Health screening 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

Temperature and pH can expedite the ability of pathogens to cause diseases in cold-water corals (CWCs). The present study employed a combination of histology and polymerase chain reaction diagnostic techniques to investigate potential pathogens present in the CWCs *Lophelia pertusa* and *Madrepora oculata* in the Porcupine Bank Canyon (PBC), NE Atlantic. No pathogen was observed in the *Madrepora* samples. Neither histology nor standard PCR detected *Vibrio* spp. in the corals, although using Illumina technology, *V. shilonii* was observed in some *L. pertusa* samples in low abundances (0.22%). A Rickettsiales-like organisms (RLOs) occurred at a prevalence of 8.0% and at a low infection intensity of 1 - 4. *Lophelia* showed a few RLOs infection from the PBC canyon head (2.7%) and high infections in the south branch (5.3%). Similarly, unidentified cells observed in *L. pertusa* from the south branch (4.0%) were more common than those found in the canyon head (1.3%) with a prevalence of 5.3%. Although the route of pathogen infection is unclear, a likely mode of entry could be associated with particulate availability and the feeding strategies of the scleractinian corals. This suggest that *L. pertusa* invests energy into an enhanced immune function and reduced susceptibility to global pathogens despite a changing ocean environment.

Keywords: *Lophelia pertusa*, Haplosporidia, *Vibrio*, Rickettsiales-like organisms, Histology, PCR, Pathogen, Cold-water corals

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1. Introduction

Studies of coral diseases allow researchers to understand the dynamics and mechanisms of pathogen infection of corals and their interaction with the environment. Coral disease may cause rapid mortality and injury of corals thereby affecting the distribution of corals [1]. Woodley et al. [2] suggested that disease study in coral species could be used as a proxy of stress conditions that the corals are exposed to in the environment, especially since corals are more susceptible to diseases when they are exposed to extreme environmental conditions. It has been recognised that stressors can change healthy coral-associated microbes to virulent coral-associated microbes [3–8]. Environmental stressors such as sedimentation, salinity, temperature, changes in pH, organic contaminants, nutrient enrichment and marine pollution can increase the virulence of pathogens in tropical and cold-water corals [3,6,9–16]. Variable genetic expressions in response to stress and coral diseases have also been described [13,17,18]. While viruses, invasive gastrovascular multicellular structures (IGMS), metaloenzymes, superoxide dismutase (SOD) and biological agents such as bacterial flagella and fungal hyphae cause virulence [19–26], organisms such as algae, sponges, helminths, coccidia, nematodes, polychaetes, copepods (including endoparasitic lamippids) and arthropods have been observed in the cells and tissues of diseased corals, although their roles in coral pathology have yet to be determined [2,12]. Moreover, predation and physical injuries also increases susceptibility to diseases in corals [27] with microbes such as viruses, bacteria, fungi, crustacea and ciliates all recognised as cause of diseases in corals [2,14,19,28–30]. Diseased corals exhibit different pathologies and show reduced reproduction, growth rate and development [2,25,31]. It has been noted that diseased/infected corals show variable immunological responses as strategies of dealing with diseases and/or pathogens [26,32–35]. In addition, corals exhibit wound repair and tissue regeneration [36,37]. Many tropical coral diseases have been described since the 1980s and include white band I & II, white plague II & III, white pox, black band, aspergillosis, bleaching, dark spots, pink-line syndrome, yellow blotch/band [11,34,38–40], while cold-water coral diseases noted include neoplasm and necrosis [2,28,41].

Different techniques are available for investigating diseases in corals. These include histopathology, immunocytochemistry, standard polymerase chain reaction (PCR), quantitative PCR (qPCR), direct sequencing, culture-dependent assays, *in situ* hybridisation (ISH) and catalysed reporter deposition fluorescence *in situ* hybridisation (CARD-FISH) techniques [9,12,17,32,38,42,43]. Histology underpins the successful highlighting of coral diseases and the interaction between pathogens and the environment [30]. Also, histology is used to determine if there is an infection, pathology associated with a pathogen and if there is a host response. A PCR-based technique is used to detect pathogens *a priori*. This method can reveal a potentially pathogenic microbial DNA or RNA gene which may or may not denote the detection of a viable pathogen [30]. In addition, some infecting microorganisms of bacterial and archaeal taxa cannot be isolated on growth media [44]. Galkiewicz et al. [40], Flannery et al. [45] and Aranguren and Figueras [46] demonstrate that a combination of at least two methods for diagnosing infections yield satisfactory results and is the approach adopted here. Historical monitoring of CWCs for pathogens and disease is non-existent/limited due to the environment and sampling effort required compared to our greater knowledge on tropical corals. In addition, there is a lack of a model coral generally accepted for use in coral diseases study [30]. Also, there remains a limitation to develop frameworks to systematically characterise coral pathologies [12,47].

Cold-water coral reefs formed by *Lophelia pertusa* (synonymised with *Desmophyllum pertusum* [48]) and *Madrepora oculata* have been recognised as hotspots of biodiversity

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comparable to tropical corals [49]. *Lophelia pertusa* growth and survival have been described to occur at temperatures between 4 - 13 °C and salinity limits of 32 - 38.8 psu [50,51]. According to [49], they can be associated with over 1,300 marine species. However generally, studies of diseases in cold-water corals are limited [2], despite their importance as ecological engineers [52]. Cold-water corals diseases have been studied from the southwest England (gorgonian *Eunicella verrucose*: [41]), Indonesia (scleractinian *Madrepora* spp.: [28]) and the Trondheimsfjord, Norway (scleractinian *Lophelia pertusa*: [43,53]). The pathogens identified (using histological and/or molecular techniques) in these studies include the prokaryote *Vibrio* spp., the petracid crustacean *Patrarca madreporae* and the prokaryote *Mycoplasma* spp. respectively.

An important marine invertebrate pathogen phylum is the obligate protozoan Haplosporidia that have caused large scale mortality events in populations of shellfish and other marine invertebrates globally since the 1970s [54–57]. Generally high temperatures (10 - 40 °C) and salinities (> 15 psu) increase haplosporidia prevalence of infection [54,58–61], however species of this pathogen group are present year-round in near shore environments and can proliferate at >3 °C [55,62]. Haplosporidia have been recognised to reduce the bioturbation capacity of some polychaetes and disrupt the population structure of some vital marine invertebrate predators [63–65]. Despite its importance to marine invertebrates' health and distribution, studies on pathogenic Haplosporidia infecting cold-water corals have yet to be carried out. Pathogenic Haplosporidia may exist as spores, plasmodia, single cell uninucleate or binucleate life stages in their hosts [54,57]. Also, as infecting microorganisms of scleractinian corals could be transported to the deep-sea as spores with terrestrial particulates [66], and cold-water corals are filter feeders/predators, it is possible that cold-water corals can become infected with Haplosporidia, hence the need for this study. These spores can remain in the environment for decades until there are favourable conditions for growth [58]. Pathogenic *Vibrio* spp. are common in the environment and most species are problematic for organisms. It is important to note that Haplosporidia and *Vibrio* spp. are problematic in a host population when they are present at very high prevalence <100% [38,41]. Gavish et al. [26] has provided evidence of pathogen infection and its systemic progression through ingestion.

The present study combines histology and PCR techniques to investigate: 1) the presence of Haplosporidia and *Vibrio* spp. in *Lophelia pertusa* in the Porcupine Bank Canyon (PBC), 2) describe other potential pathogens/parasites observed using microscopy in the tissues of *L. pertusa* and *Madrepora oculata*, and 3) the distribution of these potential pathogens in *L. pertusa* throughout the canyon.

2. Materials and Methods

2.1. Sampling and storage

The Porcupine Bank Canyon is located 310 km to the southwest coast of Ireland. Coral polyp samples (n=75) were collected during a cruise in May 2019 [67] 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 [68] (Table S1 and Figure 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 CWC frameworks have been previously noted [69]. Coral samples from the canyon head were collected from depths of between 710 - 720 m, the flank was between 688 - 693 m and the south branch were between

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600 - 610 m water depth. Generally, at the sampling depth of 600 - 720 m, the temperature and salinity ranges were 8.7 - 10 °C and 34.0 - 38.0 psu respectively. *Lophelia pertusa* and *Madrepora oculata* samples were collected with the guided manipulator arm of a remotely operated vehicle (ROV) and stored in bio boxes. 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 corals being processed and screened per site location. Coral samples collected for histology were fixed in Davidson's solution for 48 hours and transferred into 70% ethanol at room temperature for the duration of the cruise. Coral samples were decalcified with TBD-2 formic acid decalcifier (Thermo Scientific). Individual polyps were placed in an individual tissue embedding histocassette (n = 75 polyp samples).

<<<<Figure 1>>>>

2.2. Histology

Coral polyps were fixed in Davidson's solution [70]. Samples were then prepared for embedding in wax by running them through a citadel processor using a 20-hour dehydration cycle of graded volumes of ethanol (adapted from [71]). 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 [72]. Stained samples were cover-slipped and allowed to air-dry.

2.3. DNA extraction and standard Polymerase Chain Reaction (PCR)

DNA was extracted from 7 µm paraffin-embedded tissue sections. Polyp tissues were deparaffinized (Shi et al., 2002) and DNA extractions were carried out using a Qiagen DNA extraction kit (QIAamp DNA FFPE Tissue Kit). The quality and quantity of DNA were evaluated using a NanoDrop 1000 spectrophotometer (ThermoScientific). Good-quality DNA will have an A_{260}/A_{280} ratio of 1.7 - 2.0. A reading of 1.6 does not render the DNA unsuitable for any application, but lower ratios indicate more contaminants are present. Recommended and routinely used generic PCR assays (Hap1/R1, Hap1/R3, ssu980/R1) for Haplosporidia and specific (MSXA/MSXB) haplosporidian primers (Table 1) were run on extracted DNA samples following [73] and [74] while PCR for *Vibrio* spp. was performed by Kett et al. as per [75]. Positive controls i.e. Haplosporidia genomic DNA from heavily infected cockles were included for PCR runs with haplosporidian primers. All experiments were run with negative controls consisting of double distilled water (ddH_2O). Amplicons were run on a 2% agarose gel stained with 22 µl Sybr stain and run for about 35 - 45 min at 110 V for DNA detection using a UV visualiser.

2.4. DNA extraction, Illumina sequencing technology and Bioinformatics

DNA extraction was performed in a thoroughly cleaned and decontaminated laminar flow hood. Coral samples were crushed using liquid nitrogen and tools were cleaned with a 10% bleach solution and DNA AWAY® between sample preparations to avoid cross-contamination. A branch with five individual polyps was detached from each colony and used for DNA extractions. Extractions were performed using the DNeasy PowerSoil Pro kit (Qiagen) following manufacturer's protocol. DNA extractions were performed with negative controls consisting of ddH_2O. Genomic DNA samples were sent to Novogene, Cambridge, UK for amplicon-based metagenomics sequencing 16S (V3-V4). The purity and concentration of genomic DNA samples were assessed on a 1% agarose gel and Qubit Fluorometer (Invitrogen, USA) respectively. The 341 F (5'-CCTAYGGGRBGCASCAG-3') and 806 R (5'-

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GGACTACNNGGGTATCTAAT-3') primers with barcodes were used to amplify the V3-V4 region (466 bp) of the 16S rRNA gene and sequenced using the Illumina NovaSeq 6000 SP with paired ends (2 x 250 bp) strategy (Novogene, Cambridge, UK). PCR reactions were performed in a 20 µl reaction volume using the Phusion® High-Fidelity PCR Master Mix (New England Biolabs) with ~1 - 2 ng template DNA. The final primer concentration was adjusted to 0.5 µM. Amplicons were run on a 2% agarose gel for detection, desired bands were excised and purified using Qiagen Gel Extraction Kit (Qiagen, Germany).

Quality control of the raw reads (BioProject Accession Number: PRJNA729568) was performed with FastQC 0.11.9 [76], trimmed (to remove reads shorter than 200 bp, mean quality score of 30 in a sliding window of 10 bp and a maximum of two primer mismatch) with Trimmomatic 0.39 [77] and merged into contigs using FLASH 1.2.11 [78]. Quality filtering of the sequence data to remove chimeras was achieved using Dada 2 [79,80] as implemented in qualitative insights into microbial ecology (QIIME 2) v2020.8 [81]. A sample depth of 20,000 amplicon sequences was set for all the samples. Each Dada 2 amplicon sequence variants (ASVs) were utilized for taxonomic classification via the QIIME 2 feature-classifier [78]. Taxonomic assignment (using the QIIME 2 pipeline; https://docs.qiime2.org) was made from the Greengenes 13.8 database ([82]; based on 99% similarity of sequence data to the Greengenes database). Unassigned, mitochondrial and chloroplast ASVs were removed.

Table 1: Description of PCR primer-pairs, sequences of forward and reverse primers and expected products

Primer pair	Forward and Reverse	Size (bp)	reference
Hap F1/ Hap R1	F: GTT CTT TCW TGA TTC TAT GMA R: CTC AWK CTT CCA TCT GCT G	348	[73];[74]
Hap F1/Hap R3	F: GTT CTT TCW TGA TTC TAT GMA R: AKR HRT TCC TWG TTC AAG AYG A	348	[73]; [74]
SSU980/Hap R1	F: CGA AGA CGA TCA GAT ACC GTC GTA R: CTC AWK CTT CCA TCT GCT G	348	[73]; [74]
MSXA/MSXB	F: CGA CTT TGG CAT TAG GTT TCA GAC C R: ATG TGT TGG TGA CGC TAA CCG	573	[73]; [74]
Vibrio spp. F3/R3	F: CAA CAG AAG AAG CAC CGG CT R: CAC GCT TTC GCA TCT GAG TG	-	Kett et al., unpublished

3. Results

3.1. Haplosporidia and *Vibrio* spp.

DNA quantity and quality (A_{260}/A_{280} ratio) ranged between 2.2 - 11.1 ng/µl and 1.50 - 1.88 respectively. The integrity 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. Furthermore, PCR did not detect any target DNA using the haplosporidian primers (Table 1). No *Vibrio* spp. were detected in the standard PCR using the *Vibrio* spp. primers (Table 1) although *Vibrio shilonii* was observed in *L. pertusa* samples in low abundance (0.22%). using Illumina technology.

3.2. Potential pathogenic and unidentified cells tissue samples

Histological analysis revealed Rickettsiales-like organisms (RLOs) in the tissues of the *Lophelia pertusa* samples (Figure 2C and D). Overall, 8.0% (6/75) of RLOs were detected in the polyp tissues that were screened, 5.3% (4/75) were observed in the south branch whereas

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2.7% (2/75) were observed in the canyon head (Figure 1). Infection intensity identified for RLOs in individual polyps was low (1 – 4 colonies per individual). The percent prevalence of the unidentified cells was 5.3% (4/75) with 4.0% (3/75) detected in the south branch and 1.3% (1/75) in the canyon head while none were detected in corals from the canyon flank (Figure 1). The cells were bi- and multinucleate cells with well bounded membrane and showed a rounded morphology. Generally, the sizes of the cells ranged between 4.06 - 19.25 µm in diameter with an average size of 7.94 µm. The largest unidentified cell (probably a macroborer; most likely genus *Sipunculus*) was a binucleate cell with an irregular shape that is blunt at one end (posterior) and tapers at the other (anterior), with a slight depression on both sides of the trunk (and what seem to be a mouthpart/groove at the ventral side close to the anterior) (Figure 2B). The *L. pertusa* cells lining up the burrow and the acellular space that surrounds the unidentified macroborer suggests growth of the borer (Figure 2B). No potential pathogen or unidentified cells were observed in the *Madrepora* samples.

<<<<Figure 2>>>>

Generally, these potential pathogenic and/or unidentified cells were distributed in corals throughout the canyon except the canyon flank. Eleven percent of the slides contained unidentified cells (bi/multinucleate), comprising of 7% from the south branch and 4% from the canyon head samples.

4. Discussion

Temperatures beyond 3 °C and salinities above 25 psu favour proliferation and a higher rate of infection of Haplosporidia respectively [45,54,83]. Sampling of *L. pertusa* and *M. oculata* during the month of May coincided generally with the period during which Haplosporidia begin to infect their invertebrate hosts [54]. However, no Haplosporidia was observed in the sampled coral tissues even though the prevailing deep-sea mean temperature (9.3 °C) and salinity (36.3 psu) seemed optimal for the growth and infection of Haplosporidia, but generally unfavourable for *Vibro* spp. growth [21]. *Vibrio* spp. are closely associated with chitin-containing organisms (e.g., copepods) which represent one of the most important environmental reservoirs of these bacteria. They are also associated with phytoplankton as well as aquatic plants and can also infect oysters at 9 - 13°C and 19 - 20°C [84,85]. It is probable that local abiotic conditions on the high seas and in the deep ocean such as current speed and sedimentation do not favour the growth and development of the haplosporidian pathogen. Also, existing conditions may not have supported the growth and survival of the 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. Different diagnostic methods may yield different results [45,55]. Also, latent, early or light infections may be overlooked using both methods. Furthermore, where the tissue was sectioned it may not have been where the pathogen was localised in the tissues of the invertebrate host, highlighting the need for more individuals from a colony to be screened. In addition, PCR amplification is difficult to achieve on target genes (i.e., 200-500 bp) from formaldehyde fixed tissues [73] although not impossible [55].

No visible *Vibrio*-associated pathologies were observed nor were they amplified in the standard PCR. Many benign and pathogenic *Vibrio* spp. have been reported in other cold-water coral species including *L. pertusa* [44,86,87]. Moreover, using Illumina technology, we observed, based on the percent volume of DNA analysed, *V. shilonii* in low abundance (0.22%). This finding further highlights the difficulty of detecting pathogens when they occur at a very low prevalence and intensity of infection. However, the *Vibrio* spp. primers (F3/R3) are universal

for most *Vibrio* spp. and are routinely successful when screening near shore mollusca (Kett et al., unpublished as per [75]).

A Rickettsiales-like bacteria (RLOs) was observed in 8% of the slides, 5.3% in the south branch and 2.7% in the canyon head. Recent histopathological studies have described pathogenic RLOs in scleractinian corals [2,88] and has been associated with oil contamination in deep-sea waters [89]. Furthermore, RLOs bacteria have recently been observed on and/or within *L. pertusa* samples at a mean prevalence of 3.43% from the PBC (Appah et al., in review). Sequences obtained from the Illumina sequencing of *L. pertusa* had E-value of 3e-91, 100% Query Coverage and 99.47% Percent Identity to the uncultured Rickettsiales bacterium and Haplosporidium sp. endosymbiont AbFoot 16S RNA sequences in GenBank using BLAST analysis. These bacteria were detected in *Apostichopus japonicus* and *Haliotis iris*, respectively. According to Appah et al. (in review), they form the core microbiome (100% coverage of coral samples) of the coral samples. As such, the speculative presence of Rickettsiales bacteria in the coral tissues in the present study may suggest contamination of the ambient seawater by petroleum hydrocarbon. However, we have not observed any pockmarks (hydrocarbon seeps) in the area although a small sedimentary basin that may contain hydrocarbons (but has not been drilled) exists under the canyon. It is most likely that the bacteria-like cells observed (Figure 2C and D) are Rickettsiales bacteria from the same samples from the PBC. RLOs are obligate pathogens that can infect many marine organisms and cause various degrees of pathologies in the tissues of infected species [90]. The infection intensity of our coral samples was similar to the infection intensities detected in tropical corals from the Caribbean [90,91]. In the present study, gastrodermal epithelial tissues appeared sloughed and lysed with vacuolations (Figure 2D) similar to observations that were made in RLOs associated infections of *Acropora cervicornis* tissues [90]. Sloughing is characterised by lost or loose epithelial cells while necrosis is characterised by vacuolisation and cell rupture [92], and these pathologies can cause the death of the coral organism. However, not all the RLOs infected tissues showed any pathologies (Figure 2C). Also, no potential pathogen was observed in the *Madrepora* samples. The two scleractinian corals exhibit different feeding strategies [93–95] and this may be the reason why no potential pathogen was detected in *M. oculata*, since ingestion is a pathway for pathogen infection [26]. *Madrepora oculata* shows a narrower diet compared to *L. pertusa* [93,95].

The unidentified borer (Sipunculid) in the present study can be an opportunistic coloniser and not necessarily a pathogen (Figure 2B). Sipunculids are generally marine and have a wide range of habitats including inside sponges and corals [96]. Diseased, weakened tissues and/or acidified environment become vulnerable to invasion by borers [15,92]. Also, microborers are recognised agents of bioerosion in living tissues and can be the cause of diseases [92]. 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 [92].

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 sponges, coccidia, ciliates and algae have been observed associated with diseased coral tissues and have been recognised to have capabilities that drive virulence and/or causes disease in the host [2,12,27].

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 [68], 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 [26]. Also, as suggested by [66], pathogens are borne on and transported by particulates to the deep sea, 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 is based on a once off sample, which may not have coincided with a high pathogen prevalence in the PBC. Also, certain near shore Haplosporidia species that infect other invertebrate species such as bivalves can be present year-round but do peak in certain months which might be the cause for the lack of detection in the corals. Thus, 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 diseases studies by [41] and [43,53] were carried out in August 2003 and August 2004, respectively. 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 proper growth and development.

Despite the presence of RLOs and minor occurrences of *Vibrio* spp., the presence of Haplosporidia was not evident despite benthic conditions being within the pathogen's environmental tolerances. *Lophelia pertusa* and *Madrepora oculata* in this isolated canyon, remote from direct terrestrial and anthropogenic influences, appear healthy and although slightly less so in the southern branch where environmental stressors may be higher. The overall health of these reef-forming cold-water coral is encouraging and suggest that under background environmental conditions these corals can adapt to changing NE Atlantic intermediate water benthic conditions including a warming in ocean temperature [97] and consequential changes in biological productivity affect food supply for this predominant particulate organic matter (POM) filter feeders.

The combined histology and PCR screening approach used here provide an effective method for monitoring cold-water coral health with this paper offering a baseline for future studies. The continued warming of our oceans [97] and changes in ocean acidification pose significant threats for cold-water corals in the future [98,99].

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Author Contributions

JA: design, write up, data analysis, and data collection. SL: technical advice and draft editing. AL: data collection. RO'R: technical advice and draft editing. LO'R: data collection. LO: data collection. AW: technical advice and draft editing. All authors contributed to the success of the manuscript.

Additional Information

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

Competing Interests: The authors declare no competing financial interests**.**

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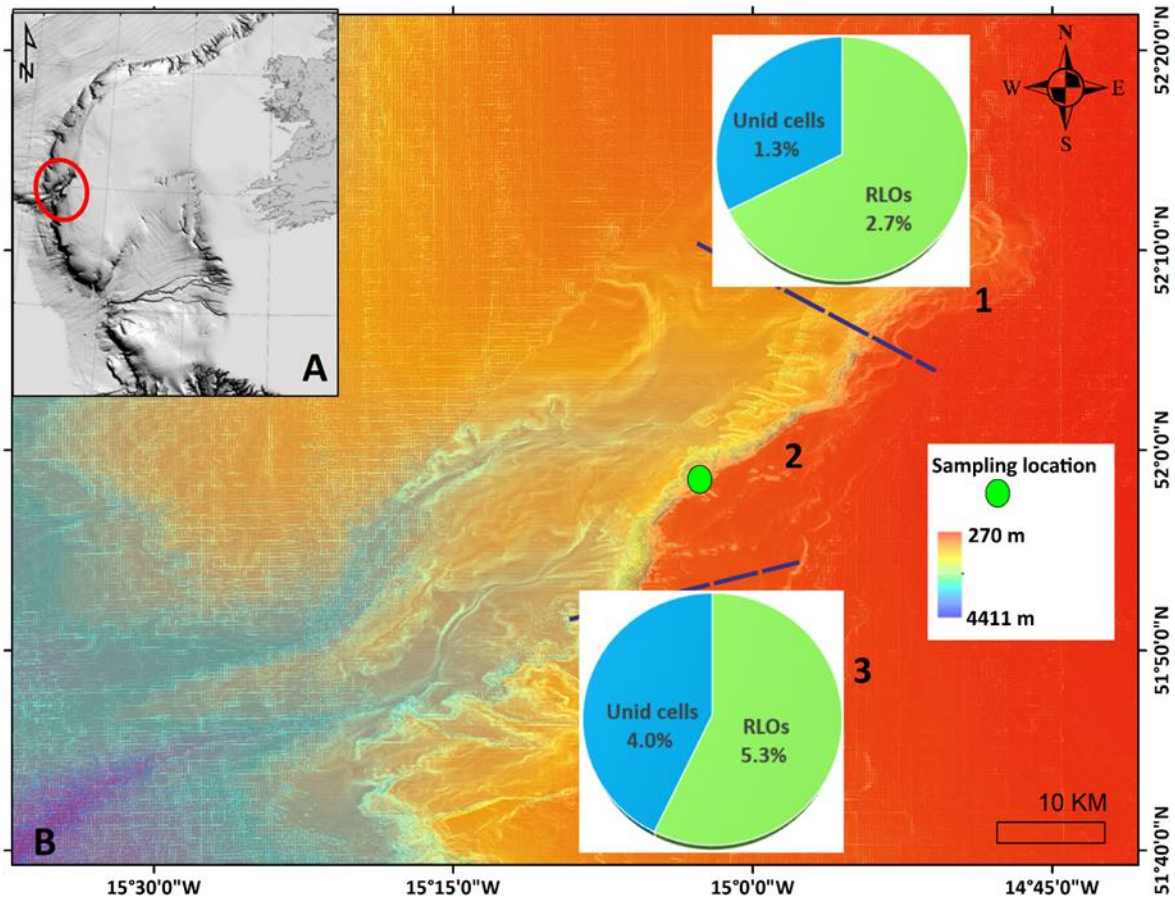

Figure 1. Location map showing: A) the Porcupine Bank Canyon (PBC) on the Irish continental margin (red ellipse), B) different areas of the PBC and the percent prevalence of Rickettsiales-like organisms and unidentified cells. The blue broken line demarcates the different areas of the PBC while the numbers 1, 2 and 3 represent canyon head, canyon flank and south branch, respectively. Unid cells = unidentified cells; RLOs = Rickettsiales-like organisms.

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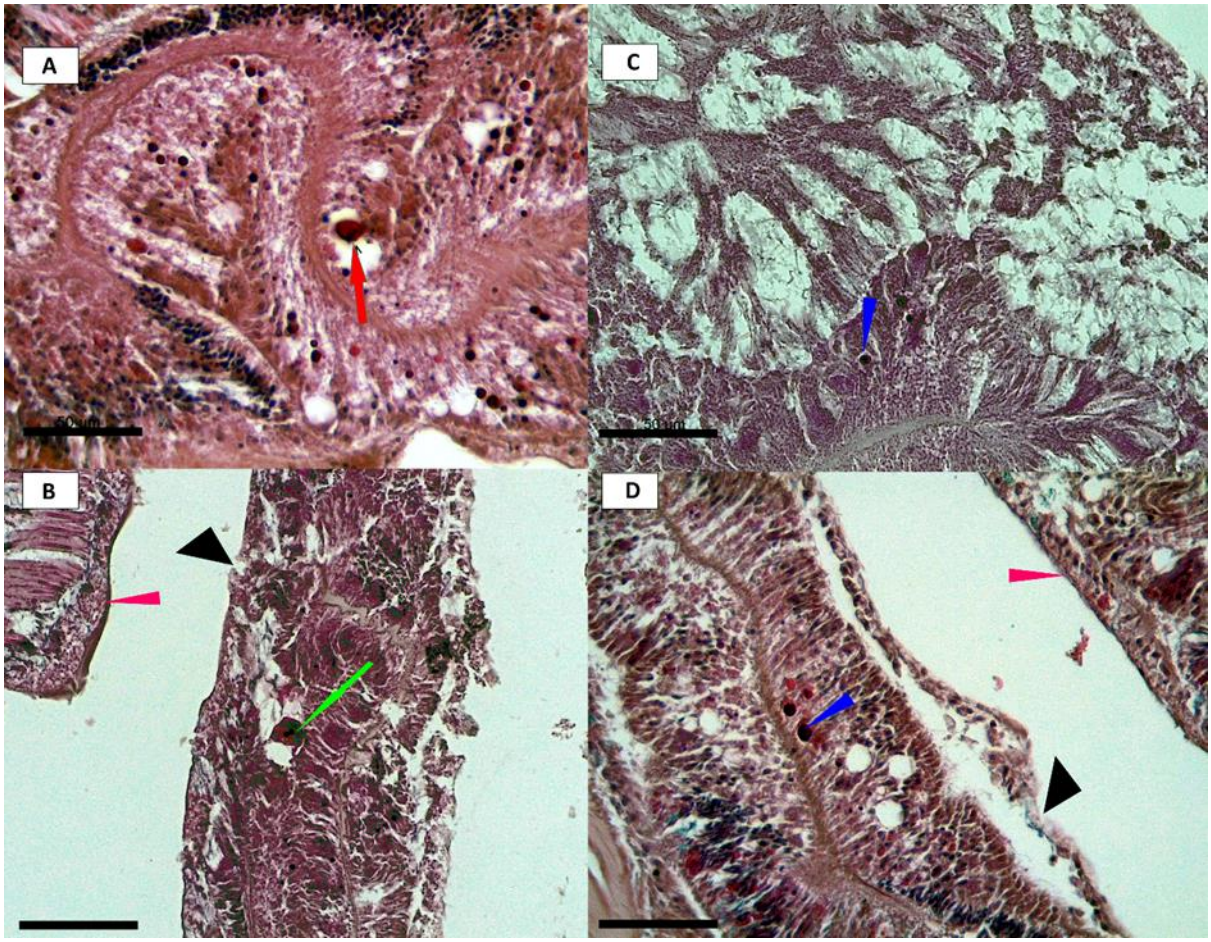

Figure 2: Histological observations of potentially harmful cells in *L. pertusa*. scale = 50 µm: A) unidentified binucleate cell, B) unidentified binucleate macroborer**,** C) Rickettsiales-like organism in epithelial tissue, but without any visible pathology, and D) Rickettsiales-like organism and vacuoles in epithelial tissue. Blue arrow = Rickettsiales-like organism (RLOs); purple arrow = intact epithelial tissue; black arrow = broken and sloughed epithelial tissue; green arrow = unidentified macroborer; red arrow = unidentified binucleate cell