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A health survey of the reef forming scleractinian cold-water corals *Lophelia pertusa* and *Madrepora oculata* in a remote submarine canyon on the European continental margin, NE Atlantic

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ABSTRACT

Monitoring of cold-water corals (CWCs) for pathogens and diseases is limited due to the environment, protected nature of the corals and their habitat and as well as the challenging and sampling effort required. It is recognised that environmental factors such as temperature and pH can expedite the ability of pathogens to cause diseases in cold-water corals therefore the characterisation of pathogen diversity, prevalence and associated pathologies is essential. The present study combined histology and polymerase chain reaction (PCR) diagnostic techniques to screen for two significant pathogen groups (bacteria of the genus *Vibrio* and the protozoan Haplosporidia) in the dominant NE Atlantic deep-water framework corals *Lophelia pertusa* (13 colonies) and *Madrepora oculata* (2 colonies) at three sampling locations (canyon head, south branch and the flank) in the Porcupine Bank Canyon (PBC), NE Atlantic. One *M. oculata* colony and four *L. pertusa* colonies were collected from both the canyon flank and the south branch whilst five *L. pertusa* colonies were collected from the canyon head. No pathogens were detected in the *M. oculata* samples. Neither histology nor PCR detected *Vibrio* spp. in *L. pertusa*, although Illumina technology used in this study to profile the CWCs microbiome, detected *V. shilonii* (0.03%) in a single *L. pertusa* individual, from the canyon head, that had also been screened in this study. A macroborer was observed at a prevalence of 0.07% at the canyon head only. Rickettsiales-like organisms (RLOs) were visualised with an overall prevalence of 40% and with a low intensity of 1 to 4 (RLO) colonies per individual polyp by histology. *L. pertusa* from the PBC canyon head had an RLO prevalence of 13.3% with the highest detection of 26.7% recorded in the south branch corals. Similarly, unidentified cells observed in *L. pertusa* from the south branch (20%) were more common than those observed in *L. pertusa* from the canyon head (6.7%). No RLOs or unidentified cells were observed in corals from the flank. Mean particulate organic matter concentration is highest in the south branch (2,612 μg l^{-1}) followed by the canyon head (1,065 μg l^{-1}) and lowest at the canyon flank (494 μg l^{-1}). Although the route of pathogen entry and the impact of RLO infection on *L. pertusa* is unclear, particulate availability and the feeding strategies employed by the scleractinian corals may be influencing their exposure to pathogens. The absence of a pathogen in *M. oculata* may be attributed to the smaller number of colonies screened or the narrower diet in *M. oculata* compared to the unrestricted diet exhibited in *L. pertusa*, if ingestion is a route of entry for pathogen groups. The findings of this study also shed some light on how environmental conditions experienced by deep sea organisms and their life strategies may be limiting pathogen diversity and prevalence.

1. Introduction

Coral pathogen and disease studies allow researchers to understand the dynamics and mechanisms of infection, their interaction with the host organism and the environment. Coral diseases may cause rapid mortality and injury of cold-water and tropical corals, thereby affecting their geographic distribution (Woodley et al., 2016). Disease studies in coral species can be used as a proxy of stress conditions that the corals

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are exposed to in the environment (Woodley et al., 2016). Environmental stressors such as sedimentation, low salinity and high temperature can change healthy coral-associated microbes to become virulent coral-associated microbes in tropical and cold-water corals (Galloway et al., 2007; Thurber et al., 2009; Mouchka et al., 2010; Vidal-Dupiol et al., 2011; La Riviere et al., 2013; Vezzulli et al., 2013; Kvitt et al., 2015). Coral disease studies have focused primarily on stony shallow water corals, with less of a focus on soft shallow water corals and even fewer studies on cold-water corals (Hall-Spencer et al., 2007; Rosenberg and Loya, 2013; Woodley et al., 2016). Historical monitoring of cold-water corals for pathogens and disease has been limited due to the environment, protected nature of the corals and their habitat, as well as the sampling effort required resulting in much less knowledge than for tropical coral health. It is recognised that there is a lack of a model species for the surveying of cold-water coral diseases (Work and Meteyer, 2014). Also, there remains a limitation to develop frameworks to systematically characterise cold-water and tropical coral pathologies (Galloway et al., 2007; Pollock et al., 2011).

Cold-water coral reefs formed by the dominant NE Atlantic framework corals *Lophelia pertusa* and *Madrepora oculata* have been recognised as hotspots for biodiversity (Roberts et al., 2006). *L. pertusa* and *M. oculata* show slow growth and survival at temperatures between 4 to 13 °C and salinities of 32 to 38.8 psu (Freiwald, 2003; Roberts et al., 2009). The two scleractinian corals live permanently in the dark and do not form associations with *Symbiodinium* spp., they also lack a carbon source and are filter feeders. Both coral species and the reefs that they form can be associated with over 1,300 marine species (Roberts et al., 2009). Studies of diseases in cold-water corals are limited, despite their importance as ecological engineers (Jonsson et al., 2004). Health surveys that have been carried out on cold-water corals include species from the southwest England (gorgonian *Eunicella verrucose*: (Hall-Spencer et al., 2007)), and Indonesia (scleractinian *Madrepora* spp. (Grygier and Cairns, 1996)). The pathogens identified (using histological and/or molecular techniques) in those studies included bacteria of the genus *Vibrio* spp., and the petracid crustacean *Patrarca madreporae* respectively. Pathogenic *Vibrio* spp. are ubiquitous in the environment, including the deep sea, and most species are problematic for organisms. A recent study isolated *Vibrio bathopelagicus* sp. nov. (closely related to pathogenic coastal *Vibrio* strains) from a water depth of 3309 m (Lasa et al., 2021), making pathogenic *Vibrio* a potential threat to deep sea ecosystems. It is recognised that Haplosporidia and *Vibrio* spp. are problematic in a host population and are associated with mortalities when they are present at very high prevalences (Bythell et al., 2002; Hall-Spencer et al., 2007).

The Haplosporidia are a significant pathogen group globally that have been associated with mortality events in near shore marine invertebrate species (Lynch et al., 2007; Arzul and Carnegie, 2015). A Haplosporidian of the rainbow abalone *Haliotis iris* that is closely related to *Bonamia exitiosa* contain within its cytoplasm a Rickettsiales-like bacteria that belongs to the spotted fever group (SFG) and is pathogenic (Hine et al., 2002). Pathogenic Haplosporidia may exist as spores, plasmodia, single cell uninucleate or binucleate life stages in their hosts (Burreson and Ford, 2004; Stentiford et al., 2013; Lynch et al., 2020). The Haplosporidian species *Urosporidium crescens* and *Haplosporidium costale* have been detected in methane seeps (Takishita et al., 2007) and their presence in the deep sea poses a possible threat to deep sea anthozoans. The genera *Urosporidium* and *Haplosporidium* are spore forming Haplosporidia (Molloy et al., 2012), which may have existed as spores due to the harsh environmental conditions that exist in methane seeps. These spores can be transported from shallow waters into the deep sea (Le Moigne, 2019) and from methane seeps into the water column. Haplosporidian spores can remain in the environment for decades until there are favourable conditions for growth and re-emergence from this dormant life stage (Ford et al., 1999; Burreson and Ford, 2004).

The order Rickettsiales contains clinically important bacterial groups (collectively called Rickettsiales-like organisms (RLOs)) that are commonly detected in the epithelial and digestive tissues of both deep and shallow marine invertebrates (Gulka et al., 1983; Castelli et al., 2016; Getchell et al., 2016). They exist in different sizes and morphological forms (Noguchi, 1926; Humphrey, 2008). The pathogenic significance of RLOs in corals is unclear due to their high prevalence in apparently healthy and diseased corals (Casas et al., 2004; Miller et al., 2014; Godoy-Vitorino et al., 2017). Detection of novel pathogens (including Haplosporidia) in marine invertebrates has become increasingly difficult due to host selection and partitioning (Lynch et al., 2020) as well as our excessively dependent use of generic molecular markers that are unable to amplify the highly divergent genes of pathogens (Hartikainen et al., 2014).

The present study combined histology and molecular techniques (standard PCR and Illumina sequencing) to investigate: 1) the presence/absence of Haplosporidia and *Vibrio* spp. in *L. pertusa* and *M. oculata* in the Porcupine Bank Canyon (PBC), 2) described other potential pathogens observed in the tissues of *L. pertusa* and *M. oculata*, and 3) report the distribution of these potential pathogens in the PBC. Findings from this study will build on the limited existing knowledge of pathogen diversity and prevalence in cold-water coral species.

2. Materials and methods

2.1. Study site, sampling and storage

The Porcupine Bank Canyon is located 310 km to the southwest of Ireland. Coral colonies (n = 15; 2 *M. oculata* and 13 *L. pertusa*) were collected during a cruise in May 2019 (Lim et al., 2019) within a 100 m periphery of each of three site locations: canyon head (52°13.6371, 14°55.5389), canyon flank (51°58.40933, 15°02.49959) and south branch (51°52.20624, 15°02.01476) in the PBC (Appah et al., 2020) (Fig. 1). The canyon head was sediment dominated and exhibited a gentle slope while the flank reveals a steep slope with exposed bedrock. The south branch is a small canyon system attached to the main canyon, where the presence of cold-water coral frameworks has been noted (Mazzini et al., 2012). Current speed in the PBC ranges from 34.1 cm s^{-1} to 114.2 cm s^{-1} and is highest at the canyon flank (31.3 cm s^{-1}) and lowest in the canyon head (18.2 cm s^{-1}) (Appah et al., 2020; Lim et al., 2020). Also, the water flow is generally uni- and bidirectional in the canyon head and flank and unidirectional in the south branch (Lim et al., 2020). Mean particulate organic matter concentration in the south branch is 2,612 μg l^{-1} in the canyon head 1,065 μg l^{-1} and in the canyon flank 494 μg l^{-1} (Appah et al., 2020).

Coral samples from the canyon head were collected from depths of between 710 and 720 m, the flank was between 688 and 693 m and the south branch were between 600 and 610 m water depth. The temperature and salinity ranges were 8.7 to 10 °C and 34.0 to 38.0 psu respectively. *L. pertusa* and *M. oculata* samples were collected with the guided manipulator arm of a remotely operated vehicle (ROV) and stored in bio boxes. For histological analysis, five colonies were collected from each of the three site locations, and a branch with five polyps was broken off from each colony resulting in twenty-five individual polyps being processed and screened per site location. One *M. oculata* colony and four *L. pertusa* colonies were collected from both the canyon flank and the south branch respectively whilst five *L. pertusa* colonies were collected from the canyon head. Additionally, five polyp samples were collected from each colony fragment for DNA extractions and Illumina sequencing. Coral samples collected for histology were fixed in Davidson's solution for 48 h and transferred into 70% ethanol at room temperature for the duration of the cruise. Davidson-fixed coral samples were used for formalin fixed paraffin-embedded (FFPE) tissues DNA extractions and standard PCR techniques. Also, coral samples collected for DNA extractions and Illumina technology were stored in 100% ethanol at − 40 °C (Straube and Juen, 2013). Coral samples were decalcified with TBD-2 formic acid decalcifier (Thermo Scientific). Each

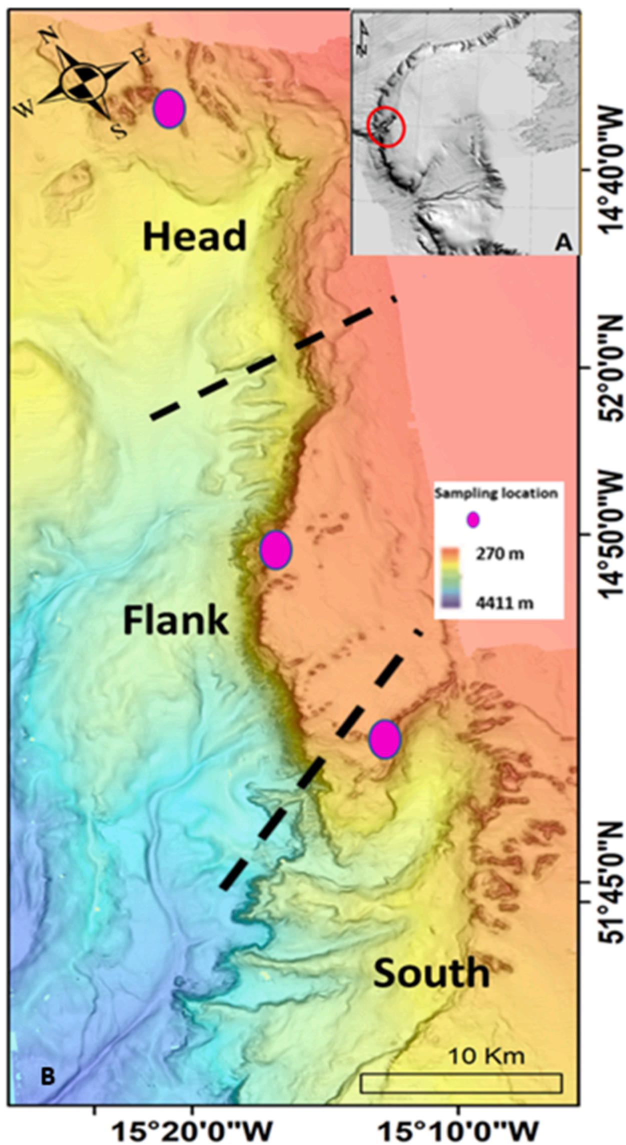

Fig. 1. Location map showing: A) the Porcupine Bank Canyon (PBC) on the Irish continental margin (red ellipse) and the, B) different areas of the PBC. The black broken line demarcates the canyon head, canyon flank and south branch in the PBC.

polyp was placed in an individual tissue embedding histocassette (n = 75 polyp samples: *L. pertusa* (n = 65 polyps) and *M. oculata* (n = 10 polyps)).

2.2. Histology

Fixed samples were then prepared for embedding in wax, for histological examination, using a 20-hr dehydration cycle of graded volumes of ethanol adapted from Howard (2004). The paraffin embedded samples were then sectioned using a Leica microtome at 5 µm, were mounted on sterile glass slides, and air dried for 7 days prior to Haemotoxylin and Eosin staining (Humason, 1962). Stained samples were cover-slipped and allowed to air-dry. Microscopic image analysis of the Haemotoxylin-Eosin slides was conducted to identify the pathogen groups, including screening of the epidermal, gastrodermal and the connective tissues of the corals. Also, DNA was isolated from Davidson-fixed, formalin-fixed paraffin embedded (FFPE) tissue sections to screen *Vibrio* spp. and *Haplosporidium* spp.

2.3. DNA extraction and standard polymerase chain reaction (PCR)

DNA was extracted from 7 µm FFPE tissue sections. Polyp tissues were deparaffinized (Shi et al., 2004) and DNA extractions were carried out using a Qiagen DNA extraction kit (QIAamp DNA FFPE Tissue Kit), following manufacturer's protocol. The quality and quantity of DNA were evaluated using a NanoDrop 1000 spectrophotometer (Thermo-Scientific). Good-quality DNA will have an A_{260}/A_{280} ratio of 1.7 to 2.0. A reading of 1.6 does not render the DNA unsuitable for any application, but lower ratios indicate that more contaminants such as proteins are present. Routinely used generic PCR assays (Hap1/R1, Hap1/*R*3, ssu980/R1) for Haplosporidia and specific (MSXA/MSXB) haplosporidian primers (Table 1) were run on extracted DNA samples following Renault et al. (2000) and Molloy et al. (2012) while PCR for *Vibrio* spp. was performed as per Notaro et al. (2021), Albuixech-Martí et al. (2021a) and Kett et al. (in review). Positive controls (i.e. Haplosporidia genomic DNA and *Vibrio* spp. from heavily infected cockles) were included for all PCRs. A negative control consisting of double distilled water (ddH_2O) was included in each PCR. Amplifications of *Haplosporidium* spp. were carried out in a total reaction volume of 20 µl, including approx. 1 µl template DNA, 4 µl of 5X green buffer, 3.2 µl of 1.25 mM dNTPs, 0.1 µl GoTaq DNA Polymerase (Advanced Biotechnologies™), 11.3 µl ddH_2O and 0.2 µl each of forward and reverse primers (Table 1). The thermal profiles comprised of one cycle of denaturation at 95 °C for 180 s followed by 35 cycles at 95 °C for 30 s, 48–59 °C for 30 s, 72 °C for 30–60 s and a final extension at 72 °C for 300 s. Amplifications of *Vibrio* spp. were carried out in a total reaction volume of 25 µl, including approx. 1 µl template DNA, 5 µl of 5X green buffer, 5 µl of 1.25 mM dNTPs, 0.1 µl GoTaq DNA Polymerase (Advanced Biotechnologies™), 0.5 µl of 2.5 mM MgCl2, 12.9 µl ddH_2O and 0.25 µl each of forward and reverse primers (Table 1). The thermal profile for *Vibrio* spp. comprises one cycle of denaturation at 95 °C for 60 s followed by 35 cycles at 94 °C for 20 s, 56 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 4200 s. Amplicons were run on a 2% agarose gel stained with 22 µl Sybr stain (Invitrogen, Paisley, UK) and run for 35 to 45 min at 110 V for detecting DNA amplicons.

Table 1
Description of PCR primer-pairs, sequences of forward and reverse primers and expected products. T_a is annealing temperature.

Primer pair	Forward and Reverse	T_a (°C)	Size (bp)	Reference
Hap F1/ Hap R1	F: GTT CTT TCW TGA TTC TAT GMA R: CTC AWK CTT CCA TCT GCT G	48	348	Renault et al. (2000); Molloy et al. (2012)
Hap F1/ Hap *R*3	F: GTT CTT TCW TGA TTC TAT GMA R: AKR HRT TCC TWG TTC AAG AYG A	48	348	Renault et al. (2000); Molloy et al. (2012)
SSU980/ Hap R1	F: CGA AGA CGA TCA GAT ACC GTC GTA R: CTC AWK CTT CCA TCT GCT G	54	348	Renault et al., (2000); Molloy et al. (2012)
MSXA/ MSXB	F: CGA CTT TGG CAT TAG GTT TCA GAC C R: ATG TGT TGG TGA CGC TAA CCG	59	573	Renault et al. (2000); Molloy et al. (2012)
Vibrio spp. F3/R3	F: CAA CAG AAG AAG CAC CGG CT R: CAC GCT TTC GCA TCT GAG TG	56	–	Albuixech-Martí et al. (2021a); Notaro et al. (2021); Kett et al. (in review)

2.4. DNA extraction, Illumina sequencing technology and bioinformatics for microbiome analysis (Appah et al., unpublished)

Coral samples were crushed using liquid nitrogen and DNA extraction was performed with DNeasy PowerSoil Pro kit (Qiagen) following manufacturer's protocol. Genomic DNA samples were sent to Novogene (Cambridge, UK) for amplicon-based metagenomics sequencing to be carried out. The purity and concentration of genomic DNA samples were assessed on a 1% agarose gel and Qubit Fluorometer (Invitrogen, USA) respectively. Amplicon products of the 16S rRNA gene at the V3-V4 region were used to prepare sequencing libraries. Amplicon products at concentrations ≥ 12 ng/μl were used to prepare sequencing libraries. PCR reactions were performed in a 20 μl reaction volume using the Phusion® High-Fidelity PCR Master Mix (New England Biolabs) with approx. 1–2 ng template DNA. The final primer concentration was adjusted to 0.5 μM. Amplicons detections were carried out on a 2% agarose gel, desired bands were excised and purified using Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing was performed using the Illumina NovaSeq 6000 SP with paired ends (2 × 250 bp) strategy (Novogene). Seven coral and six non-coral samples contained sufficient sequence data (BioProject Accession Number: PRJNA729568) for read processing, quality control and bioinformatic analysis.

2.5. Bioinformatic analysis and quality control

Raw reads were quality controlled with FastQC 0.11.9 (Andrews et al., 2014), trimmed (mean quality score of 30 in a sliding window of 10 bp) with Trimmomatic 0.39 (Bolger et al., 2014) and merged into contigs using FLASH 1.2.11 (Magoč and Salzberg, 2011). Quality filtering of the sequence data to remove chimeras was performed using Dada 2 as implemented in qualitative insights into microbial ecology (QIIME 2) v2020.8 (Bolyen et al., 2019). Sequencing data were rarefied to a sample depth of 18,000 corresponding to the least number of sequences. Each Dada 2 amplicon sequence variant (ASV) was utilized for taxonomic classification via the QIIME 2 feature-classifier (Bokulich et al., 2018) with the sklearn technique. Taxonomic assignment was made from the Greengenes 13_8 database (DeSantis et al., 2006; based on 99% similarity of sequence data to the Greengenes database). Mitochondrial, chloroplast and unassigned ASVs were removed before the analysis.

3. Results

3.1. DNA quality and quantity of the CWCs

DNA quantity and quality (A_{260}/A_{280} ratio) for paraffin-embedded tissues ranged between 2.2 and 11.1 ng/μl and 1.50 to 1.88 respectively.

DNA quantity and quality for Illumina sequencing samples ranged between 3.6 and 65.5 ng/μl and 1.79 to 1.99 respectively.

3.2. Haplosporidium spp. and Vibrio spp.polymerase chain reaction (PCR)

Neither Haplosporidia nor *Vibrio* spp. were detected in the two coral species screened by PCR using multiple primer pairs (Table 1).

3.3. Histology

The quality of the coral polyp tissues was optimal for histological analysis. Histological analysis did not reveal any Haplosporidia-like life stages (spores, plasmodia, single cells) in the cells or tissues of the coral samples. Pathological conditions associated with *Vibrio* spp. infection such as haemocyte accumulation in the connective tissues (McCleary and Henshilwood, 2015) were not observed in the samples. Sloughing was observed in 13.3% (2/15) of the coral.

RLOs were detected in the epidermal 33.3% (5/15) and gastrodermal tissues 6.7% (1/15) of the *L. pertusa* samples exclusively (Fig. 2A and B). RLOs in this study were small, ellipsoid and round with eosinophilic inclusions or granules. Overall, 40% (6/15) of RLOs were detected in the polyp tissues that were screened, 26.7% (4/15) were observed in the south branch whereas 13.3% (2/15) were observed in the canyon head (Fig. 3A). The intensity of RLOs identified in individual polyps was low (1 to 4 colonies per individual). The percent prevalence of the unidentified cells was 26.7% (4/15) with 20% (3/15) detected in the south branch and 6.7% (1/15) in the canyon head while none were detected in corals from the canyon flank (Fig. 3A and B).

Generally, the size of the unidentified cells ranged between 4.06 and 19.25 μm in diameter with an average size of 7.94 μm. Both bi- and multinucleate cells with a well bounded membrane were recorded. The unidentified binucleate cell (Fig. 2C) was slightly ellipsoid in shape. An unidentified cell (most likely a macroborer belonging to the genus *Sipunculus*), was detected in *L. pertusa* 0.07% (1/15) at the canyon head exclusively. This organism showed an irregular shape that was blunt at one end (posterior) and tapered at the other (anterior), with a slight depression on both sides of the trunk (and what seem to be a groove at the ventral side close to the anterior) (Fig. 2D). The *L. pertusa* cells associated with the burrow and the acellular space that surrounds the unidentified macroborer suggests growth of the borer (Fig. 2D).

3.4. Haplosporidium spp.and Vibrio spp.illumina sequencing

The sequences obtained from the Illumina sequencing of *L. pertusa* was identified as an uncultured Rickettsiales bacterium and *Haplosporidium* sp. endosymbiont. *Vibrio shilonii* 0.03% (1/7) was detected in a single *L. pertusa* individual from the canyon head using Illumina sequencing. The uncultured Rickettsiales bacterium 0.03% (1/7) and *Haplosporidium* sp. endosymbiont 0.03% (1/7) were detected in a single *L. pertusa* individual from the canyon head.

Sequences obtained from the Illumina sequencing of *L. pertusa* had E-value of 2e-63, 100% Query Coverage and 99.29% Percent Identity to the uncultured Rickettsiales bacterium and *Haplosporidium* sp. endosymbiont AbFoot 16S RNA sequences in GenBank using BLAST analysis.

A total of 2,586,928 raw sequences yielded 529,513 effective tags for sequence analysis after processing and quality filtering of raw reads. The removal of unassigned, mitochondrial and chloroplast ASVs resulted in the least microbial taxa (18,151) being observed in *L. pertusa* in the canyon head (52°13.62616, 14°55.54294) whilst the highest microbial taxa (73,600) were detected in *M. oculata* in the canyon flank (51°58.39740, 15°02.52970). A total of 4,494 sequence variants with mean length of 190 were recorded, whilst a total of 522,608 microbial taxa were observed in the coral and non-coral samples following the removal of unassigned, mitochondrial and chloroplast ASVs. Corals contained 344,782 ASVs and non-corals 177,826 ASVs.

4. Discussion

4.1. Tissue preservation, storage and DNA extraction

Generally, DNA extracted from Davidson-fixed paraffin embedded tissues that was used for standard PCR in this study showed DNA with low quality and quantity compared to DNA from ethanol-preserved tissues used for Illumina sequencing. As a result, the pathogen groups, RLOs and *Vibrio* spp., were not detected using DNA extracted from FFPE and standard PCR but were detected using Illumina sequencing. DNA isolated from Davidson-fixed paraffin embedded tissues can result in failure in PCR amplification due to fragmentation of DNA for histological tissue preservation (Lynch et al., 2013; Ochoa et al., 2020). Also, the time and the conditions of tissue storage can affect DNA extraction and PCR amplification (Lynch et al., 2013). Different methods of DNA extractions can cause variations in DNA quality and quantity which in turn can greatly impact sequencing results (Mygind et al., 2003; Morgan et al., 2010).

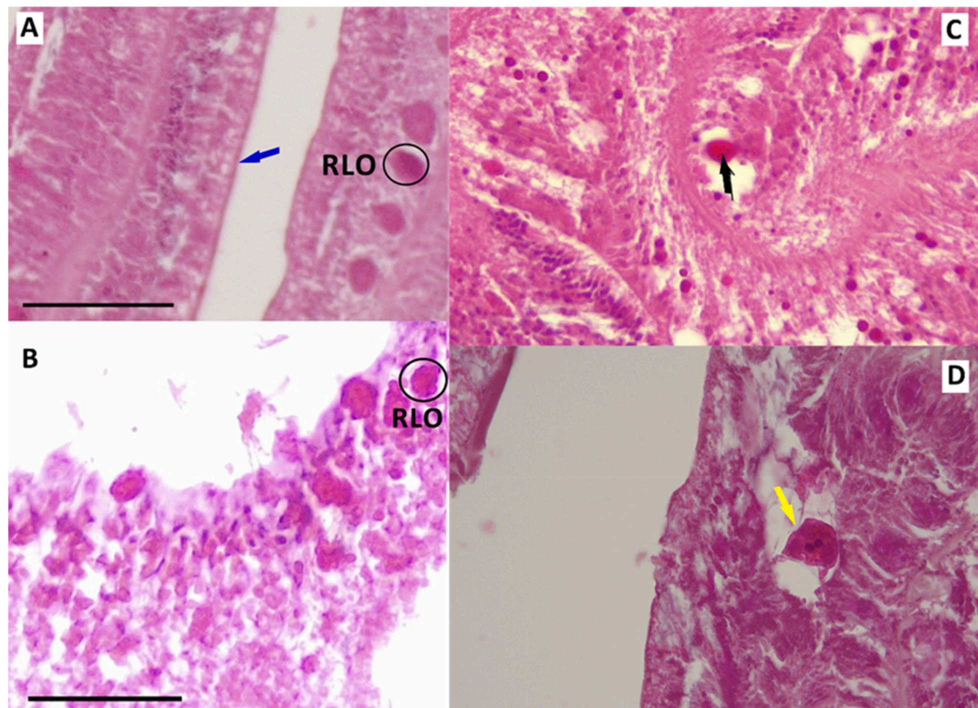

Fig. 2. Histological observations of potentially harmful cells in *L. pertusa*. scale = 50 μm: A) Rickettsiales-like organism in epithelial tissue, but without any visible pathology, B) Rickettsiales-like organism tissues without epithelium**,** C) unidentified binucleate cell, and D) unidentified macroborer. Black circle = Rickettsiales-like organism (RLOs); blue arrow = intact epithelial tissue; yellow arrow = unidentified macroborer; black arrow = unidentified binucleate cell.

4.2. Pathogen diversity and environmental factors

Pathogen diversity observed in this study was limited with two to three different pathogen groups (Illumina sequencing; RLOs and *Vibrio*) and (Histology and standard PCR; RLOs, macroborer and unidentified noncoral cells whose origin is unknown) being observed in *L. pertusa*, respectively. The low diversity of pathogens detected suggests that *L. pertusa* invests energy in an enhanced immune function, which is typical of slow-growing S-strategist organisms (Mydlarz et al., 2008; Palmer et al., 2010; Gavish et al., 2018), and may likely provide them with a reduced susceptibility to pathogen groups. RLOs generally showed low intensity in the coral tissues and were most prevalent in the south branch of the PBC. Similarly, unidentified cells were most prevalent in the south branch whereas a single macroborer was detected in the canyon head. Although the *M. oculata* specimens were sampled near the *L. pertusa*, none of these organism groups were observed in those samples. Similarly, no Haplosporidia or *Vibrio* spp. were detected in this cold-water coral species. Both scleractinian corals exhibit different feeding strategies (Mortensen, 2001; Duineveld et al., 2007; Meistertzheim et al., 2016) and this may be the reason why no potential pathogen was detected in *M. oculata*, since ingestion is a pathway for pathogen intake and subsequent infection (Lynch et al., 2007; Gavish et al., 2018). Additionally, the lack of potential pathogens in *M. oculata* can be attributed to the fewer *Madrepora* samples screened in this study because sample size is directly proportional to the probability of detecting a pathogen. *M. oculata* shows a narrower diet compared to *L. pertusa* (Mortensen, 2001; Meistertzheim et al., 2016), which may be limiting pathogen entry via ingestion.

Temperatures above 3 °C and salinities above 25 psu favour proliferation and a higher rate of infection of certain Haplosporidia respectively (Burreson and Ford, 2004; Albuixech-Martí et al., 2020). Haplosporidia were not detected in the two cold-water coral species from histological examinations and standard PCR in this study, even though the deep-sea mean temperature (9.3 °C) and salinity (36.3 psu) were optimal for the growth and infection of particular Haplosporidia species such as those belonging to the genus *Bonamia*. It is probable that local abiotic conditions on the high seas and in the deep sea such as current speed and sedimentation do not favour the development and retention of the Haplosporidia. Also, existing conditions may not have supported the growth and survival of a Haplosporidia intermediate host or vector that completes the life cycle of the pathogen. However, a negative histology or PCR result may not necessarily indicate the absence of Haplosporidia in the two deep sea coral species examined in this study. Screening for pathogens that are present at a low prevalence has been described as “looking for a needle in a haystack” even in near shore environments that are considered hot spots of infection. The probability of pathogen detection increases with the number of host specimens that are screened (Lynch et al., 2007; Albuixech-Martí et al., 2021b). Additionally, different diagnostic methods may yield different results (Lynch et al., 2013; Flannery et al., 2014) and although the extraction of DNA from FFPE samples can result in fragmented DNA being screened, the expected product size for both PCRs used in this study were small enough to be amplified (Renault et al., 2000; Lynch et al., 2013). A recent study to detect an RNA virus infecting marine shrimp using DNA from Davidson-fixed paraffin embedded tissue showed that amplicon size of approx. 230 bp detected the virus in only 4/29 samples (Ochoa et al., 2020). However, at a reduced amplicon size of approx. 150 bp, most samples were successfully amplified (Ochoa et al., 2020), suggesting that reducing amplicon size of the *Haplosporidium* from approx. 350 bp in the present study to approx. 150 bp in the future may eliminate the possibility of failure in PCR amplification of DNA from FFPE samples due to DNA fragmentation. There is generally an inherent bias towards shorter amplicons in PCR reactions. In addition, latent, early or light Haplosporidia infections may be overlooked using both methods, as has been observed in other studies (Lynch et al., 2013; Flannery et al., 2014) i.e. the part of the tissue block that was sectioned and used for histology and subsequent PCR may not have been where the pathogen was localised in the tissues of the coral species, thus highlighting the need for more individuals from a colony to be screened. Also, it is important to note that older individuals with longer exposure

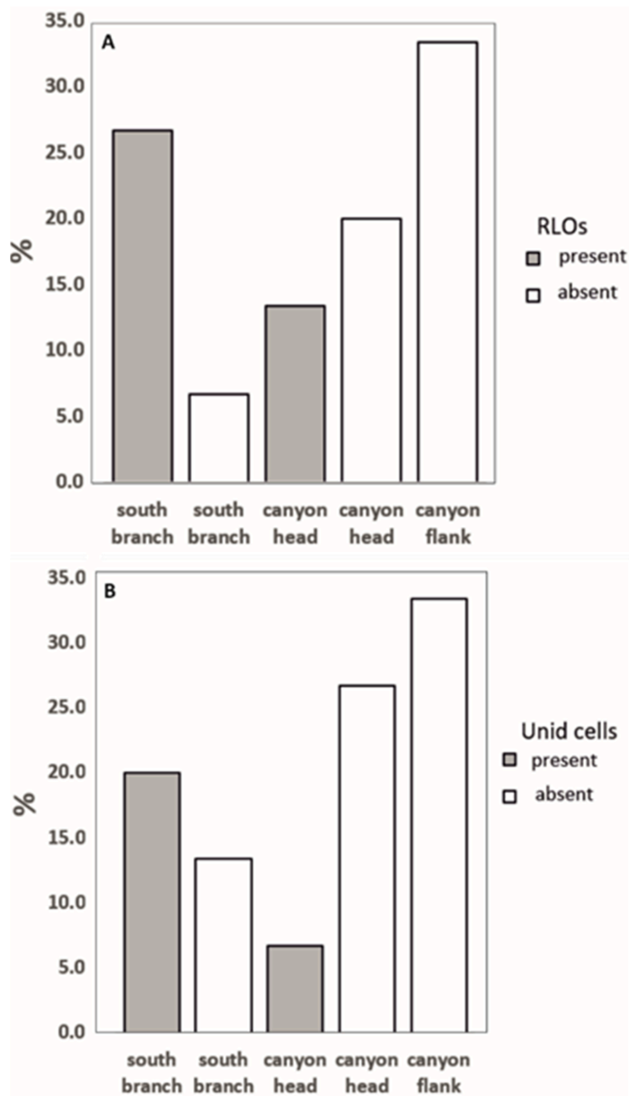

Fig. 3. The percent prevalence of: A) Rickettsiales-like organisms, and B) unidentified cells. Unid cells = unidentified cells; RLOs = Rickettsiales-like organisms. Descriptive data analyses were used, with the percentage of absence or presence for all samples analysed in the canyon head, canyon flank and the south branch summing up to 100%. The data correspond to *Lophelia pertusa*.

to pathogen groups build up resilience/resistance over time (Albuixech-Martí et al., 2020). In the present study however, sequences obtained from the Illumina sequencing of *L. pertusa* was identified as a *Haplosporidium* sp. endosymbiont that is associated with the abalone *Haliotis iris*. Of importance, their presence in cold-water corals suggests that Haplosporidia can be transported from surface waters into deep seas.

Vibrio spp. are closely associated with chitin-containing organisms (e.g. copepods), which represent one of the most important environmental reservoirs of these bacteria. Copepods have been described from different deep sea environments (Humes, 1987; Thistle and Eckman, 1990; Auel, 1999; Bode et al., 2017) and is a known food source for *L. pertusa* (Dodds et al., 2009). They are associated with phytoplankton as well as aquatic plants and can also infect oysters at temperature ranges of 9 to 13 °C and 19 to 20 °C (Health and Welfare (AHAW), 2010; Vezzulli et al., 2016). No visible *Vibrio*-associated pathologies were observed nor were they amplified in the standard PCR. This might be due to the *Vibrio* spp. being present at a low prevalence and intensity or they may simply be absent in the coral tissues. More importantly, the mean temperatures and salinities in the PBC generally are unfavourable for *Vibrio* spp. growth (Banin et al., 2003).

Recent histopathological studies have described pathogenic RLOs in scleractinian corals (Klinges et al., 2019), which have been associated with oil contamination in deep sea waters (Simister et al., 2016). Sequences obtained from the Illumina sequencing of *L. pertusa* in this study was identified as an uncultured Rickettsiales bacterium and a Rickettsiae (an endosymbiont of *Haplosporidium* sp.) contained within the cytoplasm of Haplosporidia closely related to *Bonamia exitiosa*. The uncultured Rickettsiales bacterium has been associated with the Japanese spiky sea cucumber *Apostichopus japonicus*. These RLOs are thought to possess parasitic capabilities. RLOs are obligate pathogens that can infect many marine organisms and cause various degrees of pathologies in the tissues of infected species (Miller et al., 2014). The intensity of RLOs detected in this study was similar to infection intensities described in tropical corals from the Caribbean (Miller et al., 2014; Peters, 2014). In the present study, gastrodermal epithelial tissues appeared sloughed and lysed with vacuolations similar to observations that were made in RLOs associated infections of the staghorn coral *Acropora cervicornis* tissues (Miller et al., 2014). Sloughing is characterised by lost or loose epithelial cells while necrosis is characterised by vacuolisation and cell rupture (Quéré et al., 2015), and these pathologies can cause the death of the coral organism. However, not all the RLOs infected tissues showed any pathologies in this study.

The unidentified borer (most likely of the genus *Sipunculus*) in the present study can be an opportunistic coloniser and not necessarily a pathogen. Sipunculids are generally peanut-shaped, marine and have a wide range of habitats including the soft tissues of cold-water and tropical corals (Pancucci-Papadopoulou et al., 2014; Schulze and Kawauchi, 2021). Diseased, weakened tissues and/or acidified environment become vulnerable to invasion by borers (Quéré et al., 2015). Also, microborers are recognised agents of bioerosion in living tissues and can be the cause of diseases (Quéré et al., 2015). The acellular space detected around the borer in this study can be the result of chemical secretions of the borer on the surrounding *Lophelia* cells (Quéré et al., 2015).

The importance of the unidentified cells in the coral tissues were not determined as it was beyond the scope of the present studies. However, some organisms such as coccidia, ciliates and algae (although the cells detected in this study do not look similar) have been observed associated with diseased coral tissues and have been recognised to have capabilities that drive virulence or causes disease in the host (Galloway et al., 2007; Schulze and Kawauchi, 2021).

The disproportionate distribution of RLOs and unidentified cells in the coral samples is consistent with the amount of particulate organic matter (POM) concentration throughout the PBC. The concentration of particulates measured in the PBC was highest in the south branch and least in the canyon flank (Appah et al., 2020), making POM readily available for *Lophelia* in the south branch, which can be indicative of high particulate ingestion and subsequently high infection in the south branch (Bythell et al., 2002). Pathogens are borne on and transported by particulates to the deep sea (Simister et al., 2016), so it is probable that the observed particulates disproportionately transported RLOs and unidentified cells to the corals. However, the results must be interpreted with caution as the total number of coral samples screened was constrained by the fragile and protected nature of these corals. Additionally, this survey was based on a once off sample, which may not have coincided with a high pathogen prevalence in the PBC. Certain near shore Haplosporidia species that infect other invertebrate species such as bivalves can be present year-round but do peak in particular months, which might be the cause for the lack of detection in the corals. Thus, future sampling to represent all seasons would be required to confirm this. Also, sampling and screening for pathogens in late spring (May) would be routinely used in the health surveys of marine invertebrates in near shore environments, however, this may not be the case for deep sea surveys and pathogen prevalence patterns may differ in this environment. For instance, deep-sea surveys for coral health studies by Hall-Spencer et al. (2007) were carried out in August 2003. As such it is

important that these cold-water corals are continuously monitored with the aim of evaluating their health, pathogen diversity and prevalence, as well as growth and development. Additionally, further analysis of their immune function warrants investigation to provide more knowledge on slow-growing, long-lived organisms and their susceptibility, or lack of, to pathogen groups.

5. Conclusion

Despite the presence of RLOs and the low detection of *Vibrio* spp., Haplosporidia were not detected. *L. pertusa* and *M. oculata* in this isolated canyon, remote from direct terrestrial and anthropogenic influences, appeared healthy although slightly less so in the south branch where environmental stressors such as sedimentation may be higher. The overall health of these reef-forming cold-water corals is encouraging and suggests that under current environmental conditions these corals experience low pathogen diversity.

This study not only adds to the limited knowledge that is known on the pathogens of cold-water corals, but it also provides a baseline for future studies carried out at the PBC. This will be valuable as monitoring continues moving into the future in a changing marine environment.

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.

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Additional Information

Raw sequence data used in this study can be accessed from the project link https://www.ncbi.nlm.nih.gov/sra/PRJNA729568.

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