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# Spatial variation in the gastrointestinal microbiome, diet, and nutritional condition of a juvenile flatfish among coastal habitats

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**Short title:** Spatial variation in wild fish gut microbiomes

## Abstract

Gut microbiota are important for the health, fitness and development of animal hosts, but little is known about these assemblages in wild populations of fish. Such knowledge is particularly important for juvenile life stages where nutritional intake critically determines early development, growth, and ultimately recruitment. We characterise the microbiome inhabiting the gut of young-of-the-year European plaice ('YOY plaice') on sandy beaches, their key juvenile habitat, and examine how these microbial communities vary spatially in relation to diet and nutritional condition of their plaice hosts. Body size, diet (stomach fullness and eukaryotic 18S ribosomal sequencing), nutritional condition (RNA:DNA) and gut microbiota (16S prokaryotic ribosomal sequencing) were compared in fish at two spatial scales: between beaches separated by 10s of kilometres and between sites at different depths on the same beach, separated by 10s of metres. The main microbial phyla in YOY plaice guts were Proteobacteria, Spirochaetes, Tenericutes and Verrucomicrobiae. Within the Proteobacteria there was an unusual dominance of Alphaproteobacteria. Differences in body size, diet and nutritional condition of YOY plaice between beaches were accompanied by differences in gut microbial assemblage structure. Notably, substantially reduced nutritional condition and size at one of the beaches was associated with lower stomach fullness, reduced consumption of annelids and differences in the abundance and presence of specific microbial taxa. Differences were also detected in microbial assemblages, body size, and diet between depths within the same nursery beach, although stomach fullness and nutritional condition did not vary significantly. The functional links between the environment, gut microbiota, and their hosts are potentially important mediators of the development of young fish through critical life stages. Our study indicates that these links need to be addressed at 10 km and even 10 m scales to capture the variability observed in wild populations of juvenile fish.

## Introduction

The health, fitness, and development of animals may be influenced by the microbial assemblages inhabiting their gastrointestinal tract (e.g. Bates et al. 2006, Coon et al. 2014). These gut microbiota can aid in host nutrition (Walter & Ley 2011), energy metabolism (Turnbaugh et al. 2006), immune system maturation (Mazmanian et al. 2005), and immune responses (Olsson et al. 1992, Macpherson et al. 2000, Round & Mazmanian 2009, Ingerslev et al. 2014b). Alterations in microbiome composition can influence factors such as nutrient acquisition, susceptibility to toxins and behaviour (Sekirov et al. 2010, Mangiola et al. 2016). Furthermore, gut microbial composition varies greatly due to the phylogeny (Anderson et al. 2012, Colman et al. 2012, Phillips et al. 2012) and ontogenetic development (Phillips et al. 2012) of the host as well as extrinsic environmental influences (Phillips et al. 2012, Wong & Rawls 2012, Coon et al. 2014, Dill-McFarland et al. 2014). Establishing the natural variability in the gut microbiota is therefore an important step in appreciating the role of these assemblages in the physiology, behaviour and ecology of animal populations (Talwar et al. 2018).

Our knowledge of gut microbial composition and microbiome-host interactions in fishes is developing, but remains limited. Earlier work was based on traditional culture-dependent techniques that detect only a small fraction of the microbial assemblage (Spanggaard et al. 2000, Austin 2006, Romero & Navarrete 2006, Kim et al. 2007, Aguilera et al. 2013) and suffer methodological inconsistencies that make studies incomparable (Romero et al. 2014, Givens et al. 2015). Modern sequencing technologies provide more comprehensive insights into the diversity and structure of microbial assemblages and their functional characteristics in fishes (Austin 2006, Romero & Navarrete 2006, Aguilera et al. 2013, Ghanbari et al. 2015). However, most studies to date focus on a few captive-held, cultured species such as rainbow trout (*Oncorhynchus mykiss*) or grass carp (*Cyprinus carpio*), or on developmental model-species such as zebrafish (*Danio rerio*) (Roeselers et al. 2011, Stephens et al. 2016). Far less is known about gut microbiota of wild fish, although previous studies have demonstrated marked differences from captive-held populations (Ramírez & Romero 2017).

In particular, the individual and ecological implications of variation in fish gut microbiota need to be established at key life stages. Fishes have complex life histories, across which the composition of gut microbiota can change considerably (Bakke et al. 2015, Burns et al. 2016, Llewellyn et al. 2016). Gut microbiomes in zebrafish, for example, vary considerably during development and display signature characteristics unique to each life stage (Stephens et al. 2016). The diversity and relative abundance of bacterial taxa in a range of other fish species are known to be strongly influenced by developmental events such as first feeding (Hansen et al. 1992, Bergh et al. 1994, Ingerslev et al. 2014a) and settlement (Parris et al. 2016). Younger life stages are a particularly important focus for gut microbiome studies since nutrition is critical for the early developmental processes that underpin successful recruitment into adult fish populations (Sissenwine 1984, Houde 1987, Miller et al. 1988). There is a need for fundamental information about the nature of variation in gut microbiota, accompanying changes in fish diet and nutritional condition, during early life stages when potential influence on population dynamics is large.

This study focuses on variation in the gut microbiota, diet and nutritional condition of wild populations of the juvenile life stage of European plaice *Pleuronectes platessa* L., a commercially-important flatfish species distributed across the north-east Atlantic. Following winter spawning and a pelagic larval stage, young-of-the-year European plaice (hereafter 'YOY plaice') settle onto moderately exposed sandy shores during the spring and spend their first summer in the shallows (typically < 5 m) feeding on small invertebrates (Gibson 1999). YOY plaice have been an important model for examining causes of variation in physiological performance of young fishes in the wild (reviewed in Ciotti et al. 2014). Despite extensive tidal and diel migrations across the onshore-offshore axis of beaches, juvenile plaice show strong alongshore site fidelity and marked interindividual differences in patterns of habitat use across the depth gradient (Macer 1967, Riley 1973, Burrows et al. 2004, Gibson et al. 2011). Diet and growth rate of juveniles are thought to influence survival to recruitment and therefore population dynamics of plaice, but are highly variable in time and space. In particular, applications of high-throughout, fine-resolution, RNA-based metrics of individual growth have identified substantial, but as yet unexplained variation in YOY plaice growth rate and nutritional condition at spatial scales ranging from 10s of metres to 100s of kilometres on the west coast of Scotland and Irish Sea (Ciotti et al. 2013a, Ciotti et al. 2013b, Ciotti et al. 2013c, Ciotti et al. 2014). This variability could not be explained by factors such as food availability or temperature, prompting calls for a wider consideration of underlying drivers of growth variation (Ciotti et al. 2014). The extent to which gut microbial communities may vary at these scales, and how this may interact with feeding and growth, requires clarification.

In a recent metabarcoding study, Heindler et al. (2019) reported relative homogeneity in the diets and gut microbiota of YOY plaice across four geographic regions of open coast in the eastern English Channel and southern North Sea. This study was designed to be both spatially and temporally extensive. Samples from each region were collected from 2-5 sites pooled from offshore and/or beach locations. Distances between regions varied from 10 - 20 km to as much as 150 km and samples were collected over the course of several months. Dominant components of the microbiome across these scales were found to be α-, β- and ɣ-Proteobacteria, Planctomycetia, Acidimicrobiia and chloroplasts. It remains unknown whether this microbial assemblage and its apparent spatial homogeneity is also found in other parts of the species range. Furthermore, given the spatial structuring in other aspects of juvenile plaice feeding, growth and behaviour, over as little as 100 m distances (Ciotti et al. 2013c, Ciotti et al. 2014), the potential for finer-scale spatial variation in gut microbiota needs to be tested through contemporaneous sampling at spatially delimited sampling sites. Understanding variability in the gut microbiome at contrasting spatial scales, in parallel with key aspects of feeding and growth performance, will be critical for moving towards a functional microbiomics approach capable of revealing both the drivers of microbiome variation and their bioenergetic consequences for wild populations.

This paper applies a metabarcoding approach to characterise the microbiome in YOY plaice guts at sandy beaches on the west coast of Scotland in relation to the size, diet composition, feeding success and nutritional condition of their hosts. We test how these factors vary at contrasting spatial scales, between fishes living at different water depths on the same beach (separated by 10s of metres) and at different beaches (separated by 10s of kilometres). By revealing the potential for variability in the gut microbiota, we establish factors potentially influencing the physiological performance of juvenile fishes in the wild.

## Material and methods

Variation in gut microbiota, diet, and nutritional condition of YOY plaice was examined at two spatial scales. The large scale (10s of kilometers) compared fish caught at 0.5 m depth below waterline from two nursery beaches on the west coast of Scotland: Tralee Beach ('Tralee Shallow'; 56°29'N, 05°25'W) and Caolisport Beach ('Caolisport Shallow'; 55°55'N, 05°36'W). The small scale (10s of metres within a single beach) compared fish caught at Tralee Shallow with fish from 2 m below waterline at the same beach ('Tralee Deep').YOY plaice were collected within 2 h of low water on 19th and 20th July 2016 using a 1.5 m beam trawl (6 mm mesh) towed parallel to the shore, either by hand (0.5 m depth) or by boat (2 m depth). Seventy five YOY plaice (n = 20 for Caolisport Shallow and Tralee Deep, n = 35 for Tralee Shallow) were immediately flash-frozen and stored below -70°C until processing. Bottom water temperatures and salinities measured at the time of sampling indicated that Tralee Shallow (temperature = 15.0°C , salinity = 25.3) was cooler and fresher than Caolisport Shallow (temperature = 17.4°C, salinity = 27.8). Tralee Deep was cooler and more saline (temperature = 14.1°C, salinity = 26.0) than Tralee Shallow.

### ***Size, nutritional condition and feeding success***

Size, nutritional condition and feeding success were inferred from the length and mass, RNA:DNA (Ciotti et al. 2010) and stomach fullness, respectively, in a haphazardly selected subsample of fish frozen from each site (n = 15 for Caolisport Shallow and Tralee Deep, n = 30 for Tralee Shallow). RNA:DNA is a robust and well established metric of nutritional condition which also correlates strongly with individual growth rate in early life stage fishes (Bulow 1970, Ferron & Leggett 1994, Bergeron 1997, Buckley et al. 1999, Ciotti et al. 2010). This metric has previously been applied to reveal growth dynamics of YOY plaice on the west coast of Scotland (Ciotti et al. 2010, Ciotti et al. 2013b, Ciotti et al. 2013a, Ciotti et al. 2013c) and in other areas (De Raedemaecker et al. 2012). Total length was measured from photos of fish taken upon capture (prior to freezing) using the image processing software ImageJ (Abràmoff et al. 2004). In the laboratory, frozen fish were weighed and two 0.01 g subsamples of white muscle tissue were dissected from the epaxial eyed side for RNA:DNA measurement. Stomach contents were then removed, blotted and weighed fresh.

Nucleic acids were quantified using a one-dye, two-enzyme fluorometric assay modified from Caldarone et al. (2001). Unless otherwise stated, reagents and enzymes for measurements of nucleic acids were obtained from Sigma-Aldrich (Hampshire,UK) and prepared in Tris–EDTA buffer (TE, 5 mM Tris–HCl, 0.5 mM EDTA, adjusted to pH 7.5 with NaOH). To avoid contamination with nucleases, reagents were made using diethylpyrocarbonate (DEPC)-treated ultrapure water in baked glassware.

Nucleic acids were extracted from fresh tissue in 0.23 ml 2% n-lauroylsarcosine sodium salt solution with rapid shaking at 4°C (30 min at 1,500 rpm; Eppendorf Thermomixer C, Stevenage, UK), sonication (four 4 s 20 kHz pulses; Sonics and Materials Vibra-Cell VCX130PB, Leicestershire, UK) followed by a second 30 min shake. Once digested, subsamples were diluted with 1.610 ml TE buffer, then centrifuged at 14,000 g for 15 min to precipitate cell debris. Supernatants were removed and diluted 272-fold to produce working preparations within the limits of detection at a final n-lauroylsarcosine concentration of 0.1%.

Nucleic acid concentrations in diluted supernatants were quantified by the subtraction of fluorescence attributed to RNA and DNA upon sequential digestion with RNase then DNase. Duplicate 0.075 ml aliquots of diluted supernatants were loaded into non-binding surface 96 well microplates (Corning 3650; Flintshire, UK) alongside nucleic acid standards (16S + 23S ribosomal RNA from *E. coli* MRE600, 10206938001; DNA from calf thymus, D4764) and stained with 0.075 ml 2000-fold diluted Quant-iT$^{TM}$ RiboGreen® (Invitrogen, Paisley, UK). Total fluorescence at 485 nm excitation and 520 nm emission wavelengths was then measured in a fluorometer (BMG Labtech FLUOstar Omega; Buckinghamshire, UK). Plates were incubated at 30°C inside the fluorometer for 15 min prior to all fluorescence measurements in order to stabilize temperatures between and within readings. RNA was measured as the reduction in fluorescence after digestion with RNase A (0.010 ml 0.5 U ml$^{-1}$ from bovine pancreas, R6513). DNA fluorescence was separated from background fluorescence by digestion with DNase 1 (0.010 ml 0.075 U ml$^{-1}$ from bovine pancreas, D4263) and $MgCl_2$ and $CaCl_2$ cofactors for 1 h at 37°C. RNA and DNA concentrations were calculated based on standard curves, expressed as a ratio and averaged across both duplicates and both tissue subsamples for analysis. The mean ± SD ratio of DNA to RNA standard curve slopes was 2.21 ± 0.38**.**

The mass of the stomach contents was expressed as a proportion of the total fish mass (including stomach contents; hereafter ‘stomach fullness’). Normality and homoscedasticity of residuals were assessed visually: RNA:DNA did not require transformation, but fish mass and stomach fullness were log10 and arcsine square root transformed, respectively. Differences in fish mass and stomach fullness between beaches or depths were tested with t-tests. Tests of differences in RNA:DNA between beaches or depths also included fish mass as a covariate in a 2-way general linear model. Interaction terms were tested by comparing the two-way, fully-crossed model with the additive model (containing main effects only). If the interaction term could be removed, main effects were tested by dropping them from the additive model. Models were fitted by ordinary least squares in R (R Core Team 2013). Comparisons were based on partial F-tests (Quinn & Keough 2002) and changes in Akaike’s Information Criterion (with correction for small sample sizes, AICc) (Burnham & Anderson 2002). Terms were retained if removal led to a significant drop in variance explained (α = 0.05) or an increase in AICc by more than 2.

***16S and 18S rRNA sequencing***

Gut microbiome and prey types were examined using high throughput sequencing in the remaining five fish collected from each site. Fish gastrointestinal tracts were removed whole in sterile conditions using autoclave-sterilised tools. Gut content was separated from the gut wall with the exception of three Caolisport Shallow fish which were too small. DNA extractions were performed on the gut wall (for 16S sequencing) or gut contents (for 18S sequencing) using the DNeasy™ Blood & Tissue Kit (QIAGEN, West Sussex, UK) according to the manufacturer’s recommendations. In the case of the three smaller Caolisport fish, DNA was extracted from whole gut samples (wall + gut) on which both the 16S and 18S sequencing was performed. No sample pooling was performed.

Prokaryotic 16S rRNA V3-V4 regions were amplified using fusion primers 341F (5’-[TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG]CCTACGGGNGGCWGCAG-3’) and 805R (5’-[GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG]GACTACHVGGGTATCTAATCC-3’) (Klindworth et al. 2013), which consisted of the locus-specific primer sequence ligated to Illumina adapter consensus sequence (indicated in square brackets). The eukaryotic 18S rRNA V4 region was amplified with the fusion primers F-574 (5’--[TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG]GCGGTAATTCCAGCTCCAA-3’) and R-952 (5’- -[TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG]TTGGCAAATGCTTTCGC-3’) (Hadziavdic et al. 2014).

The PCR reactions were carried out in 25 µl volumes consisting of 12.5 µl KAPA HiFi HotStart Mastermix (Roche Diagnostics), 12.5 ng genomic DNA and 1 µl forward and reverse primers (10 µM). Amplification was carried out on a Veriti 96-well thermocycler (Thermo Fisher Scientific) using the following conditions: 95°C for 3 min, followed by 25 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, with a final extension of 7 min at 72°C. Following clean-up of the resulting amplicons with 0.8x volume AMPure XP beads (Beckman Coulter Ltd), libraries were dual indexed using a Nextera XT v2 Index Kit (Illumina) for a further 8 PCR cycles. Tagged amplicon libraries were sequenced on an Illumina™ MiSeq using a MiSeq v3 Reagent Kit (Illumina) in the Environmental Genomics Facility at the University of Southampton, UK.

Sequencing reads were de-multiplexed on-instrument by the MiSeq Control Software (version 2.6) and checked for quality using FastQC version 0.11.8 (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Nextera XT sequencing adaptors and low quality 3' bases (quality threshold: 15) were trimmed using CutAdapt version 2.3 (Martin 2011). Reads shorter than 250 bp after trimming and quality filtering were discarded.

### ***16S and 18S metabarcoding analysis***

Analysis of 16S and 18S amplicon sequences was carried out with QIIME2 (Bolyen et al. 2019). Forward and reverse reads were initially trimmed with Cutadapt (Martin 2011) and merged using PandaSeq (Masella et al. 2012) with default parameters. Merged sequences were imported into QIIME2, quality filtered with "qiime quality-filter q-score-joined" and denoised with either "qiime deblur denoise-16S" or "qiime deblur denoise-other" for 16S and 18S, respectively. Phylogenetic trees were constructed from the resulting representative sequences using MAFFT for multiple sequence alignment (Katoh & Standley 2013) and FastTree (Price et al. 2010) for tree construction. To assign taxonomy to representative sequences, target regions of the 16S and 18S genes were extracted from the SILVA database (v132, ref) at 99% redundancy, and a Naïve Bayes Classifier was trained on the extracted sequences using the "feature-classifier" plugin (Bokulich et al. 2018). Host sequences and putative contaminants were removed from the 18S dataset by excluding sequences assigned to "Teleostei" and "Mammalia" using the "qiime taxa filter-table" command with "—p-exclude" option. This created a first 18S dataset called "18S_all" which also included the putative parasitic groups Apicomplexa (protozoans) and Ascaridida (Nematodes) reported in a separate section. Subsequently, to focus on fish diet, non-metazoan sequences were removed from the 18S dataset by keeping sequences assigned to "Metazoa", and also excluding parasitic sequences assigned to "Ascaridida". This generated a 18S dataset called "18S_metazoan" which included only non-parasitic metazoan sequences that was used to assess differences in diet among fish. The 16S dataset was similarly filtered to exclude sequences classified as “Chloroplast” as these likely resulted from the diet and were deemed not to be functional components of the microbiome.

Bar plots were generated with the command “qiime taxa barplot” and the resulting data were imported into R and plotted using phyloseq (McMurdie & Holmes 2013) and ggplot2 (Wickham 2016) packages. PCoA ordination plots were generated using the phyloseq function “qiime diversity core-metrics-phylogenetic” and then plotted using ggplot2. Variation in beta diversity among beaches and depths was assessed by means of PERMANOVA (default options) implemented in the “qiime diversity beta-group-significance” command. For beta-diversity analyses, the 16S feature table was rarefied to 579 sequences per sample, while the 18S feature table was rarified to 2823 sequences per sample, following inspection of the “interactive sample detail” plots from the feature-table summaries. Analysis of microbiome composition (ANCOM; Mandal et al. 2015), implemented in the “qiime composition ancom” command with default parameters and $\alpha = 0.05$, was then applied to identify features that differed in relative abundance between beaches at the same depth (Tralee Shallow vs. Caolisport Shallow) and between depths at Tralee (Tralee Shallow vs. Tralee Deep).

## Results

### ***Size, nutritional condition and feeding success***

The size and stomach fullness (estimated feeding success) of YOY plaice differed between Tralee and Caolisport and size also differed between the two depths at Tralee. Fish at Tralee Shallow were 75% longer ($t = 13$, $df = 43$, $p < 0.0001$), over five times heavier ($t = 16$, $df = 43$, $p < 0.0001$) and had much fuller stomachs ($t = 5.7$, $df = 38$, $p < 0.0001$) than those at Caolisport Shallow (Table 1; Table 2). The fish from Tralee Deep were 9% longer ($t = 2.1$, $df = 43$, $p < 0.046$) and 37% heavier ($t = 2.4$, $df = 43$, $p = 0.02$) but had similar stomach fullness ($t = 0.41$, $df = 38$, $p > 0.05$) relative to those at Tralee Shallow (Table 1; Table 2).

**Table 1:** Mean ± SD size (total length (TL) and mass), feeding success (stomach fullness), and nutritional condition (RNA:DNA) of young-of-the-year European plaice *Pleuronectes platessa* at two beaches on the west coast of Scotland in July 2016. Fish at Tralee were sampled from both 0.5 (Shallow) and 2 m (Deep) depth below waterline (n = 15 for Caolisport Shallow and Tralee Deep, n = 30 for Tralee Shallow).

| | **Caolisport Shallow** | **Tralee Shallow** | **Tralee Deep** |
|---|---|---|---|
| **Body length (mm TL)** | 35.8 ± 4.60 | 62.6 ± 7.6 | 68.5 ± 11.6 |
| **Body mass (g)** | 0.47 ± 0.17 | 2.7 ± 0.95 | 3.7 ± 1.6 |
| **Stomach fullness** | 0.0036 ± 0.0046 | 0.022 ± 0.015 | 0.025 ± 0.025 |

| | | | |
|---|---|---|---|
| **RNA:DNA** | 4.3 ± 0.55 | 8.3 ± 1.0 | 8.3 ± 0.76 |

Analysis of 2-way linear models identified a difference in RNA:DNA (nutritional condition) between Tralee and Caolisport Beaches but not between the two depths at Tralee Beach. In models comparing beaches, RNA:DNA of fish at Tralee Shallow was almost twice that at Caolisport Shallow (Figure 1; Table 1; $F_{(1,42)} = 101$, $p < 0.0001$; ΔAICc = 53), but did not vary with body mass, either as a main effect ($F_{(1.42)} = 3.04$, $p > 0.05$; ΔAICc = 0.74) or as an interaction with beach ($F_{(1,41)} = 0.37$, $p > 0.05$; ΔAICc = -2.1; Table 2). In models comparing depths at Tralee, RNA:DNA did not vary with body mass ($F_{(1,42)} = 2.3$, $p > 0.05$; ΔAICc = -0.050), depth ($F_{(1,42)} = 0.23$, $p > 0.05$; ΔAICc = -2.2) or their interaction ($F_{(1,41)} = 0.45$, $p > 0.05$; ΔAICc = -1.9; Table 2).

***18S: YOY Plaice Diet***

Sequencing of the 18S V4 region from YOY plaice gut contents yielded a total of 521,569 sequences for the “18S_metazoan” database. Beta-diversity analysis revealed a significant difference in diet assemblages between Caolisport and Tralee Beaches at 0.5 m depth (Figure 2; Table 2; PERMANOVA pseudo-F 2.2, 999 permutations, q-value = 0.016) but no difference between depths at Tralee (Figure 2, Table 2; PERMANOVA pseudo-F 0.95, 999 permutations, q-value = 0.487).

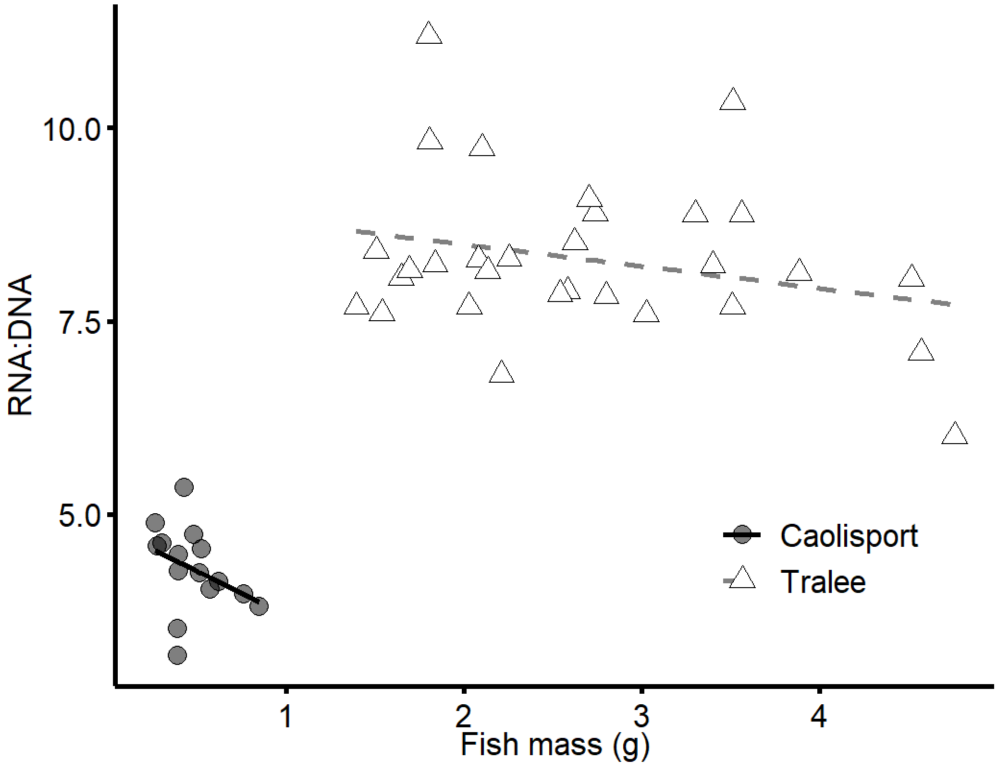

**Figure 1:** Relationship between RNA:DNA of white muscle tissue and mass of individual young-of-the-year European plaice *Pleuronectes platessa* at two beaches on the west coast of Scotland in July 2016. Fish were sampled from 0.5 m depth below waterline at both locations. Lines represent fit of 2-way general linear model.

In the "18S_metazoan" dataset, the predominant phyla were Annelida and Arthropoda comprising 55.8% and 42.8% of total sequences respectively (Figure 3). Approximately 99.5% of the annelid sequences were assigned to *Lanice conchilega*, but *Clymenura clypeata* accounted for 41.5% of sequences in one Caolisport fish (fish 71). Arthropod sequences were mostly copepods (99.7%) followed by podocopa (found in two Caolisport Shallow fish, fish 72 and 73, 0.2% of total sequences). Copepods were mostly assigned to order Harpacticoida (43.8%) but the majority (56.1%) could not be classified below the subclass level. Non-parasitic Nematode sequences were also observed (0.76% of the total sequences), but were concentrated in two Tralee samples (fish 16: 8.1%, fish 49: 89.4% of sequences for each fish). These were assigned to Chromadorida in the family Chromadoridae, a family of free-living worms commonly associated with freshwater and marine sediments. The relative abundances of known prey other than polychaetes, crustaceans and nematodes were very low, and there was a notable scarcity of molluscs (< 0.1% of total sequences).

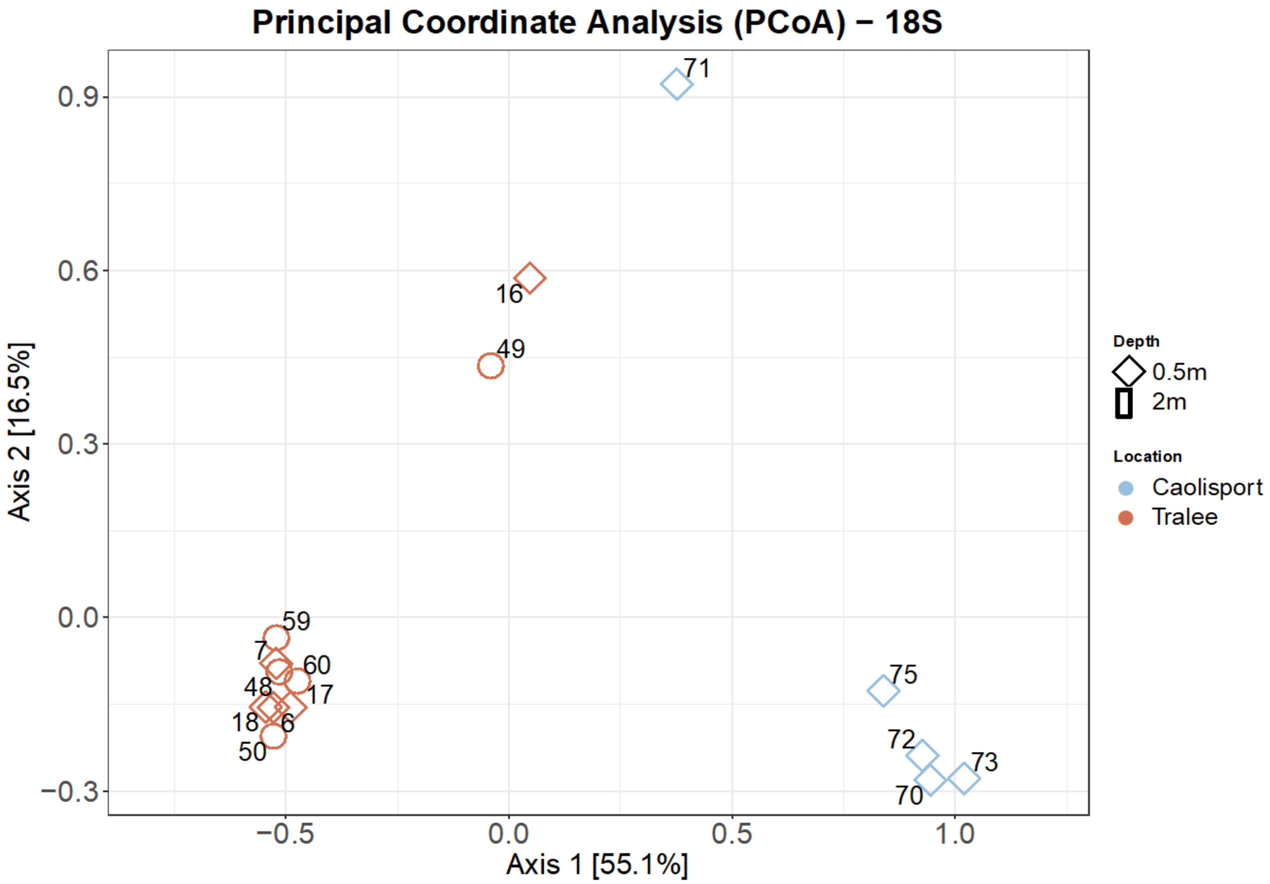

**Figure 2** Ordination of Principal Coordinate Analysis (PCoA), representing Bray-Curtis distances among prey assemblages in stomachs of individual young-of-the-year European plaice *Pleuronectes platessa* at two beaches on the west coast of Scotland in July 2016, based on the sequencing of the 18S V4 region (n = 5 from each site) using the "18S_metazoan" dataset (see Materials and Methods). Numbers associated with individual points refer to fish ID: associated body sizes are provided in Figures 3-5.

Diets of YOY plaice differed between the two beaches and two depths compared. Gut contents were dominated by crustaceans at Caolisport Shallow, annelids at Tralee Shallow and nematodes or annelids at Tralee Deep (Figure 3). ANCOM analysis indicated that phylum Annelida was significantly more abundant in Tralee Shallow fish compared to Caolisport Shallow (ANCOM W = 7); whereas no statistical difference was found for the phylum Arthropoda (W = 3; Table 2). Annelida were also significantly more abundant in fish from Tralee Shallow than in fish from Tralee Deep (W = 2; Table 2). ANCOM at a feature level suggested that *Lanice conchilega* were more abundant in fish from Tralee Shallow than Caolisport Shallow (W = ) but no differences among depths were detected.

**Figure 3:** Composition at phylum level of 18S sequences (using the "18S_metazoan" dataset; see Material and methods) from guts of young-of-the-year European plaice *Pleuronectes platessa* at two beaches on the west coast of Scotland in July 2016, based on sequencing of the 18S V4 region. Fish were sampled from 0.5 m depth below waterline at both locations and also from 2 m below waterline at Tralee (n = 5 from each site). Abundance is expressed as a percentage of the total sequence library for individual fish. Only macrofaunal phyla present at above 1% mean overall relative abundance are plotted. The excluded phyla were combined into a 'Remainder' category. Some bars do not reach 100% since they are missing the sequences that could not be identified to phylum level. Fish within sites are arranged in order of increasing size. Numbers below bars refer to fish ID with fish size (mm total length) in parentheses.

### *18S: Parasite Sequences*

Alongside diet-related taxa, the 18S libraries also contained sequences from putative parasitic groups. Within the "18S_all" dataset, Apicomplexa was the most common of these phyla and while usually present at low relative abundances of < 5% of individual fish libraries, they reached burdens > 50% in three Tralee Deep fish. Nearly all (99%) apicomplexan sequences were assigned to Coccidia (genus), a parasitic group known to infect a range of marine vertebrate and invertebrate hosts, including flatfishes and polychaetes. Putative parasitic Nematodes belonging to the family Ascaridida were also found. They were mostly present in one Tralee deep fish (sample 48, > 99% of the sequences) and in one Caolisport fish (sample 72). ANCOM analysis revealed that Coccidia sequence relative abundances did not differ between locations (Tralee Shallow vs. Caolisport Shallow, W = 0) or depths (Tralee Shallow vs. Tralee Deep, W = 0).

***16S: YOY Plaice Gut Microbiome***

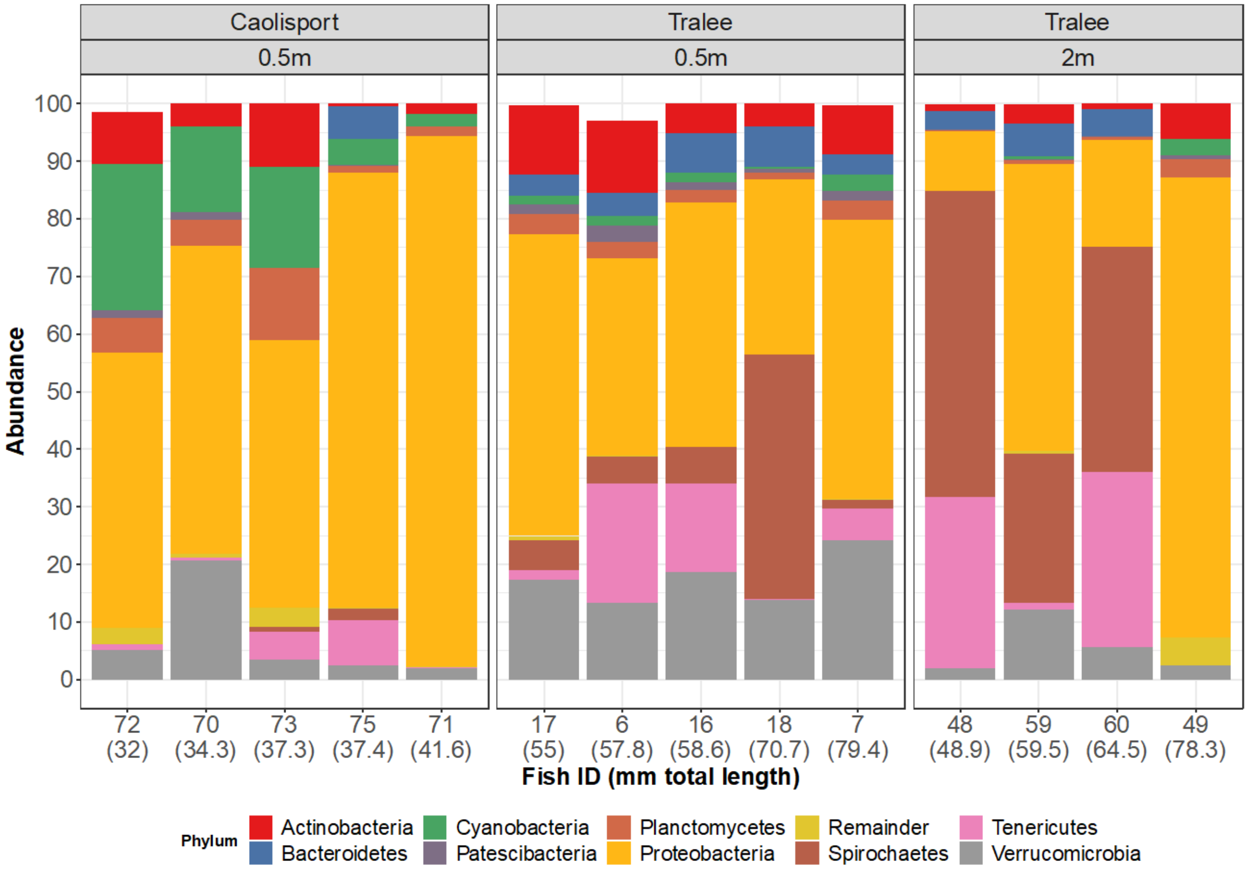

**Figure 4:** Composition at phylum level of the gut microbiota of young-of-the-year European plaice *Pleuronectes platessa* at two beaches on the west coast of Scotland in July 2016, based on the sequencing of the 16S V3-V4 regions (after excluding sequences that were assigned to "Chloroplast"). Plaice were sampled from 0.5 m depth below waterline at both locations and also from 2 m below waterline at Tralee (n = 5 from both shallow sites and n = 4 from Tralee Deep). Abundance is expressed as percentage of the total sequence library for individual fish. Only phyla present at above 0.5% mean overall relative abundance were plotted. The excluded phyla collectively represented less than 0.5% of total sequences and were combined into a 'Remainder' category. Some bars do not reach 100% since they are missing the sequences that could not be identified to phylum level. Fish within sites are arranged in order of increasing size. Numbers below bars refer to fish ID with fish size (mm total length) in parentheses.

Sequencing of the 16S V3-V4 regions from YOY plaice gut contents yielded a total of 179,769 sequences after quality filtering and denoising for a total of 1243 Operational Taxonomic Units (OTUs). One fish from Tralee Deep (fish 50) yielded a low quality 16S library (< 1000 sequences) and was removed from the analysis. Following removal of chloroplast sequences, which originally made up 96% of the cyanobacterial fraction, nine bacterial phyla, each represented at > 0.5% of total sequences accounted for 98.9% of the total sequences: Actinobacteria, Bacteroidetes, Cyanobacteria, Patescibacteria, Planctomycetes, Proteobacteria, Spirochaetes, Tenericutes, and Verrucomicrobia. Proteobacteria was the dominant phylum in the dataset, representing 45.5% of the total sequences (Figure 4). The next most abundant phyla were Spirochaetes (17.5% of total), Tenericutes (11.5% of total) and Verrucomicrobia (10.7% of total, Figure 4). All other phyla were present at < 10% of the total sequences (Figure 4). At the phylum level, ANCOM analysis revealed higher relative abundances of Cyanobacteria (W = 7) but lower abundances of Actinobacteria and Spirochaetes (W = 4 and W = 3, respectively) at Caolisport Shallow compared to Tralee Shallow (Table 2). No difference was found between the two depths at Tralee (Table 2).

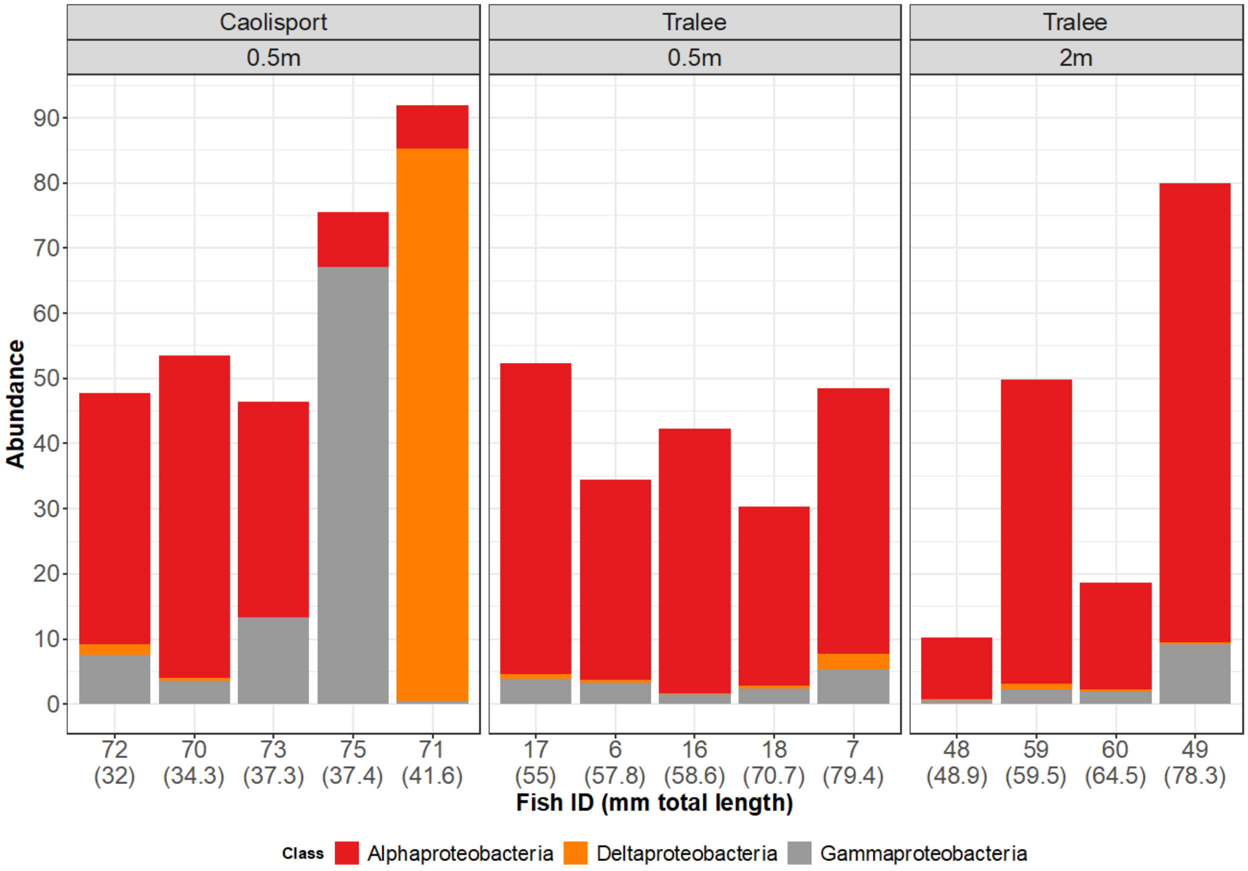

**Figure 5:** Class composition within phylum Proteobacteria in gut microbiota of young-of-the-year European plaice *Pleuronectes platessa* at two beaches on the west coast of Scotland in July 2016, based on the sequencing of the 16S V3-V4 regions. Plaice were sampled from 0.5 m depth below waterline at both locations and also from 2 m below waterline at Tralee (n = 5 from both shallow sites and n = 4 from Tralee Deep). Abundance is expressed as percentage of the total sequence library for individual fish. Fish within sites are arranged in order of increasing size. Numbers below bars refer to fish ID with fish size (mm total length) in parentheses.

Dominant classes within the phylum Proteobacteria were Alphaproteobacteria, Gammaproteobacteria, and Deltaproteobacteria (Figure 5). Alphaproteobacteria accounted for 59.7% of proteobacterial sequences overall and represented between 71.3% and 96.0% of those in individual fish libraries (median 88.6%), except in two Caolisport Shallow samples where they represented only 7.2% and 11.0% (Figure 5). The next most abundant proteobacterial class was Gammaproteobacteria (21.2% of proteobacterial sequences), which represented between 0.5% and 28.6% of proteobacterial sequences in individual fish, with the exception of one Caolisport sample in which it composed approximately 88.8% of proteobacterial sequences (Figure 5). Similarly, Deltaproteobacteria accounted for 92.1% of proteobacterial sequences in another Caolisport sample, but represented a much lower median relative abundance of 1.2% overall (Figure 5). Betaproteobacteria and Epsilionproteobacteria were not recorded.

The Alphaproteobacteria were mainly composed of members of the family Rhodobacteraceae that could not be classified to genus level (50.8% of total alphaproteobacterial sequences). The majority (69.0%) of Gammaproteobacteria sequences were assigned to *Photobacterium,* but this was largely due to high levels of Gammaproteobacteria in one sample (fish 75). In other specimens, gammaproteobacterial taxa were more varied and included Vibrionales, Cellvibrionales and Thiotrichales. Other common marine genera such as *Vibrio* and *Pseudomonas* were unusually rare (less than 0.05 % of total sequences). Deltaproteobacteria was predominantly represented by an uncultured bacterial genus from the SAR324 clade (Marine group B), which was almost exclusively responsible for the unusually high relative abundance (84% of all sequences in that library) of Deltaproteobacteria in one Caolisport sample.

Genera within the other bacterial phyla accounted for only a small proportion of the bacterial assemblage. Within the spirochaetes the most abundant genera were *Spirochaeta* (13% total sequences) and *Brevinema* (3.6% total sequences). Within the Verrucomicrobia, the most abundant family was Rubritaleaceae, with *Rubritalea*, *Luteolibacter*, *Roseibacillus*, *and Haloferula,* as well as two uncultured genera from the DEV007 family accounting for > 1% total sequences in at least one library. Tenericutes mostly consisted of *Mycoplasma*.

PCoA ordination of Bray-Curtis distances suggested that 16S microbiome compositions clustered based on sampling location (Figure 6). There was clear separation between Tralee Shallow and Caolisport Shallow samples (Figure 6; Table 2; PERMANOVA, 999 permutations, pseudo-F = 4.99, q-value = 0.009). At the feature level, ANCOM revealed that three features were differentially abundant: two Alphaproteobacteria, one of the family Rhizobiaceae (W = 499) and one Rhodobacteraceae (W = 607), which were both absent from Caolisport Shallow and present in all Tralee Shallow samples; and a Cyanobacteria of the family Chanobiaceae (W = 607) which was absent from Tralee Shallow but present in Caolisport Shallow (Table 2). In addition, separation between Tralee stations at different depths was observed along Axis 2 (Figure 6; PERMANOVA, 999 permutations, pseudo-F = 1.72, q-value = 0.009), although ANCOM did not detect specific features that differed in relative abundance between depths (Table 2).

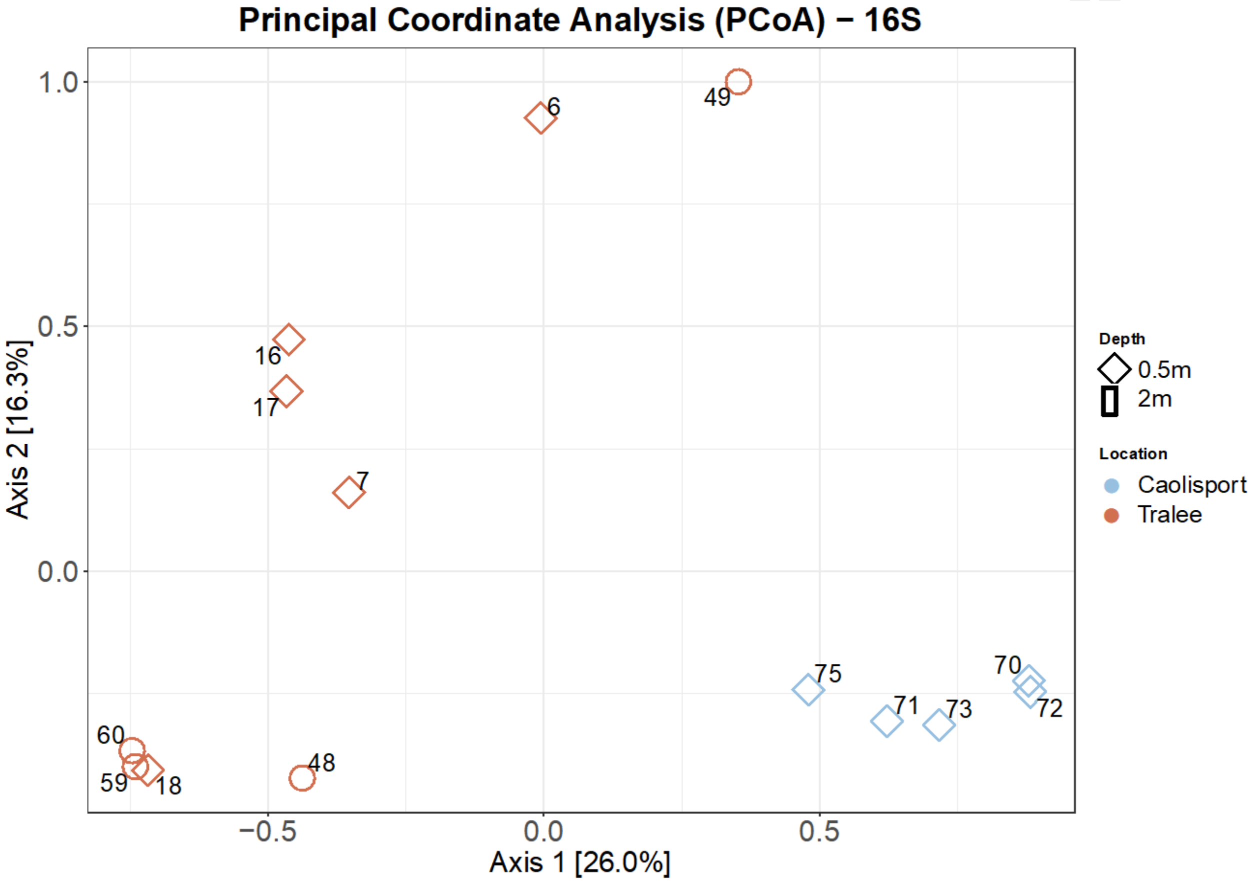

**Figure 6** Ordination of Principal Coordinate Analysis (PCoA), representing Bray-Curtis distances among gut microbiota assemblages of individual young-of-the-year European plaice *Pleuronectes platessa* at two beaches on the west coast of Scotland in July 2016, based on the sequencing of the 16S V3-V4 regions (n = 5 from both shallow sites and n = 4 from Tralee Deep). The 16S dataset was filtered to exclude sequences classified as “Chloroplast”. Numbers associated with individual points refer to fish ID: associated body sizes are provided in Figures 3-5.

**Table 2:** Spatial scales over which differences in size, diet, nutritional condition and microbiome were detected in young-of-the-year European plaice *Pleuronectes platessa* at two beaches on the west coast of Scotland in July 2016. No difference indicated by '-'.

| | **Biological parameters** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Body length and mass (Table 1)** | **Diet** | | | | **RNA:DNA (nutritional condition) (Figure 1)** | **Microbiome** | | |
| **Spatial scale** | | **Stomach fullness (feeding success) (Table 1)** | **β diversity of prey (PERMANOVA) (Figure 2)** | **Relative abundance of phyla (ANCOM) (Figure 3)** | **Relative abundance of features (ANCOM)** | | **β diversity of microbiota (PERMANOVA) (Figure 6)** | **Relative abundance of phyla (ANCOM) (Figure 4)** | **Relative abundance of features (ANCOM) (Figure 5)** |
| **Beaches (10s of km):** Tralee Shallow relative to Caolisport Shallow | 1.7 x longer 5.7 x heavier | 6.1 x fuller | Different prey assemblage | More annelids | More *Lanice conchilega* | 1.9 x higher | Different microbial assemblage | Less Cyanobacteria. More Actinobacteria and Spirochaetes. | Presence of Rhizoniaceae and Rhodobacteriaceae. Absence of Chanobiaceae |
| **Depths (10s of m):** Tralee Deep (2m) relative to Tralee Shallow (0.5m) | 1.1 x longer 1.4 x heavier | - | - | Less annelids | - | - | Different microbial assemblage | - | - |

## Discussion

Although *Pleuronectes platessa* is of considerable commercial importance and an extensively studied model species in fish ecology, factors driving variation in growth and condition of juveniles are not well resolved (Ciotti et al. 2014). In this study, we used high-throughput sequencing to characterise the diet and gut microbiome of YOY plaice at beaches on the west coast of Scotland, and to test how these vary at contrasing spatial scales in relation to the physiological status of the host. We found substantial differences in the body size, stomach fullness, nutritional condition, diet, and gut microbiome of YOY plaice between beaches separated by 10s of kilometers. Surprisingly, we also found smaller, but notable differences in the body size, diet and gut microbiome between sampling depths at the same beach, even though these were only separated by a few 10s of metres. Therefore, our study shows that there can be considerable differences in key metrics of feeding and growth at fine spatial scales along complex coastlines typical of much YOY plaice habitat (Ciotti et al. 2013c).

Our results contrast with the relative homogeneity at *ca.* 10 - 100 km spatial scales in the diets and gut microbiota of YOY plaice found by Heindler et al. (2019) in the eastern English Channel and southern North Sea. This may be due to the fact that the area studied by Heindler et al. (2019), in contrast to the west coast of Scotland, is a relatively open stretch of coast, perhaps offering more uniform environmental conditions and prey assemblages. An alternative explanation is that samples used for regional comparisons by Heindler et al. (2019) were pooled from several sites, themselves separated by tens of kilometres, and across a time window spanning several months: pooling may have smoothed out differences expected based on known small-scale (100s of metres, daily) spatiotemporal heterogeneity in the diet and growth of YOY plaice.

Diets of YOY plaice in the current study were dominated by polychaetes and crustaceans, consistent with results from studies using visual examination of gut contents from the west coast of Scotland (Edwards & Steele 1968, Steele et al. 1970, Poxton et al. 1983) and other areas (Macer 1967, Amara et al. 2001, Freitas et al. 2010, De Raedemaecker et al. 2011, Jones et al. 2020). In contrast to these previous studies, however, molluscs were conspicuously absent. Polychaete prey was almost entirely *Lanice conchilega*, a species known to form an important component of the diet at Tralee Beach (M. T. Burrows, unpublished data) but not necessarily nearby sites (Edwards & Steele 1968, Steele et al. 1970, Poxton et al. 1983). Besides resolving key prey items, 18S sequencing also identified putative parasitic taxa. The influence of parasitic infection on wild YOY plaice is unknown and would benefit from further investigation.

Diets differed considerably between beaches, with fish at Tralee having fuller stomachs and feeding almost exclusively on polychaetes and those at Caolisport preying predominantly on crustaceans. The metabarcoding study of YOY plaice by Heindler et al. (2019) found that diets along a > 100 km stretch of open coastline in the eastern English Channel and southern North Sea were remarkably stable and dominated by the crustacean *Crangon crangon* (72%) and the polychaete *Owenia fusiformis* (9%). They reported that diets changed little between spring and summer although there were small differences with body size and among years. Our results align with a growing recognition that YOY plaice are opportunistic, generalist feeders and that diets along the complex coastlines of northern Britain are highly variable at very small temporal (hours or days) and spatial (100s of metres) scales (M. T. Burrows, unpublished data; Poxton et al. 1983, Ansell & Gibson 1990, Ciotti et al. 2013c).

Our understanding of the role of the environment and prey availability on driving differences in diet and microbiota between Caolisport and Tralee is somewhat confounded by fish size as YOY plaice at Caolisport were smaller than those from Tralee. Smaller, slower growing fish at Caolisport is a consistent but as yet unexplained difference between these two sites (Ciotti et al. 2010, Ciotti et al. 2013b, Ciotti et al. 2013a, Fox et al. 2014). In this study, Caolisport fish fed primarily on copepods, while Tralee fish fed on the polychaete *Lanice conchilega*. However, there is no evidence that within each site fish were transitioning from a copepod-based to an annelid-based diet with increased size (Figure 3; fish arranged in size order). In fact, annelids (either parts or whole animals) have been previously shown to dominate the diet of the full size range of YOY plaice from Tralee (M. T. Burrows, unpublished data). Given that YOY plaice are known to exhibit considerable spatiotemporal variation in diet (Poxton et al. 1983, Ansell & Gibson 1990, Gibson 1999), we suggest that spatial differences are the cause of the diet differences, which, along with local environmental conditions and associated microbial community, are primary determinants of the gut microbiota in fishes (Talwar et al. 2018).

While this study did not examine consequences of variation in diet quality and quantity for the nutritional condition and size of juveniles at Caolisport vs. Tralee, a link seems plausible and would benefit from further investigation. Spatial variation in YOY plaice growth on the west coast of Scotland is not related to temperature, interspecific competitor density or beach productivity but is weakly and negatively related to conspecific density (Ciotti et al. 2013b, Ciotti et al. 2013a). Physical charactersitics of beaches, including wave exposure and tidal range, explain growth variation well, but the mechanisms underlying this relationship are unresolved (Ciotti et al. 2013b). Meanwhile, little is known about how variation in the quality of prey may influence growth rates. In the current study, we found that YOY plaice from Caolisport, the site with small fish in low nutitional condition and empty stomachs, consumed small crustaceans. While such prey are often abundant (Edwards & Steele 1968), they may be too small (Bregnballe 1961) or expose feeding plaice to unacceptably high levels of predation risk (Steele et al. 1970) to sustain high rates of food intake.

YOY plaice at Tralee were larger at the deeper site: this was accompanied by reduced relative abundance of annelids in their diet, but the overall diet composition, feeding success and nutritional condition did not vary. Despite some exceptions (Ciotti et al. 2013c), previous studies at Tralee (Gibson 1973, Gibson & Robb 1996, Gibson et al. 2002) and a range of other locations (Bregnballe 1961, Macer 1967, Edwards & Steele 1968, Poxton et al. 1983, Teal et al. 2008) have found a similar pattern of size difference with depth, due either to size-related differences in depth selection behaviour or to depth-related differences in growth conditions. Our study suggests that growth conditions, as reflected in stomach fullness and nutritional condition, were similar across depths. Prey quality may have differed, however, since the abundance of annelids was lower at the deeper site. We found high proportions of nematodes or platyhelminthes in stomachs of some individuals from the deeper site, although it is important to note that 18S sequencing provided relative abundances such that absolute quantities may be small if stomachs were not full. Nematodes and platyhelminthes have not previously been reported as YOY plaice prey at beaches on the west coast of Scotland (Edwards & Steele 1968, Steele et al. 1970, Poxton et al. 1983) but nematodes have been encountered in other areas (Amara et al. 2001, Heindler et al. 2019).

Overall, the gut microbiome of YOY plaice was principally composed of Proteobacteria, with a lower relative abundance of Spirochaetes, Tenericutes and Verrucomicrobiae along with minor contributions of Actinobacteria, Bacteroidetes, Cyanobacteria, Patescibacteria, and Planctomycetes. These phyla may play key roles in the nutrition, metabolism, immunity, and development of their fish hosts. Proteobacteria also dominated gut microbiota in YOY plaice from the eastern English Channel and southern North Sea (Heindler et al. 2019), but in this case the next most dominant phyla were Planctomycetes and Actinobacteria and there were also differences in the composition of minor phyla. Neither study separated gut microbiota from that found in the water, sediment or prey, and although sequencing largely focused on gut wall samples, some sequences would have been allochthonous, transient members of the gut microbial assemblage derived from prey. Despite this, we did not find marked anomalies in samples where both gut wall and contents were sequenced together or where diets differed from others at the same site (fish 16, 48, 49 and 71; Figures 3 and 4). Therefore, there is no strong evidence that the YOY plaice gut microbiome we describe derived from the prey. Indeed, Heindler et al. (2019) concluded that habitat, rather than prey composition, was the main driver of variation in YOY plaice gut microbiomes in their study.

Proteobacteria, the most common microbial phylum we encountered in YOY plaice, is an abundant and dominant member of gut microbiota in both freshwater and marine fishes (Nayak 2010, Clements et al. 2014, Givens et al. 2015). This phylum has been observed in the gut of numerous freshwater fish such as salmonids (Wong et al. 2013, Al-Hisnawi et al. 2015, Llewellyn et al. 2016) and cyprinids (Wu et al. 2010, Wu et al. 2012, Wu et al. 2013, Larsen et al. 2014), where it is a dominant element of both the transient and autochthonous microbial communities. Similarly, Givens et al. (2015) found similar results for 12 brackish water and marine bony fish species, including the flatfishes hogchocker (*Trinectes maculatus*) and southern flounder (*Paralichthys lethostigma*). High relative abundances of Proteobacteria have also been observed in the gut of other flatfishes including farmed turbot *Scophthalmus maximus* (79%; Xing et al. 2013) and wild fine flounder *Paralichthys adspersus* (68%; Ramírez & Romero 2017).

Our study of YOY plaice adds to growing evidence that the dominance of Proteobacteria is not restricted to adult fish, and also extends to earlier life stages. Bates et al. (2006) and Lan and Love (2012) observed that Proteobacteria were the most prominent taxa and numerically abundant at all stages in zebrafish juveniles. Likewise, Dulski et al. (2018) found that the core intestinal microbiome of juvenile pikeperch (*Sander lucioperca*) was predominantly Proteobacteria (92-95%), and Parris et al. (2016) reported similar results in larval and juvenile reef damselfish and cardinalfish. The relative abundance of Proteobacteria for YOY plaice in this study (29.1%) and in Heindler et al. (2019; range = 40.1 - 71.6% across regions) is lower than estimates for other fishes, although Ramírez and Romero (2017) found lower relative abundances of Proteobacteria (30% ± 24%) in aquacultured fine flounder.

The more striking difference in the gut microbiome of YOY plaice from other known fish gut microbial assemblages is in the taxonomic composition within the phylum Proteobacteria. Indeed, the majority of fish-gut microbiota studies agree that Gammaproteobacteria is the most numerically abundant protobacterial class (Desai et al. 2012, Parris et al. 2016, Dulski et al. 2018), especially in marine fishes (Ingerslev et al. 2014a). Families such as Enterobacteriaceae, Vibrionaceae, and in particular the *Vibrio* and *Photobacterium* genera, Shewanellaceae, and Alteromonadaceae usually represent major fractions of the microbial assemblages in marine fish guts, largely due to their ubiquitous distribution in marine environments (Ingerslev et al. 2014a, Givens et al. 2015). Sullam et al. (2015), for example, found a prevalence of *Vibrionales* and *Aeromonadales* in Trinidadian guppies, and Xing et al. (2013)

reported that *Vibrionales*, *Alteromonadales*, and *Enterobacteriales* were the three most abundant bacterial groups in the gut microbiome of farmed adult turbot, collectively constituting nearly 89% of bacterial sequences recovered. In the present study however, Gammaproteobacteria was dominant in only a single Caolisport individual, where *Photobacterium* represented 64% of total sequences. All the other YOY plaice gut microbiomes were instead characterised by high relative abundances of Alphaproteobacteria or, in the case of one individual, Deltaproteobacteria. Heindler et al. (2019) also found that YOY plaice gut microbiota contained relatively low Gammaproteobacteria abundances, but in their study both Alpha- and Betaproteobacteria dominated. Despite being reported in the gut microbiota of surgeonfish (Miyake et al. 2015) and in larval and juvenile reef fishes (Parris et al. 2016), Alphaproteobacteria have not commonly been encountered in the microbiome of fish. Both these previous studies suggested that Alphaproteobacteria were transient members of the gut microbiota accidently ingested along with debris. However, despite only sequencing gut contents, Alphaproteobacteria abudances were lower than in the current study suggesting that the unusual Alphaproteobacteria dominance in YOY plaice may not be solely derived from their prey.

In the current study, most Alphaproteobacteria sequences were assigned to Rhodobacteraceae: a common family and widely distributed in marine environments (Wagner-Döbler & Biebl 2006). The functional role of Rhodobacteraceae in the gut is as of yet unclear, although some members of the family have been proposed as possible probiotics for the aquaculture industry (Hjelm et al. 2004, Planas et al. 2006, Balcázar et al. 2007) owing to their production of antibacterial compounds (Hjelm et al. 2004, Wagner-Döbler & Biebl 2006, Bruhn et al. 2007). They are present in most marine environments, such as seawater or sediments, are key members of biofilms (Bruhn et al. 2007, Elifantz et al. 2013) and can occur in algae-associated microbiota (Friedrich 2012, Martin et al. 2015).

Verrucomicrobia is a commonly reported phylum in gut microbiome studies of marine adult and juvenile fishes, but is usually rare and present at much lower relative abundances than in the present study (Ingerslev et al. 2014a, Ghanbari et al. 2015, Givens et al. 2015). It is not a dominant taxon in YOY plaice in the eastern English Channel and southern North Sea (Heindler et al. 2019) nor in reports on other marine flatfishes, such as fine flounder (Ramírez & Romero 2017).

We found that the phylum Tenericutes was represented primarily by the genus *Mycoplasma* in YOY plaice. *Mycoplasma* are abundant members of gut microbiota in numerous vertebrate hosts (Whitcomb & Tully 1989, Giebel et al. 1990, Razin et al. 1998, Clemente et al. 2012) and have been found to dominate in the gut of Atlantic salmon

(*Salmo salar*; Holben et al. 2002), farmed rainbow trout (*Oncorhynchus mykiss*; Lyons et al. 2017), Trinidadian guppies (*Poecilia reticulata*; Sullam et al. 2015), and the long-jawed mudsucker (*Gillichtythys mirabilis*; Bano et al. 2007). *Tenericutes* has been reported at lower relative abundances in the guts of several other fishes (Moran et al. 2005, Kim et al. 2007, Sukhanova et al. 2014), but was not an important component in the previous study of YOY plaice (Heindler et al. 2019). Most known species of *Mycoplasma* are pathogens or parasites (Razin et al. 1998, Dandekar et al. 2002), although other species seem to have no harmful effects on their hosts and *Mycoplasma* may be normal members of the gut microbiota in some fish (Giebel et al. 1990, Bano et al. 2007).

The physiological performance of wild fish is determined by interactions between the external environment, the individual and its gastrointestinal microbiota (Talwar et al. 2018). Characterising geographic variation within and among wild animal populations can be an informative step in the challenging task of building a functional understanding of the influence that the gut microbiome has on physiological performance. Our paper demonstrates that the gut microbiome in YOY plaice along the west coast of Scotland varies at 10 km scales, that differences can also be distinguished at 10 m scales and furthermore that this variability is accompanied by variations in key aspects of fish feeding and bioenergetics at similar geographic scales (Table 2). The processes driving variation in gut microbiomes of coastal fishes, and potentially the influence of the gut microbiome on physiological performance, therefore appear to operate, and should be studied, at as little as 10 m scales. Although we did not examine the correlation among factors directly, substantially reduced nutritional condition and size at one of the beaches (Caolisport Shallow) was associated with lower stomach fullness, reduced consumption of annelids and differences in the abundance and presence of specific microbial taxa: Cyanobacteria, Actinobacteria, Spirochaetes, Rhizobiaceae, Rhodobacteraceae and Chanobiaceae.

Our conclusion that processes determining the diet, microbiota, and the associated nutritional condition of juvenile fish operate at fine spatial scales forms an important departure point for future studies to understand drivers of variation in physiological performance of young fish *in situ*. The next step could be a much larger study involving extensive sampling to better partition the variation across different spatial scales. There is also a need for targeted lab and field manipulations as well as mensurative experiments to establish the relative contribution of the environment, host and diet in driving variations in the microbiome and to quantify the influences of these variations on YOY plaice physiology. Ultimately, by resolving how environmental, host-specific and feeding-related factors interact to shape gut microbial assemblages and identifying resulting consequences for host performance, we stand to elucidate fundamental processes underpinning the dynamics of wild fish populations (Talwar et al. 2018).

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