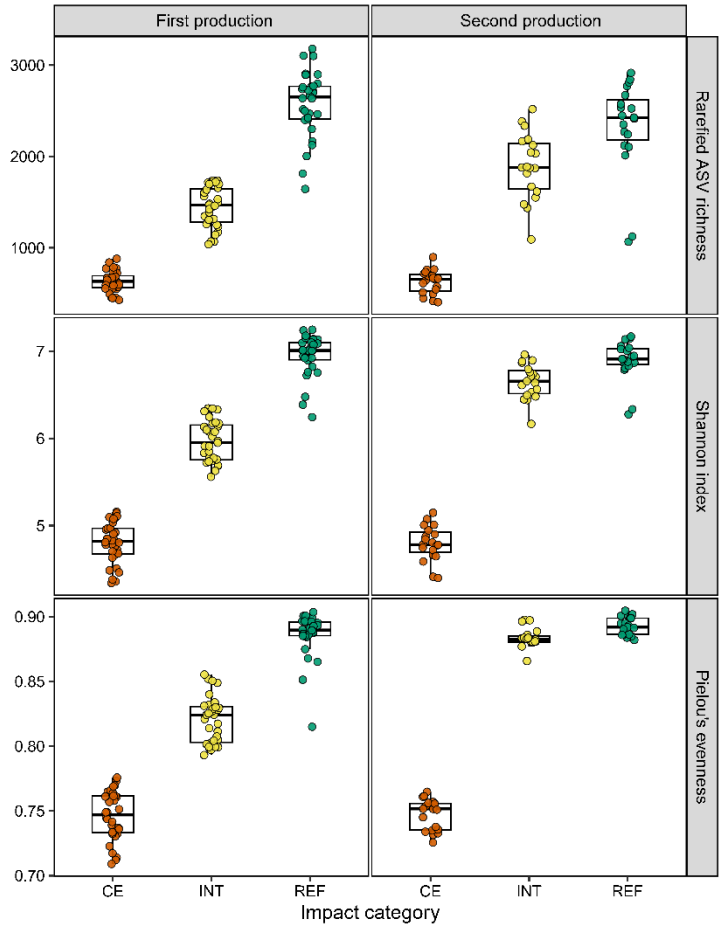


Figure 3. *Alpha diversity of sediment bacterial communities across impact categories (CE, INT, and REF) and production cycles. Panels show rarefied ASV richness, Shannon diversity index, and Pielou's evenness for the first and second production cycles. Boxplots represent the median and interquartile range. Points represent individual samples. Across both production cycles, all pairwise comparisons among impact categories were significant for all alpha diversity metrics (pairwise Wilcoxon rank-sum tests with Benjamini-Hochberg correction, $p < 0.001$). Pairwise significance annotations are not shown in the figure to preserve visual clarity.*

The non-metric multidimensional scaling (NMDS) analysis of beta diversity evidenced a high-resolution power (NMDS stress value = 0.044 in both panels) of benthic bacterial assemblages in the distinct clustering of sites with different degrees of organic enrichment (Fig. 4). Within each cycle, bacterial assemblages completely segregated along the first axis, corresponding to the nutrient enrichment gradient (Fig. 4). While CE samples clustered tightly together on the ordination space, REF samples were distributed along the second axis, with INT samples forming an intermediate group in the middle.

Month centroids showed rather short trajectories, indicating limited temporal displacement relative to the strong spatial structuring imposed by enrichment. CE centroids remained nearly stationary across months, while INT and REF centroids displayed gradual directional shifts along MDS2. In the second production cycle, INT and REF trajectories followed a partial arc from February to August, reflecting a progressive but non-cyclical temporal change in assemblage composition. The ordination pattern was consistent with that of the first cycle, although assemblages at the INT site were positioned closer to the REF

cluster, corresponding to the initial community state following a nine-month fallow period preceding the second production cycle.

In addition, environmental vector fitting indicated that both LOI and IQI were significantly correlated with patterns in bacterial assemblage composition (envfit, $p < 0.05$ for both). The LOI vector aligned along the negative end of the first NMDS axis (MDS1), while the IQI vector was oriented toward the positive end of the same axis (Fig. 4). LOI and IQI were significantly and inversely correlated (Spearman's $\rho = -0.64$, $p < 0.001$), indicating that they describe related aspects of the enrichment gradient. The correlation was moderate ($R^2 = 0.55$), and variance inflation factors (VIF = 2.23 for both) were below thresholds of concern, suggesting that multicollinearity was limited. Both vectors were therefore retained in the NMDS for orientation purposes, as they jointly capture the gradient in sediment organic enrichment and benthic condition.

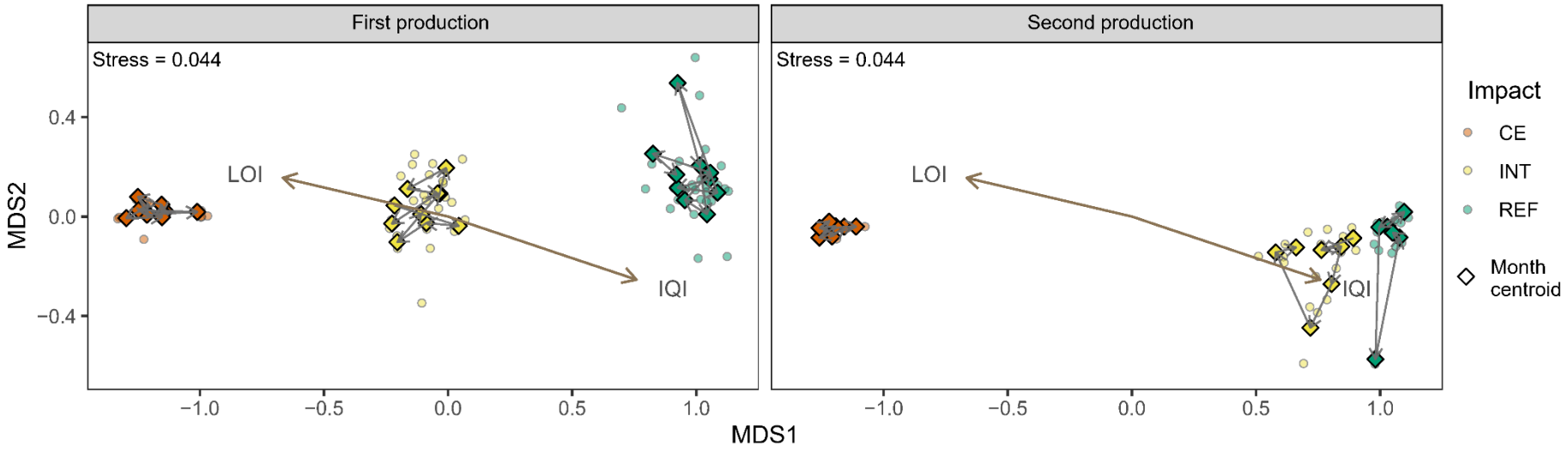


Figure 4. *Non-metric multidimensional scaling (NMDS) plot based on the Bray-Curtis dissimilarities of bacterial assemblages along a nutrient enrichment gradient across two production cycles. Transparent points represent individual samples. Diamonds indicate monthly centroids connected by arrows to illustrate temporal trajectories from the first to the last sampling month within each site. Brown arrows for LOI and IQI represent significant environmental vectors (envfit, $p < 0.05$) aligned with the enrichment gradient. CE = cage edge; INT = intermediate impact zone; REF = reference.*

Moreover, the results of PERMANOVA further validated that organic enrichment remained the dominant factor structuring bacterial assemblage composition ($R^2 = 0.589$, $p < 0.001$; Table 1), followed by production cycle ($R^2 = 0.050$, $p < 0.001$). Temperature and biomonitoring operation phase (peak vs. non-peak) explained smaller but significant proportions of variation ($R^2 = 0.010$ and 0.011, respectively; $p < 0.01$ for both). The interaction between temperature and sampling phase was not significant ($p = 0.24$), indicating that the compositional differences between peak and non-peak periods were not temperature-dependent. The residual variance remained at 34%, indicating that the majority of the variation was captured by the included factors, with only a minor proportion left unexplained.

Table 1. Results of the PERMANOVA analysis showing the differences in bacterial assemblage composition across the three impact categories (CE, INT, and REF), two production cycles and four seasons, as well as the explained variations by each factor.

Sample comparison	Df	Sum of Squares	R^2	F	Pr(>F)
Impact	2	21.54	0.589	125.40	0.001
ProductionCycle	1	1.85	0.051	21.48	0.001
Temp	1	0.37	0.010	4.34	0.009
PeakBiomass	1	0.41	0.011	4.81	0.006
PeakBiomass×Temp	1	0.11	0.003	1.24	0.243
Residual	143	12.28	0.336		
Total	149	36.56	1.000		

Ecological trajectories of bacterial assemblages across sites and production cycles

Ecological trajectory analysis allowed us to assess the magnitude (trajectory length), linearity (directionality), and stability (internal variance) of assemblage changes across two salmon production cycles at sites with varying levels of organic enrichment (Table 2). Across both production cycles, the REF site consistently exhibited the longest trajectories, with the first cycle showing nearly double the path length of the second. Directionality remained stable between cycles, while internal variance slightly decreased, indicating a reduction in temporal variability over time. At the INT site, the total path length was intermediate relative to the other

sites and declined by over one-third between cycles. Directionality values remained moderately high, and internal variance remained relatively stable. These metrics indicate a modest but consistent temporal shift across cycles. The CE site displayed the shortest trajectories among the three sites, with a nearly 50% reduction in path length from the first to the second cycle. Directionality was highest in the first cycle but declined slightly thereafter. Internal variance was moderate in the first cycle and dropped further in the second, indicating a shift toward greater consistency. The relatively short but highly directed trajectory in the first cycle, coupled with elevated variance, indicates that assemblage composition underwent rapid but irregular shifts, consistent with an unstable response under high-impact conditions.

In addition, we used linear models to assess whether bacterial communities were moving directionally away from their initial state, and at what rate. Two main outputs were considered: the initial offset, indicating how much a site already differed from the reference community at the beginning of each cycle, and the slope, representing the rate of directional change over survey time. We observed that at the REF site, slope estimates for both production cycles were near zero and statistically non-significant ($p > 0.3$), indicating no directional trend and suggesting temporal stability. Similarly, the INT site showed no significant trend in the first cycle, although the positive slope suggested a non-significant emerging change. However, in the second cycle, we detected a significant positive slope ($p < 0.001$), indicating a strong directional shift in assemblage structure over time at this intermediate-impact site.

At the CE site, the initial offset was slightly negative in both production cycles (Table 2), indicating that the community started marginally closer to the reference condition. During the first cycle, the slope was positive and statistically significant ($p < 0.001$), indicating a strong directional change over time. In the second cycle, the slope remained positive but was not significant ($p = 0.32$), suggesting that the directional trend weakened or saturated.

Table 2. Summary of ecological trajectory metrics and linear model results for bacterial community change across two salmon production cycles at three impact categories (CE, INT, and REF). Trajectory metrics include total path length (magnitude of change), directionality (linearity of change), and internal variance (temporal consistency). Linear model outputs include the initial offset (distance from the starting point at the beginning of the cycle), slope (rate of change over time), and associated *p*-value. Asterisks denote statistical significance ($p < 0.001$).

Trajectory metrics					Linear model		
Impact	**Production**	**Length**	**Directionality**	**Internal variance**	**Initial offset**	**Slope**	***p*-value**
REF	First	11.362	0.344	0.102	0.036	0.0005	0.804
	Second	6.240	0.341	0.080	0.091	-0.0040	0.329
INT	First	9.110	0.359	0.076	0.026	0.0041	0.225
	Second	5.751	0.360	0.077	-0.359	0.0326	4.01E-08***
CE	First	5.254	0.391	0.059	-0.013	0.0184	2.15E-08***
	Second	2.848	0.367	0.040	-0.004	0.0017	0.32

3.2.5 Discussion

As understanding temporal dynamics becomes increasingly important in evaluating environmental change, this study examined not only whether bacterial assemblages differed across sites and seasons, but also how they evolved through time. By tracking community trajectories across two aquaculture production cycles, we found that bacterial communities at impacted sites exhibited more directional and cumulative paths, suggesting progressive divergence from reference conditions likely driven by increasing organic load. In contrast, reference sites showed less directional and more stable community patterns. These findings underscore the stronger role of organic enrichment compared to seasonality in shaping sedimentary bacterial community structure over time.

Taxonomic transitions in bacterial assemblages across the impact gradient and production cycles

Shifts in bacterial assemblage structure along organic enrichment gradients from salmon aquaculture are well documented and have been discussed in detail previously (Dowle et al., 2015; Keeley et al., 2018; Stoeck et al., 2018; Verhoeven et al., 2018; Frühe et al., 2021b; Wilding et al., 2023; Oladi et al., 2024). This section contains descriptions of the autecology of selected bacterial families that exhibited significant changes in relative abundance from the highly impacted CE site to the REF station. While inferring ecological properties at the family level can be challenging due to the often high functional and phylogenetic diversity among constituent genera and species, some families display relatively well-conserved ecological roles that allow for meaningful interpretation in the context of organic enrichment gradients.

Members of the family Sulfurovaceae, currently represented mainly by the genus *Sulfurovum*, are typically involved in sulfur oxidation, often coupled to nitrate or oxygen

reduction (Pettersen et al., 2022), and are abundant in sulfate-rich environments (Hamilton et al., 2014; Aranda et al., 2015). This taxon is a well-established indicator of sediments that are highly impacted by aquaculture activities (Aranda et al., 2015; Choi et al., 2018; Pettersen et al., 2022; Oladi and Stoeck, 2026). Similarly, the family Desulfocapsaceae primarily contains sulfate-reducing bacteria widely associated with high-organic, low-oxygen marine sediments (Kovacik et al., 2006; Sun et al., 2015; Song et al., 2021). While earlier studies reported enrichment of Desulfobulbaceae at aquaculture sites (e.g., Frühe et al., 2021b; Aylagas et al., 2017; Verhoeven et al., 2018), recent phylogenomic revisions have reassigned several of these genera (e.g., *Desulfocapsa*) to Desulfocapsaceae (Waite et al., 2020), without substantially altering their inferred ecological roles in anaerobic sulfur cycling and organic matter degradation (Wasmund et al., 2017).

The family Caldilineaceae is typically associated with anaerobic degradation of organic matter in marine sediments, where it couples organic breakdown to iron reduction as part of the redox cascade of terminal electron acceptors (Canfield et al., 1993; Hyun et al., 2009; Ape et al., 2019; Duchinski et al., 2019). This metabolic capacity makes them well-suited to nutrient-enriched environments with elevated iron concentrations, such as sediments beneath salmon cages, where iron can accumulate from uneaten feed.

Representatives of the family Pirellulaceae are heterotrophic bacteria (Lage et al., 2019; Kulichevskaya et al., 2022; Vitorino and Lage, 2022) that often thrive in low-nutrient, oligotrophic environments (Kaboré et al., 2020), typically inhabiting well-oxygenated sediments. Such conditions are generally restricted to the upper oxic layer of sediments and tend to diminish under high organic loading due to accelerated oxygen depletion (Jørgensen and Revsbech, 1985; Holmer et al., 2005). Likewise, Hyphomicrobiaceae (Alphaproteobacteria) includes predominantly aerobic chemoheterotrophs, many of which are adapted to oligotrophic, oxygen-rich conditions (Oren and Xu, 2014).

While the ecological traits of these families suggest relatively predictable responses to enrichment or oxic conditions, their temporal trajectories are more complex to interpret. A central challenge is distinguishing whether observed changes reflect natural seasonal turnover (Gilbert et al., 2012; Fuhrman et al., 2015) or progressive shifts driven by the cumulative accumulation of organic matter from aquaculture. The latter can alter sediment biogeochemistry by lowering oxygen, increasing sulfide concentrations, and compressing oxic layers, thereby favouring enrichment-tolerant taxa such as Sulfurovaceae and

Desulfocapsaceae, while disadvantaging oxygen-sensitive groups such as Pirellulaceae and Hyphomicrobiaceae (Holmer et al., 2005; Keeley et al., 2014; Cranford et al., 2022). Distinguishing between seasonal variability and cumulative enrichment is therefore critical for interpreting temporal trends and assessing ecological quality.

Enrichment-tolerant families such as Sulfurovaceae and Desulfocapsaceae were consistently dominant at the CE and INT sites across both production cycles, underlining their strong association with organically enriched, reduced sediments. This pattern was also observed at this farm during a separate production cycle (Oladi et al., 2024). At the INT site, however, their relative abundances were significantly higher during the first cycle compared to the second. This decline may be linked to the longer nine-month fallow period preceding the second cycle, which likely promoted partial sediment re-oxygenation and organic matter degradation, thereby reducing the competitive advantage of strictly enrichment-tolerant taxa (Keeley et al., 2014). In contrast, Caldilineaceae were restricted to the CE site, with a marked decline in relative abundance toward less enriched sites, in line with observations by Frühe et al. (2021b). Their relative abundance did not differ significantly between production cycles, indicating that their occurrence at CE may reflect stable enrichment conditions rather than temporal variation.

In contrast to the impacted sites, aerobic families such as Pirellulaceae and Hyphomicrobiaceae remained relatively stable across production cycles. Pirellulaceae showed consistent abundances, reflecting stable oxic conditions at the reference station, while Hyphomicrobiaceae exhibited only a slight, non-ecologically meaningful increase during the second cycle. These stable patterns reinforce the interpretation that seasonal variation exerted only a minor influence on bacterial assemblages in our system, whereas the cumulative effects of organic loading were the primary driver of temporal shifts at impacted sites.

Alpha and beta diversity responses in bacterial assemblages across the impact gradient and production cycles

The observed patterns in bacterial assemblage composition were also reflected in alpha diversity metrics. This pattern suggests that environmental disturbances associated with salmon farming reduce bacterial diversity in sediments, consistent with previous reports of diversity loss under increased organic loading (Dowle et al., 2015; Verhoeven et al., 2018; Dully et al., 2021; Frühe et al., 2021b; Johansen et al., 2024). However, it is important to note that such differences in diversity may partly reflect the compositional nature of amplicon sequencing

data, where strong dominance by a few enrichment-tolerant taxa can reduce the apparent detection of rarer groups following rarefaction. Nevertheless, the consistent pattern across multiple diversity metrics and sites supports a genuine ecological signal of reduced evenness and compositional simplification under high organic loading.

It is worth noting, however, that some studies have found either no significant differences or even lower richness at reference sites (Bissett et al., 2007; Zhang et al., 2008; Kawahara et al., 2009; Hornick and Buschmann, 2018). Such discrepancies may arise not only from site-specific factors such as sediment type, hydrodynamics, or background nutrient conditions (Bissett et al., 2006; Tamminen et al., 2011), and from differences in aquaculture practices and disturbance intensity (Hornick and Buschmann, 2018), but also from temporal variability. The comparatively weak seasonal signal observed here may partly reflect the buffering capacity of sediment environments, where temperature fluctuations and other short-term environmental changes are often less pronounced than in the overlying water column, potentially contributing to greater temporal stability of benthic bacterial assemblages (Taberlet et al., 2018; Jiao et al., 2020). Seasonal fluctuations or production-cycle dynamics can transiently reshape sediment bacterial diversity patterns (Quero et al., 2020; Johansen et al., 2024), meaning that observed differences may partly reflect the timing of sampling in addition to spatial drivers.

Interestingly, temporal comparisons revealed no significant differences in diversity metrics between production cycles at the CE and REF sites, whereas the INT site exhibited significantly higher richness, Shannon diversity, and evenness during the second cycle. This pattern likely reflects partial recovery of sediment conditions following the extended fallow period, which may have promoted re-oxygenation and organic matter degradation. Although the intermediate site was relocated slightly closer to the cages (which would typically increase enrichment pressure), the rise in diversity suggests that recovery associated with the extended fallow period may have outweighed the effect of increased proximity to production activity. Similar trends have been reported for both macrofaunal and microbial assemblages, where fallowing promotes recolonization and partial restoration of benthic communities (Keeley et al., 2014, 2019; Verhoeven et al., 2018). These results indicate that intermediate sites represent transitional zones where bacterial diversity is especially responsive to changes in management intensity, contrasting with the constrained conditions at the fish cages and the relative stability at reference sites.

Beta diversity patterns further reinforced the dominance of organic enrichment in structuring benthic bacterial assemblages. CE assemblages clustered tightly, indicating a narrow set of enrichment-tolerant taxa (Holmer et al., 2005; Dowle et al., 2015; Stoeck et al., 2018; Keeley et al., 2018), while reference sites were more heterogeneous and dynamic. Monthly centroid trajectories showed little directional change at CE, confirming that persistent enrichment constrains natural seasonal variability. In contrast, the partial arc-shaped trajectories observed at INT and REF during the second cycle suggest a modest re-emergence of temperature-linked cyclicity following prolonged fallowing. This partial return of seasonal responsiveness highlights the potential for management actions, such as optimized fallow periods, to restore natural benthic dynamics.

Environmental vector fitting strengthened these ecological interpretations. The alignment of LOI with the enrichment gradient confirms that accumulated organic matter underpins community turnover, while the opposite orientation of IQI indicates that reference-like communities coincide with improved benthic ecological status. These findings are consistent with recent efforts to integrate microbial and macrofaunal indicators (Aylagas et al., 2017; Stoeck et al., 2018; Cordier, 2020; Oladi et al., 2024), including emerging approaches that link sequencing-derived bacterial indicators to quantitative molecular assessment frameworks (Oladi and Stoeck, 2026). Together, these developments highlight the strong potential of bacterial assemblage data to complement, and potentially streamline, conventional benthic monitoring frameworks, although further intercalibration and regulatory validation are still required before widespread replacement of macrofauna-based indices can be achieved.

Statistical analyses corroborated these visual patterns, confirming the predominance of organic enrichment as the main driver of bacterial assemblage structure, with smaller yet significant effects of production cycle, temperature, and biomonitoring operation phase (peak vs. non-peak). The remaining unexplained variation likely reflects additional environmental heterogeneity and stochastic microbial dynamics not explicitly captured in the model, including local sediment conditions, hydrodynamics, and other unmeasured physicochemical variables (Fagervold et al., 2014; Chen et al., 2021; Lin et al., 2023). The non-significant temperature-by-peak interaction indicates that seasonal variability did not confound enrichment signals in sediments, consistent with Wyness et al. (2026), who detected no seasonal bias in large-scale eDNA assessments. Consequently, monitoring efforts can be confidently aligned with operational schedules without concern for strong seasonal bias. This outcome strengthens the case for incorporating eDNA-based bacterial assemblage data into standardized environmental

assessments, where their temporal consistency offers a clear advantage for practical implementation.

Temporal trajectories of bacterial assemblages under aquaculture disturbance

Ecological trajectory analysis further illustrated how bacterial assemblages evolved under contrasting impact regimes. At the REF site, trajectories were comparatively long but lacked consistent direction, indicating natural background variability without cumulative drift in assemblage composition. This pattern demonstrates a resilient microbial community capable of absorbing natural fluctuations, thus providing a dynamic baseline for interpreting disturbance-induced change (Scheffer et al., 2001; Keeley et al., 2019). This has practical implications for monitoring, as it highlights the importance of robust reference conditions for ecological monitoring.

In contrast, the CE assemblages rapidly converged along short, highly directed trajectories, reflecting reduced temporal variability and flexibility in community responses under chronic organic loading (Allison and Martiny, 2008; Shade et al., 2012). These findings align with theories of microbial stability and alternative community states, where disturbance can restrict community pathways and promote convergence toward enrichment-tolerant assemblages (Scheffer et al., 2001; Fukami and Nakajima, 2011).

The INT site demonstrated the most dynamic response, initially resisting directional drift, but ultimately showing a marked, statistically significant shift at a critical threshold, where cumulative organic enrichment began to dominate over background variability (Fukami and Nakajima, 2011; Hanusch et al., 2022; Blake et al., 2024). This shift from stochastic to deterministic processes shows how chronic perturbation can override seasonal drivers and restructure microbial succession (Gilbert et al., 2012; Teeling et al., 2016). Management interventions, including fallowing and farm relocation, likely contributed to this sensitivity, illustrating that intermediate systems may exist in a fragile balance (metastable state), where even small perturbations in disturbance intensity or duration can push communities toward alternative configurations (Abreu et al., 2020; Philippot et al., 2021). Therefore, even though a short recovery window may open, enrichment pressure might quickly take over again. This also means that recovery does not simply reverse the changes caused by degradation. Instead, communities may follow different pathways back, a point that is highly relevant for restoration and adaptive management in aquaculture systems, because simply reducing the impact (e.g., longer fallow) doesn’t guarantee a full return to baseline (Shade et al., 2012).

Taken together, these results substantiate that cumulative organic enrichment from aquaculture shapes not just microbial assemblage composition but also the predictability and stability of microbial succession (Sturbois et al., 2021). The progressive constraint of bacterial assemblages at enriched sites reflects a reduction in ecological flexibility, with convergence toward disturbance-tolerant assemblages that persists across seasonal transitions (Scheffer et al., 2001; Fukami and Nakajima, 2011). Such temporal stability has critical implications for developing robust bacterial bioindicators, as it suggests that impact-related signals remain detectable regardless of sampling season or operational timing. This reinforces the practical utility of trajectory-based and eDNA-informed approaches for regulatory monitoring, where reliable detection of cumulative impacts is essential for adaptive, evidence-based aquaculture management. Nevertheless, the extent of temporal stability and the trajectory of bacterial community succession are likely to vary among sites depending on local environmental conditions, including hydrodynamics, sediment characteristics, depositional regime, farm management practices, and fallowing history (Cromey et al., 2022; Kalantzi and Karakassis, 2006). However, minimizing methodological biases in sequencing and sampling remains essential to ensure that observed temporal patterns reflect ecological processes rather than analytical artifacts (Dully et al., 2021; Oladi et al., 2024; Ashley-Wheeler et al., 2026). Incorporating such considerations will improve the robustness of trajectory-based approaches and support their integration into ecological quality assessments.

3.2.6 Conclusion

This study demonstrates that organic enrichment is the primary driver of bacterial assemblage dynamics in salmon farm sediments, consistently exerting a stronger influence than seasonal variability. Assemblages at reference sites remained resilient but variable, while those at the cage edge stabilized into enrichment-tolerant states. In contrast, at intermediate sites, assemblage dynamics proved especially sensitive to management, with extended fallowing and farm relocation influencing the shift from stochastic to directional trajectories. These findings highlight that cumulative disturbance not only alters assemblage composition but also constrains stability and predictability, reshaping microbial succession under prolonged aquaculture pressure. Subtle directional shifts observed in ordination-based trajectories suggest that while temporal movement occurs, it remains limited relative to the strong spatial structuring imposed by enrichment. This consistency across months and production cycles reinforces that seasonal fluctuations do not obscure enrichment-driven patterns. Although

further replication across farms and production cycles is needed to confirm the generality of these findings, our results support trajectory-based approaches as a promising advance toward developing robust, time-aware bacterial bioindicators. Such indicators could provide earlier and more reliable signals of environmental degradation or recovery, offering a valuable step toward integrating microbial metrics into ecological quality assessments that inform adaptive management and promote sustainable aquaculture.

Author Contributions

Mahshid Oladi: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review and editing; Adam Wyness: Data curation, Investigation, Methodology, Validation, Writing – review and editing; Thomas A. Wilding: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Validation, Writing – review and editing; Hans-Werner Breiner: Data curation, Investigation; Thorsten Stoeck: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Validation, Writing – review and editing.

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Conflict of Interest Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

Sequence data are available from NCBI SRA under BioProject ID PRJNA1353086.

3.2.7 References

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CHAPTER III

IDENTIFICATION OF ROBUST BACTERIAL INDICATORS FOR AQUACULTURE BIOMONITORING

4.1 Abstract

Although numerous analytical approaches have been developed to identify bacterial bioindicators from metabarcoding data, differences among analytical methods can lead to inconsistent indicator selection, therefore limiting confidence in candidate taxa for biomonitoring applications. This chapter evaluated the reproducibility of bacterial bioindicator selection by applying three complementary analytical approaches, i.e. Indicator Species Analysis (IndVal), MaAsLin2, and Threshold Indicator Taxa Analysis (TITAN), across two independent Scottish sediment metabarcoding datasets encompassing contrasting sampling designs and environmental gradients. Indicator selection was performed at amplicon sequence variant (ASV) resolution arend compared within and between datasets using sequence identity and taxonomic assignment. All three approaches identified large numbers of significant bacterial indicators, although each captured different aspects of community responses to organic enrichment. Consensus analysis substantially reduced the number of candidate indicators while retaining ASVs that exhibited reproducible ecological responses across methods and datasets. Sulfurovaceae, Flavobacteriaceae, BD2-2 and sulfate-reducing lineages consistently characterized organically enriched sediments, whereas Hyphomicrobiaceae, Anaerolineaceae and the Desulfuromonadales lineage Sva1033 were repeatedly associated with higher ecological quality. Several indicator ASVs were represented by identical nucleotide sequences in both datasets and showed agreement with previously reported bacterial indicators, demonstrating a high degree of reproducibility. In contrast, some bacterial families contained ASVs associated with opposing ecological conditions, highlighting the importance of sequence-level rather than higher taxonomic resolution for indicator selection. These findings demonstrate that robust bacterial bioindicators are most reliably identified through the integration of complementary statistical approaches combined with independent dataset validation and ecological interpretation. The resulting consensus ASVs provide a transferable set of candidate indicators for the future development of standardized molecular indices and targeted quantitative assays, representing an important step towards the routine application of bacterial metabarcoding in salmon aquaculture biomonitoring.

Keywords: Bacteria, Bioindicators, Ecological Threshold, Biomonitoring, Aquaculture.

4.2 Background

The rapid expansion of marine finfish aquaculture has intensified the need for reliable environmental monitoring to ensure sustainable production while minimizing impacts on surrounding ecosystems (Martinez-Porchas and Martinez-Cordova, 2012). Organic waste derived from uneaten feed and fish faeces alters sediment biogeochemistry, leading to oxygen depletion, sulphide accumulation and shifts in nutrient cycling that profoundly influence benthic ecosystem functioning (Hargrave et al., 2008; Holmer et al., 2005). While these physicochemical variables provide valuable information on the intensity of environmental disturbance, they primarily represent a snapshot of environmental conditions and may exhibit substantial spatial and temporal variability. In contrast, biological communities integrate the cumulative effects of environmental change over ecologically relevant timescales, reflecting the net outcome of multiple interacting stressors on ecosystem structure and function (Holt and Miller, 2011; Parmar et al., 2016). Consequently, biological indicators have become the foundation of environmental biomonitoring programmes because they provide a direct measure of ecological condition by integrating the cumulative effects of environmental change, rather than simply quantifying the environmental drivers that influence it (van de Kamp et al., 2023).

In aquaculture monitoring, the search for biological indicators has historically focused on benthic macrofaunal communities (e.g., Mirto et al., 2012; Stojanović et al., 2017). Species exhibiting predictable responses to organic enrichment form the basis of widely adopted biotic indices, such as AZTI's Marine Biotic Index (AMBI) and Infaunal Quality Index (IQI), which are routinely used to classify ecological status and support regulatory decision-making (Borja et al., 2000; Culhane et al., 2014). The development of high-throughput sequencing technologies has subsequently expanded this concept to microbial communities, with numerous studies identifying bacterial taxa associated with gradients of organic enrichment beneath fish farms (Stoeck et al., 2018; Verhoeven et al., 2018; Aylagas et al., 2021; Frühe et al., 2021b; Leontidou et al., 2023; Rubel et al., 2025). Among these studies, bacterial groups such as members of the Sulfurovaceae, Desulfocapsaceae, Caldilineaceae and Flavobacteriaceae have repeatedly been associated with organically enriched sediments (Aranda et al., 2015; Choi et al., 2018, 2022; Frühe et al., 2021b; Pettersen et al., 2022), whereas taxa including Pirellulaceae, Hyphomicrobiaceae and Desulfuromonadaceae are more frequently associated with reference conditions (Aylagas et al., 2017; Frühe et al., 2021b). Despite these recurring patterns, the proposed indicator taxa often differ among studies,

highlighting the need for systematic evaluation of their robustness across datasets and analytical frameworks.

For microbial bioindicators to be suitable for routine environmental monitoring, they should satisfy several complementary criteria. They should respond predictably to ecological disturbance (i.e., sensitivity; Holt and Miller, 2010), be consistently detected across spatial and temporal scales (Nikinmaa, 2014), possess ecological relevance that enables interpretation of their responses, and remain robust to methodological sources of variation introduced during sampling and laboratory processing. The methodological and temporal robustness of bacterial metabarcoding were established in Chapters I and II of this thesis. Building upon this foundation, the present chapter focuses on identifying bacterial taxa that fulfil these criteria and therefore represent strong candidates for routine environmental biomonitoring.

A variety of statistical approaches have been developed to identify microbial bioindicators from metabarcoding datasets. These approaches differ fundamentally in the ecological characteristics they evaluate. Broadly, these include (i) association-based methods, such as Indicator Species Analysis (IndVal), which identify taxa exhibiting significant relationships with ecological quality classes or environmental gradients (Bakker, 2008; De Caceres and Legendre, 2009); (ii) regression-based association models, such as MaAsLin2, which detect taxa associated with environmental variables while accounting for potential confounding factors (Mallick et al., 2021); (iii) threshold-based methods, such as Threshold Indicator Taxa Analysis (TITAN), which identify taxa exhibiting abrupt responses along continuous disturbance gradients (Baker and King, 2010); and (iv) predictive approaches, including Random Forests (Breiman, 2001), which rank taxa according to their ability to classify ecological status (Rubel et al., 2025). More recently, differential abundance methods originally developed for transcriptomics, such as ALDEx2 (Fernandes et al., 2013) or DeSeq2 (Love et al., 2014), have also been applied to metabarcoding datasets to identify taxa associated with environmental disturbance. Overall, these approaches have substantially advanced microbial indicator discovery and identified numerous bacterial taxa associated with aquaculture-derived organic enrichment.

Although these methods have collectively identified numerous candidate bioindicators, they are founded on different statistical principles and emphasize different ecological characteristics. IndVal prioritizes taxa that are characteristic of particular ecological quality classes (De Caceres and Legendre, 2009), MaAsLin2 identifies taxa displaying consistent

relationships with environmental variables (Mallick et al., 2021), whereas TITAN identifies taxa exhibiting threshold-like responses along disturbance gradients (Baker and King, 2010). Consequently, different analytical approaches frequently recover different candidate indicators, and relatively few studies have evaluated whether taxa identified by one framework are consistently supported by complementary analytical methods or reproduced across independent datasets. This lack of cross-method and cross-dataset validation limits confidence in the ecological robustness and transferability of proposed microbial bioindicators.

Therefore, the aim of this chapter was to identify robust bacterial bioindicators of aquaculture-derived organic enrichment by integrating complementary analytical approaches across two independent metabarcoding datasets and comparing the resulting candidate taxa with those previously identified from an independent metagenomic dataset (Rubel et al., 2025). Specifically, IndVal, MaAsLin2 and TITAN were applied to identify taxa exhibiting ecological specificity, consistent environmental associations and threshold responses, respectively. By combining these complementary lines of evidence, this study sought to identify bacterial indicators that are ecologically meaningful, analytically robust and transferable across independent monitoring datasets.

4.3 Methods

4.3.1 Study datasets

The first dataset (henceforth Dataset 1) comprised the final bacterial ASV table generated in Chapter II of this thesis. The dataset consisted of 150 sediment eDNA samples collected monthly over two Atlantic salmon production cycles (11 months during the first production cycle and seven months during the second), spanning three impact categories i.e. cage edge (CE), intermediate impact (INT), and reference (REF). Associated environmental metadata included IQI values derived from conventional macrofaunal monitoring. IQI was calculated from macrobenthic communities provided by Ocean Ecology (Oban, UK) using the IQI v2 workbook (Phillips et al., 2014). As demonstrated in Chapter II, IQI was strongly associated with bacterial community composition and explained a substantial proportion of the observed variation in community structure. Owing to its temporal resolution and well-characterized environmental gradients, Dataset 1 was used to identify bacterial taxa exhibiting consistent associations with ecological quality over time.

The second dataset (henceforth Dataset 2) comprised the bacterial ASV table generated by Wyness et al. (2026). This dataset consisted of 744 sediment eDNA samples collected from six local authorities and 75 farm sites across Scotland. In contrast to Dataset 1, which provided repeated temporal observations at a single farm, Dataset 2 encompassed a broad geographical range of farms and environmental conditions, thereby enabling the spatial validation and transferability of candidate bacterial indicators. Unlike Dataset 1, samples were not assigned to predefined impact categories but were instead associated with IQI values derived from conventional macrofaunal monitoring, allowing bacterial taxa to be evaluated against a continuous measure of ecological quality across diverse monitoring sites.

4.3.2 Indicator identification

To identify robust bacterial bioindicators of aquaculture-derived organic enrichment, three complementary analytical approaches were applied to evaluate different ecological characteristics of candidate indicator taxa. ASV abundance tables were first converted to relative abundances and filtered to retain only ASVs occurring in at least 10% of samples, thereby reducing the influence of extremely rare taxa and improving the robustness of subsequent analyses. Each analytical approach was applied independently to both datasets.

(i) *Ecological specificity*. Indicator Species Analysis (IndVal) was used to identify bacterial ASVs that were significantly associated with each ecological condition by jointly evaluating their specificity (uniqueness to a condition) and fidelity (frequency of occurrence within that condition). To improve ecological interpretability and ensure sufficient sample representation within each group, IQI values were aggregated into three ecological condition classes representing impacted, intermediate, and good conditions. Analyses were performed using the R package 'indicspecies' (De Caceres and Legendre, 2009) with the group-equalized IndVal statistic (IndVal.g) and 999 permutations. Statistical significance was assessed using both nominal p-values and Benjamini-Hochberg false discovery rate (FDR)-adjusted *p*-values.

(ii) *Environmental associations*. Associations between bacterial ASVs and environmental variables were evaluated using the R package 'MaAsLin2' (Mallick et al., 2021). A model was fitted using IQI as a fixed effect to identify bacterial taxa consistently associated with ecological quality. ASVs with Benjamini-Hochberg FDR-adjusted *q*-values < 0.05 were considered significantly associated with the corresponding environmental gradient.

(iii) *Ecological thresholds*. Threshold Indicator Taxa Analysis (TITAN; Baker and King, 2010) was used to identify taxa exhibiting abrupt abundance shifts along continuous gradients of organic enrichment (IQI). Analyses were performed using the R package 'TITAN2'. Purity and reliability thresholds were both set to 0.95, with 100 bootstrap replicates and 100 permutations used to assess the robustness of indicator responses.

4.3.3 Consensus indicator selection

Candidate bacterial indicators were selected through a stepwise filtering procedure designed to prioritize ecologically relevant and reproducible taxa. First, for each analytical approach (IndVal, MaAsLin2 and TITAN), significant ASVs were ranked according to their relative abundance, and the 100 most abundant significant ASVs were retained for further evaluation. This abundance-based filtering was applied to minimize the influence of rare taxa that, although statistically significant, are less suitable as robust biomonitoring indicators. ASVs lacking taxonomic assignment below the family level were subsequently excluded to facilitate biological interpretation and comparison among methods and datasets.

The remaining ASVs were then prioritized according to method-specific performance metrics. For Indicator Species Analysis (IndVal), significant ASVs (FDR-adjusted $p < 0.05$) were ranked according to their indicator statistic (IndVal "*stat*"), with higher values indicating greater specificity and fidelity to a particular ecological condition. For MaAsLin2, significant ASVs ($q < 0.05$) were ranked according to the absolute value of their regression coefficients, representing the strength of association with the environmental variable. For TITAN, ASVs meeting the predefined purity and reliability thresholds (≥ 0.95) were ranked according to their standardized z-scores, with larger absolute values indicating stronger threshold responses along the environmental gradient.

Candidate ASVs were subsequently compared among the three analytical approaches within each dataset to identify indicators consistently supported by multiple methods. Consensus indicators were then evaluated across Dataset 1 and Dataset 2. Because ASV identifiers are unique to each dataset, cross-dataset reproducibility was assessed by comparing the representative nucleotide sequences of candidate ASVs rather than their ASV names. In doing so, the sequences were exported in FASTA format and combined into a single sequence dataset. Multiple sequence alignment was performed using MAFFT v7 (Katoh et al., 2019) with default parameters. The aligned sequences were imported into R, where pairwise nucleotide distances were calculated using the "ape" package (Paradis and Schliep, 2019) under

the uncorrected p-distance ("raw") model. Pairwise sequence identity (%) was calculated as 100 × (1 – raw distance). For each candidate ASV from Dataset 1, the corresponding sequence exhibiting the highest identity in Dataset 2 was identified. Sequence similarity was subsequently interpreted together with taxonomic assignment and ecological association to distinguish identical sequence variants from closely related bacterial lineages recovered independently in both datasets.

An unrooted maximum-likelihood phylogenetic tree was reconstructed from the aligned sequences of consensus indicator ASVs in phangorn under the GTR substitution model with 500 bootstrap replicates. The tree was visualized using ggtree, with indicator direction, dataset of origin, and family annotations.

Finally, the resulting priority indicator taxa were compared with bacterial indicators previously identified by Rubel et al. (2025) via Indval and Random Forest approaches using shotgun metagenomic sequencing. Agreement between metabarcoding- and metagenomics-derived indicator sets provided an independent assessment of indicator transferability across sequencing technologies and informed the selection of bacterial targets for targeted dPCR assay development in Chapter IV.

4.4 Results

4.4.1 Overview of bacterial ASV filtering and analytical workflow

After filtering to retain ASVs present in at least 10% of samples, 4,321 ASVs (out of 29,113) remained in Dataset 1 and 1,915 ASVs (out of 42,583) remained in Dataset 2 for subsequent analyses. The filtered ASV tables were subsequently transformed (relative abundances) and analysed using three complementary analytical approaches targeting different ecological characteristics of bacterial indicators, namely ecological specificity (IndVal), environmental associations (MaAsLin2), and ecological threshold responses (TITAN).

4.4.2 Ecological specificity (IndVal)

In total, Indicator Species Analysis identified 2,725 and 1,718 ASVs with p_{BH} <0.05 in Dataset 1 and Dataset 2, respectively. In Dataset 1, 70% of indicators were associated with good ecological conditions (1,905 ASVs), followed by impacted (545 ASVs; 20%) and moderate conditions (276 ASVs; 10%). A similar pattern was observed in Dataset 2, where 1,040 ASVs were associated with good conditions, compared with 458 and 220 ASVs

associated with impacted and moderate conditions, respectively. The highest IndVal value (output as *stat*) ranged from 0.95 to 0.36 and from 0.87 to 0.25 in Dataset 1 and 2, respectively. Across both datasets, indicator ASVs were assigned to 134 and 110 bacterial families, respectively. Tables containing all significant ASVs, their association with ecological condition, stat value, and their taxonomy are presented in Supplementary File S1. In both datasets, among the 30 families containing the highest numbers of significant indicator ASVs, some were predominantly associated with either impacted or good ecological conditions, whereas others contained indicators associated with multiple ecological states (Supplementary Figs. S1 and S2).

To facilitate detailed examination of the most abundant and strongly associated indicator taxa, subsequent analyses focused on the 100 most abundant significant indicators that could be assigned below the family level and exhibited IndVal statistics ≥0.65. This filtering resulted in 66 (34 impacted and 28 good) and 50 (29 impacted and 21 good) selected indicator ASVs in Datasets 1 and 2, respectively. The taxonomic composition of these selected indicator ASVs was subsequently summarised at the family level (Figure 10). While several bacterial families contained indicator ASVs associated with both ecological conditions, others exhibited a clear predominance of indicators associated with either impacted or good conditions.

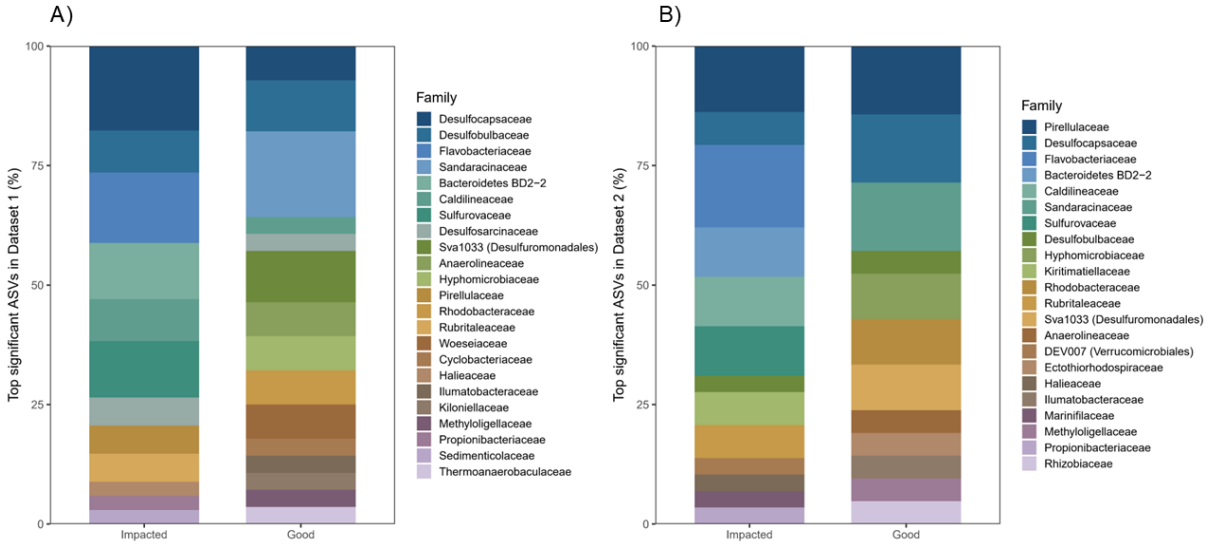


Figure 10. Family-level taxonomic composition of the selected IndVal indicator ASVs in (A) Dataset 1 and (B) Dataset 2. *Stacked bars show the proportional representation of bacterial families among indicator ASVs associated with impacted and good ecological conditions.*

In both datasets, Desulfocapsaceae and Desulfobulbaceae contained indicator ASVs associated with both impacted and good ecological conditions. In contrast, several families showed a consistent association with a single ecological condition across datasets. Sulfurovaceae, Flavobacteriaceae, Bacteroidetes BD2-2, and Propionibacteriaceae were represented almost exclusively by impacted-associated indicator ASVs, whereas Hyphomicrobiaceae, Ilumatobacteraceae, Anaerolineaceae, and Sva1033 (Desulfuromonadales) were dominated by good-condition indicators. Overall, the family-level composition of the selected indicator ASVs was highly consistent between the two datasets despite differences in the relative contribution of individual families.

4.4.3 Environmental associations (MaAsLin2)

MaAsLin2 identified 3,642 and 1,735 significant ASVs ($q < 0.05$) associated with IQI, in Datasets 1 and 2, respectively. In Dataset 1, 584 ASVs exhibited negative associations with IQI (coef < 0), whereas 3058 exhibited positive associations (coef > 0). Similarly, Dataset 2 contained 502 negatively associated and 1,233 positively associated ASVs. Across both datasets, the significant IQI-associated ASVs were assigned to 144 and 110 bacterial families, respectively. Tables containing all significant ASVs, their coefficient estimates, and taxonomic assignments are provided in Supplementary File S2. In both datasets, among the 30 families containing the highest numbers of significant IQI-associated ASVs, several showed strongly directional responses, with most ASVs exhibiting either positive or negative associations with IQI, whereas other families contained ASVs responding in both directions (Supplementary Figs. S3 and S4).

Following the predefined filtering criteria, 57 (29 negatively and 28 positively associated with IQI) and 54 (28 negatively and 26 positively associated with IQI) candidate ASVs remained in Datasets 1 and 2, respectively. The distribution of MaAsLin2 regression coefficients for the selected ASVs is shown in Figures 11 and 12, with point size representing the mean relative abundance of each ASV across all samples.

Consistent with IndVal results, in both Datasets, negatively associated ASVs were dominated by members of Flavobacteriaceae, Sulfurovaceae, and Bacteroidetes BD2-2, whereas positively associated ASVs were primarily affiliated with Hyphomicrobiaceae, Anaerolineaceae, Rhodobacteraceae, Ilumatobacteraceae, and Sva1033 (Desulfuromonadales). Most selected ASVs exhibited relatively small regression coefficients (approximately -0.02 to

+0.016 in Dataset 1 and -0.008 to +0.005 in Dataset 2), although considerable variation in mean relative abundance was observed among taxa (Figs. 11 and 12). Overall, the dominant family-level patterns identified by MaAsLin2 showed strong agreement with those obtained using Indicator Species Analysis, with negatively associated ASVs corresponding largely to families characteristic of impacted sediments and positively associated ASVs corresponding to families characteristic of good ecological conditions.

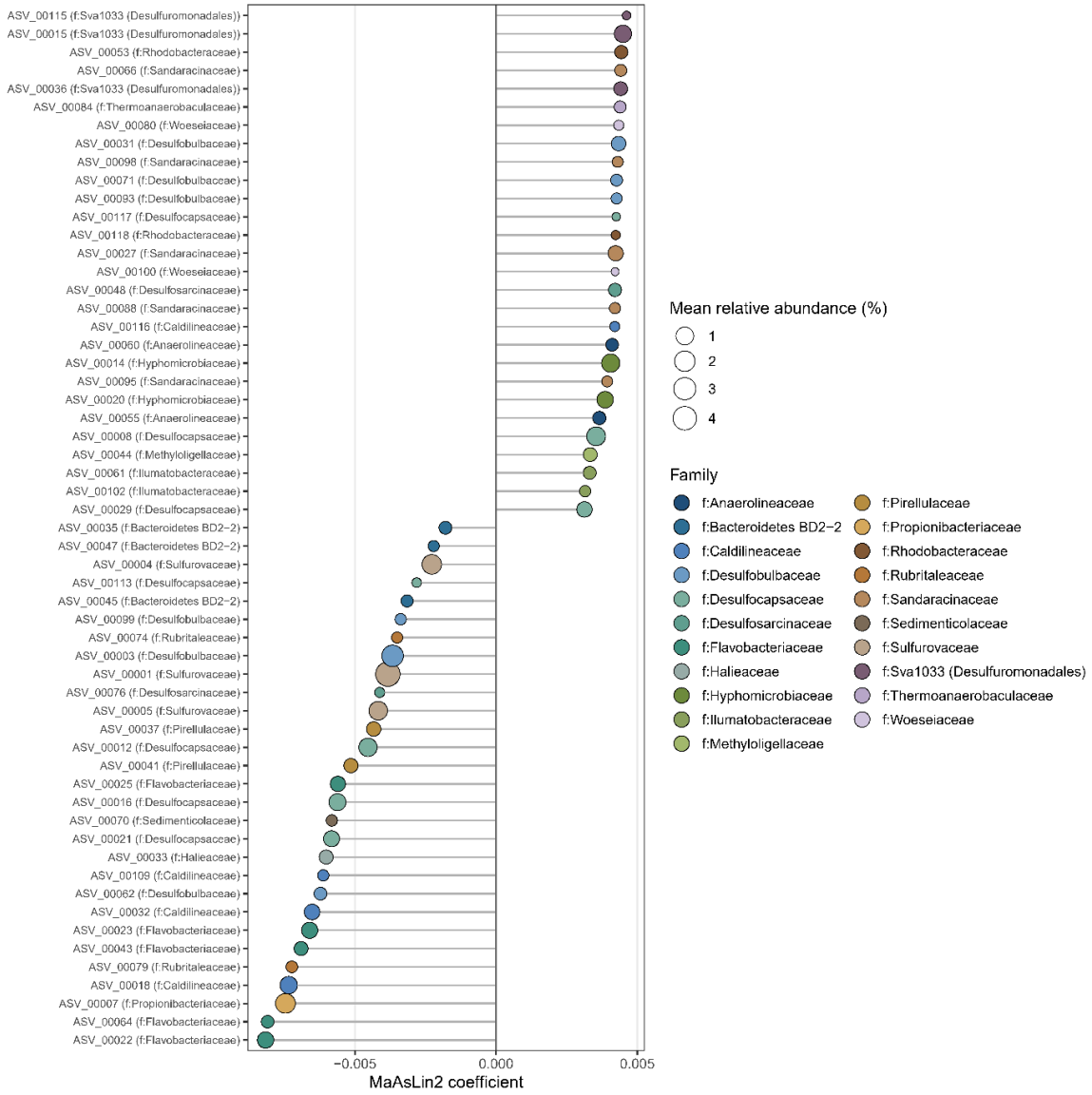


Figure 11. Selected bacterial ASVs significantly associated with IQI identified by MaAsLin2 in Dataset 1. *Lollipop plots show the regression coefficient of each selected ASV, with negative coefficients indicating association with impacted conditions and positive coefficients indicating association with good ecological conditions. Point size represents the mean relative abundance of each ASV across all samples, and point colour indicates bacterial family. The displayed ASVs comprise the subset of the 100 most abundant significant ASVs retained after filtering for family-level taxonomic assignment and occurrence in more than 50% of samples.*



Figure 12. Selected bacterial ASVs significantly associated with IQI identified by MaAsLin2 in Dataset 2. *Horizontal lollipop plots show the regression coefficient of each selected ASV, with negative coefficients indicating association with impacted conditions and positive coefficients indicating association with good ecological conditions. Point size represents the mean relative abundance of each ASV across all samples, and point colour indicates bacterial family. The displayed ASVs comprise the subset of the 100 most abundant significant ASVs retained after filtering for family-level taxonomic assignment and occurrence in more than 50% of samples.*

4.4.4 Ecological thresholds (TITAN)

In total, TITAN identified 3,947 and 1,759 bacterial ASVs exhibiting significant threshold responses along the IQI gradient in Datasets 1 and 2, respectively. Of these, 666 and 529 ASVs exhibited decreasing responses (z-), whereas 3,281 and 1,230 exhibited increasing responses (z+) in Datasets 1 and 2, respectively. These thresholds represent the points along the IQI gradient where bacterial communities undergo their most pronounced compositional changes. Complete lists of significant ASVs, their threshold statistics, and taxonomic assignments are provided in Supplementary File S3.

At the community level, TITAN identified clear transition points along the IQI gradient in both datasets (Figure 13A and Figure 14A). In Dataset 1, the threshold for decreasing responders occurred at an IQI of 0.591, whereas increasing responders showed a threshold at 0.637. Corresponding thresholds in Dataset 2 occurred at slightly lower IQI values (0.543 and 0.621, respectively; Table 1). Bootstrap confidence intervals were narrow around the estimated thresholds, indicating that community-level change points were consistently identified across bootstrap replicates.

Table 1. Community-level TITAN thresholds (filtered Sum(z) change points) and the 95% bootstrap intervals for decreasing (z-) and increasing (z+) bacterial indicator ASVs along IQI gradient both Datasets.

	z- threshold	z- 95% bootstrap interval	z+ threshold	z+ 95% bootstrap interval
Dataset 1	0.591	0.417-0.595	0.637	0.589-0.666
Dataset 2	0.543	0.434-0.543	0.621	0.580-0.671

To facilitate biological interpretation, the analysis focused on the most abundant and prevalent indicator ASVs. Following filtering for taxonomic assignment, occurrence (≥50% of samples), and maximum purity and reliability (both = 1), 62 ASVs (31 decreasing and 31 increasing responders) were retained in Dataset 1 and 66 ASVs (33 decreasing and 33 increasing responders) in Dataset 2. Within each response direction, ASVs were ranked by TITAN z-score, and the strongest indicators were selected for visualisation (Figure 13B and 14B).

Most selected ASVs changed close to the community-level thresholds, although individual taxa exhibited some variation in the exact IQI value at which they responded. In both datasets, increasing responders were concentrated around the community threshold, whereas decreasing responders showed greater variability, with several taxa responding earlier or later along the environmental gradient. In particular, in Dataset 2, one Sulfurovaceae ASV exhibited an early change point below IQI 0.30, whereas some Sulfurovaceae and Desulfobulbaceae ASVs showed later changes at approximately IQI 0.65-0.67. Thus, although the community-level thresholds were relatively well defined, individual indicator ASVs differed in the ecological condition at which their strongest responses occurred. All in all, despite this variability, the same bacterial families, including Sulfurovaceae, Desulfobulbaceae, Flavobacteriaceae, Anaerolineaceae, and Hyphomicrobiaceae, were consistently represented among the strongest threshold indicators in both datasets.

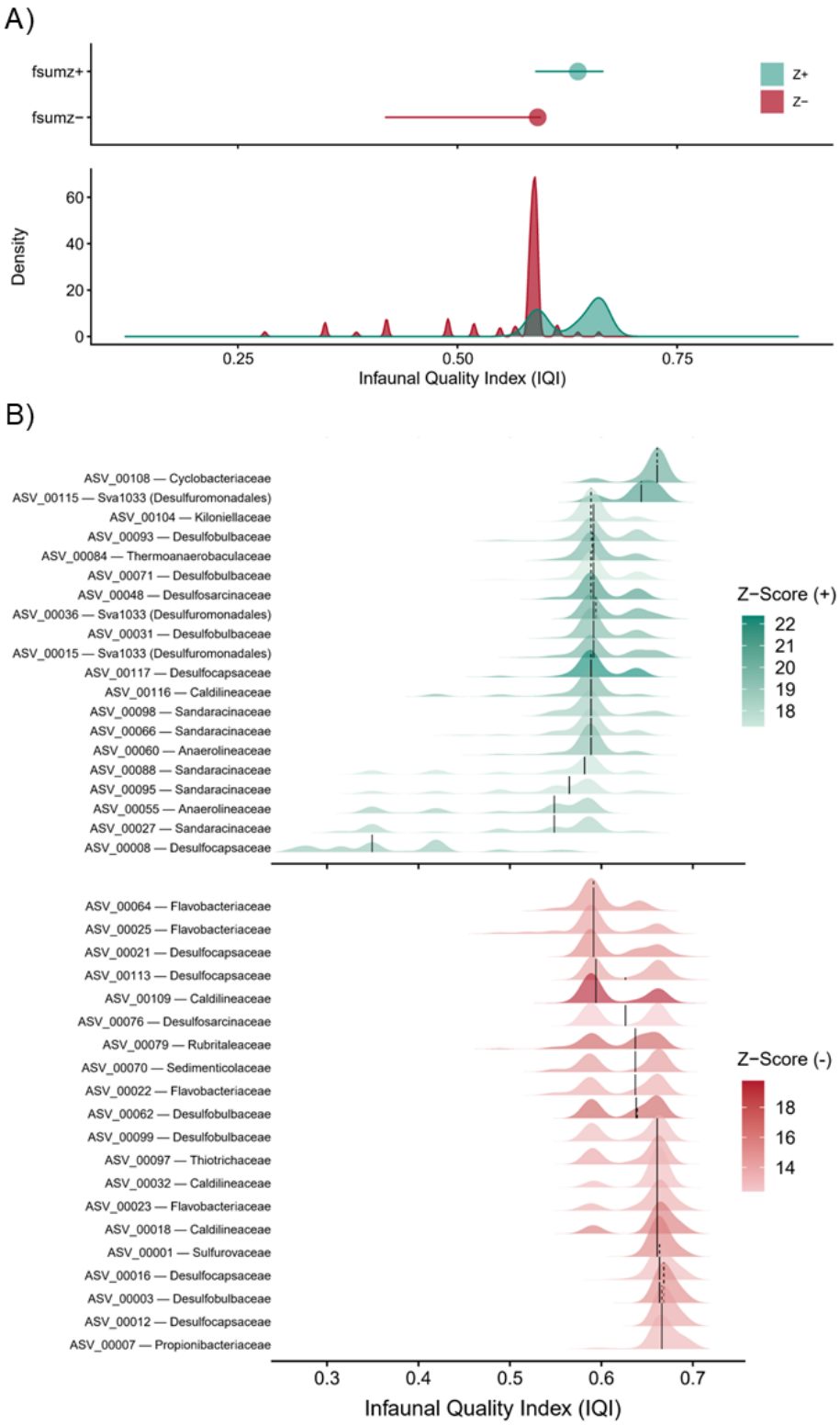


Figure 13. Community- and taxon-level threshold responses of bacterial ASVs along the IQI gradient identified by TITAN in Dataset 1. *(A) Community-level threshold estimates for decreasing (z-) and increasing (z+) responders showing filtered community change points (points and 95% bootstrap intervals) and bootstrap density distributions. (B) Bootstrap distributions of change points for the most abundant indicator ASVs (20 decreasing and 20 increasing responders) ranked by z-score. Vertical solid lines indicate the observed change point estimates, while dashed lines indicate the median bootstrap change points.*

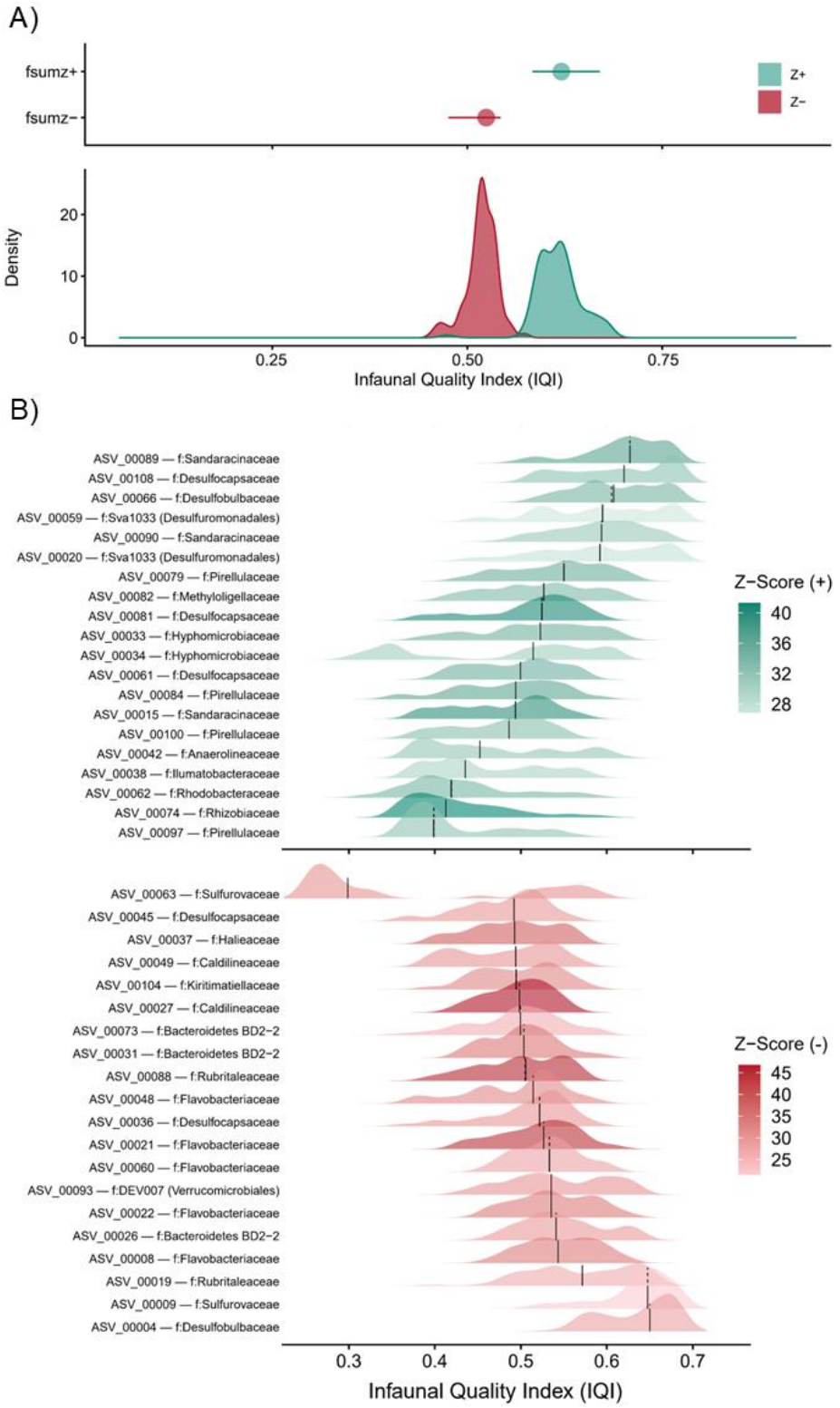


Figure 14. Community- and taxon-level threshold responses of bacterial ASVs along the IQI gradient identified by TITAN in Dataset 2*. (A) Community-level threshold estimates for decreasing (z-) and increasing (z+) responders showing filtered community change points (points and 95% bootstrap intervals) and bootstrap density distributions. (B) Bootstrap distributions of change points for the most abundant indicator ASVs (20 decreasing and 20 increasing responders) ranked by z-score. Vertical solid lines indicate the observed change point estimates, while dashed lines indicate the median bootstrap change points.*

4.4.5 Consensus indicator taxa across analytical approaches

Since ASV identifiers were generated independently for each dataset, correspondence between datasets was assessed using nucleotide sequence similarity rather than ASV labels. Consensus was first evaluated within each dataset by identifying ASVs detected by all three analytical approaches (IndVal, MaAsLin2, and TITAN). Cross-dataset consensus was then assessed by comparing the nucleotide sequences of these consensus ASVs to identify identical or highly similar sequence variants occurring in both datasets.

A total of 2,486 and 1,628 ASVs were identified as significant by all three analytical approaches in Datasets 1 and 2, respectively. A complete list of these ASVs and their assigned family is provided in Supplementary File S4. Looking at the selected ASVs from the 100 most abundant with known taxonomy, 53 and 42 ASVs were consistently identified by all three methods in Datasets 1 and 2, respectively, representing the highest-confidence consensus indicators (Supplementary Tables S1 and S2).

Comparison of the consensus indicator ASVs between datasets revealed a high degree of consistency despite differences in sampling design, geographic coverage, and independent ASV inference. Several consensus indicators were represented by identical nucleotide sequences in both datasets, demonstrating that the same bacterial sequence variants were repeatedly identified as indicators. Additional consensus indicators exhibited very high sequence similarity (>97%), differing by only one or a few nucleotide substitutions while remaining assigned to the same bacterial families.

The shared consensus indicator ASVs are summarized in Table 2. Most exhibited 100% sequence identity between datasets, while the remaining matches shared greater than 97% sequence similarity. The majority of these conserved indicators were associated with impacted ecological conditions and belonged predominantly to the families Sulfurovaceae, Flavobacteriaceae, Desulfobulbaceae, Caldilineaceae, and Bacteroidetes BD2-2. In contrast, consensus indicators associated with good ecological conditions were primarily affiliated with Hyphomicrobiaceae, Sandaracinaceae, Anaerolineaceae, and Sva1033 (Desulfuromonadales).

Table 2. List of consensus indicator taxa from both datasets, which had more than 97% sequence similarity. In addition, the exact sequence matches that were identified as indicator ASV by IndVal and Random Forest approaches in Rubel et al. (2025) are marked.

D1 ASV	D2 ASV	Family	Seq. identity %	Ecological direction	Exact match in Rubel et al. (2025)
ASV_00001	ASV_00001	f:Sulfurovaceae	100	Impacted	
ASV_00003	ASV_00004	f:Desulfobulbaceae	100	Impacted	
ASV_00005	ASV_00009	f:Sulfurovaceae	100	Impacted	✓
ASV_00016	ASV_00045	f:Desulfocapsaceae	100	Impacted	
ASV_00018	ASV_00027	f:Caldilineaceae	100	Impacted	
ASV_00021	ASV_00036	f:Desulfocapsaceae	100	Impacted	
ASV_00022	ASV_00048	f:Flavobacteriaceae	100	Impacted	✓
ASV_00023	ASV_00022	f:Flavobacteriaceae	100	Impacted	
ASV_00025	ASV_00008	f:Flavobacteriaceae	100	Impacted	
ASV_00032	ASV_00049	f:Caldilineaceae	100	Impacted	
ASV_00033	ASV_00037	f:Halieaceae	100	Impacted	
ASV_00037	ASV_00032	f:Pirellulaceae	100	Impacted	
ASV_00041	ASV_00041	f:Pirellulaceae	100	Impacted	
ASV_00045	ASV_00031	f:Bacteroidetes BD2-2	100	Impacted	✓
ASV_00047	ASV_00026	f:Bacteroidetes BD2-2	100	Impacted	
ASV_00062	ASV_00004	f:Desulfobulbaceae	100	Impacted	
ASV_00074	ASV_00019	f:Rubritaleaceae	100	Impacted	
ASV_00099	ASV_00004	f:Desulfobulbaceae	99.53	Impacted	
ASV_00004	ASV_00001	f:Sulfurovaceae	98.25	Impacted	
ASV_00064	ASV_00060	f:Flavobacteriaceae	97.89	Impacted	
ASV_00109	ASV_00027	f:Caldilineaceae	97.75	Impacted	
ASV_00014	ASV_00034	f:Hyphomicrobiaceae	100	Good	
ASV_00015	ASV_00020	f:Sva1033 (Desulfuromonadales)	100	Good	✓
ASV_00020	ASV_00033	f:Hyphomicrobiaceae	100	Good	
ASV_00027	ASV_00015	f:Sandaracinaceae	100	Good	
ASV_00036	ASV_00059	f:Sva1033 (Desulfuromonadales)	100	Good	✓
ASV_00044	ASV_00082	f:Methyloligellaceae	100	Good	
ASV_00055	ASV_00042	f:Anaerolineaceae	100	Good	
ASV_00088	ASV_00090	f:Sandaracinaceae	100	Good	
ASV_00095	ASV_00089	f:Sandaracinaceae	100	Good	
ASV_00117	ASV_00061	f:Desulfocapsaceae	100	Good	
ASV_00066	ASV_00090	f:Sandaracinaceae	99.76	Good	
ASV_00098	ASV_00090	f:Sandaracinaceae	99.76	Good	
ASV_00060	ASV_00042	f:Anaerolineaceae	98.51	Good	
ASV_00008	ASV_00081	f:Desulfocapsaceae	97.45	Good	

In addition, four of the consensus indicator ASVs were identical to bacterial indicators previously identified by Rubel et al. (2025) using an independent analytical framework combining IndVal analysis and Random Forest classification (Table 2). These included three indicators associated with impacted conditions (Sulfurovaceae, Flavobacteriaceae, and Bacteroidetes BD2-2) and one indicator associated with good ecological condition (Sva1033 - Desulfuromonadales).

Phylogenetic reconstruction of the consensus indicator ASVs demonstrated that indicators identified independently in both datasets clustered consistently within major bacterial families (Figure 15). Closely related ASVs originating from Dataset 1 and Dataset 2 frequently formed paired or neighboring branches, supporting the sequence-based correspondence identified during the cross-dataset comparison. Impact-associated indicators were concentrated within families previously linked to organically enriched sediments, including Sulfurovaceae, Flavobacteriaceae, Caldilineaceae, and Desulfobulbaceae. In contrast, indicators associated with good ecological condition were predominantly represented by Anaerolineaceae, Sandaracinaceae, Hyphomicrobiaceae, Sva1033 (Desulfuromonadales), and Methyloligellaceae. Notably, Desulfocapsaceae contained ASVs associated with both impacted and good ecological conditions, suggesting ecological diversification among closely related taxa and highlighting the importance of ASV-level rather than family-level resolution for bacterial indicator development.

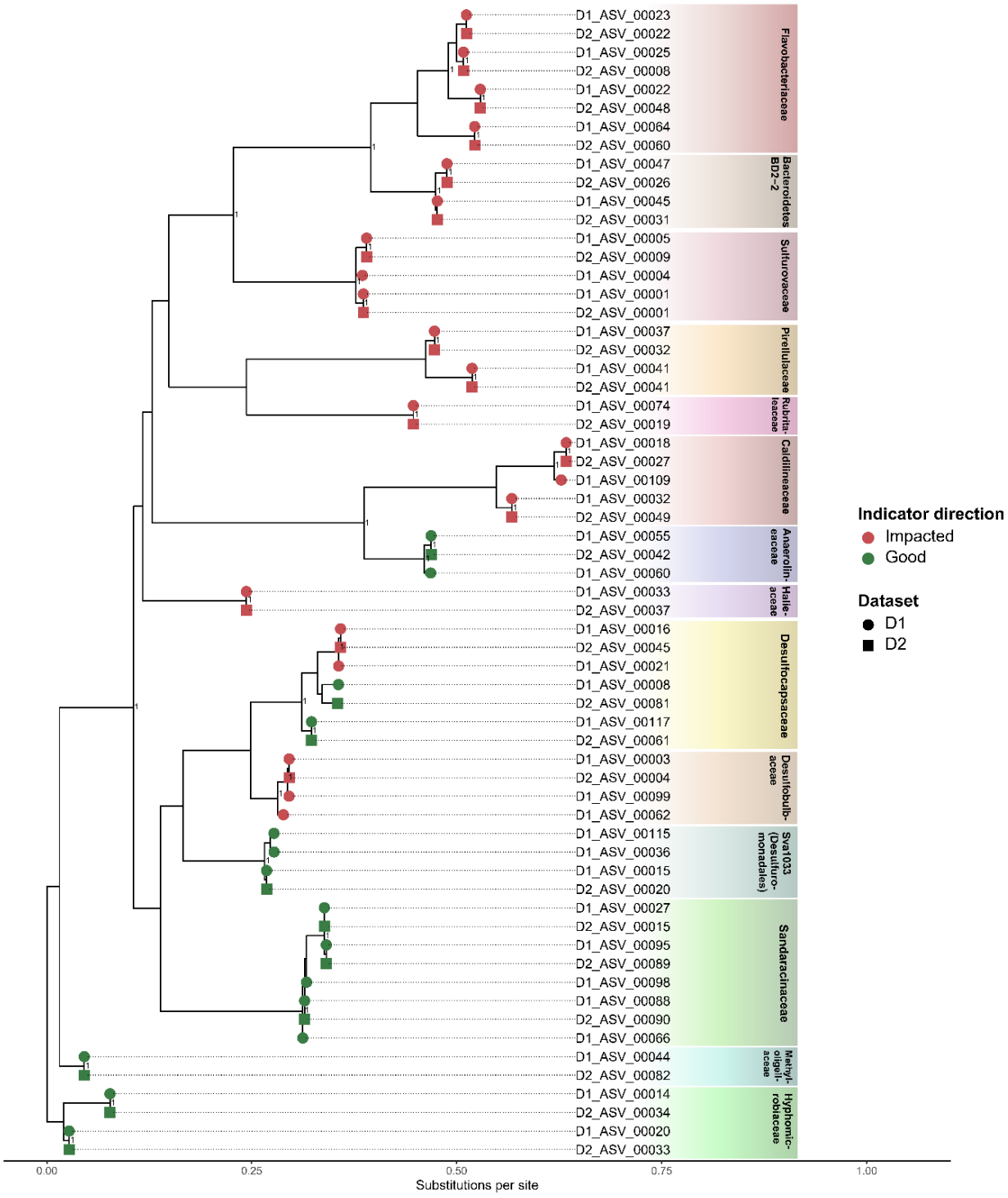


Figure 15. Unrooted maximum-likelihood phylogenetic tree of consensus bacterial indicator ASVs identified across Dataset 1 and Dataset 2. *The tree was reconstructed from aligned 16S rRNA gene (V3–V4) sequences of consensus indicators, with branch support estimated from 500 bootstrap replicates (values ≥70% shown). Tip symbols indicate the dataset of origin, while tip colors represent the ecological direction of each indicator. ASVs are grouped by bacterial family, shown as colored annotation blocks, illustrating the distribution of consensus indicators across major taxonomic groups.*

The repeated recovery of identical or highly similar bacterial sequence variants across independent datasets and complementary analytical approaches demonstrates a high degree of consistency in indicator identification.

4.5 Discussion

4.5.1 Analytical consensus identifies robust bacterial bioindicators

One of the principal findings of this chapter is that three conceptually distinct analytical approaches consistently identified the same bacterial lineages as indicators of ecological condition. Although IndVal, MaAsLin2 and TITAN quantify different properties of the relationship between species and the environment (i.e., ecological specificity, continuous environmental associations and threshold responses, respectively), they converged on remarkably similar indicator communities. Importantly, the three approaches also provided complementary ecological perspectives. IndVal identified taxa that were characteristic of particular ecological states, MaAsLin2 quantified gradual changes in abundance along the continuous IQI gradient, and TITAN identified bacterial taxa exhibiting abrupt responses at ecological transition points. Together, these approaches describe not only which bacterial taxa are associated with environmental quality, but also how they respond as ecological conditions change.

A wide range of statistical approaches has been developed to identify microbial bioindicators from metabarcoding datasets, including association-based methods such as Indicator Species Analysis, gradient-based approaches such as TITAN, machine learning algorithms including Random Forest, and differential abundance methods originally developed for transcriptomics (e.g., Alonso et al., 2022; Keeley et al., 2018; Lanzén et al., 2021; Lee et al., 2023; Leontidou et al., 2023; Rubel et al., 2025). Because these approaches quantify different aspects of species-environment relationships and rely on distinct statistical assumptions, they often identify overlapping, but not identical, sets of candidate indicators. Consequently, previous studies have largely focused on evaluating the predictive performance of individual methods or determining which analytical framework performs best for environmental assessment (e.g., Lanzén et al., 2021; Frühe et al., 2021a). Rather than attempting to identify a single superior analytical approach, the present study adopted an integrative framework to identify bacterial taxa whose ecological responses were consistently supported across methods. We argue that bacterial taxa independently supported by multiple

analytical approaches are less likely to represent method-specific statistical artefacts and more likely to reflect genuine ecological responses to organic enrichment.

Consistent with this expectation, the three analytical approaches did not produce entirely independent sets of candidate taxa, but repeatedly identified ASVs belonging to the same bacterial families associated with either organically enriched or environmentally healthy sediments. This convergence was particularly evident for families such as Sulfurovaceae, Flavobacteriaceae, Hyphomicrobiaceae and Anaerolineaceae, which were consistently recovered despite substantial differences in the underlying statistical principles. Importantly, each analytical approach evaluated a different characteristic of the relationship between bacterial taxa and ecological condition. Consequently, consensus indicators satisfied multiple independent criteria expected of robust bioindicators: they were ecologically specific to particular environmental conditions (IndVal), exhibited consistent responses along the ecological quality gradient (MaAsLin2), and showed well-defined threshold responses at ecological transition points (TITAN).

The threshold responses identified by TITAN are particularly informative from a biomonitoring perspective because they indicate that some bacterial taxa do not simply increase or decrease gradually with organic enrichment, but instead respond disproportionately once ecological conditions pass specific transition points. Such nonlinear responses are consistent with the progressive deterioration of sediment biogeochemistry, whereby increasing organic loading ultimately drives shifts in dominant microbial metabolisms as oxygen becomes depleted and sulfate reduction intensifies (Bisset et al., 2008; Choi et al., 2022). These threshold responses therefore have the potential to identify ecological tipping points and may provide early warning of transitions between ecological quality classes (Liu et al., 2020). In contrast, the continuous associations identified by MaAsLin2 demonstrate that many consensus indicators respond progressively across the entire IQI gradient, making them particularly suitable for the development of quantitative molecular indices capable of tracking incremental changes in ecological condition (Mallick et al., 2021). IndVal complements these approaches by identifying taxa that are both frequent and abundant within particular ecological states, thereby providing ecologically interpretable indicators that facilitate environmental classification (Dufrêne and Legendre, 1997). Together, these complementary lines of evidence provide substantially greater confidence in the ecological relevance and biomonitoring potential of the identified bacterial taxa than could be achieved using any single analytical approach alone.

4.5.2 Consistency across independent datasets demonstrates transferability

Transferability is one of the principal requirements for developing microbial bioindicators, because taxa identified in a single dataset may reflect local environmental conditions, seasonal patterns, or study-specific design rather than broadly reproducible ecological responses (Astudillo-García et al., 2019). Previous work has therefore emphasized the need to evaluate microbial indicators across seasons, locations, and independent environmental settings before they can be considered suitable for general biomonitoring applications (Fontaine et al., 2023).

The use of two independent datasets in the present study provided complementary tests of indicator robustness. Dataset 1 evaluated the temporal consistency of bacterial indicators by following a single salmon farm over multiple sampling campaigns spanning different stages of the production cycle. In contrast, Dataset 2 assessed spatial transferability across a broad range of Scottish salmon farms encompassing diverse environmental settings and operational conditions. This distinction is important because microbial indicators intended for routine monitoring must be reproducible across both temporal and spatial sources of variation (Fontaine et al., 2023).

Despite these contrasting study designs, remarkably similar bacterial lineages were consistently identified by all three analytical approaches. Families associated with organically enriched sediments, including Sulfurovaceae, Flavobacteriaceae and Bacteroidetes BD2-2, together with families associated with environmentally healthy sediments, such as Hyphomicrobiaceae, Anaerolineaceae and Sva1033 (Desulfuromonadaceae), were repeatedly recovered in both datasets. Moreover, comparison at the nucleotide sequence level demonstrated that several consensus indicators were represented by identical ASVs, while some others exhibited greater than 97% sequence similarity despite being inferred independently. This level of agreement is particularly noteworthy because ASVs were generated separately for each dataset, meaning that correspondence was established through sequence identity rather than shared ASV labels.

These findings are consistent with a growing body of evidence demonstrating that relatively compact sets of bacterial indicators can reliably capture ecological condition across diverse environmental settings. Fontaine et al. (2023) showed that accurate biological status classification along the Danube River could be achieved using only a small number of highly informative ASVs, highlighting redundancy among bacterial indicators and suggesting that

robust ecological assessment does not require large indicator sets. Their study further demonstrated that bacterial communities retained predictive information across both spatial and temporal gradients, emphasizing the importance of evaluating microbial indicators beyond individual sampling locations (Fontaine et al., 2023). Similarly, Hermans et al. (2024) reported that bacterial community composition accurately reflected land-use intensity across more than 200 stream sites spanning approximately 1000 km, indicating that bacterial community signatures remain sufficiently stable to discriminate ecological conditions despite substantial environmental heterogeneity.

Comparable observations have also been reported for marine aquaculture. Frühe et al. (2021b) analysed bacterial communities from ten salmon farms distributed across four major salmon-producing countries and identified a relatively small set of bacterial families that consistently characterized high-, moderate-, and low-impact sediments despite differences in geography, season, sediment type, and farming conditions. Together, these studies suggest that bacterial communities contain reproducible ecological signals that can be detected across broad spatial and environmental gradients (Frühe et al. 2021b).

Our findings complement and strengthen this evidence by demonstrating that similar bacterial lineages, and in several cases, identical nucleotide sequences, were independently recovered from two separate metabarcoding datasets generated from different sampling designs. Whereas previous studies demonstrated transferability within single, albeit geographically extensive, datasets, our study demonstrates reproducibility across independent datasets that separately evaluated temporal consistency (repeated sampling of a single farm) and spatial consistency (multiple farms across Scotland). The convergence of the same indicator taxa across these complementary dimensions suggests that they represent general ecological responses to organic enrichment rather than dataset-specific patterns, providing strong support for their application in routine environmental monitoring.

4.5.3 Ecological significance of the dominant indicator lineages

The bacterial lineages associated with impacted conditions in the present study are ecologically consistent with the progressive changes in sediment biogeochemistry caused by the deposition of uneaten feed and faecal material beneath salmon cages (Hargrave et al., 2008; Holmer et al., 2005). Microbial degradation of this organic matter increases oxygen demand, lowers redox potential and promotes the accumulation of reduced compounds, particularly sulfide (Reimers et al., 2013; Kawahara et al., 2008). These conditions favour organisms

involved in anaerobic organic-matter degradation and sulfur cycling, while excluding many taxa associated with more oxygenated sediments (Hornick and Buschmann, 2018). The repeated recovery of Sulfurovaceae, Flavobacteriaceae, BD2-2, Caldilineaceae and several sulfate-reducing lineages therefore reflects different but closely connected components of the microbial response to organic enrichment. Previous aquaculture studies similarly concluded that bacterial communities are particularly responsive to this gradient because the taxa mediating organic carbon degradation and terminal electron-accepting processes change directly as sediment conditions become increasingly reduced (Holmer et al., 2005; Frühe et al., 2021a, b).

Among these lineages, Sulfurovaceae represented one of the most consistent indicators of impacted conditions in the present study. Members of this family, including *Sulfurovum*, commonly exploit reduced sulfur compounds generated under oxygen-limited conditions and are therefore favored near the interface between sulfidic sediments and residual oxygen or alternative electron acceptors (Miranda et al., 2021). Their repeated identification in both datasets and by all three analytical approaches suggests that sulfur oxidation is a particularly stable ecological feature of organically enriched salmon-farm sediments. This interpretation is strongly supported by previous work (Keeley et al., 2021; Lanzén et al., 2021; Johansen et al., 2024; Rubel et al., 2025). Lanzén et al. (2021) identified *Sulfurovum* sequence variants among taxa assigned to tolerant or opportunistic ecological groups, while Rubel et al. (2025) recovered Sulfurovaceae as an indicator in nearly every combination of country, sequencing technology and indicator-selection method examined. In the latter study, Sulfurovaceae was identified in all analytical combinations except one Scottish metabarcoding-IndicSpecies dataset, indicating that its indicator value was retained across Scottish and Norwegian farms and across both metabarcoding and metagenomic data (Rubel et al., 2025).

The prominence of sulfur-oxidizer Sulfurovaceae should not be interpreted independently of sulfate-reducing bacteria, because sulfur oxidizers depend on the reduced sulfur compounds produced during anaerobic organic-matter mineralization (Ghosh and Dam, 2009; Nguyen et al., 2022). In the present study, ASVs assigned to Desulfobulbaceae and Desulfocapsaceae were also associated with impacted conditions, although some members of these families exhibited more variable responses. Frühe et al. (2021b) identified Desulfobulbaceae, Desulfobacteraceae and Desulfarculaceae as significantly enriched at high-impact salmon-farm sites across ten farms in four countries. These taxa participate in sulfate reduction and organic-matter turnover, producing sulfide as an end product under anoxic

conditions. It should be noted, however, that recent phylogenomic revisions have reassigned several genera previously classified within Desulfobulbaceae, including *Desulfocapsa*, to the newly established Desulfocapsaceae (Waite et al., 2020). Consequently, comparisons among studies should be interpreted in the context of evolving bacterial taxonomy rather than as evidence of conflicting ecological patterns. Importantly, this taxonomic revision does not alter the recognized ecological role of these lineages in anaerobic sulfur cycling and organic matter degradation (Wasmund et al., 2017). Desulfobulbaceae were also detected by Rubel et al. (2025) as indicators in all metagenomic datasets, with a weaker representation in metabarcoding data. Overall, the co-occurrence of sulfur-oxidizing and sulfate-reducing lineages suggests that the indicators identified here do not merely reflect the presence of deposited organic material, but the development of an intensified and internally coupled sediment sulfur cycle (Choi et al., 2018).

Flavobacteriaceae formed a second prominent group of impacted-condition indicators. They belong to the Bacteroidetes phylum, historically referred to as the Cytophaga-Flavobacterium-Bacteroidetes (CFB) group, whose members occupy a wide range of ecological niches and exhibit considerable metabolic versatility, particularly in the degradation of complex organic matter (Weller et al., 2000; Bernardet and Nakagawa, 2006; Bisset et al., 2008). Members of this family are widely distributed across marine and other aquatic environments and exhibit considerable metabolic versatility, enabling them to occupy a broad range of ecological niches (García-López et al., 2019; Miranda et al., 2021). Their repeated association with organically enriched sediments in the present study is therefore consistent with their ability to respond to changing environmental conditions associated with salmon farming (Bisset et al., 2008). Frühe et al. (2021) likewise identified Flavobacteriaceae as both significantly more abundant at high-impact sites and among the strongest family-level predictors of aquaculture disturbance across Norway, Scotland, New Zealand and Canada. However, other studies have reported contrasting abundance patterns. For example, Miranda et al. (2021) observed lower relative abundances of Flavobacteriaceae near salmon cages despite an overall increase in other members of the class Bacteroidia during periods of peak farm biomass. This apparent discrepancy likely reflects the considerable ecological diversity within Flavobacteriaceae, as different genera exhibit distinct environmental preferences.

The Bacteroidetes BD2-2 group provides further evidence for the importance of heterotrophic microbial communities in organically enriched sediments. In the present study, BD2-2 was consistently associated with impacted conditions in both datasets. Similar

observations have been reported from other marine environments influenced by human activities (Kolda et al., 2020; Pearman et al., 2020; Sun et al., 2023; Johansen et al., 2024). For example, Sun et al. (2023) identified Bacteroidetes BD2-2 as one of the bacterial taxa significantly enriched in fishery and aquaculture zones compared with marine protected and shipping areas. In their study, BD2-2 was recovered as one of the bacterial families contributing most strongly to random forest classification of anthropogenic activity zones, indicating that this lineage consistently responds to environmental conditions associated with intensive aquaculture (Sun et al., 2023). Although the ecological roles of the BD2-2 lineage remain less well resolved than those of sulfur-cycling bacteria such as Sulfurovaceae, its repeated recovery across independent studies supports its robustness and transferability as a biomonitoring indicator.

In contrast to the indicator lineages associated with organic enrichment, several bacterial families were consistently linked to sediments of higher ecological quality. These taxa are likely to represent microbial communities adapted to relatively stable, oxygenated sediments with lower organic loading, where nutrient cycling proceeds under less reduced conditions. The repeated recovery of Hyphomicrobiaceae, Anaerolineaceae, Pirellulaceae, Ilumatobacteraceae and members of the Desulfuromonadales (Sva1033) across both datasets suggests that bacterial communities associated with environmentally healthier sediments are also highly reproducible, despite differences in sampling design and geographic extent.

Among these, Hyphomicrobiaceae emerged as one of the most consistent indicators of high ecological quality. Members of this family are widespread marine Alphaproteobacteria that contribute to carbon and nitrogen cycling and are commonly associated with stable sediment environments (Oren and Xu, 2014; Aylagas et al., 2017). Similar observations have been reported by Leontidou et al. (2023), who identified Hyphomicrobiaceae as characteristic of reference sediments and suggested that the family represents a sensitive indicator of good environmental quality in Mediterranean fish farms. Likewise, Frühe et al. (2021b) identified Hyphomicrobiaceae among the bacterial families consistently associated with low-impact sediments across salmon farms distributed throughout four major aquaculture-producing countries, supporting the view that this lineage represents a robust indicator of relatively undisturbed benthic conditions. Together with the present findings, these studies suggest that Hyphomicrobiaceae respond negatively to increasing organic enrichment and may therefore provide a useful complement to enrichment-associated indicators when assessing ecological status.

Anaerolineaceae also showed a consistent positive association with ecological quality, although their ecological interpretation is somewhat less straightforward. Members of this family are anaerobic heterotrophs that participate in the degradation of organic compounds and have been implicated in nitrogen cycling through dissimilatory nitrate reduction to ammonium under facultatively hypoxic conditions (Zhu et al., 2018; Zhang et al., 2020; Zhang et al., 2022; Ghosh et al., 2025). However, their occurrence spans a broad range of sedimentary environments, indicating considerable ecological diversity within the family. In aquaculture sediments, increasing organic loading drives a progressive succession of microbial metabolisms, ultimately leading to sulfate reduction and sulfide accumulation as terminal anaerobic processes (Vasquez-Cardenas et al., 2022). As sulfate reduction becomes the dominant terminal anaerobic process, sulfur-cycling microorganisms increasingly outcompete lineages occupying earlier stages of organic matter degradation (Kawahara et al., 2008). This ecological succession provides a plausible explanation for the positive association between Anaerolineaceae and higher-IQI sediments observed in the present study, suggesting that the ASVs identified here are associated with moderately reduced sediments that have not yet transitioned to the sulfide-dominated conditions typical of heavily enriched sediments. Their response therefore appears to reflect a particular stage of sediment redox development rather than a universal preference for either impacted or pristine environments.

Among the reference-associated indicators, the uncultured Sva1033 lineage (Order Desulfuromonadales), affiliated with the family Desulfuromonadaceae, was consistently associated with higher ecological quality in both datasets. Although relatively little is known about the ecology of Sva1033 specifically, experimental studies have shown that this lineage participates in acetate oxidation coupled primarily to iron reduction, with evidence suggesting a potential capacity for manganese reduction under appropriate geochemical conditions (Aromokeye et al., 2021; Wunder et al., 2024). Members of the broader Desulfuromonadaceae are therefore commonly associated with metal-reducing metabolisms operating under suboxic to anoxic conditions, where their distribution is strongly influenced by the availability of alternative terminal electron acceptors (Guo et al., 2021; Soares et al., 2024). This ecological framework provides a plausible explanation for the positive association between Desulfuromonadaceae-affiliated Sva1033 and higher IQI values observed in the present study. In contrast to the strongly reduced, sulfide-dominated conditions beneath salmon cages, reference sediments retain relatively more oxidized or suboxic environments that can support iron- and metal-reducing guilds (Kolda et al., 2020). This interpretation helps reconcile

apparently contradictory findings reported in the literature, where in some studies members of Desulfuromonadaceae were characteristic of low-impact sediments (Dowle et al., 2015; Aylagas et al., 2017; Frühe et al., 2021b), whereas other studies reported them as enriched in organically disturbed environments (Elsabé et al., 2012; Aranda et al., 2015). Consequently, the ecological interpretation of Desulfuromonadaceae (particularly the Sva1033 lineage) should be considered in the context of sediment redox conditions rather than viewed as a universal indicator of either pristine or impacted environments.

Despite the valuable ecological context provided by family-level classifications, the contrasting responses observed among some families, such as Desulfocapsaceae, demonstrate that higher taxonomic ranks can mask substantial ecological heterogeneity. Similar limitations have been highlighted in microbial ecology more broadly, where physiological diversity frequently occurs among closely related microorganisms despite shared taxonomic affiliation (Escalas et al., 2019; Djemiel et al., 2022). In the present study, resolving bacterial indicators at the ASV level enabled the discrimination of closely related sequence variants exhibiting contrasting associations with ecological quality, thereby providing substantially greater ecological specificity than would have been achieved through family-level analyses alone. Consequently, robust bacterial bioindicators should be selected at sequence resolution while using higher-level taxonomy to provide ecological interpretation.

4.5.4 Implications for biomonitoring

A major challenge in developing microbial biomonitoring tools is distinguishing bacterial taxa that consistently respond to environmental disturbance from those whose apparent indicator value reflects dataset-specific characteristics, analytical choices or stochastic variation. The present study demonstrates that robust indicator selection should not rely solely on statistical significance obtained from a single analytical approach. Instead, confidence in candidate indicators is substantially strengthened when consistent responses are observed across complementary statistical methods, independent datasets encompassing multiple farms, sampling designs, and environmental conditions. Although both datasets originated from Scottish salmon farms, they differed substantially in spatial coverage and environmental heterogeneity, thereby providing an important first assessment of indicator reproducibility under independent field conditions. Nevertheless, further validation across additional aquaculture regions, production systems and biogeographic settings will be necessary to determine the global transferability of the proposed indicator set.

Rather than proposing a single "best" statistical method, the results suggest that complementary analytical approaches capture different aspects of bacterial responses to environmental gradients. Their integration increases confidence that retained ASVs represent reproducible ecological responses rather than method-specific artefacts, thereby reducing the risk of selecting indicators that perform poorly when applied to new monitoring datasets. The final consensus set therefore provides a practical pool of candidate bioindicators for the further development of metabarcoding-based assessment tools. Because these ASVs displayed consistent responses across analytical approaches and independent datasets, they are stronger candidates for inclusion in molecular indices or targeted quantitative assays than indicators identified within individual studies alone. However, operational implementation will require further evaluation of their sensitivity, specificity, detection thresholds and performance across laboratories and geographic regions. Subject to such validation, sequence-resolved bacterial indicators could support increasingly standardized, reproducible and quantitative assessment of benthic impacts associated with salmon aquaculture.

4.6 Contribution to the Thesis

This chapter advanced the development of bacterial metabarcoding for aquaculture biomonitoring by establishing a consensus-based framework for selecting robust, sequence-resolved bioindicators. By integrating three complementary analytical approaches across two independent Scottish datasets, it distinguished reproducible ecological responses from method- and dataset-specific associations and identified bacterial ASVs consistently associated with impacted and reference conditions. The agreement observed across analytical methods, datasets and ecological interpretations demonstrates that reliable indicator selection requires both statistical consensus and independent validation. Together, these findings extend the thesis from demonstrating the methodological and temporal robustness of bacterial community patterns to identifying specific bacterial sequences that can be taken forward for quantitative validation and the eventual development of operational monitoring tools.

4.7 References

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CHAPTER IV

QUANTITATIVE VALIDATION OF BACTERIAL BIOINDICATORS USING DIGITAL PCR

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5.1 Summary

5.1.1 Background

The previous chapters demonstrated that sediment bacterial communities respond consistently to gradients of organic enrichment associated with salmon aquaculture. Chapter III further identified bacterial amplicon sequence variants (ASVs) with consistent associations with ecological quality across complementary statistical approaches and independent Scottish datasets. These findings established a robust pool of candidate bacterial bioindicators. However, their implementation in routine biomonitoring requires methods capable of detecting individual indicator taxa quantitatively without the need for high-throughput sequencing.

Metabarcoding provides relative abundance estimates for thousands of taxa simultaneously and is well suited for community-level ecological assessment (Baird and Hajibabaei, 2012). However, sequencing workflows remain relatively expensive, require specialized bioinformatic processing, and are not always practical for rapid routine monitoring or targeted validation of specific indicators (Piper et al., 2019). Consequently, once robust bacterial bioindicators have been identified, the next step towards operational molecular biomonitoring is the development of targeted assays capable of quantifying these organisms directly in environmental samples.

Digital PCR (dPCR) has emerged as a promising technology for this purpose. Unlike conventional quantitative PCR, dPCR partitions each reaction into thousands of individual microreactions, enabling absolute quantification of target DNA molecules through Poisson statistics without the need for calibration curves (Guri et al., 2024; Dias et al., 2025). The approach provides high analytical sensitivity, is relatively resistant to PCR inhibition, and is particularly well suited for low-abundance targets commonly encountered in environmental DNA samples (Mauvisseau et al., 2019; Guri et al., 2024; Gross et al., 2026).

This chapter therefore represents the final step in translating bacterial metabarcoding into an operational biomonitoring tool. Building on the indicator ASVs identified in Chapter III, species-specific dPCR assays were developed, analytically validated and applied to environmental sediment samples collected from Scottish salmon farms spanning a gradient of ecological quality. The study served as a proof of concept to determine whether sequencing-derived bacterial indicators exhibit quantitative abundance changes that correspond with conventional macrofaunal assessments and therefore have potential for future incorporation into routine environmental monitoring.

5.1.2 Methods

Three indicator ASVs were selected as a proof-of-concept panel to evaluate the feasibility of quantitative validation using dPCR. Selection was based on the consensus indicator analyses presented in Chapter III and further supported by Rubel et al. (2025). The panel comprised two indicators associated with organically enriched sediments (*Sulfurovum* sp. and *Cytophaga* sp., which are often associated with organic enrichment; Bernardet and Nakagawa, 2006; Bisset et al., 2008; Choi et al., 2022) and one reference-associated indicator belonging to the family Desulfuromonadaceae. Accordingly, species-specific primer pairs were designed using BLAST and Primer-BLAST (Camacho et al., 2009), experimentally validated using conventional PCR and Sanger sequencing, and subsequently transferred to a digital PCR platform.

Assays were optimized using synthetic double-stranded DNA fragments (gBlocks) and environmental DNA extracts. Annealing temperature gradients were evaluated to maximize amplification efficiency while ensuring clear separation between positive and negative partitions. Analytical performance of each assay was assessed using serial dilutions of synthetic DNA to determine the limit of detection (LOD) and limit of quantification (LOQ). Detection limits were established from replicate dilution series, while quantification precision was evaluated using the coefficient of variation across technical replicates, with a threshold of ≤35% defining the LOQ (Klymus et al., 2020).

Following assay validation, the dPCR assays were applied to environmental sediment samples collected during routine regulatory monitoring of Scottish salmon farms and previously described by Wyness et al. (2026). Three farms within the Highland region were selected to represent a gradient of ecological quality based on the Infaunal Quality Index (IQI). For each farm, two independent sediment samples were analysed from each of three ecological quality classes (poor, moderate and good), resulting in a total of 18 environmental samples. Each sample was analysed in three technical dPCR replicates, and target abundance was expressed as gene copy numbers per reaction.

Technical replicates were averaged prior to statistical analysis. Gene copy numbers were log-transformed to improve normality, and relationships between bacterial abundance and continuous IQI values were evaluated using Spearman's rank correlation and linear regression models that included farm as a fixed effect. Potential differences in ASV-IQI relationships among farms were assessed by testing interaction terms between IQI and farm. Differences

among ordered ecological quality classes were examined using the Jonckheere-Terpstra trend test (Jonckheere, 1954; Terpstra, 1952), and changes in bacterial abundance were summarized as fold changes between poor- and high-quality sediments. Where multiple indicator ASVs were analysed simultaneously, *p*-values were adjusted using the Holm procedure (Holm, 1979). All statistical analyses and graphical visualizations were performed in R (R Core Team, 2022).

5.1.3 Results and Discussion

The objective of this chapter was to evaluate whether bacterial indicator ASVs identified through sequencing could be translated into quantitative molecular biomarkers using dPCR. Three bacterial indicators previously associated with contrasting levels of organic enrichment were selected as a proof of concept, and species-specific dPCR assays were developed, analytically validated and applied to environmental sediment samples spanning a gradient of ecological quality. A summary of the analytical validation, ecological responses and overall performance of the three bacterial indicators is presented in Figure 16.

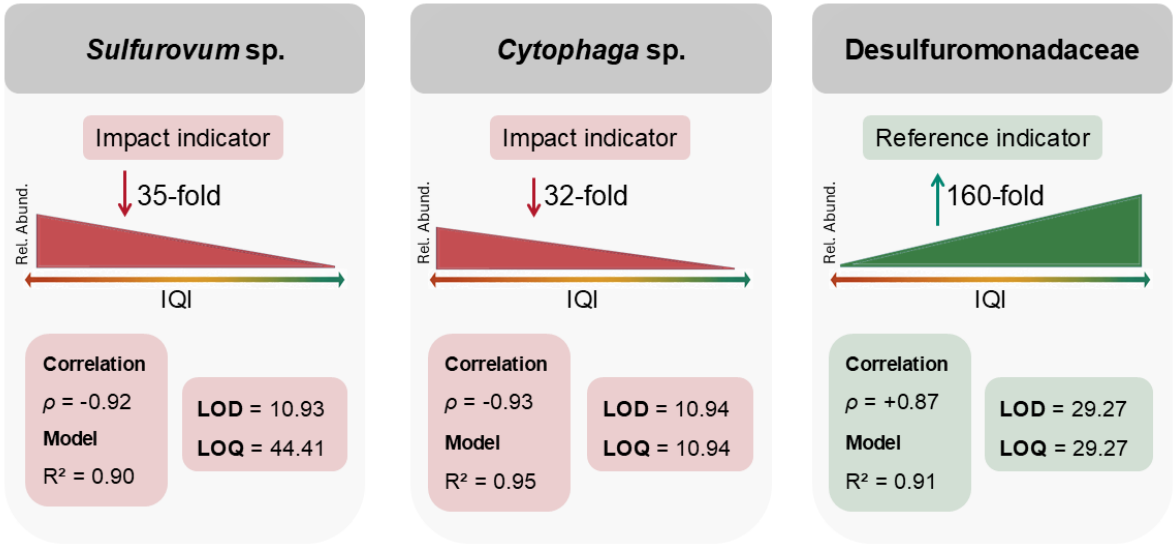


Figure 16. Summary of digital PCR validation of bacterial indicator ASVs identified in Chapter III. *Three bacterial indicators were translated into species-specific digital PCR assays and evaluated as proof-of-concept quantitative biomarkers of sediment ecological quality. The figure summarizes the ecological response of each indicator across the Infaunal Quality Index (IQI) gradient, including the direction and magnitude of abundance change (fold change), correlation with IQI (Spearman's ρ and linear model R²), and analytical assay performance (limit of detection, LOD; limit of quantification, LOQ).*

All three assays demonstrated robust analytical performance. Primer specificity was confirmed through cloning and Sanger sequencing, while assay optimization established low LOD (10.9-29.3 copies reaction^{-1}) and LOQ (10.9-44.4 copies reaction^{-1}) suitable for environmental samples. Quantification precision improved rapidly with increasing target concentration, with coefficients of variation at the LOQ ranging from only 6-26%, well below the predefined acceptance threshold of 35%. Following LOQ-based filtering, all environmental samples were retained for *Sulfurovum* sp. and *Cytophaga* sp., while only four samples fell below the quantification limit for Desulfuromonadaceae. These results demonstrate that sequencing-derived bacterial indicators can be translated into sensitive and reproducible dPCR assays suitable for environmental applications.

Application of the assays to Scottish salmon farm sediments revealed clear abundance changes across ecological quality gradients. *Sulfurovum* sp. and *Cytophaga* sp. exhibited approximately 35-fold and 32-fold higher median copy numbers, respectively, in poor-quality sediments than in high-quality sediments. In contrast, the reference-associated Desulfuromonadaceae increased by approximately 160-fold towards high-quality sediments. Overall, target abundances ranged from 62-16,273 copies reaction^{-1} for *Sulfurovum* sp., 95-13,248 copies reaction^{-1} for *Cytophaga* sp., and 3-2,683 copies reaction^{-1} for Desulfuromonadaceae, demonstrating that the assays captured large abundance changes across the environmental gradient.

The strongest evidence supporting the utility of these assays was the close relationship between bacterial abundance and environmental quality. All three indicators showed highly significant correlations with IQI (*Sulfurovum* sp.: ρ = -0.920; *Cytophaga* sp.: ρ = -0.930; Desulfuromonadaceae: ρ = 0.874; all adjusted $p < 0.001$). Linear models explained 90–95% of the variation in ASV abundance (R^2 = 0.899-0.945), while no significant IQI × farm interactions were detected, indicating that indicator responses were highly consistent among the three study sites.

This study demonstrates that bacterial indicators identified through metabarcoding correspond to genuine changes in absolute population abundance rather than solely to compositional changes in sequencing data. The enrichment-associated indicators (*Sulfurovum* sp. and *Cytophaga* sp.) consistently declined with improving ecological quality, whereas the reference-associated Desulfuromonadaceae increased towards less impacted sediments. These relationships were highly reproducible across farms and closely reflected established redox-

driven ecological succession beneath salmon aquaculture installations. The successful translation of sequencing-derived ASVs into validated dPCR assays demonstrates that targeted molecular quantification can provide robust ecological information while avoiding the compositional limitations inherent to metabarcoding. Together, these findings establish dPCR as a promising complementary tool for quantitative assessment of bacterial bioindicators in benthic environmental monitoring.

5.1.4 Contribution to the Thesis

This chapter represents the final step in translating bacterial metabarcoding into a practical biomonitoring framework. While Chapter I demonstrated that bacterial metabarcoding is methodologically robust, Chapter II established that bacterial communities respond consistently to organic enrichment despite natural variability, and Chapter III identified robust bacterial indicator ASVs across complementary analytical approaches and independent datasets. Building on these findings, the present chapter demonstrated that selected indicator ASVs can be quantified directly using dPCR and exhibit predictable changes in absolute abundance across ecological quality gradients. This provides proof of concept that sequencing-derived bacterial indicators can be transformed into rapid, targeted molecular assays suitable for environmental monitoring. Together, these findings bridge the gap between microbial community profiling and quantitative biomonitoring, advancing bacterial eDNA from indicator discovery towards operational implementation in regulatory assessment.

5.1.5 References

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5.2 Publication

Digital PCR assays quantify bacterial bioindicators across environmental quality gradients in salmon aquaculture sediments

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5.2.1 Abstract

Organic enrichment beneath marine finfish aquaculture installations alters benthic ecosystem structure and sediment biogeochemistry, creating pronounced environmental gradients that require effective and scalable monitoring tools. High-throughput sequencing has revealed consistent associations between benthic bacterial communities and organic enrichment beneath salmon farms, yet regulatory implementation remains constrained by the compositional nature of sequencing data. Relative read abundances do not directly demonstrate whether candidate indicator taxa undergo absolute population shifts along impact gradients, limiting their deployment as quantitative decision-support tools. Here, we test whether sequencing-derived bacterial bioindicators reflect measurable abundance changes under real-world aquaculture conditions. Bacterial amplicon sequence variants (ASVs) previously associated with contrasting levels of sediment enrichment were targeted using ASV-specific digital PCR (dPCR) assays and quantified across multiple Scottish salmon farms spanning a gradient of ecological quality defined by the Infaunal Quality Index. Enrichment-associated taxa showed significant declines in absolute copy number as organic enrichment decreased, whereas a reference-associated indicator signature increased toward less enriched environments. These abundance-quality relationships were consistent across farms and aligned with redox-structured sediment succession, indicating that sequencing-derived indicators correspond to genuine population shifts rather than purely relative community dynamics. Targeted dPCR thereby provides a practical bridge between microbial community profiling and quantitative marine aquaculture impact assessment. Beyond relative abundance patterns, targeted bacterial indicators offer a transferable, mechanistically grounded approach for strengthening benthic marine monitoring frameworks.

Keywords: eDNA, Absolute quantification, Digital PCR, Salmon farm, Biomonitoring.

5.2.2 Introduction

Marine finfish aquaculture, and salmon farming in particular, exerts strong, spatially constrained pressures on benthic ecosystems through the sustained deposition of organic matter and associated nutrients beneath and around production cages. These inputs generate pronounced enrichment gradients in sediments, driving predictable changes in benthic ecosystem structure and sediment biogeochemistry (Brooks and Mahnken, 2003; Hargrave et al., 2008; Cranford et al., 2022). As organic loading increases, intensified microbial respiration reduces oxygen penetration depths and promotes a progression from oxic to suboxic and ultimately sulfidic conditions in surface sediments, fundamentally reorganizing benthic communities and redox-sensitive biogeochemical processes along impact gradients (Holmer et al., 2005). Given the ecological consequences of this redox restructuring, regulatory frameworks in many countries mandate routine biomonitoring of sediments surrounding salmon farms to assess environmental status, ensure compliance with environmental quality standards, and mitigate ecological degradation (Wilson et al., 2009).

Benthic biomonitoring around salmon aquaculture sites has traditionally been based on macrofaunal community indices that quantify changes in sediment condition along organic enrichment gradients (e.g., Mirto et al., 2012; Stojanović et al., 2017; Bouchet et al., 2020). Central to this approach is the AZTI Marine Biotic Index (AMBI), which classifies taxa according to their sensitivity to organic disturbance (Borja et al., 2000). When combined with measures of species richness and evenness, AMBI forms the basis of composite indices designed to assess the ecological status of marine benthic environments (Muxika et al., 2007). These composite indices can be further integrated within the Infaunal Quality Index (IQI), which serves as the principal regulatory metric for determining ecological quality (EQ) status at salmon farming sites and underpins routine environmental compliance assessments (Culhane et al., 2014).

Despite their widespread use and long regulatory track record, benthos-based assessments have practical limitations in the context of intensive salmon aquaculture, including high analytical costs, labour-intensive processing, and long turnaround times, which restrict temporal resolution (Pawlowski et al., 2018). In addition, these indices are based on presence-

abundance data and may be relatively insensitive to subtle or early community shifts. In this context, recent advances in environmental DNA (eDNA) metabarcoding have emerged as a complementary approach, enabling rapid and high-throughput characterization of benthic communities directly from sediment DNA, including taxa that are difficult to detect using morphology-based methods (Pochon et al., 2015; Pawlowski et al., 2016; Stoeck et al., 2018; Pearman et al., 2020; He et al., 2021). Among these, bacterial metabarcodes usually obtained from the 16S rRNA gene have consistently demonstrated particularly strong and reproducible responses to organic enrichment gradients in aquaculture sediments, supporting more accurate inference of EQ than other organismal groups (Dowle et al., 2015; Keeley et al., 2018; Stoeck et al., 2018; Verhoeven et al., 2018; Aylagas et al., 2021; Frühe et al., 2021b; Wilding et al., 2023; Oladi et al., 2024). Metabarcoding of bacterial communities combined with statistical approaches such as regression spline analyses (Keeley et al., 2018) or supervised machine learning (Leontidou et al., 2023; Rubel et al., 2025; Wyness et al., 2026) has been successfully established to infer molecular proxies of established macrofaunal indices and to classify EQ status.

Beyond community-level assessments, these approaches have increasingly been used to identify specific microbial amplicon sequence variants (ASVs) as bioindicators associated with organic enrichment gradients in aquaculture-impacted sediments (Verhoeven et al., 2018; Keeley et al., 2018; Leontidou et al., 2023; Johansen et al., 2024). Candidate indicators from marker-gene data may be affected by amplification biases, incomplete reference databases, and low phylogenetic resolution. Therefore, a recent study (Rubel et al., 2025) used shotgun metagenomics to independently validate metabarcoding-based indicator selection, increasing confidence in their ecological relevance and use for targeted assays.

To further exploit such candidate bioindicator signatures for applied biomonitoring, the next logical step is the development of assays which allow quantification of these bioindicators in environmental samples (Netzer et al., 2021; Stoeck et al., 2024). For a molecular bioindicator signature to serve as an independent, quantitative decision-support tool in aquaculture management, it is essential that the selected bioindicator demonstrates absolute changes in population abundance across gradients of organic enrichment. One of the best options at hand for quantification of targeted genes in an environmental sample is digital PCR (dPCR). This technology enables direct enumeration of target DNA molecules without reliance on standard curves and is less sensitive to amplification efficiency and inhibition than conventional quantitative PCR (Nathan et al., 2014; Guri et al., 2024; Dias et al., 2025).

Our proof-of-concept study was undertaken to assess whether bacterial indicators identified through sequencing can serve as reliable and quantifiable proxies for environmental quality in practical coastal aquaculture settings, and to determine their potential integration into routine monitoring protocols for aquaculture management. In specific, we aimed (i) to quantify selected bacterial indicator ASVs in Scottish salmon farm sediments using dPCR to determine whether sequence-derived indicators reflect absolute population shifts along organic enrichment gradients, and (ii) to evaluate how these quantitative signals correspond with conventional environmental quality classifications across impact gradients.

5.2.3 Material and methods

Primer and dPCR assay design

Candidate bacterial indicator ASVs for benthic enrichment of organic material derived from salmon aquaculture were selected from a previous study (Rubel et al., 2025). This study was chosen because it combined metagenomic and metabarcoding approaches to identify bacterial indicators across near- and far-field salmon aquaculture installations in Scotland, thereby providing a robust, cross-validated set of candidate taxa relevant to the present study system.

Indicator ASVs were selected based on consistent ranking across complementary statistical approaches, including indicator value analysis and random forest classification (see Rubel et al., 2025 for details). From the resulting candidate list, we selected taxa that were among the highest-ranking indicators consistently associated with high-impact (high organic enrichment) conditions (Supplementary Table S1). These taxa were identified as *Sulfurovum* sp. and *Cytophaga* sp. (ASVs 04 and 22, respectively, in Rubel et al., 2025). Furthermore, as a control, we also included one indicator with high sensitivity to organic enrichment, namely an unidentified bacterial species of the family Desulfuromonadaceae (ASV 24 in Rubel et al., 2025). These targets were intended as a proof-of-concept ASV set for evaluating whether sequencing-derived bacterial indicators could be translated into quantitative, absolute abundance measurements using dPCR, rather than as a proposed minimal monitoring panel.

For PCR primer design, these candidate indicator ASV sequences were first subjected to a Basic Local Alignment Search Tool (BLAST) analysis (Camacho et al., 2009) via the NCBI BLAST web interface (NCBI BLAST, accessed August 2025) to identify closely related

reference sequences with ≥99% sequence similarity to the target ASVs. These complete reference sequences (Supplementary Table S1) were then used as templates for primer design using NCBI Primer-BLAST (Ye et al., 2012), which integrates Primer3 for primer generation and BLAST for in silico specificity assessment (Untergasser et al., 2012). Design parameters included a target primer length of ~20 bp (range 18-24 bp), melting temperature of 60 ± 3 °C, GC content of 40-60%, and an expected amplicon size of 100–180 bp suitable for dPCR analysis. Primer specificity was evaluated against the NCBI core nucleotide database with the search restricted to bacterial sequences, and primer pairs showing potential off-target amplification outside the intended taxonomic group were excluded. For each ASV, we chose a primer pair based on highest predicted specificity, low self-complementarity, coverage of distinct regions of the target sequence and optimal PCR product size for dPCR (Table 1). Primers were synthesized by Biomers GmbH (Ulm, Germany).

Table 1. Designed primer pairs and assay characteristics for three indicator bacterial ASVs.

Target	Assigned taxonomy	Forward primer (5'->3')	Reverse primer (5'->3')	T_m (F/R °C)	GC % (F/R)	Amplicon length (bp)
ASV04	*Sulfurovum*	TAAACATTCCGCCTGGGGAG	GCACCTCTCCATCTCTGGTG	59.75/59.82	55/60	158
ASV22	*Cytophaga*	GCGTTTGTAACCTACCCTTTAACT	AGCCGTTACCTCACCAACTA	59.24/58.36	41.67/50	151
ASV24	Desulfuromonadaceae	AGAACGAACGTTGGCGGTAT	GAGGGCGTAGGCTTATCAGG	60.04/59.68	50/60	165

Primer validation and specificity testing

Candidate primer pairs were initially evaluated using end-point PCR to assess amplification performance and specificity. PCR reactions were performed using OneTaq® DNA Polymerase (New England Biolabs, USA), with environmental DNA extracts from Scottish salmon farm sediment samples, isolated using the DNeasy PowerSoil Kit (Qiagen), used as template material. Each reaction contained 10 µl 5× Standard Taq-Polymerase Buffer, 2 µl of forward primer (4 µM), 2 µl of reverse primer (4 µM), 0.25 µl of 5 U Taq Polymerase, 1 µl of dNTPs, and 1 µl of template DNA (50 ng/µl) in a total reaction volume of 50 µL. Negative controls without template DNA were included in all reactions. Thermocycling conditions consisted of an initial denaturation step at 94 °C for 30 s, followed by 30 cycles of 94 °C for 30 s, 52 °C for 30 s, and 68 °C for 30 s, with a final extension at 68 °C for 5 min. To determine optimal annealing conditions for subsequent dPCR analysis, gradient PCR was performed across a temperature range of 51-67 °C. Amplification products were visualized by agarose gel electrophoresis (1.5% w/v agarose, 1× TAE buffer, 20 min at 90 V) using a 100 bp DNA ladder (New England Biolabs, USA) as a size standard to confirm expected amplicon size and assess specificity. Successfully amplified products (clear and clean target band with correct expected size on agarose gel) were purified using the MinElute PCR Purification Kit (Qiagen, Germany) for downstream validation of primer performance.

To verify the specificity of candidate primer pairs, PCR amplicons were cloned and Sanger sequenced. Amplified products were ligated into the pGEM®-T Easy vector (Promega, USA) and transformed into competent *Escherichia coli* cells following the manufacturer's instructions. Positive clones were screened by colony PCR using universal M13 forward (5′-GTAAAACGACGGCCAG-3′) and M13 reverse (5′-CAGGAAACAGCTATGAC-3′) primers. PCR reactions were carried out using OneTaq® DNA Polymerase (New England Biolabs, USA) following the manufacturer's protocol and thermocycling conditions described above, with products validated by agarose gel electrophoresis. Colonies yielding a single amplicon corresponding to the expected insert size flanked by the M13 priming sites were selected for plasmid purification using the Monarch's Plasmid Miniprep Kit (NEB, Ipswich, MA, USA). Plasmid insert sequences were confirmed by Sanger sequencing (StarSEQ, Mainz, Germany). Obtained sequences were aligned with the target ASV sequences to verify identity, and representative sequences were additionally compared against the NCBI nucleotide database using BLAST to assess target specificity.

Assay optimization for digital PCR

dPCR analyses were performed using the QIAcuity Nanoplate Digital PCR System (Qiagen, Germany) with 26k 24-well nanoplates, each generating approximately 26,000 partitions per reaction. dPCR reactions were prepared using QIAcuity EvaGreen Mastermix with ASV-specific primers at a final concentration of 0.4 µM and analyzed according to the manufacturer's instructions. Non-template controls were included in all runs to monitor potential contamination.

Annealing temperature optimization was performed using both synthetic DNA templates and environmental DNA extracts. Synthetic double-stranded DNA fragments (gBlocks; Integrated DNA Technologies, USA) containing the target amplicon sequences were used to establish assay performance under controlled conditions. Sequences and characteristics of synthetic DNA templates used for assay optimization and analytical validation are provided in Supplementary Table S2. Temperature gradients ranging from 52-64 °C were evaluated to maximize amplification efficiency and achieve clear separation between positive and negative partitions. An annealing temperature of 58.5 °C provided optimal amplification efficiency and clear partition separation for all three retained assays. These conditions were subsequently verified using representative eDNA extracts to ensure robust assay performance in sediment-derived matrices. Likewise, the analytical performance of validated primers was evaluated using synthetic target DNA (gBlocks). gBlocks were reconstituted according to the manufacturer's instructions, and serial dilutions were prepared to generate templates spanning a wide range of target concentrations. Expected copy numbers were calculated based on fragment length and DNA mass using Avogadro's constant (6.022×10^{23} mol^{-1}).

The limit of detection (LOD) and limit of quantification (LOQ) were determined empirically using replicate dilution series of synthetic target DNA. Serial dilutions (1:5) were analyzed under optimized assay conditions with ten technical replicates per dilution. Copy numbers were obtained from instrument output and expressed as copies per reaction. For each dilution level, the detection rate was calculated as the proportion of technical replicates yielding at least one positive partition. The LOD was defined as the lowest target concentration detected in at least one technical replicate (Klymus et al., 2019). Quantification precision was assessed by calculating the coefficient of variation (CV) of measured copy numbers across replicates at each dilution level. The LOQ was defined as the lowest concentration at which quantification

precision met the predefined acceptance criterion of CV ≤35%, consistent with commonly applied thresholds for dPCR assay validation (Klymus et al., 2019).

Application of dPCR assays in environmental samples

- Samples used in this study

Sediment samples for this study originated from regulatory compliance surveys conducted at Scottish salmon farms and previously described in Wyness et al. (2026). Sampling followed the standardized operating protocol published by the Scottish Environment Protection Agency (SEPA, 2023). Sediments were collected using Van Veen grabs along monitoring transects spanning near- and far-field positions relative to production cages. For each grab, six surface sediment scrapes (three from each side of the grab) were pooled into a single sterile tube for eDNA analysis. Samples used in the present study originated from single survey time points, and each grab sample was treated as an independent observation in downstream analyses.

For this test-case study, samples from three farms within the Highland local authority were selected to cover a gradient of EQ based on Infaunal Quality Index (IQI) values (Table 2). Samples spanned low (<0.44), moderate (~0.64), and high (>0.75) IQI values, representing aggregated regulatory EQ status classes used for analytical purposes (Culhane et al., 2014), and corresponding to high, moderate, and low levels of benthic organic enrichment, respectively. For each farm, two independent samples were selected from each EQ class, resulting in six samples per farm and 18 samples in total. IQI scores were obtained from Wyness et al. (2026), where IQI as a measure of EQ status was determined from benthic macrofaunal inventory and sediment characteristics of these samples following standard regulatory procedures (Phillips et al., 2014; UKTAG, 2014).

The datasets used in Rubel et al. (2025) and Wyness et al. (2026) are comparable because both were derived from marine sediments beneath and surrounding Atlantic salmon aquaculture installations in Scotland and represent similar gradients of organic enrichment and ecological quality. While individual sites may vary in local sediment characteristics, hydrodynamic conditions, and farm management regimes, both studies highlight the same underlying ecological processes linked to organic loading, including redox-driven sediment restructuring. Therefore, the use of an independent dataset for validation provides a robust test

of whether sequencing-derived bacterial indicators identified by Rubel et al. (2025) are transferable across sites and environmental contexts.

Table 2. Overview of sediment samples used for digital PCR validation of ASV indicators across Scottish salmon farms. Sampling dates, environmental quality (EQ) classes, and corresponding Infaunal Quality Index (IQI) values are shown. IQI values were obtained from Wyness et al. (2026), where EQ status was determined based on benthic macrofaunal assemblages and sediment characteristics.

Loch Torridon			**Loch Kishorn**			**Loch Sunart**		
Sampling date	EQ class	IQI	Sampling date	EQ class	IQI	Sampling date	EQ class	IQI
January 2023	low	0.17	September 2023	low	0.30	March 2022	low	0.13
		0.21			0.38			0.18
	moderate	0.50		moderate	0.66		moderate	0.58
		0.55			0.67			0.62
	high	0.81		high	0.85		high	0.77
		0.86			0.90			0.86

- Digital PCR quantification of bacterial targets in environmental samples

Quantification of indicator ASVs in environmental DNA extracts was performed using assay-specific conditions determined during optimization. Environmental samples were analyzed in three technical replicates, and non-template controls were included in all runs to monitor contamination. DNA template concentrations were standardized to a concentration of 1 ng μL^{-1}, quantified using a Quantus Fluorometer (Promega, USA), to ensure consistent DNA input across reactions and minimize variability associated with sediment-derived DNA extracts. Reactions were prepared in a final volume of 40 µL per reaction, containing QIAcuity EvaGreen Mastermix (13.3 µL), ASV-specific primers (0.4 µM final concentration), template DNA (1 µL at 1 ng μL^{-1}), and nuclease-free water to volume. Nanoplates were then sealed and run on the QIAcuity One platform with thermal cycling conditions consisted of an initial enzyme activation step at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 58.5 °C for 15 s, a final extension step at 72 °C for 15 s and a final cool down for 5 min at 40 °C. Replicate consistency and partition clustering were evaluated during data processing to assess amplification performance and identify potential inhibition effects. After

amplification, the nanoplates were imaged in the green fluorescence channel, using an exposure time of 250 ms and a gain setting of 3 to detect the EvaGreen® signal.

Following amplification, positive and negative partitions were identified using the QIAcuity analysis software with automatic thresholding. Target concentrations were calculated using Poisson statistics via the platform's standard analysis pipeline. Results were expressed as gene copy numbers per reaction (corresponding to 1 ng input DNA) for subsequent comparison across samples and environmental quality classes.

Statistical analysis

dPCR technical replicates were averaged at the sample level prior to analysis. No failed reactions were observed. Replicate variability was assessed based on the dispersion of replicate copy number estimates, which was generally low across samples, indicating consistent amplification. No systematic outlier removal was applied prior to averaging. Target copy numbers, expressed as gene copies per reaction, were $\log_{10}$-transformed ($\log_{10}[x+1]$) to improve normality and variance homogeneity. Associations between ASV copy numbers and continuous IQI values were assessed using Spearman's rank correlation. To quantify the relationship between ASV abundance and environmental quality while accounting for site effects, linear models were fitted with log-transformed copy number as the response variable and IQI and farm included as fixed factors. To assess whether ASV-IQI relationships differed among farms, an interaction term (IQI × farm) was tested by comparing models with and without the interaction using an F-test (ANOVA). Differences across ordered environmental quality classes were assessed using the Jonckheere–Terpstra trend test (Jonckheere, 1954; Terpstra, 1952). Effect sizes across classes were summarised as fold-changes in median ASV copy numbers between low- and high-quality sediments. Where multiple ASVs were analyzed, *p*-values were adjusted using the Holm method (Holm, 1979) to account for multiple testing and to control the family-wise error rate due to the small number of targeted hypotheses tested.

All statistical analyses were conducted in R v.4.3.1 (R Core Team, 2023). Data processing and visualization were performed using the packages tidyverse (Wickham et al., 2019) and ggplot2 (Wickham et al., 2016). Spearman correlations and linear models were implemented using base R (stats package), and ordered trend analyses were conducted using the DescTools package (Signorell, 2025). P-value adjustments were performed using the Holm method implemented in stats.

5.2.4 Results

Analytical performance of dPCR assays (LOD, LOQ)

All three validated assays demonstrated reliable detection across a dynamic range, with limits of detection (LOD) ranging from 10.93 to 29.27 copies per reaction depending on the target (Table 3). Limits of quantification (LOQ), defined as the lowest concentration yielding quantification precision with a coefficient of variation (CV) ≤ 35%, ranged from 10.94 to 44.41 copies per reaction.

Table 3. Analytical performance characteristics of validated dPCR assays.

Target	LOD (copies/reaction)	LOQ (copies/reaction)	LOQ CV
***Sulfurovum* sp.**	10.93	44.41	0.21
***Cytophaga* sp.**	10.94	10.94	0.26
Desulfuromonadaceae	29.27	29.27	0.06

Quantification precision improved with increasing target concentration, with coefficients of variation (CV) decreasing sharply as concentrations increased beyond the LOQ and stabilizing at higher copy numbers (Fig. 1). For each assay, the LOQ defined the lower bound of reliable quantification, below which variability increased and detection became less consistent. Environmental samples yielding copy numbers below the assay-specific LOQ were therefore excluded from correlation analyses to ensure that statistical relationships were evaluated within the validated dynamic range. After LOQ-based filtering, all samples were retained for *Sulfurovum* sp. and *Cytophaga* sp. (n = 18), whereas four samples for Desulfuromonadaceae fell below the LOQ, resulting in a reduced sample size (n = 14) for this target. Within this range, all retained assays demonstrated stable and reproducible quantification across concentrations representative of field samples.

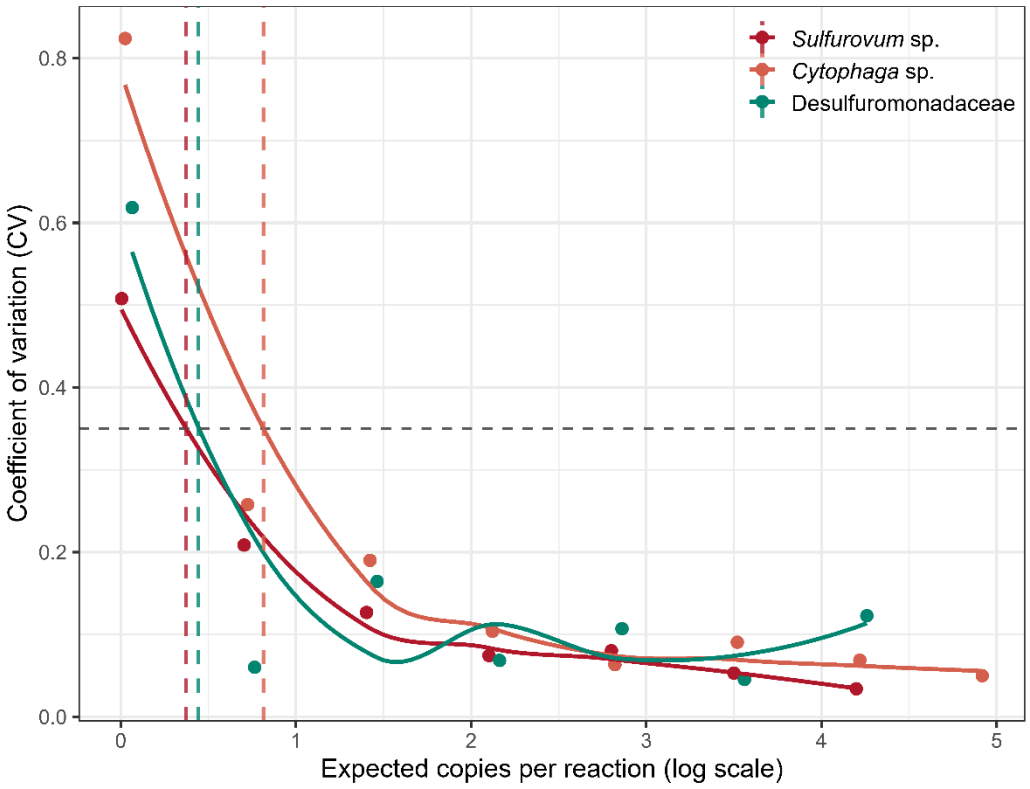


Fig. 1. Analytical precision of validated dPCR assays across dilution series. *The coefficient of variation (CV) is plotted against expected target copy numbers per reaction (log_{10} scale) for the three indicator ASVs. Points represent individual dilution levels (mean of technical replicates), and solid lines indicate fitted smoothing curves. The horizontal dashed line marks the predefined LOQ threshold (CV = 0.35). Vertical dashed lines indicate the concentration corresponding to the determined LOQ for each assay.*

Quantification of ASV copy numbers in environmental samples

All three validated assays successfully quantified target ASVs across the majority of environmental sediment samples analyzed. Following exclusion of measurements below assay-specific LOQ thresholds, copy numbers remained within the validated dynamic range for nearly all samples. Copy numbers spanned several orders of magnitude, ranging from 61.9 to 16,272.6 copies per reaction for *Sulfurovum* sp., 94.9 to 13,247.5 copies per reaction for *Cytophaga* sp., and 3.4 to 2,682.6 copies per reaction for Desulfuromonadaceae.

Distinct abundance patterns were observed across EQ classes. *Sulfurovum* sp. copy numbers were highest in low-quality sediments (median: 6554 copies per reaction, range: 2,671-16,273) and decreased progressively toward moderate (1,365; 554-2,509) and high-

quality conditions (185; 62-318). A similar trend was observed for *Cytophaga* sp., with median copy numbers declining from 5,250 copies per reaction (range: 1,662-13,248) in low-quality sediments to 886 (327-1,205) and 164 (94.9-292) in moderate and high-quality sediments, respectively. In contrast, the reference-associated Desulfuromonadaceae exhibited the opposite pattern, with the lowest copy numbers observed in impacted sediments (median: 12.7; range: 3.41-139), increasing to 654 (33.1-1,144) in moderate conditions and reaching the highest abundance in high-quality sediments (median: 2,082; range: 464-2,683). Copy number distributions across EQ classes are shown on a $\log_{10}$ scale in Fig. 2 to facilitate visualization across the observed dynamic range.

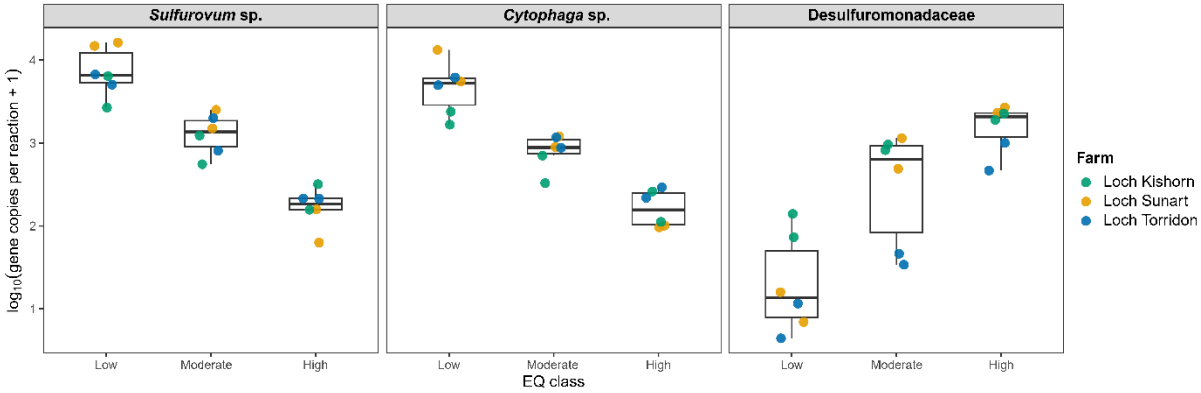


Fig. 2. Distribution of $\log_{10}$-transformed ASV copy numbers across environmental quality classes (low, moderate, high EQ). *Points represent individual samples coloured by farm. Boxplots summarize the median and interquartile range. These patterns correspond to significant monotonic trends detected by Jonckheere-Terpstra tests.*

Distinct effect sizes were observed across EQ classes. Median copy numbers of *Sulfurovum* sp. were approximately 35-fold higher in low-quality sediments compared to high-quality sediments (6,554 vs 185 copies per reaction). Similarly, *Cytophaga* sp. exhibited a ~32-fold increase under low-IQI conditions (5,250 vs 164 copies). In contrast, Desulfuromonadaceae displayed the opposite pattern, with median abundance approximately 160-fold higher in high-quality sediments relative to impacted sites (2,082 vs 12.7 copies).

Relationship between ASV abundance and environmental quality

Absolute quantification of indicator ASVs using dPCR revealed strong and highly significant relationships with IQI values across all study sites (Fig. 3). Using all samples (n =

18 per ASV), Spearman's rank correlations showed significant monotonic relationships for all three targets after Holm correction (*Sulfurovum* sp.: ρ = -0.920, p_{Holm} < 0001; *Cytophaga* sp.: ρ = -0.930, p_{Holm} < 0001; Desulfuromonadaceae: ρ = 0.874, p_{Holm} < 0001). To evaluate whether these relationships were influenced by measurements near the quantification limit, correlations were repeated after excluding environmental measurements below the assay-specific LOQ. LOQ filtering did not materially change the strength of the enrichment-associated relationships (*Sulfurovum* sp.: ρ = -0.920, p_{Holm} < 0.0001; *Cytophaga* sp.: ρ = -0.930, p_{Holm} < 0.001; n = 18 for both). For Desulfuromonadaceae, the positive association with IQI remained significant but was reduced in strength after LOQ filtering (ρ = 0.758, p_{Holm} = 0.001; n = 14).

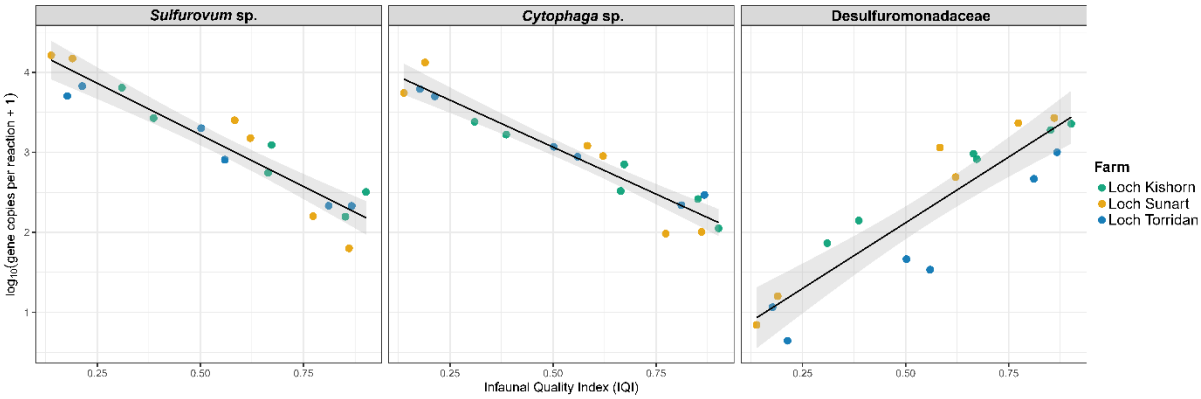


Fig. 3. Relationship between log_{10}-transformed ASV copy number and IQI values. *Points represent individual sediment samples coloured by farm. All quantified samples are shown for visualization. Lines indicate linear regression fits with 95% confidence intervals.*

Linear models fitted to log-transformed copy numbers corroborated these patterns. IQI explained a large proportion of variance in ASV abundance (R^2 = 0.899-0.945). *Sulfurovum* sp. and *Cytophaga* sp. exhibited significant negative slopes with increasing IQI (β = -2.629 and -2.337; $p_{Holm} = 2.66 \times 10^{-8}$ and 1.24×10^{-8}, respectively), while Desulfuromonadaceae showed a significant positive slope (β = 3.160; $p_{Holm} = 1.66 \times 10^{-9}$). Including farm as a fixed factor did not materially alter model fit, and IQI × farm interaction terms were not significant for any ASV ($p_{Holm} \geq 0.392$), indicating no detectable site-specific differences in slope.

Consistent with these patterns, ASV copy numbers differed significantly across ordered EQ classes. Jonckheere-Terpstra tests identified significant ordered trends for all three ASVs ($p_{Holm} \leq 0.001$), confirming monotonic relationships between ASV abundance and EQ class.

5.2.5 Discussion

Ecological plausibility of enrichment- and reference-associated bacterial ASVs

Organic enrichment beneath salmon cages does not simply increase microbial biomass (Dowle et al., 2015); it reorganizes benthic communities along a predictable biogeochemical trajectory. Sustained deposition of organic matter intensifies microbial respiration, progressively depletes oxygen, restructures electron acceptor availability, and drives sediments from oxic through suboxic to sulfidic states (Holmer et al., 2005; Frühe et al., 2021b; Johansen et al., 2024). This redox succession reorganizes microbial functional guilds, favouring taxa adapted to specific positions along the electron acceptor cascade (Dowle et al., 2015; Vasquez-Cardenas et al., 2022). The ASVs validated here occupy distinct niches along this continuum, providing a mechanistic explanation for their indicator behaviour across the EQ gradient.

The bacterial ASV affiliated with the genus *Sulfurovum* (Campylobacterota, formerly Epsilonproteobacteria; Waite et al., 2017) showed a clear enrichment under low IQI conditions and declined in relative abundance toward stations with higher EQ. Members of *Sulfurovum* are chemolithoautotrophic sulfur-oxidizing bacteria (Pettersen et al., 2022), capable of oxidizing reduced sulfur compounds such as sulfide and thiosulfate using oxygen or nitrate as terminal electron acceptors (Hamilton et al., 2014; Aranda et al., 2015). Consistent with this ecology, *Sulfurovum* and related Sulfurovaceae have been reported to proliferate beneath finfish aquaculture installations and in other organic-rich coastal environments characterized by high organic loading and the accumulation of reduced sulfur species (Aranda et al., 2015; Choi et al., 2018, 2022; Pettersen et al., 2022). Their association with dynamic redox interfaces and coupled iron-sulfur cycling makes them characteristic inhabitants of enrichment-driven geochemical gradients (Choi et al., 2022). Consistent with these ecological expectations, Sulfurovaceae emerged as enrichment-associated indicators across nearly all metagenomic and metabarcoding datasets examined by Rubel et al. (2025), underscoring the robustness of this taxonomic group as a signal of organic impact. These observations indicate that the enrichment-associated response of *Sulfurovum* sp. under low IQI conditions is ecologically coherent with elevated organic inputs, enhanced anaerobic mineralization, and increased sulfide production in impacted farm sediments.

Similarly, the ASV assigned to the genus *Cytophaga* exhibited markedly higher copy numbers in low-quality sediments and declined toward stations with higher ecological status. Members of *Cytophaga* are heterotrophic degraders of dissolved and particulate organic matter,

including polymers such as chitin and proteins (Cottrell and Kirchman, 2000; Wörner and Pester, 2019). Their enrichment in organically loaded sediments beneath fish farms and in eutrophic coastal systems is well documented (Vezzulli et al., 2002; Danovaro et al., 2003; Bissett, 2004; Kawahara et al., 2009; Verhoeven et al., 2018). Their proliferation under low IQI conditions is therefore consistent with the increased availability of labile organic substrates derived from uneaten feed and faecal deposition beneath aquaculture installations, which are known to stimulate heterotrophic microbial activity in enriched sediments (Hargrave et al., 2008; Holmer et al., 2005). Together with previous observations of *Cytophaga* enrichment under finfish aquaculture, the consistent negative relationship between *Cytophaga* sp. copy number and EQ across farms supports its suitability as an enrichment-responsive bacterial indicator.

In contrast, the bacterial ASV affiliated with the family Desulfuromonadaceae exhibited higher abundance in sediments classified as high EQ and declined toward more impacted, low-IQI stations. Members of this family are commonly associated with iron- and sulfur-reducing metabolisms operating under suboxic to anoxic conditions, and their distribution is strongly structured by the availability of alternative electron acceptors (Guo et al., 2021; Soares et al., 2024). Experimental and field studies indicate that such taxa often occupy intermediate redox niches preceding the establishment of fully sulfidic conditions dominated by complete sulfate reducers (Cord-Ruwisch et al., 1988; Nguyen-tiêt et al., 2025).

Within aquaculture enrichment gradients, reference sediments may maintain more extended suboxic zones that support iron- and sulfur-reducing guilds, whereas cage-proximal sediments subjected to sustained organic loading tend to progress toward sulfide-dominated geochemistry. Under such conditions, classical sulfate reducers often become dominant, potentially constraining Desulfuromonadaceae populations. The positive association between Desulfuromonadaceae and IQI observed here is therefore consistent with redox stratification linked to organic loading intensity, rather than disturbance per se. This context-dependent behaviour helps reconcile contrasting reports in the literature, where Desulfuromonadaceae have been described as enriched in undisturbed sediments in some systems (Dowle et al., 2015; Aylagas et al., 2017; Frühe et al., 2021b), yet also detected in organically impacted environments elsewhere (Elsabé et al., 2012; Aranda et al., 2015). Collectively, these findings suggest that Desulfuromonadaceae should be interpreted cautiously as reference-associated indicators, as their directionality appears contingent upon local sediment biogeochemistry and redox zonation.

Based on the established ecological properties of the here evaluated three indicator ASVs, and on their previously reported distribution in near- and far-field sediments associated with salmon aquaculture operations, it can be concluded that the absolute 16S rRNA gene abundances quantified using our tailored dPCR assays are entirely consistent with the anticipated relative abundance distributions of these indicators. These results confirm that the observed quantitative signals align with known ecological behaviors, thereby validating (i) the dPCR assays, and, (ii) the use of these ASVs as robust indicators of organic enrichment in benthic environments.

From relative signals to quantitative ecological indicators

Given that these ASVs occupy defined ecological positions along a redox continuum, their indicator value should manifest as absolute population shifts detectable beyond proportional sequencing signals. Although high-throughput sequencing has fundamentally reshaped aquaculture biomonitoring (e.g., Dowle et al., 2015; Keeley et al., 2018; Laroche et al., 2018; Stoeck et al., 2018; Cordier, 2020; Wilding et al., 2023; Oladi et al., 2024; Wyness et al., 2026), with metabarcoding and metagenomic-derived ASVs repeatedly emerging as strong predictors of EQ (Verhoeven et al., 2018; Frühe et al., 2021a; Rubel et al., 2025), such associations remain inherently compositional (Gloor et al., 2017). By applying dPCR to sequence-derived candidates, the present study provides direct evidence that these sequence-based signals correspond to quantitative changes in bacterial abundance. The enrichment-associated taxa declined in absolute copy number with improving sediment quality, whereas the reference-associated ASV increased toward higher IQI conditions. These monotonic relationships were strong across the full gradient and explained a substantial proportion of variance in sediment condition. Crucially, the direction and magnitude of these responses were consistent among farms, indicating that the observed patterns are not artefacts of site-specific community structure but reflect broader ecological processes associated with organic enrichment.

Absolute quantification strengthens the ecological interpretation of bacterial indicators. It confirms that predictive ASVs identified in metabarcoding and metagenomic frameworks (Rubel et al., 2025) are not merely statistical correlates shaped by relative abundance constraints, but represent populations that expand or contract in response to enrichment-driven biogeochemical succession. dPCR does not replace sequencing as a discovery platform; rather, it operationalizes sequence-derived candidates into standardized, transferable measurements

that are independent of library size variation and amplification bias (Borchardt et al., 2021). In doing so, it provides a direct bridge between ecological understanding and regulatory application.

Ecological context and methodological considerations

Although the quantitative relationships observed here were consistent across farms, bacterial indicators are inherently embedded within sediment biogeochemistry. Organic enrichment does not produce a uniform endpoint; rather, it drives systems along trajectories shaped by hydrodynamics, sediment grain size, oxygen penetration depth, and production phase (Robinson et al., 2016; Shi et al., 2021; Oladi et al., under review). Indicator taxa therefore respond not only to enrichment intensity but also to the spatial configuration of redox zones (Robinson et al., 2016; Shi et al., 2021). This context dependency is particularly evident for metabolically versatile groups such as Desulfuromonadaceae, whose capacity for extracellular electron transfer and use of insoluble electron acceptors allows occupation of multiple redox niches (Guo et al., 2021; Soares et al., 2024). Absolute quantification clarifies population-level responses but does not eliminate the need to interpret indicators within their geochemical setting.

Beyond ecological variability, several methodological considerations warrant attention. dPCR quantifies gene copy numbers rather than cell counts, and copy number variation among genomes can introduce proportional differences that are independent of biomass. In addition, extracellular DNA persistence and variable extraction efficiency may influence measured concentrations, particularly in fine-grained or organic-rich sediments (Corinaldesi et al., 2005). Although standardized DNA input and dilution-based assay validation reduce analytical variability, they cannot fully resolve these sources of uncertainty. Quantitative bacterial indicators should therefore be interpreted as robust ecological proxies rather than direct measures of microbial biomass.

In addition to these methodological considerations, the scope of the present study should also be considered when interpreting the broader applicability of the results. The study focused on a relatively small number of target ASVs and samples collected from multiple farms within a single geographic region, allowing robust proof-of-concept validation under a consistent environmental framework, but further evaluation across broader environmental settings and production systems will be important to assess generality. While the selected

indicators demonstrated consistent quantitative relationships with ecological quality, their ecological interpretation remains context dependent and linked to local sediment biogeochemistry and redox structure. Furthermore, because dPCR quantifies gene copy numbers rather than direct microbial biomass, quantitative shifts should be interpreted as ecological proxies of enrichment-associated community restructuring. Finally, the present study represents a cross-sectional assessment, and future time-series analyses spanning production cycles and fallowing periods will be essential to evaluate the temporal stability and predictive robustness of these indicators under dynamic aquaculture conditions.

5.2.6 Conclusion

By moving from relative sequence signals to absolute quantification, this study demonstrates that selected bacterial ASVs function as measurable ecological indicators of organic enrichment in salmon farm sediments. The validated targets exhibited consistent, gradient-aligned abundance responses across independent farms, indicating that sequencing-derived indicators reflect genuine population shifts associated with redox restructuring of organically enriched sediments rather than purely compositional artefacts. In doing so, this work establishes a practical bridge between high-resolution microbial community profiling and operational biomonitoring, showing that targeted dPCR assays can translate ecological knowledge into standardized, transferable measurements of benthic condition under aquaculture influence.

Beyond proof of concept, these findings provide a scalable foundation for expanding indicator panels and integrating quantitative bacterial metrics into structured aquaculture impact assessment workflows. With continued validation across environmental settings and production cycles, such approaches offer a realistic pathway toward rapid, cost-efficient evaluation of benthic conditions that remains grounded in ecological process. In this context, quantitative bacterial indicators have the potential to contribute to strengthening marine environmental monitoring frameworks for aquaculture-affected sediments.

Author contributions: CRediT

Mahshid Oladi: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review and editing; Thorsten Stoeck:

Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Resources, Validation, Writing – review and editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data are provided in the manuscript and in the Supplementary Online Material.

5.2.7 References

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SYNTHESIS AND OUTLOOK

6.1 Building the scientific foundation for bacterial biomonitoring

Environmental biomonitoring is entering a period of fundamental transitions. For several decades, assessments of ecological condition have depended largely on the collection, sorting, and morphological identification of organisms (e.g., Pereira, 1997; Mirto et al., 2012). These approaches have provided the foundations of modern environmental regulation, but they are labor-intensive, dependent on increasingly scarce taxonomic expertise, and often too slow to support responsive environmental management (Pawlowski et al., 2018). Molecular methods have begun to change this pathway. By recovering ecological information directly from environmental DNA, metabarcoding has created the possibility of assessing entire biological communities rapidly, reproducibly, and at scales that would be difficult to achieve using conventional taxonomy alone (Baird and Hajibabaei, 2012).

This transition is no longer limited to methodological demonstrations. Across aquatic biomonitoring, molecular information is being translated into ecological-quality assessments through several parallel strategies. Taxonomy-based approaches seek to reconstruct established biotic indices from DNA-derived community data (Borja et al., 2024), while de novo approaches use molecular assemblages as independent indicators of environmental condition (e.g., Keeley et al., 2018; Stoeck et al., 2018). Supervised machine-learning models provide a third route by learning the relationship between eDNA community profiles and existing regulatory metrics (Wyness et al., 2026). Together, these developments signal a shift from asking whether molecular methods can detect ecological change to determining how they can be calibrated, validated, and incorporated into established assessment systems. Molecular and morphological benthic indices can already produce comparable classifications, but further calibration is needed to ensure that they assign samples to equivalent ecological status classes (Borja et al., 2024).

Marine aquaculture, and particularly salmon farming in Scotland, is at the forefront of this transition. In 2023, the Scottish Environment Protection Agency (SEPA) introduced guidance allowing standardized bacterial DNA analyses to contribute directly to compliance assessment of seabed mixing-zone limits (SEPA, 2023). Under this approach, bacterial community data are converted into predicted infaunal quality index values using a machine-learning algorithm trained against paired macrofaunal assessments. This represents an important departure from the conventional role of eDNA as an experimental or supplementary method; under defined conditions, molecular data can now substitute for invertebrate analyses

in a regulatory assessment pathway. Nevertheless, the guidance remains deliberately precautionary, and conventional macrofaunal assessments are still recommended in case predicted IQI values fall close to the regulatory compliance threshold.

The development of eDNA2IQI has advanced this pathway further by integrating standardized sample collection, bacterial metabarcoding, automated bioinformatic processing, and random-forest prediction within a single reproducible framework (Wyness et al., 2026). Trained using 745 paired eDNA and macrofaunal samples collected from 75 Scottish aquaculture sites, the model reproduced conventional IQI patterns across diverse geographic and environmental settings and performed strongly when tested on complete farm surveys. Its implementation as an accessible R package demonstrates that one of the principal barriers to molecular biomonitoring, which is the conversion of complex sequence data into an interpretable regulatory output, can be substantially reduced through workflow standardization and automation. However, Wyness et al. (2026) also demonstrated systematic overestimation at low IQI values and underestimation at high IQI values, together with weaker discrimination between adjacent ecological classes at the extremes of the gradient. These findings highlight that, despite substantial progress towards operational implementation, further calibration remains necessary before molecular approaches can be considered fully equivalent to conventional benthic assessment.

The transition toward molecular monitoring is therefore already underway, but it is not yet complete. The central challenge is no longer simply to demonstrate that bacterial communities respond to aquaculture-derived organic enrichment, nor is it only to show that an algorithm can reproduce a conventional ecological-quality score. The deeper challenge is to demonstrate why molecular assessments are reliable, under which methodological and ecological conditions they remain reliable, which biological signals underpin their predictions, and how those signals can be validated independently of the models through which they were discovered. Without this foundation, molecular monitoring risks functioning as a highly accurate predictive system whose ecological mechanisms remain only partially resolved.

The central contribution of this thesis is therefore not another demonstration that bacterial metabarcoding has potential for aquaculture monitoring. That potential has already been convincingly established, and in Scotland it has begun to enter regulatory practice. Instead, this work addresses the scientific foundation needed to understand why molecular biomonitoring works, when it can be trusted, and how sequencing-derived ecological signals

can be translated into dependable monitoring tools. As illustrated in Figure 17, the progression presented throughout this thesis reflects its central theme, *"moving bacterial biomonitoring from promise to practice"*.

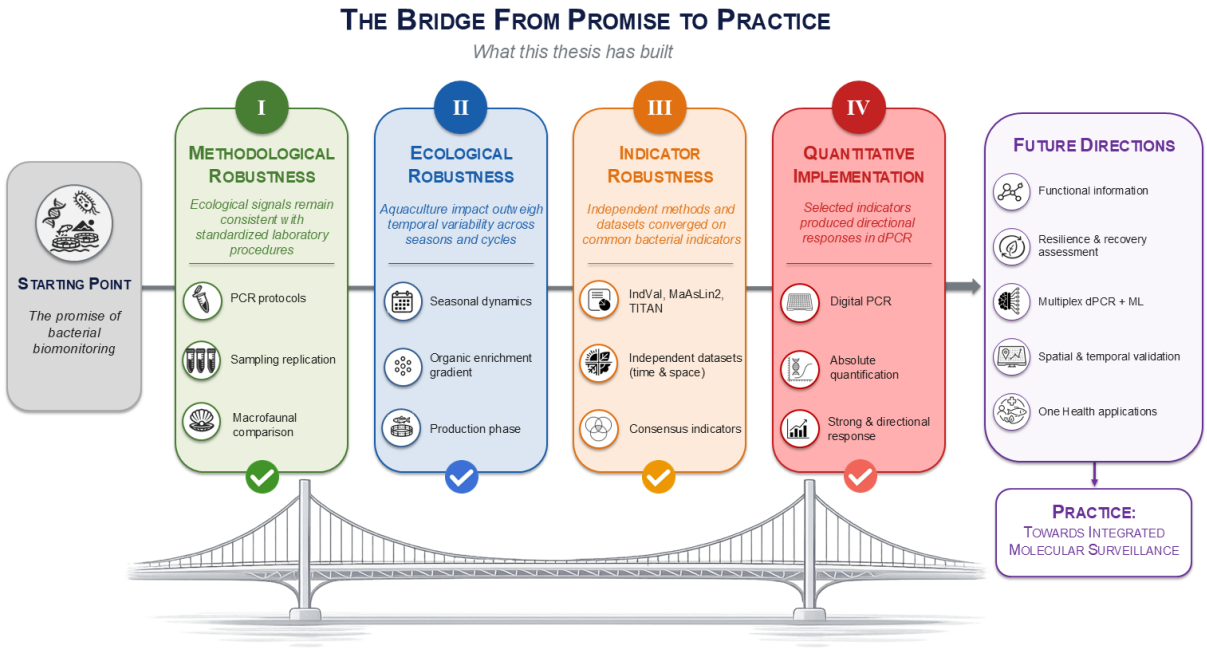


Figure 17. Conceptual overview of the scientific progression presented in this thesis. *The framework illustrates the transition from the current state of bacterial biomonitoring towards future routine molecular environmental surveillance. The thesis addresses four key components required to bridge this transition: methodological robustness, ecological robustness, reproducible indicator selection, and targeted molecular quantification. Together, these advances establish the foundation for future developments including functional interpretation, predictive ecosystem assessment, multiplex molecular assays, and integrated molecular surveillance.*

6.2 *Outcomes of this thesis*

The development of bacterial biomonitoring has progressed rapidly over the last decade. High-throughput sequencing has demonstrated that bacterial communities respond sensitively to environmental disturbance (e.g., Stoeck et al., 2018; Frühe et al., 2021b), while recent machine-learning approaches have shown that these responses can predict ecological quality with remarkable accuracy (Wyness et al., 2026). Nevertheless, reliable implementation requires considerably more than predictive performance alone. Effective environmental monitoring depends not only on the ability to detect ecological change, but also on maximizing the impact-related signal relative to unrelated methodological and ecological noise, thereby

ensuring that observed patterns are both reproducible and biologically meaningful (Wilding et al., 2023). Consistent with this view, early reviews of eDNA monitoring emphasized that successful implementation would require a better understanding of methodological uncertainty, natural temporal variability, and the reproducibility of molecular workflows before these approaches could be confidently adopted for environmental monitoring (Mathieu et al., 2020). The chapters presented in this thesis directly address several of these challenges by evaluating methodological robustness, quantifying temporal variability under operational monitoring conditions, identifying reproducible bacterial indicators, and demonstrating the feasibility of translating these indicators into quantitative molecular assays.

A fundamental requirement of any environmental monitoring approach is analytical robustness, meaning that methodological variation should remain smaller than the ecological signal of interest (Mathieu et al., 2020). This thesis demonstrated that achieving this robustness depends on standardized laboratory workflows. Although alternative PCR amplification protocols produced measurable differences in bacterial diversity estimates and influenced community composition, the study identified the need for a consistent PCR protocol to ensure comparable biomonitoring results across laboratories. In contrast, sampling-related variability in bacterial eDNA was comparable to that observed for conventional macrofaunal monitoring, indicating that the molecular approach does not introduce greater background uncertainty than the current regulatory standard. Together, these findings demonstrate that, when standardized laboratory procedures are applied, bacterial metabarcoding provides reproducible ecological assessments suitable for routine environmental monitoring.

Analytical robustness alone, however, is not sufficient for environmental monitoring. A method that performs well under laboratory conditions must also remain reliable under the natural variability characteristic of marine ecosystems (Wilding et al., 2023). This is particularly relevant for aquaculture routine biomonitoring. Existing regulatory programmes typically conduct environmental surveys at defined stages of the production cycle, particularly around peak biomass regardless of the season. Consequently, uncertainty regarding the influence of natural temporal variability has raised concerns about whether bacterial biomonitoring would require dedicated, multi-season sampling, potentially limiting its integration into existing monitoring frameworks. The results presented here suggest that such additional sampling requirements are unlikely to be necessary. By showing that bacterial community responses to organic enrichment remain consistent across production cycles and seasons, the work provides evidence that molecular monitoring can be integrated into existing

monitoring schedules without requiring additional sampling campaigns. Consequently, bacterial biomonitoring has the potential to be implemented within current operational frameworks rather than replacing them with new sampling strategies. These findings reduce one of the principal sources of uncertainty associated with bacterial metabarcoding and provide confidence that standardized molecular workflows can support reproducible environmental assessments suitable for regulatory biomonitoring.

Another important barrier to the adoption of bacterial biomonitoring is the uncertainty surrounding the reproducibility of indicator selection. Candidate indicators often depend on the analytical approach used or the characteristics of individual datasets, limiting confidence in their broader applicability (Nearing et al., 2022). By integrating complementary statistical approaches and evaluating indicator consistency across independent datasets, this thesis demonstrates that robust bacterial indicators can be identified despite differences in methodology and study system. This provides greater confidence that indicator selection can move beyond dataset-specific observations towards reproducible bioindicators suitable for routine monitoring and future assay development.

While high-throughput sequencing provides powerful opportunities for indicator discovery, routine monitoring requires practical methods for quantifying those indicators. By translating sequencing-derived bacterial indicators into dPCR assays, this thesis demonstrates a pathway from exploratory metabarcoding towards targeted molecular monitoring. Although further validation is required before widespread implementation, this proof-of-concept illustrates how bacterial indicators identified through metabarcoding can be converted into rapid, quantitative assays suitable for future environmental monitoring applications.

Taken together, the contributions of this thesis extend beyond individual methodological advances and the identification of bacterial indicator taxa. Instead, they provide complementary scientific foundations for the development of an integrated molecular biomonitoring platform for aquaculture. By addressing methodological robustness, ecological interpretation, reproducible indicator discovery, and quantitative validation, this work establishes several of the essential components required for translating bacterial metabarcoding from a research tool into an operational monitoring technology. Importantly, these components should not be viewed as independent developments, but as interconnected stages in the evolution of molecular biomonitoring. Reliable implementation depends not only on accurate prediction of ecological status, but also on the confidence that molecular signals are

analytically robust, ecologically meaningful, reproducible across datasets, and amenable to standardized quantitative measurement. Together, these attributes provide the scientific basis upon which future monitoring platforms can be developed and rigorously evaluated. Rather than representing the endpoint of bacterial biomonitoring development, this thesis provides an evidence-based foundation for its next phase: the integration of validated molecular indicators into practical, cost- and time-efficient tools capable of supporting routine environmental assessment and regulatory decision-making in marine aquaculture.

6.3 Advancing towards routine biomonitoring

The work presented in this thesis demonstrates that bacterial metabarcoding has progressed beyond proof-of-concept towards a practical monitoring approach. Methodological robustness, ecological reliability, reproducible indicator discovery, and quantitative validation as a whole address several of the major scientific barriers that have historically limited the implementation of molecular biomonitoring (Mathieu et al., 2020). Nevertheless, successful adoption within regulatory monitoring programmes requires additional developments extending beyond the identification of responsive bacterial communities.

A first priority is the establishment of standardized operating procedures suitable for regulatory implementation. Over the past decade, substantial progress has been made towards standardizing individual components of the bacterial metabarcoding workflow, including sampling strategy, sample preservation, DNA extraction, PCR amplification, bioinformatic processing and interlaboratory validation (e.g., Dully et al., 2021a, b, c; Hestetun et al., 2021; Ruan et al., 2022; Pawlowski et al., 2022; Ashley-Wheeler et al., 2026). Together with previous efforts to standardize individual components of the metabarcoding workflow, the work presented in this thesis provides additional evidence that methodological uncertainty can be sufficiently constrained to allow robust ecological interpretation. Nevertheless, the remaining challenge is therefore to a lesser extend the optimization of individual methodological steps, and to a greater extend their integration into harmonized regulatory frameworks. This will require internationally accepted protocols and quality standards to ensure comparable ecological assessments across laboratories and regulatory programmes.

A second priority is the continued validation of bacterial indicators across broader environmental settings. Although several indicator taxa were consistently identified across independent datasets and analytical approaches, both datasets originated from temperate

Scottish salmon aquaculture systems. Consequently, further validation across different geographic regions, climatic zones, sediment types, hydrodynamic conditions and aquaculture systems will be important to determine the extent to which these bacterial indicators represent broadly transferable responses to organic enrichment or region-specific ecological associations (Hargrave, 2010). It has been shown that ecosystem characteristics, local environmental conditions and farm management practices can strongly influence ecological responses to organic enrichment, highlighting the importance of evaluating indicators across contrasting monitoring environments (Macleod et al., 2004). Furthermore, while the relatively weak seasonal influence observed in this thesis is consistent with the integrative nature of sediment microbial communities used in routine benthic biomonitoring, it remains unclear whether the balance between seasonal variability and organic enrichment is maintained under different climatic regimes, where stronger fluctuations in temperature, hydrology and primary production may exert a greater influence on microbial community dynamics (Jiang et al., 2024). Together, such studies will help define the environmental boundaries within which bacterial indicators can be applied with confidence and establish their broader applicability for routine biomonitoring.

A third priority is the development of integrated molecular monitoring frameworks that balance the comprehensive information provided by high-throughput sequencing with the practical requirements of routine monitoring. While metabarcoding will remain indispensable for discovering and periodically refining bacterial indicators, routine regulatory assessments may not always require characterization of complete microbial communities. Instead, future monitoring frameworks are likely to combine complementary molecular approaches, with high-throughput sequencing supporting indicator discovery and targeted methods such as dPCR enabling rapid quantification of validated indicator taxa. Such integrated indicator frameworks could simplify laboratory workflows while retaining the ecological information most relevant for environmental assessment (Astudillo-García et al., 2019; Wang et al., 2025). The dPCR assays developed in this thesis illustrate one possible pathway towards this transition. However, expanding the number of validated assays, evaluating multiplex detection strategies, and assessing long-term analytical performance under routine laboratory conditions will be important next steps before widespread implementation can be considered.

Finally, successful implementation will require not only robust laboratory methods but also transparent frameworks for interpreting molecular data within regulatory decision-making. Recent advances in machine-learning models capable of predicting ecological quality from

bacterial community composition illustrate the considerable potential of automated data interpretation (Wyness et al., 2026). Future monitoring frameworks are likely to integrate standardized laboratory workflows, validated bacterial indicators, targeted molecular assays and predictive analytical tools within unified decision-support systems. More broadly, environmental monitoring is increasingly moving towards integrated indicator frameworks that combine complementary molecular, biological, chemical and physical indicators to provide a more comprehensive assessment of ecosystem condition and support ecosystem-based aquaculture management (Rector et al., 2022; Cuff et al., 2023). Importantly, these developments should be viewed as complementary to existing monitoring programmes, enabling molecular approaches to strengthen rather than replace established ecological assessments.

6.4 Future perspectives

6.4.1 From indicators to ecosystem processes

While the present thesis demonstrates that taxonomic bacterial indicators provide robust and reproducible assessments of benthic ecological condition, future developments in molecular biomonitoring are likely to place increasing emphasis on the ecological processes that these indicators represent. The bacterial taxa identified throughout this thesis were consistently associated with metabolic functions linked to organic enrichment, including sulfur oxidation, sulfate reduction and anaerobic organic matter degradation, suggesting that microbial indicators capture not only changes in community composition but also shifts in ecosystem functioning (Paerl et al., 2003). Future monitoring frameworks may therefore increasingly move beyond identifying individual taxa towards understanding the functional traits and biogeochemical processes that underpin ecological responses to environmental disturbance (Astudillo-García et al., 2019).

Recent advances in shotgun metagenomics and other multi-omics approaches provide new opportunities to strengthen this mechanistic understanding by linking taxonomic composition with functional genes, metabolic pathways and microbial activity (Mallick et al., 2021; Rubel et al., 2025). Although these approaches remain less practical than metabarcoding for routine regulatory monitoring because of their higher costs and analytical complexity, they offer powerful discovery platforms for identifying functionally informative biomarkers and improving the ecological interpretation of molecular indicators. Integrating taxonomic and

functional information may also improve the transferability of bacterial indicators across different environmental settings by focusing on conserved ecosystem functions rather than individual taxa, which often vary among regions and habitats. In this way, future molecular biomonitoring may evolve from describing microbial community composition towards directly assessing the ecological processes that regulate sediment health and ecosystem functioning.

6.4.2 Towards predictive biomonitoring

Conventional benthic monitoring primarily provides a snapshot of ecological condition at the time of sampling. However, the rapid turnover and high environmental responsiveness of microbial communities offer the potential to move beyond static assessments towards predictive biomonitoring capable of identifying ecological change before it becomes apparent through conventional indicators. Rather than simply classifying sediments as being in good or poor ecological condition, future monitoring frameworks may increasingly seek to evaluate ecological trajectories, resilience and recovery, providing managers with information not only on the current state of benthic ecosystems but also on their capacity to withstand or recover from environmental disturbance (Verhoeven et al., 2018).

The temporal analyses presented in this thesis demonstrate that bacterial communities respond consistently to changes in organic enrichment while remaining comparatively robust to seasonal variability under the temperate conditions investigated. These characteristics suggest that microbial indicators could be particularly well suited for monitoring ecological recovery during fallowing periods, evaluating the effectiveness of management interventions and identifying early deviations from expected recovery trajectories (Verhoeven et al., 2018). Similarly, bacterial community dynamics may provide valuable insights into ecosystem resilience by revealing whether sediments maintain stable functional states under increasing environmental pressure or approach ecological thresholds beyond which recovery becomes more difficult (Shade et al., 2012; Philippot et al., 2021).

The integration of long-term molecular monitoring with environmental metadata, production records and predictive analytical approaches may further enable the development of decision-support systems capable of forecasting ecological responses before environmental deterioration becomes established. Rather than reacting to declining ecological status, future biomonitoring programmes may increasingly support proactive management by identifying

early warning signals of ecosystem change and informing adaptive management strategies that reduce environmental impacts before regulatory thresholds are exceeded.

6.4.3 Integrated molecular environmental surveillance

As molecular technologies continue to mature, future biomonitoring programmes are likely to evolve beyond individual analytical methods towards integrated molecular surveillance frameworks capable of supporting routine environmental management (Rector et al., 2022). Rather than relying on a single monitoring approach, future systems may combine complementary molecular technologies, each fulfilling a distinct role within the monitoring workflow (Cuff et al., 2023). High-throughput sequencing is likely to remain indispensable for the discovery and periodic refinement of bacterial indicators, while targeted approaches such as multiplex dPCR offer a practical route for rapidly quantifying validated indicator panels during routine monitoring (Tiwari et al., 2022). Coupled with standardized laboratory workflows and transparent analytical pipelines, these complementary technologies have the potential to balance ecological resolution with the speed, reproducibility and cost-effectiveness required for regulatory implementation.

The growing availability of large molecular datasets also creates new opportunities for integrating predictive analytical approaches into environmental decision-making. Machine-learning models trained on validated ecological indicators may increasingly support the interpretation of complex microbial datasets, transforming molecular observations into transparent assessments of ecological quality, recovery potential and emerging environmental risk (Hino et al., 2018; Keck et al., 2023). Rather than replacing expert judgment, it can help translate complex molecular data into information that supports environmental management and regulatory decision-making. Together, standardized laboratory methods, targeted molecular assays, comprehensive sequencing datasets and predictive analytical frameworks may ultimately form integrated monitoring systems capable of continuously improving as new ecological knowledge becomes available.

Beyond benthic ecological assessment, the same molecular framework may ultimately support broader environmental surveillance within aquaculture. The principles established throughout this thesis, i.e. robust molecular workflows, reproducible indicator selection and targeted quantitative assays, could be extended to simultaneously monitor additional microbial indicators relevant to ecosystem and aquaculture health, including opportunistic pathogens,

antimicrobial resistance genes and other functionally important microbial groups (Bentzon-Tilia et al., 2016). Such integrated surveillance systems would provide a more holistic understanding of environmental condition while reducing the need for multiple independent monitoring programmes.

Ultimately, this progression reflects a broader transition in environmental biomonitoring, from describing ecosystem condition towards continuously monitoring ecosystem function, environmental health and biological risk within a unified molecular framework. By integrating ecological indicators, targeted molecular diagnostics and predictive analytical tools, future monitoring programmes have the potential to support not only sustainable aquaculture management but also broader "*One Health*" objectives, recognizing the close interdependence between environmental integrity, animal health and human well-being.

6.5 Conclusions

The promise of molecular biomonitoring has never been in doubt. It is now well-known that microbial communities respond rapidly and consistently to environmental disturbance, offering unprecedented opportunities to assess ecosystem health. However, translating this promise into practice has proven considerably more challenging. Before bacterial metabarcoding can become part of regulatory practice, confidence is needed that molecular measurements are robust to methodological choices, distinguish ecological change from natural variability, identify reproducible indicators and can be translated into practical monitoring tools. This thesis was designed to address these challenges, and the findings herein provide the scientific confidence required to move bacterial metabarcoding from an emerging research approach towards a practical biomonitoring tool for salmon aquaculture. This confidence is built upon four complementary pillars.

The first pillar of this confidence is analytical robustness, which was achieved through demonstrating that methodological variation can be effectively managed through standardized workflows, thereby providing an essential foundation for routine molecular biomonitoring. The second pillar is the ecological robustness, evidenced by showing that bacterial communities provide stable indicators of ecological condition despite natural temporal variability, while consistently reflecting management-related changes associated with organic enrichment. This is particularly intriguing, because it suggests that bacterial metabarcoding can provide reliable

ecological assessments under realistic operational monitoring conditions. The third pillar is the indicator reproducibility, supported by the strong agreement among analytical approaches and the recovery of highly similar indicator communities across datasets. This consensus-based strategy increases confidence that selected indicators reflect consistent ecological responses rather than methodological artefacts. The fourth pillar is the translation into practice, achieved through developing and validating targeted dPCR assays for selected indicator taxa. This combination of discovery-based sequencing with targeted molecular quantification represents an important step towards practical implementation, allowing validated indicators to be measured using workflows that are compatible with routine environmental assessment.

Taken together, the findings presented in this thesis demonstrate that bacterial metabarcoding has progressed beyond proof-of-concept towards a robust, reproducible and practically applicable framework for benthic biomonitoring in salmon aquaculture. By addressing the methodological, ecological and analytical requirements necessary for routine implementation, this work provides a comprehensive framework that bridges fundamental microbial ecology with applied environmental management. In doing so, it strengthens the scientific foundation for integrating molecular approaches into routine biomonitoring programmes and represents an important step towards the next generation of ecosystem-based aquaculture monitoring.

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SUMMARY

The expansion of salmon aquaculture has increased the need for reliable and efficient approaches to assess its environmental impacts. Routine compliance monitoring of salmon farms has traditionally relied on morphology-based assessment of benthic macrofaunal communities, which requires considerable taxonomic expertise, time, and financial resources. Advances in environmental DNA (eDNA) metabarcoding have demonstrated that sediment bacterial communities respond rapidly and consistently to aquaculture-derived organic enrichment, and molecular approaches are beginning to be incorporated into environmental monitoring frameworks. However, their application in routine regulatory assessment remains comparatively limited. Broader implementation requires confidence that bacterial indicators are analytically robust, remain reliable despite natural ecological variability, are reproducible across analytical approaches and datasets, and can ultimately be translated into practical monitoring tools. The overarching aim of this thesis was therefore to evaluate whether sediment bacterial communities can provide reliable measures of benthic ecological condition under the methodological and environmental variability encountered in salmon aquaculture monitoring, and whether robust bacterial indicators can ultimately be translated into practical quantitative tools. To address this aim, bacterial community responses were examined across methodological treatments, temporal and environmental gradients, complementary indicator-selection approaches, and independent datasets.

Methodological robustness was evaluated by comparing PCR amplification protocols and quantifying sampling-related variability relative to conventional macrofaunal monitoring. Temporal dynamics were investigated across two salmon production cycles to determine the relative influence of aquaculture impact and natural seasonal variability on bacterial community structure. Reproducible bacterial indicators were subsequently identified using three complementary analytical approaches and compared across two independent Scottish aquaculture datasets. Finally, selected indicators were developed into targeted digital PCR (dPCR) assays and quantified across samples representing different levels of benthic ecological quality.

The findings demonstrated that methodological standardization is a prerequisite for robust bacterial biomonitoring. Although alternative PCR amplification protocols produced substantial differences in bacterial diversity estimates, they yielded highly consistent ecological patterns across the organic enrichment gradient. Likewise, sampling-related variability in bacterial eDNA was comparable to that observed for conventional macrofaunal monitoring, indicating that the molecular approach did not introduce greater background variability than the established monitoring method. This consistency extended beyond methodological variation to temporal ecological dynamics. Across two salmon production cycles, organic enrichment remained the dominant driver of bacterial community structure

(59%), whereas temperature variations explained a considerably smaller proportion of community change (1%). Bacterial communities also reflected changes associated with farm management, particularly at intermediate impact levels.

Importantly, the ecological signal was reproducible at the level of individual indicators. The three indicator-selection approaches converged on highly similar sets of enrichment-responsive taxa within each dataset, and several indicators were recovered across both independent datasets. Selected indicators were successfully quantified using dPCR, and their absolute abundances followed the expected ecological quality gradients across independent salmon farm samples. These results demonstrate that sequencing-derived bacterial indicators can be converted into quantitative molecular targets.

Overall, this thesis advances bacterial biomonitoring beyond the demonstration of ecological potential towards the requirements of practical application. The findings show that bacterial responses to aquaculture-derived organic enrichment remain interpretable despite important sources of methodological and natural variability, that reproducible indicators can be identified across complementary analytical approaches and independent datasets, and that these indicators can be translated from sequencing data into quantitative molecular targets. Importantly, the contribution of this work lies not in proposing bacterial metabarcoding as a replacement for established benthic assessment, but in strengthening the scientific basis for its integration into routine environmental monitoring. By connecting methodological evaluation, ecological validation, reproducible indicator discovery, and targeted quantification within a single framework, this thesis helps bridge a gap that has persisted between microbial ecology and its practical application in environmental assessment. It therefore contributes both to the development of bacterial bioindicators as reliable indicators of benthic ecological condition and to building the scientific foundation and confidence required to advance molecular biomonitoring "*from promise to practice*" in salmon aquaculture.

ZUSAMMENFASSUNG

Die Ausweitung der Lachsaquakultur erhöht den Bedarf an zuverlässigen und effizienten Verfahren zur Bewertung ihrer Umweltauswirkungen. Die konventionelle Untersuchung benthischer Makrofaunagemeinschaften ist jedoch zeit- und kostenintensiv und erfordert umfangreiche taxonomische Expertise. eDNA-Metabarcoding hat gezeigt, dass bakterielle Sedimentgemeinschaften schnell und konsistent auf organische Anreicherung reagieren, seine routinemäßige Anwendung erfordert jedoch methodisch robuste, ökologisch zuverlässige und reproduzierbare Indikatoren. Ziel dieser Dissertation war daher zu untersuchen, ob bakterielle Gemeinschaften trotz methodischer und ökologischer Variabilität zuverlässige Informationen über den ökologischen Zustand des Benthos liefern und in praktische quantitative Monitoringinstrumente überführt werden können.

Die methodische Robustheit wurde durch den Vergleich verschiedener PCR-Amplifikationsprotokolle und die Quantifizierung der probenahmebedingten Variabilität im Vergleich zur konventionellen Makrofauna-basierten Überwachung untersucht. Die zeitliche Dynamik wurde über zwei Produktionszyklen hinweg analysiert, um den relativen Einfluss der Aquakulturbelastung und der natürlichen saisonalen Variabilität auf die Struktur der bakteriellen Gemeinschaften zu bestimmen. Anschließend wurden mithilfe dreier komplementärer analytischer Ansätze reproduzierbare bakterielle Indikatoren identifiziert und zwischen zwei unabhängigen schottischen Aquakulturdatensätzen verglichen. Abschließend wurden für ausgewählte Indikatoren spezifische Digital-PCR-Assays (dPCR-Assays) entwickelt und diese Indikatoren in Proben quantifiziert, die unterschiedliche Stufen des ökologischen Zustands des Benthos repräsentierten.

Die Ergebnisse zeigten, dass die methodische Standardisierung eine Voraussetzung für ein robustes bakterielles Biomonitoring ist. Obwohl unterschiedliche PCR-Amplifikationsprotokolle zu erheblichen Unterschieden in den Schätzungen der bakteriellen Diversität führten, ergaben sie entlang des Gradienten der organischen Anreicherung weitgehend übereinstimmende ökologische Muster. Auch die probenahmebedingte Variabilität der bakteriellen eDNA war mit derjenigen der konventionellen Makrofauna-basierten Überwachung vergleichbar. Dies zeigt, dass der molekulare Ansatz keine höhere Hintergrundvariabilität verursachte als das etablierte Monitoringverfahren. Diese Konsistenz zeigte sich über die methodische Variation hinaus auch in der zeitlichen ökologischen Dynamik. Über zwei Produktionszyklen hinweg blieb die organische Anreicherung der dominierende Einflussfaktor auf die Struktur der bakteriellen Gemeinschaften und erklärte 59 % der Variation, während Temperaturschwankungen einen deutlich geringeren Anteil der Veränderungen erklärten (1 %). Die bakteriellen Gemeinschaften spiegelten zudem Veränderungen infolge des Farmmanagements wider, insbesondere bei mittleren Belastungsstufen.

Von besonderer Bedeutung war, dass das ökologische Signal auch auf der Ebene einzelner Indikatoren reproduzierbar war. Die drei Verfahren zur Indikatorauswahl führten innerhalb jedes Datensatzes zu weitgehend übereinstimmenden Gruppen von Bakterientaxa, die auf organische Anreicherung reagierten, und mehrere Indikatoren wurden in beiden unabhängigen Datensätzen identifiziert. Ausgewählte Indikatoren konnten erfolgreich mittels dPCR quantifiziert werden, wobei ihre absoluten Häufigkeiten in unabhängigen Proben von Lachsfarmen die erwarteten Gradienten des ökologischen Zustands des Benthos widerspiegelten. Diese Ergebnisse zeigen, dass aus Sequenzierungsdaten abgeleitete bakterielle Indikatoren in quantitative molekulare Zielgrößen überführt werden können.

Insgesamt führt diese Dissertation das bakterielle Biomonitoring über den bloßen Nachweis seines ökologischen Potenzials hinaus und richtet den Blick auf die Anforderungen einer praktischen Anwendung. Die Ergebnisse zeigen, dass bakterielle Reaktionen auf die durch Aquakultur verursachte organische Anreicherung trotz wesentlicher methodischer und natürlicher Variabilität ökologisch interpretierbar bleiben, dass sich reproduzierbare Indikatoren mithilfe komplementärer analytischer Ansätze und unabhängiger Datensätze identifizieren lassen und dass diese Indikatoren aus Sequenzierungsdaten in quantitative molekulare Zielgrößen überführt werden können. Der Beitrag dieser Arbeit besteht dabei nicht darin, das bakterielle Metabarcoding als Ersatz für etablierte Verfahren zur Bewertung des Benthos vorzuschlagen, sondern darin, die wissenschaftliche Grundlage für seine Integration in die routinemäßige Umweltüberwachung zu stärken. Durch die Verknüpfung methodischer Überprüfung, ökologischer Validierung, reproduzierbarer Indikatoridentifizierung und gezielter Quantifizierung innerhalb eines gemeinsamen Rahmens trägt diese Dissertation dazu bei, die bestehende Lücke zwischen mikrobieller Ökologie und ihrer praktischen Anwendung in der Umweltbewertung zu schließen. Damit leistet sie sowohl einen Beitrag zur Entwicklung bakterieller Bioindikatoren als zuverlässige Indikatoren für den ökologischen Zustand des Benthos als auch zum Aufbau der wissenschaftlichen Grundlage und des notwendigen Vertrauens, um das molekulare Biomonitoring in der Lachsaquakultur „*vom Potenzial zur Praxis*“ weiterzuentwickeln.

APPENDIX

10.1 Declaration of Originality

Mahshid Oladi Kalarijani

From Promise to Practice: Advancing Bacterial Biomonitoring of Salmon Aquaculture

Hiermit erkläre ich wahrheitsgemäß, dass ich diese Dissertation eigenständig und ohne unzulässige Hilfe dritter angefertigt habe. Alle für die Arbeit benutzen Hilfsmittel und Hilfestellungen wurden sachgemäß angegeben und die Dissertation enthält eine detaillierte Darlegung des Eigenanteils. Die vorliegende Dissertation oder Teile hiervon wurden bisher nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung bei einem anderen Fachbereich oder einer anderen Hochschule eingereicht.

I hereby declare that I prepared this cumulative dissertation independently and without any unauthorized assistance from third parties. All resources and assistance used in preparing this dissertation have been properly acknowledged, and the dissertation contains a detailed account of my individual contributions. Neither this dissertation nor any part of it has previously been submitted as an examination paper for a state or other academic examination to another department or institution of higher education.

10.2 Statement of Contribution

Mahshid Oladi Kalarijani

From Promise to Practice: Advancing Bacterial Biomonitoring of Salmon Aquaculture

Chapter I

Publication: **Oladi, M.**, Wilding, T. A., Wyness, A., & Stoeck, T. (2024). Towards a Standardized Operating Procedure for eDNA-Based Biomonitoring in Coastal Marine Salmon Aquaculture. Environmental DNA, 6(5), e70014.

Statement: My contribution to this publication included the laboratory procedures, sequence quality control, bioinformatic processing, data curation, formal analyses, preparation of all figures, and drafting some parts and revising the manuscript.

Chapter II

Publication: **Oladi, M.**, Wyness, A., Wilding, T. A., Breiner H.W., & Stoeck, T. (2024). eDNA-Based Temporal Trajectories of Bacterial Communities in Salmon Farm Sediments: Disentangling Seasonal Variability from Aquaculture Impacts. Environmental DNA, 8(4), e70339.

Statement: My contribution to this publication includes participation in one sampling campaign, laboratory work conducted with assistance from a coauthor (H.W. Breiner), downstream analyses including sequence quality control, bioinformatic processing, data curation, and formal analyses, preparation of all figures, and drafting and revising the manuscript.

Chapter III *(unpublished)*

Statement: I independently developed the chapter concept, selected and implemented the methodology, conducted all formal analyses, validated the results, prepared all figures, and wrote the chapter.

Chapter IV

Publication: **Oladi, M.**, & Stoeck, T. (2026). Digital PCR Assays Quantify Bacterial Bioindicators Across Environmental Quality Gradients in Salmon Aquaculture Sediments. Aquaculture, 623, 744179.

Statement: My contribution to this publication includes all laboratory procedures, data curation, formal analyses, generating all figures and writing the original draft and reviewing the manuscript.

10.3 Declaration of Used Tools and Assistance

Mahshid Oladi Kalarijani

From Promise to Practice: Advancing Bacterial Biomonitoring of Salmon Aquaculture

Software and devices used for processing the data, conducting statistical analyses, and generating the figures are indicated in each chapter and respective publications. Furthermore, the following software was used for this work:

Microsoft Excel 365 Version 2606 and Microsoft Word 365 Version 2606 were used for preparing tables and writing this thesis, respectively. Microsoft PowerPoint 365 Version 2606 and Inkscape were used to assemble the figures. ChatGPT V.5.5 was used to improve readability and language, as well as generating some graphics for the introduction.

10.4 Table Of Abbreviations

AI	Artificial Intelligence	**IQI**	Infaunal Quality Index
AMBI	AZTI's Marine Biotic Index	**ITI**	Infaunal Trophic Index
ANOVA	Analysis of Variance	**LCA**	Last Common Ancestor
ASV	Amplicon Sequence Variant	**LOD**	Limit of Detection
AZE	Allowable Zone of Effect	**LOI**	Loss on Ignition
BC	Bray-Curtis	**LOQ**	Limit of Quantification
BH	Benjamini-Hochberg	**$MgCl_2$**	Magnesium Chloride
BLAST	Basic Local Alignment Search Tool	**NCBI**	National Center for Biotechnology Information
bp	Base Pair	**NMBAQCS**	National Marine Biological Quality Control Scheme
CE	Cage Edge	**NMDS**	Non-metric Multidimensional Scaling
CFB	Cytophaga-Flavobacterium-Bacteroidetes	**PCoA**	Principal Coordinates Analysis
CV	Coefficient of Variation	**PCR**	Polymerase Chain Reaction
DADA2	Divisive Amplicon Denoising Algorithm	**PERMANOVA**	Permutational Multivariate Analysis of Variance
DNA	Deoxyribonucleic Acid	**qPCR**	Quantitative Polymerase Chain Reaction
dNTP	Deoxyribonucleoside Triphosphate	**QRS**	Quantile Regression Spline
dPCR	Digital Polymerase Chain Reaction	**REF**	Reference
eDNA	Environmental Deoxyribonucleic Acid	**rRNA**	Ribosomal Ribonucleic Acid
EIA	Environmental Impact Assessments	**SDG**	Sustainable Development Goals
EQ	Environmental Quality	**SEPA**	Scottish Environment Protection Agency
FDR	False Discovery Rate	**SOP**	Standardized Operating Protocol
GTR	General Time Reversible	**SSU**	Small Subunit
HiFi	High Fidelity	**Temp**	Temperature
HTS	High-throughput Sequencing	**TITAN**	Threshold Indicator Taxa Analysis
ID	Identification	**UK**	United Kingdom
IndVal	Indicator Species Analysis	**VIF**	Variance Inflation Factor
INT	Intermediate-impacted	**WFD**	Water Framework Directive

***Note**: The Scottish salmon farms investigated in this thesis must stay anonymous due to farm operator guidelines and are therefore abbreviated with codenames throughout the text and publications (CRE and DUN). However, environmental attributes and descriptions of the farms are given in the associated publications.*

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SUPPLEMENTARY MATERIALS OF CHAPTER I

Supplementary File S1.* The R script used to process the data and prepare visualizations.

Supplementary File S2.* Input data tables used to perform the analyses and generate the figures are presented in multiple sheets in the provided Excel file.

**These Microsoft Excel files contain extensive data tables that could not be included in the printed version of the dissertation because of their size. The files are permanently available through Zenodo at https://doi.org/10.5281/zenodo.21790914*

Supplementary Table S1

Table S1. Number of sequences after each cleaning step in the DADA2 pipeline for both PCR protocols at CRE and DUN farms. In addition, alpha diversity indices for the rarefied data (to 5,000 reads) as well as the p-value of the significance testing between the two PCR protocols are provided.

	CRE			**DUN**		
	Protocol 1	Protocol 2	*p*-value	Protocol 1	Protocol 2	*p*-value
Sequencing overview						
Raw reads	5,039,438	2,058,600		5,985,090	1,985,085	
Reads post DADA2 filtering (% reads lost)	3,720,402 (26.2)	1,549,758 (24.7)		4,333,175 (27.6)	1,394,658 (29.7)	
Reads post DADA2 merging (% reads lost)	3,204,762 (13.9)	1,128,353 (27.2)		3,610,005 (16.7)	1,004,932 (28)	
Reads post chimera removal (% chimera)	3,055,544 (4.7)	1,093,002 (3.1)		3,416,069 (5.4)	800,165 (20.4)	
Reads post singleton and contaminant removal (% reads lost)	2,994,443 (2)	1,065,722 (2.5)		3,339,143 (2.3)	780,899 (2.4)	
Number of ASVs	33,859	14,130		36,490	11,769	
Number of non-bacteria* ASVs (%)	150 (0.4)	94 (0.7)		218 (0.6)	120 (1.0)	
ASVs post rarefying to 5,000 reads	13,441	8,608		15,126	8,452	
Rarefied (5,000 reads) alpha diversity indices (min-max)						
Richness	611 – 2,782	515 – 1,657	<0.001	1,171 – 2,362	728 – 1,345	<0.001
Shannon diversity	4.3 – 7.5	4.7 – 6.9	0.02	5.8 – 7.3	5.5 – 6.7	<0.001
Evenness	0.68 – 0.95	0.76 – 0.94	0.9996	0.82 – 0.94	0.84 – 0.93	0.5747

* Archaea and unassigned ASVs

Supplementary Figure S1

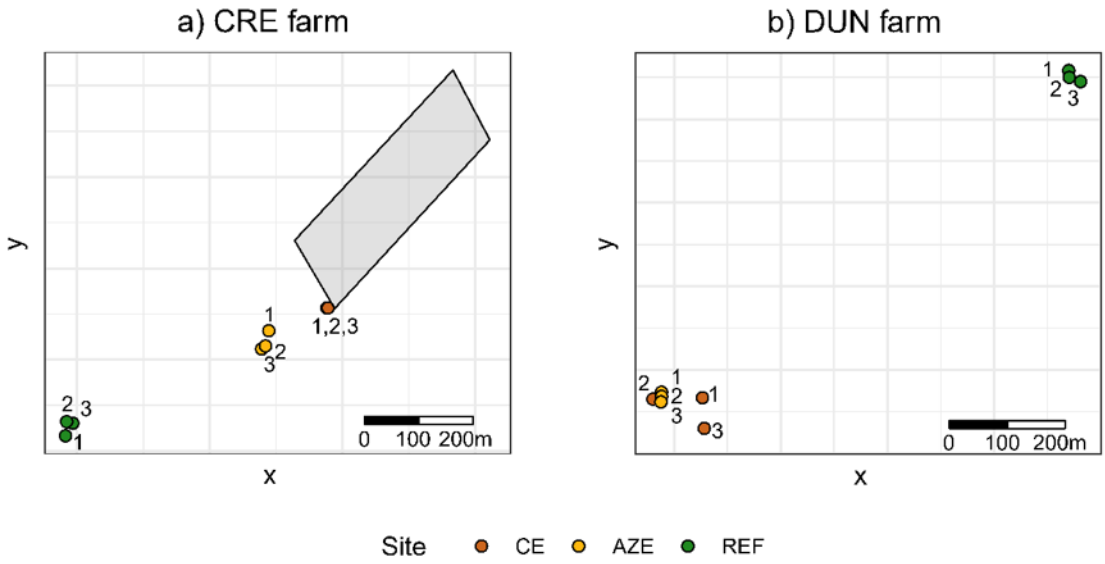


Fig. S1. Location of the sampled Van Veen grabs in both a) CRE and b) DUN farms. Numbers in the plots represent the grab numbers. Lat = Latitude; Long = Longitude. The grey area indicates the approximate location of the CRE farm. Note that there were no cages in DUN farm at the time of sampling; hence, it is not represented in the figure.

Supplementary Figure S2

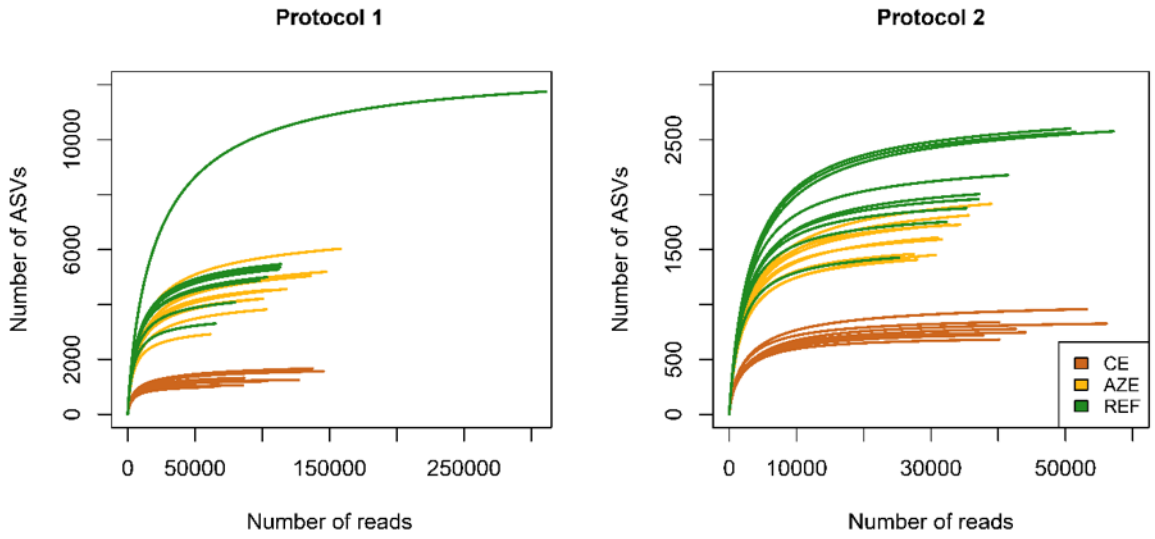


Fig. S2. Rarefaction curves representing the relationship between sequencing depth (number of reads per sample) and the number of unique amplicon sequence variants (ASVs) identified using PCR protocol 1 (left) and PCR protocol 2 (right) in the CRE farm. CE= cage edge; AZE= allowable zone of effect; REF= reference.

Supplementary Figure S3

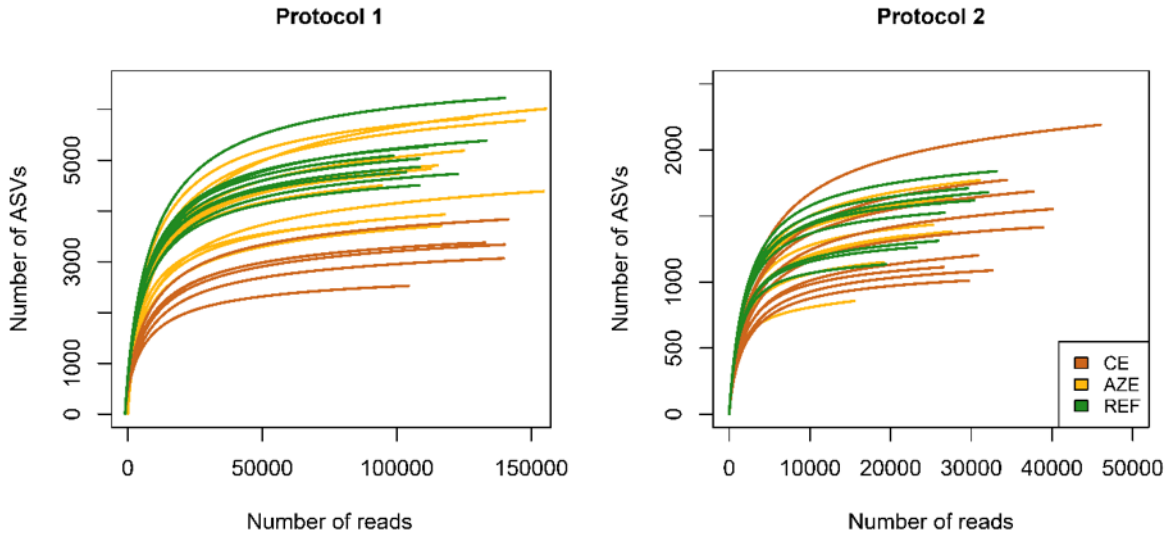


Fig. S3. Rarefaction curves representing the relationship between sequencing depth (number of reads per sample) and the number of unique amplicon sequence variants (ASVs) identified using PCR protocol 1 (left) and PCR protocol 2 (right) in the DUN farm. CE= cage edge; AZE= allowable zone of effect; REF= reference.

Supplementary Figure S4

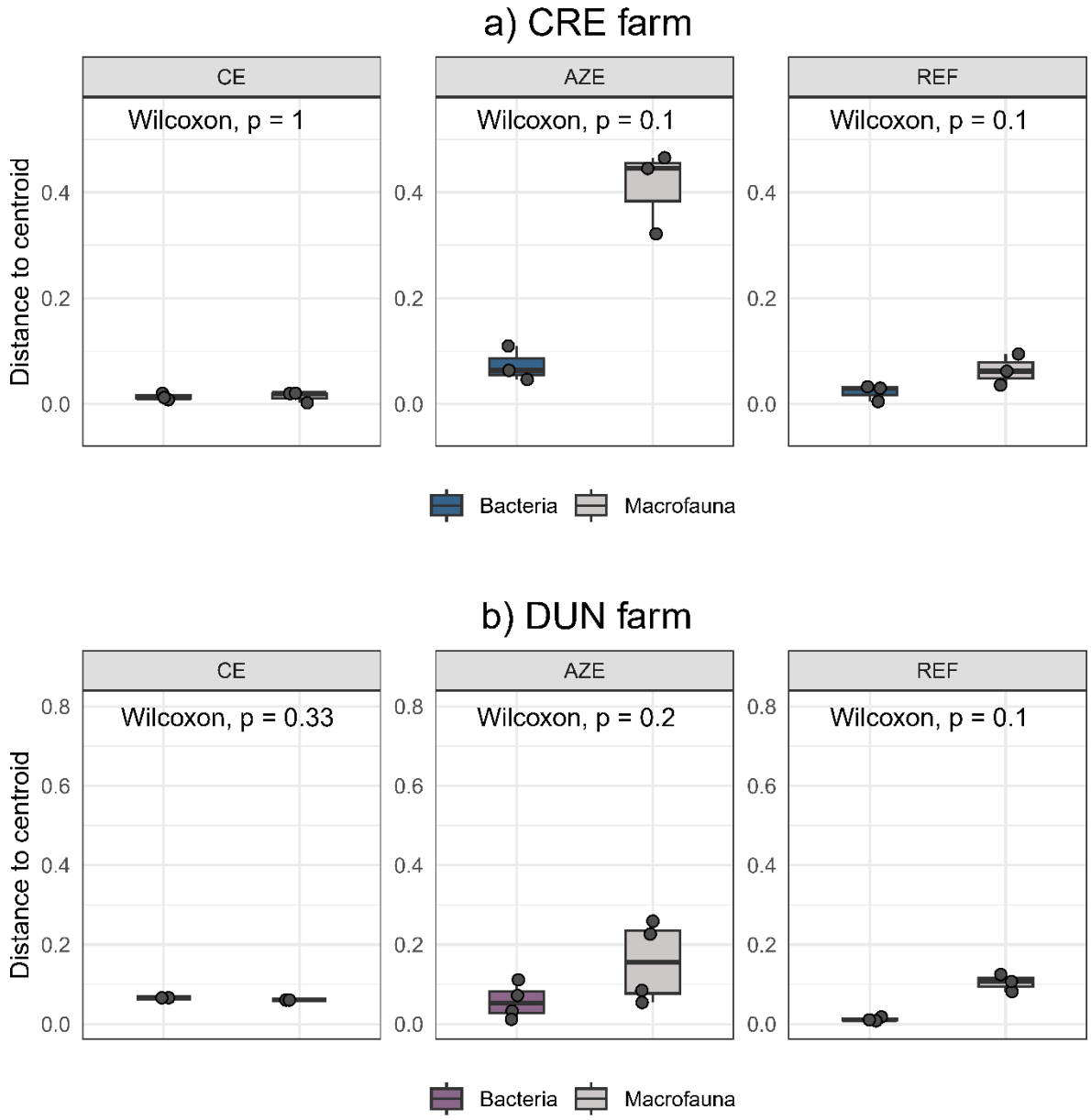


Fig. S4. Comparison of the sampling noise between macrofauna and bacteria represented by Bray-Curtis distances of each sample to their group centroids (each site separately), as well as the corresponding significance test results (Wilcoxon *p*-values) in both a) CRE and b) DUN farms.

SUPPLEMENTARY MATERIALS OF CHAPTER II

Supplementary Figure S1

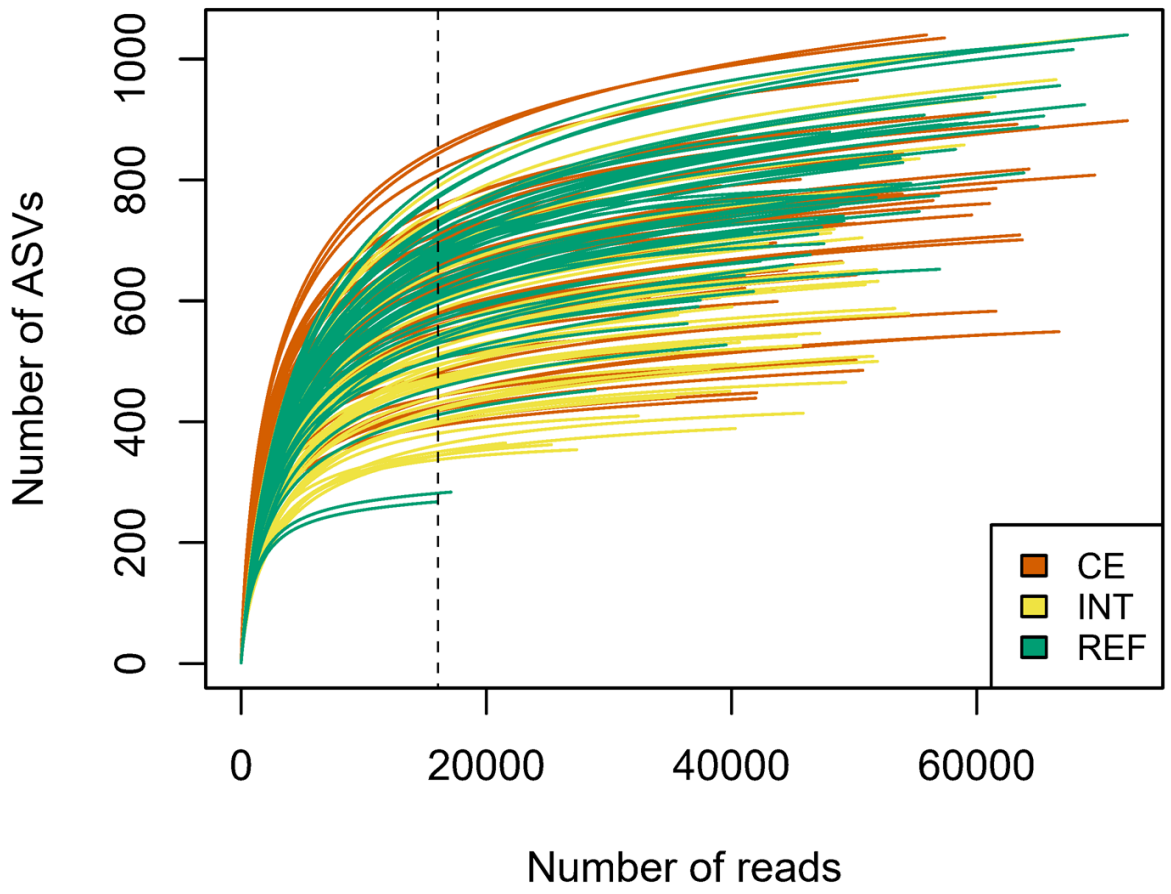


Fig. S1. Rarefaction curve representing the relationship between sequencing depth (number of reads per sample) and the number of unique amplicon sequence variants (ASVs). CE= cage edge. INT= intermediate impact zone. REF= reference.

SUPPLEMENTARY MATERIALS OF CHAPTER III

Supplementary File S1.* Results of IndVal analysis containing all significant ASVs, their association with ecological condition, stat value, and their taxonomy. Sheet 1 contains the results from Dataset 1 and Sheet 2 contains the results of Dataset 2.

Supplementary File S2.* Results of MaAsLin2 analysis containing all significant ASVs, their their coefficient estimates, and their taxonomy. Sheet 1 contains the results from Dataset 1 and Sheet 2 contains the results of Dataset 2.

Supplementary File S3.* Results of TITAN analysis containing all significant ASVs, their threshold statistics, and their taxonomy. Sheet 1 contains the results from Dataset 1 and Sheet 2 contains the results of Dataset 2.

Supplementary File S4.* Complete list of consensus significant ASVs identified by all three approaches and their assigned family-level taxonomy.Sheet 1 contains the results from Dataset 1 and Sheet 2 contains the results of Dataset 2.

**These Microsoft Excel files contain extensive data tables that could not be included in the printed version of the dissertation because of their size. The files are permanently available through Zenodo at https://doi.org/10.5281/zenodo.21790914*

Supplementary Figure S1

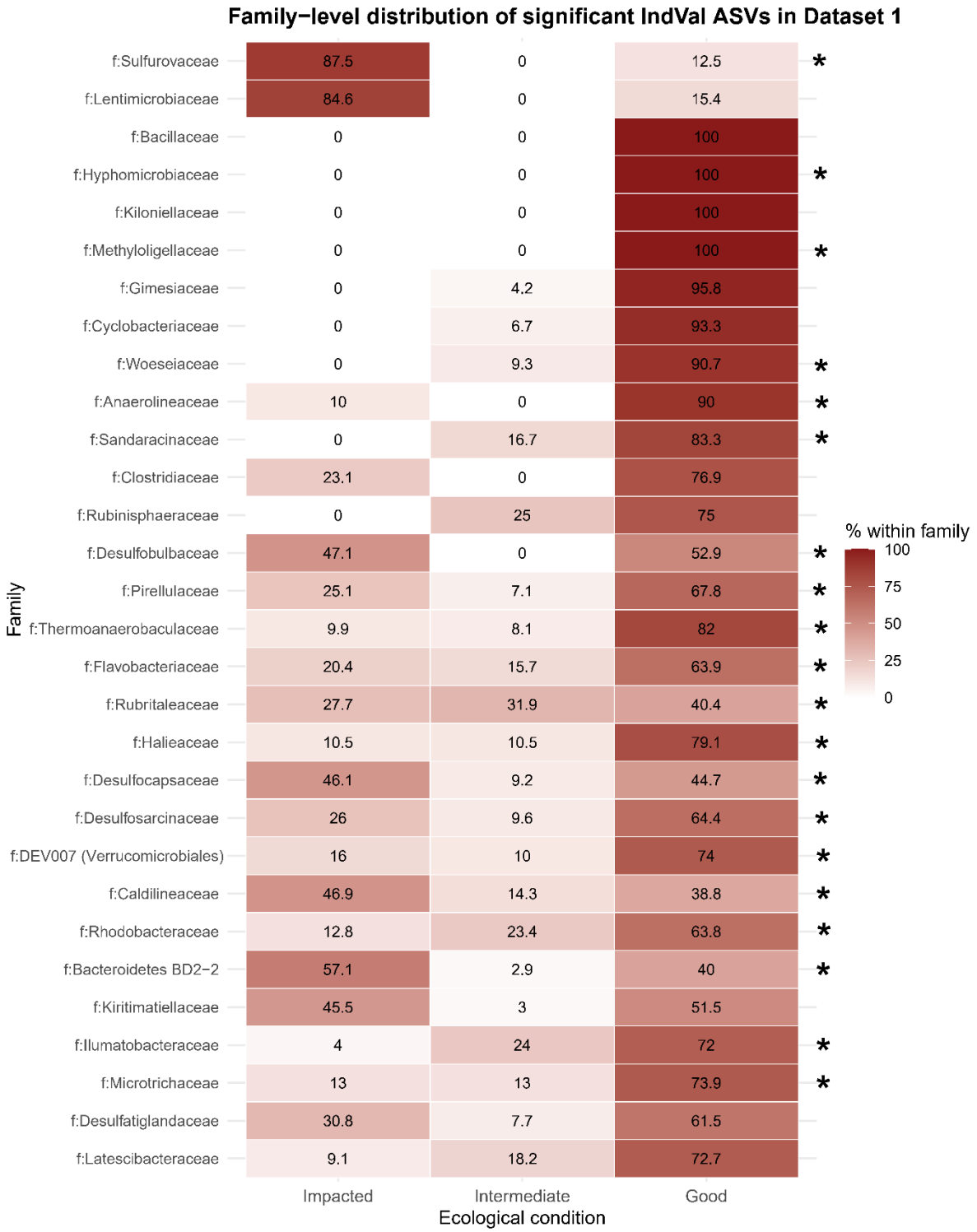


Fig. S1. Family-level distribution of significant bacterial indicator ASVs identified by Indicator Species Analysis (IndVal) in Dataset 1. Heatmap colors represent the percentage of significant indicator ASVs within each bacterial family assigned to impacted, moderate, or good ecological conditions. The 30 families containing the highest numbers of significant indicator ASVs are shown. An asterisk (*) denotes families with a mean relative abundance exceeding 1% across the complete dataset.

Supplementary Figure S2

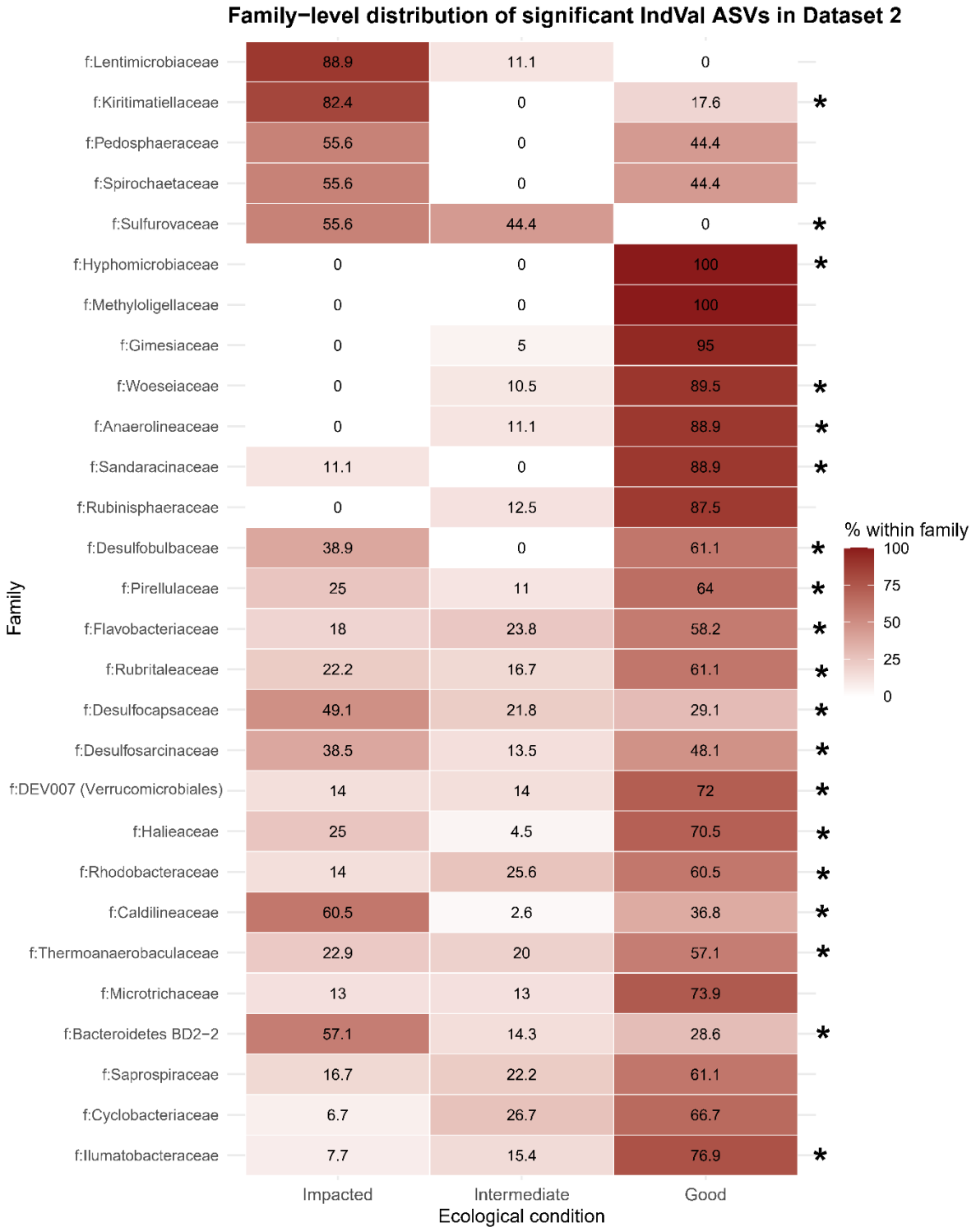


Fig. S2. Family-level distribution of significant bacterial indicator ASVs identified by Indicator Species Analysis (IndVal) in Dataset 2. Heatmap colors represent the percentage of significant indicator ASVs within each bacterial family assigned to impacted, moderate, or good ecological conditions. The 30 families containing the highest numbers of significant indicator ASVs are shown. An asterisk (*) denotes families with a mean relative abundance exceeding 1% across the complete dataset.

Supplementary Figure S3

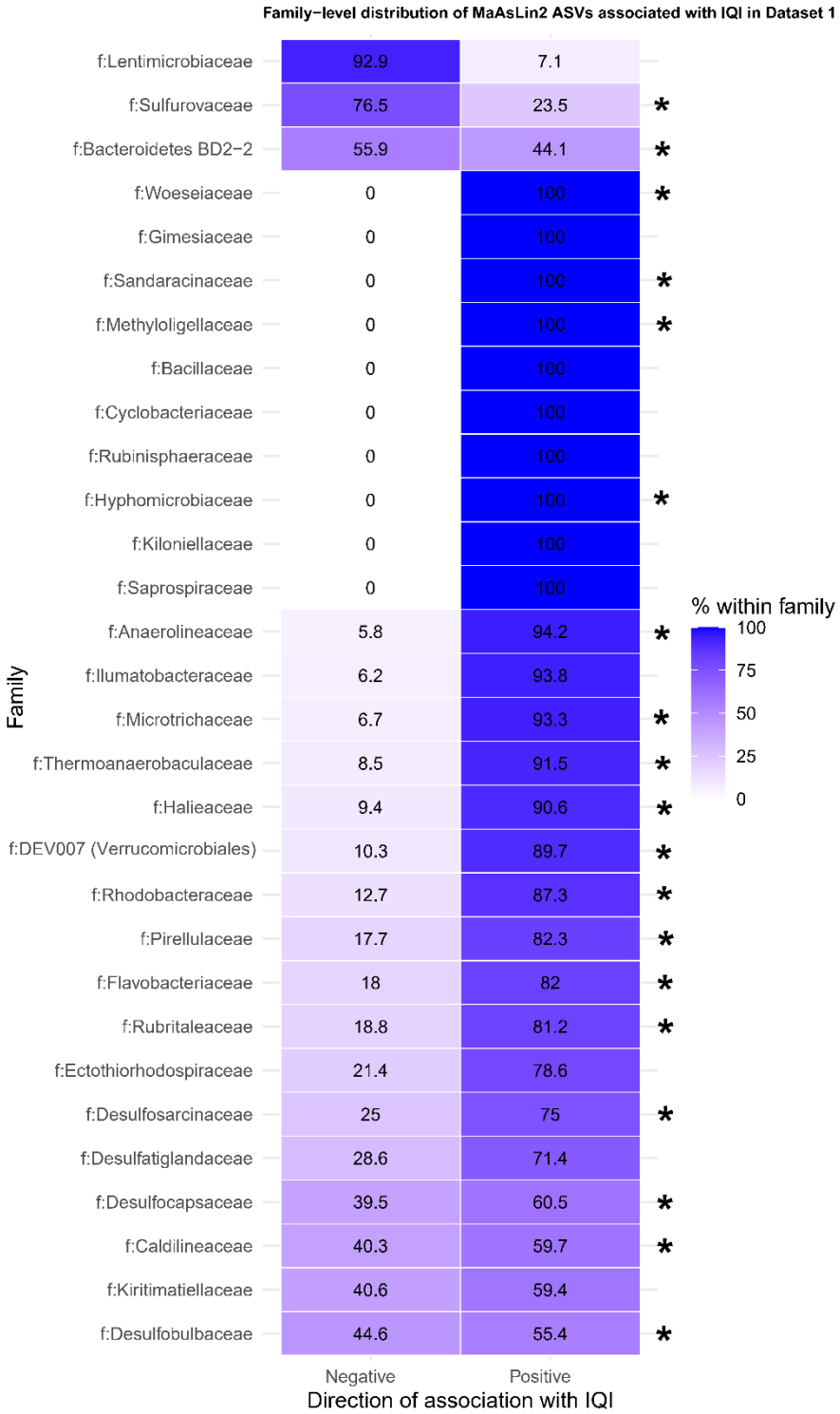


Fig. S3. Family-level distribution of significant bacterial ASVs associated with IQI identified by MaAsLin2 in Dataset 1. Heatmap colors represent the percentage of significant ASVs within each bacterial family exhibiting negative or positive associations with IQI, based on the direction of the MaAsLin2 regression coefficient. The 30 families containing the highest numbers of significant IQI-associated ASVs are shown. An asterisk (*) denotes families with a mean relative abundance exceeding 1% across the complete dataset.

Supplementary Figure S4

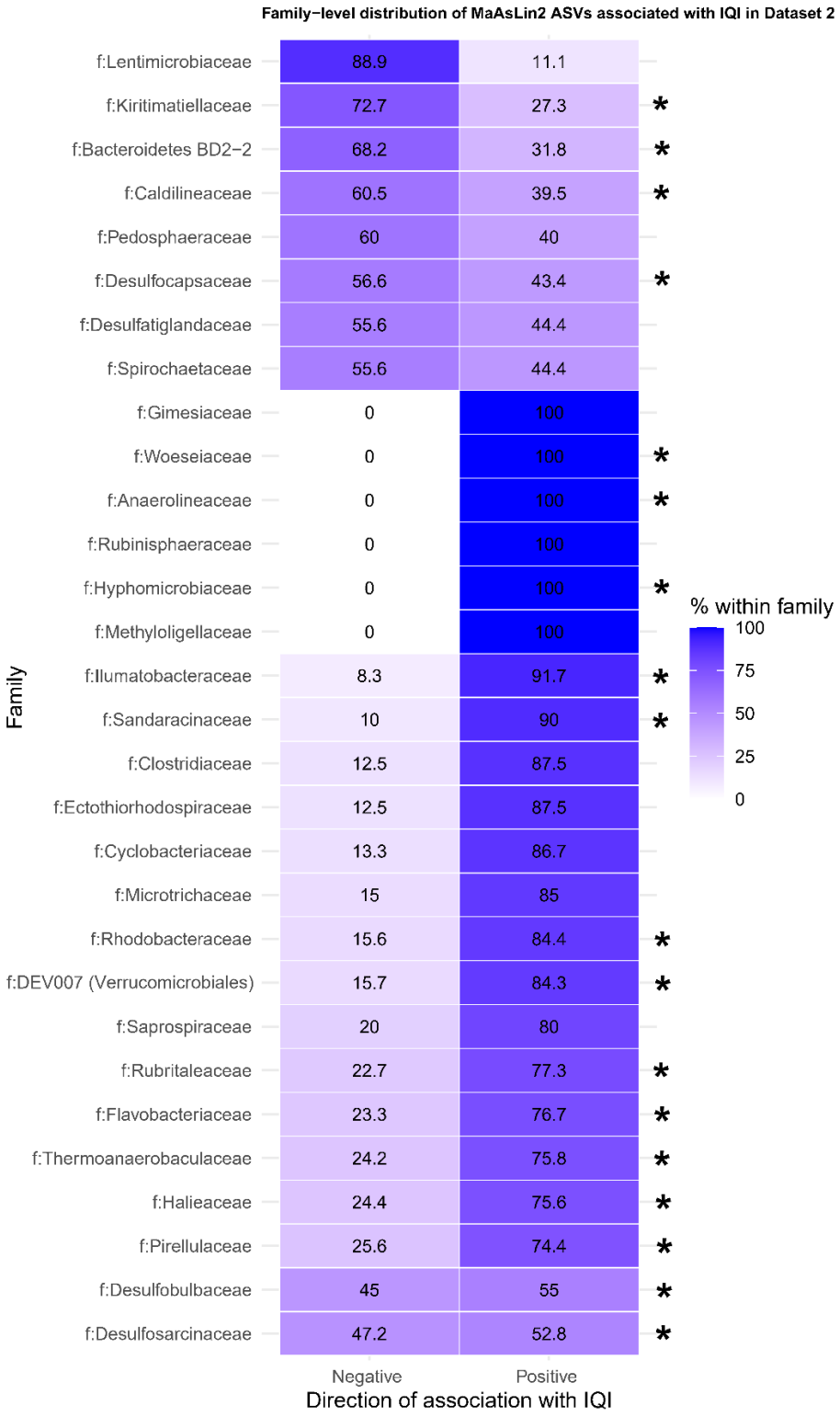


Fig. S3. Family-level distribution of significant bacterial ASVs associated with IQI identified by MaAsLin2 in Dataset 2. Heatmap colors represent the percentage of significant ASVs within each bacterial family exhibiting negative or positive associations with IQI, based on the direction of the MaAsLin2 regression coefficient. The 30 families containing the highest numbers of significant IQI-associated ASVs are shown. An asterisk (*) denotes families with a mean relative abundance exceeding 1% across the complete dataset.

Supplementary Table S1

Table S1. List of consensus significant ASVs identified by all three approaches (IndVal, MaAsLin2, and TITAN), the direction of association, and their family assignment in Dataset 1.

ASV	Direction	Family
ASV_00001	Impacted	f:Sulfurovaceae
ASV_00003	Impacted	f:Desulfobulbaceae
ASV_00004	Impacted	f:Sulfurovaceae
ASV_00005	Impacted	f:Sulfurovaceae
ASV_00007	Impacted	f:Propionibacteriaceae
ASV_00012	Impacted	f:Desulfocapsaceae
ASV_00016	Impacted	f:Desulfocapsaceae
ASV_00018	Impacted	f:Caldilineaceae
ASV_00021	Impacted	f:Desulfocapsaceae
ASV_00022	Impacted	f:Flavobacteriaceae
ASV_00023	Impacted	f:Flavobacteriaceae
ASV_00025	Impacted	f:Flavobacteriaceae
ASV_00032	Impacted	f:Caldilineaceae
ASV_00033	Impacted	f:Halieaceae
ASV_00037	Impacted	f:Pirellulaceae
ASV_00041	Impacted	f:Pirellulaceae
ASV_00045	Impacted	f:Bacteroidetes BD2-2
ASV_00047	Impacted	f:Bacteroidetes BD2-2
ASV_00062	Impacted	f:Desulfobulbaceae
ASV_00064	Impacted	f:Flavobacteriaceae
ASV_00070	Impacted	f:Sedimenticolaceae
ASV_00074	Impacted	f:Rubritaleaceae
ASV_00076	Impacted	f:Desulfosarcinaceae
ASV_00079	Impacted	f:Rubritaleaceae
ASV_00099	Impacted	f:Desulfobulbaceae
ASV_00109	Impacted	f:Caldilineaceae
ASV_00113	Impacted	f:Desulfocapsaceae
ASV_00008	Good	f:Desulfocapsaceae
ASV_00014	Good	f:Hyphomicrobiaceae
ASV_00015	Good	f:Sva1033 (Desulfuromonadales)
ASV_00020	Good	f:Hyphomicrobiaceae

ASV_00027	Good	f:Sandaracinaceae
ASV_00031	Good	f:Desulfobulbaceae
ASV_00036	Good	f:Sva1033 (Desulfuromonadales)
ASV_00044	Good	f:Methyloligellaceae
ASV_00048	Good	f:Desulfosarcinaceae
ASV_00053	Good	f:Rhodobacteraceae
ASV_00055	Good	f:Anaerolineaceae
ASV_00060	Good	f:Anaerolineaceae
ASV_00066	Good	f:Sandaracinaceae
ASV_00071	Good	f:Desulfobulbaceae
ASV_00080	Good	f:Woeseiaceae
ASV_00084	Good	f:Thermoanaerobaculaceae
ASV_00088	Good	f:Sandaracinaceae
ASV_00093	Good	f:Desulfobulbaceae
ASV_00095	Good	f:Sandaracinaceae
ASV_00098	Good	f:Sandaracinaceae
ASV_00100	Good	f:Woeseiaceae
ASV_00102	Good	f:Ilumatobacteraceae
ASV_00115	Good	f:Sva1033 (Desulfuromonadales)
ASV_00116	Good	f:Caldilineaceae
ASV_00117	Good	f:Desulfocapsaceae
ASV_00118	Good	f:Rhodobacteraceae

Supplementary Table S2

Table S2. List of consensus significant ASVs identified by all three approaches (IndVal, MaAsLin2, and TITAN), the direction of association, and their family assignment in Dataset 2.

ASV	Direction	Family
ASV_00001	Impacted	f:Sulfurovaceae
ASV_00004	Impacted	f:Desulfobulbaceae
ASV_00008	Impacted	f:Flavobacteriaceae
ASV_00009	Impacted	f:Sulfurovaceae
ASV_00019	Impacted	f:Rubritaleaceae
ASV_00021	Impacted	f:Flavobacteriaceae
ASV_00022	Impacted	f:Flavobacteriaceae
ASV_00025	Impacted	f:Pirellulaceae
ASV_00026	Impacted	f:Bacteroidetes BD2-2
ASV_00027	Impacted	f:Caldilineaceae
ASV_00031	Impacted	f:Bacteroidetes BD2-2
ASV_00032	Impacted	f:Pirellulaceae
ASV_00036	Impacted	f:Desulfocapsaceae
ASV_00037	Impacted	f:Halieaceae
ASV_00041	Impacted	f:Pirellulaceae
ASV_00043	Impacted	f:Marinifilaceae
ASV_00045	Impacted	f:Desulfocapsaceae
ASV_00048	Impacted	f:Flavobacteriaceae
ASV_00049	Impacted	f:Caldilineaceae
ASV_00060	Impacted	f:Flavobacteriaceae
ASV_00064	Impacted	f:Caldilineaceae
ASV_00073	Impacted	f:Bacteroidetes BD2-2
ASV_00093	Impacted	f:DEV007 (Verrucomicrobiales)
ASV_00104	Impacted	f:Kiritimatiellaceae
ASV_00015	Good	f:Sandaracinaceae
ASV_00020	Good	f:Sva1033 (Desulfuromonadales)
ASV_00033	Good	f:Hyphomicrobiaceae
ASV_00034	Good	f:Hyphomicrobiaceae
ASV_00038	Good	f:Ilumatobacteraceae
ASV_00039	Good	f:Rhodobacteraceae
ASV_00042	Good	f:Anaerolineaceae

ASV_00059	Good	f:Sva1033 (Desulfuromonadales)
ASV_00061	Good	f:Desulfocapsaceae
ASV_00062	Good	f:Rhodobacteraceae
ASV_00074	Good	f:Rhizobiaceae
ASV_00079	Good	f:Pirellulaceae
ASV_00081	Good	f:Desulfocapsaceae
ASV_00082	Good	f:Methyloligellaceae
ASV_00084	Good	f:Pirellulaceae
ASV_00089	Good	f:Sandaracinaceae
ASV_00090	Good	f:Sandaracinaceae
ASV_00100	Good	f:Pirellulaceae

SUPPLEMENTARY MATERIALS OF CHAPTER IV

Supplementary Table S1

Table S1. Selected ASVs based on high statistical rankings resulted from two methods (IndVal and RF) in Rubel et al. (2025).

ASV	s.bad	s.good	stat	p.value	p.value.bh	min RF variable importance	Taxonomy	Acession number	Similarity score
ASV_0000004	1	0	0.727214289	0.0001	0.002881818	0.230532526	*Sulfurovum*	KC545727.1	99.50%
ASV_0000022	1	0	0.938880425	0.0001	0.002881818	7.015420569	*Cytophaga*	JF743580.1	99.29%
ASV_0000024	0	1	0.820035998	0.0001	0.002881818	1.314896011	Desulfuromonadaceae	JF806801.1	100.00%

Supplementary Table S2

Table S2. Sequences and characteristics of synthetic DNA templates (gBlocks) used for dPCR assay optimization and analytical validation**.**

	Target sequence length	Molecular weight (g/mol)	GC Content	Sequence
ASV_04_gBlocks	158	97500.1	47.47%	TAAACATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATAGACGGGACCCGCACAAGTGGTGGAGCATGTGGTTTAATTCGAAGATACGCGAAGAACCTTACTAGCCTTGACATATATTAGAACTCACCAGAGATGGAGAGGTGC
ASV_22_gBlocks	151	93166.4	41.72%	GCGTTTGTACCCTACTTAACTGGAGAATAGCCCGAAGAATTCGGATTAATACTCCATAACACTATGATCTGGCATCTTATTATAGTTAAAGCTCCGGCGGTTAAGATGGCATGCGTAACATTAGATAGTTGGTGAGGTAACGGCT
ASV_24_gBlocks	165	101825.9	47.88%	AGAACGAACGTTGGCGGTATGCTTAACACATGCAAGTCGTACGTGAAAGCTTCCTTCGGGAAGTGATTAAAGTGGCGCACGGGTGAGTAACACGTGGATAATCTACCCGACAGTCAGGGATAACATTCGAAGGAATGCTAATACCTGATAAGCCTACGCCCTC

CURRICULUM VITAE

PERSONAL DETAILS

FIRST NAME	**MAHSHID**
LAST NAME	**OLADI KALARIJANI**
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EDUCATION

2022-present	**Doctoral Researcher** Department of Ecology (AG Stoeck), Rheinland-Pfälzische Technische Universität, Kaiserslautern, Germany
2014 - 2017	**M.Sc. in Marine Biology** Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran *Graduated with honors as the highest-ranking student in the program*
2008 - 2012	**B.Sc. in Animal Biology** Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran

ACADEMIC AND RESEARCH EXPERIENCES

2018-2024	**Editorial Board Member** Environmental Sciences Journal, Environmental Sciences Research Institute, Shahid Beheshti University, Tehran, Iran
2021	**Guest Researcher** Department of Ecology (AG Stoeck), Rheinland-Pfälzische Technische Universität, Kaiserslautern, Germany
2016- 2021	**Research Assistant** Department of Aquatic Biology and Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran
2016-2019	**Co-supervision of undergraduate and master's research projects** Marine Biology, Shahid Beheshti University, Tehran, Iran

TEACHING EXPERIENCES

2024-present	**Instructor** Seminars "Current topics in Molecular Ecology" and "Modern Methods in Molecular Ecology", Department of Ecology, Rheinland-Pfälzische Technische Universität, Kaiserslautern, Germany
2023-present	**Instructor** Reading course "Aquatic Microbial Ecology", Department of Ecology, Rheinland-Pfälzische Technische Universität, Kaiserslautern, Germany

2020 | **Lecturer**
Regional Online Workshop on Trends of Coral Reefs of the Persian Gulf and the Gulf of Oman, Tehran, Iran

2019 | **Teaching Assistant**
Phytoplankton Laboratory, Department of Aquatic Biology and Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran

PUBLICATIONS

2026 | **Oladi, M.** & Stoeck, T. Digital PCR assays quantify bacterial bioindicators across environmental quality gradients in salmon aquaculture sediments. Aquaculture, 623: 744179.

2026 | **Oladi, M.**, Wyness, A., Wilding, T.A., Breiner, H.W. & Stoeck, T. eDNA-Based Temporal Trajectories of Bacterial Communities in Salmon Farm Sediments: Disentangling Seasonal Variability from Aquaculture Impacts. Environmental DNA, 8(4): e70339.

2024 | **Oladi, M.**, Wilding, T.A., Wyness, A. & Stoeck, T. Towards a Standardized Operating Procedure for eDNA-Based Biomonitoring in Coastal Marine Salmon Aquaculture. Environmental DNA, 6(5): e70014

2022 | **Oladi, M.**, Leontidou, K., Stoeck, T. & Shokri, M.R. Environmental DNA-based profiling of benthic bacterial and eukaryote communities along a crude oil spill gradient in a coral reef in the Persian Gulf. Marine Pollution Bulletin, 184: 114143

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2021 | **Oladi, M.** & Shokri M.R., Multiple benthic indicators are efficient for health assessment of coral reefs subjected to petroleum hydrocarbons contamination: A case study in the Persian Gulf. Journal of Hazardous Materials, 409: 124993

2021 | **Oladi, M.**, Rouzbehani, S., Ahmadzadeh, F. & Ghazilou, A., Dynamics of *Dipsastraea pallida*-symbiont association following bleaching events across the northern Persian Gulf and Gulf of Oman. Symbiosis, 84(2): 141-149

2020 | Shokri M.R. & **Oladi, M.**, New record of azooxanthellate *Dendrophyllia cribrosa* (Scleractinia: Dendrophylliidae) in the Persian Gulf. Spixiana, 44(1): 11-16

2020 | Ahmadzadeh, F., Shahrokhi, G., Saberi-Pirooz, R., **Oladi, M.**, Taati, M., et al., Alborz Heritage: Geographic distribution and genetic differentiation of the Iranian *Paradactylodon* (Amphibia: Hynobiidae). Amphibia-Reptilia, 41(4): 519-534

2019 | **Oladi, M.**, Shokri, M.R. & Rajabi Maham, H., Extremophile symbionts in extreme environments; a contribution to the diversity of Symbiodiniaceae across the northern Persian Gulf and Gulf of Oman. Journal of Sea Research, 144: 105-111

2019 | Jaberimanesh, Z., **Oladi, M.**, Nasrolahi, A. & Ahmadzadeh, F., Presence of *Amphibalanus eburneus* (Crustacea, Cirripedia) in Gomishan Wetland: Molecular and morphological evidence of a new introduction to the southern Caspian Sea. Regional Studies in Marine Science, 25: 100469

2019 | Saberi-Pirooz, R., Shahriari, A., Ahmadzadeh, F., Javidkar, M., **Oladi, M.** & Asadi Aghbolaghi, M., Dispersal beyond geographic barriers: a contribution to the phylogeny and demographic history of *Pristurus rupestris* Blanford, 1874 (Squamata: Sphaerodactylidae) from southern Iran. Zoology, 134: 8-15

2019 | **Oladi, M.**, Saberi-Pirooz, R., Ahmadzadeh, F. & Ghazilou, A., Further documentation of the Indian frogfish, *Antennarius indicus* (Teleostei: Lophiiformes), from the ROPME Sea Area; a molecular and morphological approach. Thalassas, 35(2): 503-509

2018 | **Oladi, M.**, Nasrolahi, A., Ahmadzadeh, F., Alizadeh, O. & Krug, P., Molecular confirmation of the occurrence of *Elysia* cf. *tomentosa* (Mollusca: Heterobranchia) in the Persian Gulf. Zoology in the Middle East, 64(2): 124-130

2018 | **Oladi, M.**, Shokri, M.R. & Rajabi Maham, H., The correlation between bleaching and morphological condition and population dynamics of symbiotic algae with *Acropora downingi* Wallace, 1999 in Qeshm Island [in Persian]. Journal of Marine Biology, 10(2): 1-10

2017 | **Oladi, M.**, Shokri, M.R., & Rajabi Maham, H., Application of the Coral Health Chart to determine bleaching status of *Acropora downingi* in a subtropical coral reef. Ocean Science Journal, 52(2): 267–275

2017 | **Oladi, M.**, Badri, S., Nateghi Shahrokni, S.A. & Ghazilou, A., Observation of the eye-attacking behaviour of Koi *Cyprinus carpio* var. *koi* in a koi–goldfish polyculture system. Journal of Applied Animal Research, 45(1): 215-216

Honors and Awards

2025 | Awarded RPTU Mobility Funding to attend SymbioMar Workshop at CRIMAC International School, Amendolara, Italy

2021 | Awarded DAAD Short-Term Research Grant for a six-month research stay at the University of Kaiserslautern, Germany

2017 | Winner of the Best Oral Presentation Award at the 1st International Conference on Oceanography for West Asia held in Tehran, Iran

Oral Presentations

2025 | eDNA on trial: Toward a standard protocol for biomonitoring in salmon farming.
Aquaculture Europe, Valencia, Spain

2025 | Environmental DNA-based profiling of benthic bacterial and eukaryote communities along a crude oil spill gradient in a coral reef in the Persian Gulf.
AquaEcOmics, Évian-les-Bains, France

2017 | Distinct symbiont types in three different hard coral hosts from Qeshm Island: Proof of stable associations between hosts and particular zooxanthellae.
The 1st International Conference on Oceanography for West Asia, Tehran, Iran

2016 | Comparison of Density and Mitotic Index of Symbiotic Algae in Coral Reefs of Kharku and Qeshm Island.
Annual International Congress on Biology, Agriculture, Tourism and Sustainable Development, Tehran, Iran

Selected Poster Presentations

2025	Stress Signals from the Seafloor: eDNA Reveals Microbial Community Shifts in Coral Reef Sediments. CRIMAC International School - SymbioMar Workshop, Amendolara, Italy
2021	A combination of benthic indicators is efficient for health assessment of coral reefs subjected to oil pollution. The 14th International Coral Reef Symposium, Bremen, Germany
2018	Disparate types of symbiotic algae occur within branching and massive corals of Chabahar Bay: an implication for different response of corals to environmental stress. The 20th National and 8th International Congress on Biology, Maragheh, Iran
2017	Mutual Participation of *Symbiodinium* Clade D and C in Increasing Coral Reef's Resilience to Environmental Stressors in the northern Persian Gulf and Gulf of Oman. The 1st Bilateral German-Iranian workshop on Response of coastal and pelagic marine ecosystems to environmental stressors in the Persian Gulf, Kish Island, Iran
2017	Identification of Endosymbionts in Corals from Chabahar. The 2nd GCC Marine Biotechnology Conference: Emerging opportunities and future perspectives, Muscat, Oman

Further Education and Training

2020	Completed "Molecular Phylogenetics 1" 4-day training course, Tehran, Iran
2020	Completed "Molecular Phylogenetics 2" 6-day training course, Tehran, Iran
2019	Completed "OTGA/INIOAS – Remote Sensing of Coral Reefs" Training Course, Tehran, Iran
2018	Completed "OTGA/INIOAS - Marine Biogeographic Data processing using OBIS" Training Course, Tehran, Iran
2014	PADI Advanced Open Water Diver

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