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Investigation of *Capitella* spp. symbionts in the context of varying anthropic pressures: First occurrence of a transient advantageous epibiosis with the giant bacteria *Thiomargarita* sp. to survive seasonal increases of sulfides in sediments

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Non epibiotic *Capitella* (80% of the population)

Epibiotic *Capitella* (20% of the population)

Thiomargarita

Anthropized site

Non anthropized site

Eyes

1 mm

Non epibiotic *Capitella* (100% of the population)

Epibiotic *Capitella*

Thiomargarita

Diversity of the associated microbes

Epibiosis with *Thiomargarita* sp.

Worm's survival to sulfides

Surviving

Time (h)

Graphical Abstract
Highlights

• Local sediment geochemistry and diversity of symbionts associated to a common coastal worm are compared between specimens from an anthropized versus a non-anthropized site

• A peculiar development of a transient epibiosis with the giant sulfur oxidizing bacteria *Thiomargarita* spp was observed in *Capitella* spp. exposed to high level of sulfides

• The transient epibiosis allows the worms to survive toxic levels of sulfides during the summer

• This is the first evidence of an adaptive advantage of a facultative ectosymbiosis to face changing habitats
Abstract

Capitella spp. is considered as an important ecological indicator of eutrophication due to its high densities in organic-rich, reduced, and sometimes polluted coastal ecosystems. We investigated whether such ability to cope with adverse ecological contexts might be a response to the microorganisms these worms are associated with. In populations from the French Atlantic coast (Roscoff, Brittany), we observed an epibiotic association covering the tegument of 20-30% specimens from an anthropized site while individuals from a reference, non-anthropized site were devoid of any visible epibions. Using RNAseq, molecular and microscopic analyses, we described and compared the microbial communities associated with the epibiotic versus the non-epibiotic specimens at both locations. Interestingly, data showed that the epibiosis is characterized by sulfur-oxidizing bacteria amongst which the giant bacterium Thiomargarita sp., to date only described in deep sea habitats. Survey of Capitella combined with the geochemical analysis of their sediment revealed that epibiotic specimens are always found in muds with the highest concentration of sulfides, mostly during the summer. Concomitantly, tolerance tests demonstrated that the acquisition of epibions increased survival against toxic level of sulfides. Overall, the present data highlight for the first time a peculiar plastic adaptation to seasonal variations of the habitat based on a transcients epibiosis allowing a coastal species to survive temporary harsher conditions.

INTRODUCTION

The past decades have seen an increasing number of studies with the aim of characterizing the biology of bacterial symbions in a wide variety of invertebrates and plants, as well as their role on community structure and ecosystem functioning (Brooks et al., 2017; Carrier and Reitzel, 2017; Ferrari and Vavre, 2011; Gilbert et al., 2015; Moran and Wernegreen, 2000). It is now widely admitted that symbiotic associations can be responsible for some
of the most noticeable changes in phenotypes, as they constitute a low-cost source of evolutionary innovation for their host (Margulis, 1991). The very short generation time of associated microorganisms could allow a faster acclimatization of the host to changing environments than the fixation of favorable alleles in the host genome, and therefore accelerate the acquisition of new phenotypes more adapted to novel ecological conditions. For instance, it is now well established that diagnostic traits of numerous symbiotic species are in fact a response to the microorganisms they are associated with (McFall-Ngai, 2008; McFall-Ngai et al., 2013). Symbioses have been shown to affect adaptive traits, from trophic niche (Kohl et al., 2014) to temperature dependence (Morsy et al., 2010), salinity tolerance (Nougué et al., 2015), resistance to oxidative stress (Richier et al., 2005), or resistance against pathogens (Kaltenpoth and Engl, 2014; Tasiemski et al., 2015) that may have an early effect during organism development (Gasnier-Fauchet et al., 1986; Gilbert et al., 2015). Consequently, understanding the adaptation of marine species to changing environments requires the further exploration of how the environment impacts the host-symbiont associations and their evolution for either endo- or ecto-symbioses (epibiosis).

Until now, the symbiotic microflora of marine animals was often considered as a random consortium (McFall-Ngai, 2008). However, multiple lines of evidence show that this microflora corresponds in fact to a highly specialized microbial community forming a specific and stable symbiosis with its host, with dedicated roles. The discovery of the association of chemoautotrophic bacteria with the deep-sea hydrothermal vent tube worm, *Riftia pachyptila* revolutionized our view about the morphological and physiological impact of bacteria on the host (Bright and Lallier, 2010; Cavanaugh et al., 1981; Felbeck, 1981). Chemoautotrophic bacteria use sulfur compounds, particularly hydrogen sulfide, a chemical highly toxic to most known organisms, to produce organic material through the process of chemosynthesis. Interestingly, *R. pachyptila* develops from a non-symbiotic
trochophore larva, which enters juvenile development, becoming sessile, and subsequently acquiring symbiotic bacteria through skin infection. After chemosynthetic bacteria are established in the midgut of the juveniles, it undergoes substantial remodelling and enlargement to become the trophosome, while the remainder of the digestive tract fully disappears in adults (Stewart and Cavanaugh, 2006). Lacking a mouth and a gut and being unable to obtain organic compounds by diffusion, adults gain the latter via sulfur oxidation-CO$_2$ fixation driven by the endosymbionts confined into peculiar cells (namely bacteriocytes) of the trophosome. The tubeworm depends completely on the chemosynthetic bacteria for the byproducts of their carbon fixation cycles needed for its growth. Reciprocally, endosymbionts rely on _R. pachyptila_ for the assimilation of nutrients needed for the array of metabolic reactions they employ (Bright and Lallier, 2010).

Soon after the first description of chemosynthetic symbiosis, additional thiotrophic symbioses were described at oxic–anoxic interfaces of more accessible coastal shallow-waters also recognized as chemosynthetic based ecosystems (Dubilier et al., 2008; Petersen et al., 2011; Stewart et al., 2005). Gutless oligochaetes’ (annelids) and stilbonematids’ (nematodes) symbioses are among them and constitute a remarkably well-described and interesting mode of nutrition (Bulgheresi, 2016; Dubilier et al., 2006; Polz et al., 1992). More recently, the nematode _Metoncholaimus albidus_, reported in the Roscoff Harbor (Brittany, France), has also been shown to be associated with distinct microbial communities known to be involved in sulfur metabolism (Bellec et al., 2019).

Marine worms belonging to the genus _Capitella_ represent the most common component species of benthic communities in organically enriched ecosystems throughout the world (Kitamori, 1975; Pearson and Rosenberg, 1978; Reish, 1979). This so-called sediment “black zone” - previously considered to be azoic – is characterized by strongly reducing, micro- to anaerobic conditions with high concentrations of reduced sulfur species like
dissolved sulfides and polysulfides, thiols... and sulfide precipitates such as MeS (where Me can be Fe, Pb, Zn, Cd...), Fe₂S₄ and FeS₂ (Wood, 1992). Differential tolerance to sulfide has been observed between sibling species of *Capitella*, leading to the hypothesis that these ecophysiological differences were genetically fixed and that sulfidic environments could have been the driving force of such species diversification (Gamenick et al., 1998). The tolerance to sulfides in *Capitella* sp1 from North America (subsequently identified as *Capitella teleta* (Blake et al., 2009)) was evaluated through experimental exposure of the annelids to H₂S under laboratory conditions. Sulfide concentrations up to 2mM were considered as a cue for *Capitella* sp1 larval settlement (Cuomo, 1985) whereas those exceeding 10mM were detrimental to their survival (Dubilier et al 1988). The presence of sulfides up to 7mM was also shown to favor the burrowing activity of adults thus stimulating the respiratory activities of the bacteria associated with the mucus-lined burrow of the worm in soft agar microcosm (Wada et al., 2006). This was coupled with an enhanced growth and survival rates of the adults observed in sediments supplied with sulfides for 6 weeks (Tsutsumi et al., 2001). Consequently, *Capitella* species does not seem to favor organically enriched sediment with sulfides but rather prefers the environments that sulfides provide. As mentioned before, hydrogen sulfide can be exploited for the chemosynthesis of organic matter by chemoautotrophic bacteria. *Capitella* species are not gutless worms and an examination of *Capitella* sp. I for the presence of enzymes commonly associated with chemoautotrophic bacteria ~40 years ago has led to the conclusion by the authors that adults were not associated with chemoautotrophic symbionts (Cavanaugh, 1983; Cuomo, 1985).

The main purpose of this paper was to explore both the microhabitat and microbial diversities associated with the complex of *Capitella* species recently identified as “*Capitella* spp. from the English Channel” (Boidin-Wichlacz et al., Under review) to first report
whether such host-symbiont interactions (notably with chemoautotrophic bacteria) exist
and to evaluate secondarily whether changing environmental conditions, and especially
sulfides can affect these associations and the worm tolerance to this chemical.
For this end, *Capitella* specimens collected from two sites with differing levels of anthropic
influence and sulfides were compared. Biogeochemical characteristics of the sites were
documented, and microbial communities associated with specimens of *Capitella* sp. were
assessed using a RNASeq-based approach. Finally, the cost versus benefit of the transient
association with sulfur-oxidizing ectosymbionts was studied in animals exposed to lethal
doses of sulfides.

1. MATERIALS AND METHODS

1.1. Specimen collection

Sediment and *Capitella* specimens were collected together at two different sites: the
Roscoff Harbor and Le Laber near Roscoff (Brittany, France). For the « Tolerance tests to
experimental exposure to sulfides” worms were only sampled at the Roscoff Harbor.
A map with the GPS coordinates is presented in Fig. 1. The sampling dates and locations for
each experiment as well as the number of collected worms are detailed in the
supplementary data (Table S1). *Capitella* spp. were collected at low tide. At both locations,
*Capitella* individuals were abundant, representing the most dominant species in the Roscoff
Harbor, and with abundance similar to that of oligochaetes in the nearby site Le Laber. The
sediment was sieved on a 500 µm mesh in the field and animals were brought back to the
laboratory for sorting under a dissecting microscope.

1.2. Sediments
The methods used to determine the “Total metal concentrations”, the “Carbon and nitrogen contents”, the “AVS, CRS and HCl-extractable metals” and the “Granulometry” of the sediments are provided as supplementary data.

**Sampling and pretreatments** – Sediments of the two study sites were characterized in terms of trace metals concentrations (total metals and metals extracted with 1M HCl), reduced sulfur species content (AVS: Acid Volatile Sulfides and CRS: Chromium Reducible Sulfur), dissolved sulfides and additional environmental parameters. Sediments were collected using a 5 cm long (for the top 0–5-cm surface layer of sediment) or along cores of 35 cm long (for sediment profiles) using Perspex tubes (internal diameter: 7.5 cm). Cores sampled with the Perspex tubes were put into a glove box, previously flushed with nitrogen, and sliced every 1 cm at both sites. Each sediment sample was then stored under nitrogen untreated in a plastic bag at -18°C prior to perform AVS, CRS and metal analyses. A slice of each core was also dried to measure granulometry and total carbon and nitrogen contents.

Additional sediment cores were sampled for exposure to DGT (Diffusive Gradients in Thin films) - AgI passive samplers used for dissolved sulfide determination.

**Enrichment factor and toxicity index calculation** – The enrichment factor (EF) normalized towards aluminum (Al) has been used to compare the level of metal pollution between our sediment samples. This factor is defined as follows:

\[
EF = \frac{[Me]_{\text{sample}}}{[Al]_{\text{sample}}} \times \frac{[Me]_{\text{reference}}}{[Al]_{\text{reference}}}
\]

Where \([Me]_{\text{sample}}\) and \([Me]_{\text{reference}}\) are the concentrations of metal (Me: Cd, Co, Cu, Ni, Pb or Zn) in our samples and in the reference material, respectively (Audry et al., 2004; Davide et al., 2003). To avoid using average world values for the reference material that do not
reflect the local geology of the area studied, reference geochemical background values from pristine loess deep horizons in the North of France (Boulogne, Gravelines and Authie) has been considered (Sterckeman et al., 2006).

The toxicity index (TI) was calculated as the ratio SEM/AVS to predict metal sediment toxicity towards benthic invertebrate species (Ankley et al., 1993). Its relevance has been demonstrated via toxicity tests on several benthic organisms (notably the polychaetes Capitella capitata and Neanthes arenaceodentata), in natura or through experimental exposure to contaminants (Lee et al., 2000). For each sample, the TI has been calculated, according to the following relation: TI = log ([SEM]/[AVS]) (Ankley et al., 1993). Previous studies have shown that sediments with TI > 0 are toxic for animals whereas sediments with TI ≤ 0 are not (Hansen et al., 2005). AVS and SEM data of the 5 first cm of the sediment were used to calculate the TI values for both study sites over a period of time from 28 of July to 8 of December 2015.

Dissolved Sulfides – Dissolved sulfides were measured using DGT-AgI probes (Gao et al., 2009). Briefly, dissolved sulfides were measured from a coloration which turns from white to black when forming Ag₂S with sulfides after diffusing from pore-water through an acetate cellulose filter (0.45 μm pore size) into a polyacrylamide gel containing the AgI precipitate. After a known exposure time of the filter in pore-water samples, the precipitate is scanned using a commercial flatbed scanner and color intensity is then digitized and calibrated to calculate the concentrations initially present (Lourino-Cabana et al., 2014; Teasdale et al., 1999). Calibration of the DGT-AgI probes in standard sulfide solutions were performed using the same conditions.

1.3. Microbial communities associated with the worms

worms sampling – For the RNAseq, animals collected in 2013, were checked for filamentous epibionts under the microscope and separated into three groups: 1/ non
epibiotic animals from the Le Laber 2/ non epibiotic animals from the Roscoff Harbor and
3/ epibiotic animals from the Roscoff Harbor (Fig 2B). For each group, 30 individuals were
placed in RNA-later. At the time of sampling for transcriptome sequencing, Capitella
covered by epibionts were only found at the Roscoff Harbor site; no epibiotic individuals
were found in Le Laber. For the morphological analyses, five specimens of each group were
fixed in glutaraldehyde 2.5% for electron microscopy and five were fixed in
paraformaldehyde 4%, for fluorescence in situ hybridization in 2013 and in 2014.

**Seasonal survey of associations with Thiomargarita on Capitella spp** – From March to
December 2015, samples were collected at two-week intervals from both Le Laber and the
Roscoff Harbor sites (19 sampling events per site). Each individual worm was then
preserved in 85% ethanol. Fifty-two individuals were used for the genetic analysis (see
below) and the remaining worms were later observed individually under a dissection
microscope to check for presence of epibiotic microorganisms and measure the width of
the body at the fifth setiger (Pardo et al., 2010). In total, 5900 worms were sampled (with
150-160 worms collected at each sampling event at each site). To obtain a better estimation
of the association prevalence among the worms, the association (presence/absence) of
large epibiotic microorganisms was modeled as a Bernoulli random variable through a
generalized linear model (GLM) with binomial error and logit link between the explanatory
variables and their effect on the association probability. We built 166 different GLM based
on the “complete model”, which incorporated the effects of site (Le Laber vs. Roscoff
Harbor), worm size and Julian date (number of days since last change of year). The other
165 models were obtained as the sub-models nested within the complete one (i.e. models
lacking one or more explanatory variables or interactions thereof). The goodness-of-fit of
each model and its corrected Akaike Information Criterion (AICc) were computed and
models were ranked from best to worst following increasing values of AICc. To obtain a
more robust estimation of model predictions, model averaging procedures were used
based on the Akaike weight of each model (Burnham et al., 2011). For all these statistical
analyses, R (v 3.2.3) was used with package ‘fields’ to make the heatmaps and package
‘MuMIn’ for automated model goodness-of-fit comparisons and model averaging.

1.4. Morphological observations of associated microorganisms

Optical microscopy - For each sample of Le Laber and the Roscoff Harbor, worms with and
without large epibionts were examined alive or fixed (paraformaldehyde 4%) using an
optical microscope (Zeiss Axio Imager M2) and a stereomicroscope (Zeiss Stemi 305).

Electron microscopy of the epibiotic microflora - Specimens of the three groups (epibiotic
from the Roscoff Harbor and non-epibiotic from the Roscoff Harbor or from Le Laber) fixed
in 2.5% glutaraldehyde were dehydrated in a series of ethanol solutions of progressively
increasing concentrations (75–100%), critical-point-dried with a Balzers SCD 30
(temperature 37°C and pressure 70 kg cm⁻²), mounted on stubs, covered with a layer of 10–
20 nm of gold, and observed under the SEM using a JEOL JSM-840A Scanning Electron
Microscope at 20 kV accelerating voltage.

Fluorescence in situ hybridization (FISH) of epibiotic microflora - FISH experiments
were performed using generalist probes targeting Eubacteria (EUB338),
Gammaproteobacteria (GAM42), and the probe NON338 (antisense of EUB338) as a
negative control {Amann, 1990 #159}. All hybridizations were conducted using 30%
formamide at 46°C for 3 hours, followed by a 15 minutes rinse in appropriate buffer using
the protocol described in (Duperron, 2017). FISH hybridizations were performed on whole
specimens of Capitella fixed in paraformaldehyde 4% to visualize epibionts, as well as on
8µm-thick cross sections of specimens that were previously embedded in Steedman Wax
as described in (Duperron et al., 2008), using DAPI as a background stain. Hybridized
samples were visualized under a BX61 epifluorescence microscope (Olympus, Japan).
1.5. **Assessing microorganism’s biodiversity associated with *Capitella* by RNAseq sequencing**

**RNA extraction and sequencing** – To assess microorganisms co-occurring with *Capitella*, RNAs from the three groups (see worm sampling) were extracted and sequenced to obtain transcriptomes representative of eukaryotes and prokaryotes associated with the worms. The total RNAs of each group were extracted with the TRI-Reagent solution (Sigma), following the manufacturer's protocol. The RNAs were re-suspended in DEPC-treated water and the quality and quantity were evaluated on a Nanodrop. An Illumina library was prepared for each of the three groups. Each library was sequenced on one lane of HiSeq 2000 (100 million clusters, 2x100 bases paired-end). RNAseq sequencing was performed at Genoscreen (Lille, France).

**Assembly and determination of the abundance of assembled contigs** – The analyses were all carried out in the Galaxy environment and the computing power was provided by the ABiMS platform (Station Biologique de Roscoff, France). The 100-bp paired-ends reads for each group were first filtered for quality with Prinseq-lite, and the pairs of sequences of sufficient quality were established (GetPairs) (Schmieder and Edwards, 2011). The ribosomal sequences were separated from the remaining sequences based on similarity with a rRNA database (riboPicker) (Schmieder et al., 2011). These reads targeted rRNA of both the hosts and the associated microfauna (typically about 25 million paired reads per library) were then assembled with Trinity after normalization to reduce the size of the dataset. This was performed on the three libraries and the resulting contigs were concatenated. Redundancy was removed with CAP3 (Huang and Madan, 1999). The final assembly of rRNA sequences was then used as a reference for quantification of the contigs for each habitat-driven library of worms with RSEM (Li and Dewey, 2011). The results were normalized for the size of the contigs, and the sequencing effort, and are expressed in...
Fragment Per Kilobase of transcript per Million reads of sequencing (FPKM). The closest sequences in GenBank were identified by Blastn and the identifier recovered for all contigs (Altschul et al., 1997).

1.6. Molecular identification of the large epibionts using 16S rRNA

Clone libraries of the 16SrRNA-encoding gene were built from 4 specimens, 2 displaying and 2 devoid of large epibionts using standard bacterial 16SrRNA primers 8F and 1492R as described in (Duperron et al., 2005). Among the distinct bacterial sequences identified, one found only in specimens displaying epibionts was used to design specific primers targeting these epibionts (Forward 5′- GCTGGTCTGAGGACGAAC-3′; Reverse 3′- TTCATGGAGTCGAGTTGCAG-5) with the Primer3 Input software (http://frodo.wi.mit.edu/cgi-bin/ primer3/primer3www.cgi).

Large epibionts were also isolated from debris pellets after centrifuging each worm of the 2015 collection (at 4000 rpm for 5 min) in an ethanol solution as they immediately detach from Capitella in presence of ethanol. Microbial DNA was extracted using the NucleoSpin Tissue kit for bacteria (Macherey-Nagel) according to the manufacturer’s instructions, and amplified with a GoTaq® G2 DNA Polymerase (Promega) using Thiomargarita-specific primers. Reaction mixture for PCR amplification contained 10 µM of each primer, 10 µM of each (dNTP), 1X Go Taq® Flexi buffer (Promega), and 5U of GoTaq G2 Flexi DNA polymerase (Promega). The final volume was adjusted to 25 µl with water. DNA amplification was performed under the following conditions: (1) An initial denaturation step at 95°C for 3 min without enzyme, followed by (2) a series of 39 cycles of denaturation at 95°C for 45 s, of annealing at 55°C for 45 s, and elongation at 72°C for 1 min with the enzyme, and (3) a final elongation step at 72°C for 7 min. PCR products were purified with the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) and were then sequenced according to the Sanger method on a 310 ABI prism (Applied Biosystems).
Sequence alignments and phylogenetic tree: Sequences were aligned using ClustalX (Larkin et al., 2007). A 16S rRNA dataset was built by collecting sequences available from Thiomargarita and related groups. Phylogenetic relationships were estimated based on maximum likelihood using a General Time Reversible (GTR) model and a 5-category discrete Gamma distribution of rates with invariants. Positions with gaps and missing data were not used, resulting in a 1107-bp dataset. Phylogenetic reconstructions were generated using the software MEGA 7 (Kumar et al., 2016).

1.7. Genotyping of epibiotic and non-epibiotic Capitella individuals

DNA extraction and barcoding – After the epibiont recovery, fifty-two Capitella collected during our 2015 temporal survey (see before) in both Le Laber and the Roscoff Harbor were used entirely for DNA extraction using a NucleoSpin Tissue XS (Macherey-Nagel) according to manufacturer's protocol. A 569 bp fragment of the cytochrome oxidase subunit 1 (Cox-1) mitochondrial gene was then amplified using Capitella-specific primers CO1F and CO1R: Forward 5’- GTACAGAACTTGCGCTTCCT-3’ and Reverse 5’- CCACCACGTAGGATCAAA -3’. Amplifications were carried out with a GoTaq® G2 DNA Polymerase (Promega). Reaction mixture for PCR amplification contained 10 µM of each primer, 10 µM of each desoxynucleotide triphosphate (dNTP), 1X Go Taq® Flexi buffer (Promega), and 5U of GoTaq G2 Flexi DNA polymerase (Promega). The final volume was adjusted to 25 µl with sterile water. DNA amplification was performed on a Thermocycler (Eppendorf) with the following conditions: (1) an initial denaturation step at 95°C for 15 min without enzyme, followed by (2) a series of 39 cycles of denaturation at 95°C for 30 s, of annealing at 56°C for 30 s, and elongation at 72°C for 1 min with the enzyme, and (3) a final elongation step at 72°C for 5 min. The PCR products were then visualized onto a 1.5% agarose gel with ethidium bromide following electrophoresis at 100 volts for half an hour. PCR products were then purified with nucleofast 96 PCR cleanup kit and then Sanger-
sequenced on an ABI 3100 using BigDye (PerkinElmer) terminator chemistry following the manufacturer’s protocol. (Applied Biosystems, Foster City, CA).

**Sequence analysis** – Chromatograms were checked manually using SeqScape V2.5. The sequence data were aligned manually with BioEdit v.7.2.5. Maximum likelihood tree reconstructions were performed on our subset of barcoded specimens and additional referenced sequences from Genbank using the software Mega7 following the HKY model of substitutions with the pairwise deletion option (Kumar et al., 2016) to check whether *Capitella* spp. populations found at Le Laber and at the Roscoff Harbor represent cryptic species.

### 1.8. Tolerance tests to experimental exposure to sulfides

Animals collected in July 2020 at the Roscoff Harbor were checked for filamentous epibionts under the microscope and then split into two groups: non-epibiotic *Capitella* (3 batches of 10 individuals each) and epibiotic *Capitella* (3 batches of 10 individuals each). Each batch was placed in a petri dish (35mm) containing 2 mL of artificial seawater (Instant Ocean). The 3 “non epibiotic” batches and the 3 “epibiotic” batches were separately exposed to increasing concentrations of sulfides (batch 0 mM, 1 mM and 3 mM of Na$_2$S 9H$_2$O (SIGMA) in artificial seawater (Instant Ocean® Sea Salt) for 4 days in a moisture chamber in the dark at 16°C. Mortality was assessed every 3 hours, dead animals were counted and immediately removed. The sulfide concentration was also measured and adjusted when required at the same intervals by using the N, N-dimethyl-p-phenylenediamine colorimetric method (Walkley and Black, 2003).

Survivorship data were analyzed through Cox proportional hazard models (Andersen and Gill, 1982), using the ‘coxph’ function within the ‘survival’ package in R programming language (Jackson, 2016). All survival data were analyzed together (same mortality baseline) for the sake of effect comparability. Mortality was assumed to depend on the
phenotype of the worms (epibiotic and non-epibiotic) and the concentration of sulfides (0, 2 and 3mM). We used robust variance estimation (Horvitz-Thompson estimate) assuming correlation among individuals from the same batch (same experiment x same phenotype x same treatment).

2. RESULTS

3.1. Geochemical characterization of sediments in both sites

General parameters - Sediment granulometry was very similar for both sites (Fig. S1): silts (2-63 µm) are the most abundant fraction (40-50%), and their proportions increased toward the sediment-water interface. In the fine fraction, smaller than 63 µm, the amount of Ca, Fe and Al were higher at the Roscoff Harbor than at Le Laber, suggesting that sandy particles, less reactive than clays, carbonates and iron oxides, were more frequent in sediments of Le Laber (Table 1). In the Roscoff Harbor, the layer with the highest proportion of silts extends to a depth of about 3 cm when compared with the site Le Laber (less than 2 cm depth). In this top layer, organic and inorganic carbon contents were greater at the Roscoff Harbor (Table S2). Total nitrogen contents however, are very similar. At sediment depth greater than 3.5 cm, no significant difference between the two sites was noticeable.

Reduced Sulfur Species – At the time when the worms were collected for NGS sequencing (October 2013), the two locations greatly differed by the amount of sulfide in the upper layer of the sediment (Fig. 2A). At the water-sediment interface, the concentrations of solid reduced sulfur species increased in sediments of Roscoff Harbor but not at Le Laber. At one cm depth, concentrations of reduced sulfur species were 5-6 times higher at the Roscoff Harbor than at Le Laber site. Below the depth of 3 cm, concentrations of AVS (the less stable fraction of solid reduced sulfur to oxidation) and CRS (the less reactive fraction of solid
reduced sulfur) ranged from 141 to 978 mgS kg\(^{-1}\) and from 447 and 712 mgS kg\(^{-1}\) for the Roscoff Harbor and Le Laber sites, respectively.

A survey of dissolved sulfide concentrations performed two years later (from July to December 2015) monitored with DGT-Agl probes showed that these species were more abundant in a deeper part of the cores (i.e. below 4-5 cm depth). Interestingly, sulfide concentrations were on average higher at the Roscoff Harbor (from 8.2 to 11.60 mg L\(^{-1}\)) than at Le Laber (from 0.58 to 5.52 mg L\(^{-1}\)) (Fig. S2), in a way similar to the AVS and CRS concentrations. More precisely, in the first 3 cm, where the worms live, the inter-site differences were even more marked, with levels ranging between 1.08 and 5.75 mg L\(^{-1}\) for the Roscoff Harbor as opposed to 0 and 0.27 mg L\(^{-1}\) for Le Laber (Fig. S2 and Table 2).

**Trace metals** - Total metal concentrations (Cd, Co, Cr, Cu, Mn, Ni, Pb and Zn) in the sediments clearly indicate that the Roscoff Harbor was enriched in metals when compared to Le Laber site (Table 1 and S2), especially in Cu for which the ratio reaches 6.1 (Roscoff Harbor/Le Laber). Following the normalization of values, it is worth noting that all the EF values in the sediments of Le Laber site were lower than or equal to 1, excepted for Pb (EF = 1.4). These findings clearly suggest that sediments from Le Laber can be used as an unmodified environment regarding metal concentrations. Conversely, the EF values for Cd, Cu, Zn and Pb were much higher (3.5, 2.8, 2 and 1.6) in the Roscoff Harbor, suggesting a low to moderate anthropic contamination of sediments.

The toxicity index (TI) was calculated for each sample (averaged over the first 5 cm of sediment) from July to December 2015 (Fig. S3). During this period, all TI values were below zero, indicating that no significant toxicity has been encountered in sediments (i.e. most of trace metals are efficiently trapped by sulfides forming AVS). The bioavailability of metals should therefore be extremely limited as sulfides are in excess. The TI values are
however consistently greater in the Roscoff Harbor throughout the sampling period because of higher Zn concentrations.

3.2. Micro-organisms associated with *Capitella* differ between localities and associated habitats

**Morphological observations** – In October 2013 (RNAseq sampling date), around 20% of worms exhibited an epibiosis with long white hair-like projection at the Roscoff Harbor (Figs. 2B, 3B, D). This association was also observed in some *Capitella* worms from Le Laber during the 2015 temporal survey of the two localities, notably during the summer period. Electron microscopy and FISH hybridizations using the probe EUB338 evidenced dense assemblages of filamentous structures (Figs 3D, 4A), with small bacteria attached to larger and more visible ones strongly anchored in the tegument (Figs. 3B, 4B, C) of the epibiotic *Capitella* worms only (Fig. 4, Figs. 3F, G). The larger epibiotic microorganisms were easily observable under transmission or light microscopy (Fig. 3A-D) displaying a size reaching 50 microns from basal to apical ends with refringent cytoplasmic inclusions resembling sulfur granules typical of sulfur-oxidizing bacteria, the lack of a nucleus based on DAPI staining (Fig. 4) and the presence of a large vacuole in the center of the cells (Figs 4D, E). Large *Capitella* epibionts also displayed a larger basal bacterium with an elongated rod shape, atop of which a second, spherical-to-elongate bacterium is budding. A few worms were also parasitized by nematodes (*Trophomera sp.*) living in the coelomic cavity of the worm (Figs.S4D, E), by vorticellid ciliates attached to the tegument (Figs. S4A, B, C) or by gut gregarines (*Ancora saggitata*) (Figs. S4F, G).

**Abundance of symbionts lineages based on RNAseq data** – The most abundant assembled sequences regroup three different *Capitella* rRNAs as expected (Boidin-Wichlacz et al., Under review). These sequences were not considered in the following analyses. The other recovered contigs corresponded to organisms associated to *Capitella,*
which could be either epibionts (tegument), part of the gut contents, or parasites. In the
following analyses, we only considered contigs with abundances greater than 100 FPKM in
at least one of the libraries. Some of these may correspond to different fragments of the
same organism (e.g. fragment of 28S, another fragment of 28S, fragment of 18S, etc.).

The sequence assembly followed by quantification allowed us to identify contigs
corresponding to associated organisms that are found in all three groups (1/ non epibiotic
Capitella from Le Laber 2/ non epibiotic Capitella from the Roscoff Harbor and 3/ epibiotic
Capitella from the Roscoff Harbor) but in variable abundances (Table 3), contigs that are
more common at Le Laber (Table 4), and contigs that are more abundant in the group
corresponding to animals with epibiotic microorganisms (Table 5). Capitella from the three
compared groups are host to a variety of eukaryotes at intermediate occurrence (Tables 3-
5).

Among the organisms found in all three groups in variable proportions, there is a total of
51 contigs (Table 3). The great majority of these organisms are eukaryotes (86.3%), in
particular apicomplexan fish parasites (31.8%) for which Capitella could be an
intermediary host (e.g. Eimeria leucisci, Sphaerospora dicentrarchi, Kudoa iwatai,
Sarcocystis sp). The contig that ranks second in Table 3 corresponds to the known parasitic
gut gregarine Ancora sagittata (Apicomplexa, Ancoridae) (Simdyanov et al., 2017)
specifically associated to Capitella (Fig. 4). Other abundant types of organisms are ciliates
(e.g. Vorticella sp.), nematodes, and annelids that could be part of the gut contents
(Paramphinome jeffreysi, Tubificoides brownie; Fig. 3). Bacteria occupy lower ranks in this
list (ranks 28, 31, 34, 35, 44, 49, and 51), which could reflect their lesser abundance and
also the fact they are single-celled (as opposed to most eukaryotes mentioned earlier). The
15 top-ranking contigs are usually more abundant in the epibiotic animals from the Roscoff
Harbor, with the exception of ranks 1 (a polychaete, possibly from the gut contents), 6 and
11 (a nematode) that are more abundant in the animals from the control site. The animals from the Roscoff Harbor that do not exhibit an epibiosis tend to have low or very low abundances of these contigs. The animals from Le Laber site exhibited a series of taxa corresponding to the contigs that are found in very low abundances in the Roscoff Harbor (Table 4). Six of these eight contigs correspond to apicomplexan parasites, the two remaining ones correspond to a bivalve (likely found in the gut contents), and Corynebacterium. The contigs that are found in much greater abundance in epibiotic animals (Table 5) differed greatly from the organisms identified in Tables 3 and 4. The Capitella specimens from the two other groups (non-epibiotic from the Roscoff Harbor and le Laber) have very low abundances of these contigs (FPKM ≤ 20). 31 out of the 38 contigs (81.6%) correspond to bacteria, mostly within the Gammaproteobacteria. Eight of these bacterial contigs (21%) correspond to sulfur-oxidizing bacteria (Thiomargarita, the most abundant, and Thioalkalivibrio). Six of the bacterial contigs (15.8%) correspond to mollicutes, a group usually found in the guts of invertebrates.

3.3. Phylogenetic affiliation of the large bacterial epibiont to the genus Thiomargarita

A near full length 16S rRNA-encoding sequence (6 reads) affiliated with Thiomargarita was identified in clone libraries from the two specimens displaying the large epibiont (Table S5). The same sequence was successfully amplified from isolated large epibionts using Thiomargarita-specific primers, supporting that this sequence actually corresponds to the large epibiont morphotype. Analysis performed on the near full-length Thiomargarita sequences indicate a single 16S rRNA phylotype that shared 98 % of sequence identity and clustered in a 100% bootstrap-supported clade with sequences of Candidatus ‘Thiomargarita nelsonii’ recovered from the
Costa Rica margin and the Namibian upwelling zone. This clade is distinct from that containing "Candidatus 'T. namibiensis'" (Salman et al., 2011) (Fig. 5). The sequence was registered in GenBank (accession number MZ053470).

3.4. Prevalence of the epibiosis with *Thiomargarita* according to the season, and the size and gender of *Capitella*’ worms

Observed prevalence of *Thiomargarita* fluctuated between zero and 0.44 among sampling dates (average over the year: 0.10), with 95% of observations between zero and 0.31 and a median prevalence of 0.08. Worm size as estimated from the width at the fifth setiger varied between 0.19 and 1.56 mm (average: 0.54 mm) with a slightly fluctuating average value (between 0.43 and 0.68 mm), without any clear temporal trend. The numbers of males, females and undetermined individuals also do not vary much between sampling dates (Fig. S7). A statistical analysis of time-series was performed using the association occurrences as a quantitative variable and the sampling date, size and gender of the worm as explanatory variables. Overall, the probability of association with *Thiomargarita* increases in summer and increases with the worm’s size (Fig. 6). Independently of worm’s size, this probability is also higher for males and undetermined individuals than for females (Figs. S5 and S6). As many models have comparable AICc and Akaike values (Table S6), model predictions have been explored using the Akaike-weighted average of all tested models (Figs. 6, S5 and S6). The analysis of evidence ratios (ratio of Akaike weights of models incorporating or not the focal variable) of all explanatory variables (Table S7) indicates that all variables have likely effects, except ‘site’ (implausible effect), and ‘sex:date’, ‘sex:date²’ and ‘sex:size:date²’ interactions (only plausible effects) using the vocabulary of (Massol et al., 2007).
3.5. Prevalence of the epibiosis with Thiomargarita according to Capitella genotypes

As we know that Capitella spp. from the Roscoff Harbor and Laber represent a complex of three cryptic species (Boidin-Wichlacz et al., Under review), series of individuals with and without epibionts from Le Laber and the Roscoff Harbor were barcoded using the mitochondrial Cox-1 gene to test whether the epibiotic phenotype was species-specific. The obtained phylogenetic tree (Fig. 7) confirmed the co-occurrence of the three different mitochondrial lineages (C-Channel1, C-Channel2 and C-Atlantic) in our set of epibiotic and non-epibiotic worms. The two most closely related species (C-Channel1 and C-Atlantic: see (Boidin-Wichlacz et al., Under review)) dominate the assemblage and correspond to about 90% of the sampling. The epibiosis with the Thiomargarita-like epsilon proteobacteria was checked and is present in all of the mitochondrial lineages examined, including the rarer C-Channel2 one.

3.5. Sulfide tolerance of non-epibiotic versus epibiotic Capitella spp.

A tolerance assay was performed on adult worms from the Roscoff Harbor presenting the epibiotic and non-epibiotic phenotypes, exposed to 0,1 and 3 mM of sulfides. As shown in figure 8, both phenotypes survive to a 3 mM exposure for 1 day (23h30). After this delay, non-epibiotic worms (NE) start immediately to die reaching a 50% mortality after 48h. On the contrary, epibiotic worms (E) first die after an additional 36h delay (first death observed at 58h30) and reached the 50% mortality following a 88h post exposure to 3 mM. In both cases, NE and E all die following a post exposure to 3 mM of 88h and 91h, respectively while non-exposed individuals (0 mM) remain alive until the end of the experiment (104h). A dose-dependent effect was observable, with a shift of the 1 mM mortality curve in NE when compared to the 3 mM curve showing a better survival of this
later group to a 1mM than to a 3 mM exposure. No mortality was observed in E exposed to 1 mM during the allotted time.

4. DISCUSSION

The appearance of animals exhibiting an epibiosis is concomitant with a higher level of sulfides

Capitella worms from the English Channel, which also represent three distinct mitochondrial lineages (Boidin-Wichlacz et al., Under review) are opportunistic species that occupy the top 5 cm of sediment of estuaries and polluted harbors: a black zone (named thiobiome) rich in organic matter especially in the muddy sediments. The surveyed sites are enriched in silts, with a high concentration of organic carbon in the Roscoff Harbor. Concentrations, availability and lability of metals estimated through SEM were greater in the Roscoff Harbor than in the Laber site (excepted for Cr) without reaching levels of contamination as high as those reported in industrialized harbor of the Northern France (e.g. Boulogne Harbor (Table S4)(Cuvillier-Hot et al., 2018) (Fig. S2)). Although concentrations of ETM slightly varied during the monitoring period, the sediments from both sites never reached the threshold of the toxicity index (calculated at a macroscopic scale from about 1 g of sediments) classically used to investigate polluted environments. By contrast, the two sites colonized by the worms strongly exhibited spatial and/or temporal differences in AVS concentrations reaching highly toxic levels for most organisms including other Capitella species from different locations (higher than 10mM) (Dubilier, 1988). This could be explained by differences in the hydrologic conditions and the anthropogenic contamination between the two sites over the year. The seasonal survey shows that sulfide production takes place throughout the year in the Roscoff Harbor while it mostly occurs in the summer period at Le Laber. In the Harbor sediments, the important
input of organic matter linked to anthropogenic activities and anthropization processes results in the production of high quantities of AVS through the bioreduction of sulfates by the Sulfate-Reducing Bacteria (SRB) (https://doi.org/10.1016/j.scitotenv.2018.08.278; https://doi.org/10.1016/S0967-0637(02)00092-4). The confinement of the Roscoff Harbor added to the accumulation of cadavers of crabs due to fishing offloading activities in this zone, promotes green algal proliferation and a high retention of organic matter (with enrichments in TOC and nitrogen contents), and, as a consequence, a greater production of sulfide due to microbial degradation over the year when compared with Le Laber. By contrast, although not affected by off falls, the site of the Laber is subjected to a short and local eutrophication due a river input that favors intense proliferation of benthic algae at the surface of the sediment in this area during the summer period. By being open to the ocean, tidal currents renew twice a day the oxygenation of the water sediment interface of the Le Laber site, promoting the quick reoxidation of AVS (https://doi.org/10.1016/j.oceano.2018.03.003; doi 10.1007/s10498-005-4574-2).

These differential sediment compositions qualitatively and quantitatively are likely to change the community structure of micro- and macroorganisms co-inhabiting with Capitella. Concomitantly with these geochemistry differences over the year and space, we observed two distinct phenotypes of Capitella worms from the English Channel, which are co-occurring independently between at least three genetic lineages of the worm: one characterized by a tegument covered by a consortium of large filamentous bacteria and another one with an epidermis perfectly clean of any microorganisms as checked by electron microscopy and confirmed by PCR and RNASeq. Epibiosis with the large filamentous bacteria were only observed in sediments where the sulfide concentrations reach levels known to be toxic for other Capitella species (Cuomo, 1985). Under these conditions, the prevalence of the epibiotic association is around 20-30% and mostly affects
larger individuals. Trace metals do not seem to affect the epibiosis, since during our survey over the year 2015, the appearance of animals exhibiting an epibiosis increased concomitantly with the level of sulfides in the site Le Laber.

Distinct prokaryotic and eukaryotic associations with the host phenotypes

We assessed the diversity of microorganisms associated with the worm using a RNAseq approach on animals with and without epibionts in the two distinct nearby habitats. First assignments of contig sequences shown that these small worms are associated with a wide variety of prokaryotes and eukaryotes. The composition of the associated communities clearly varies according to the environmental setting. All animals used for the RNAseq study were collected at the same time of the year. Although in the three groups (i.e. Le Laber worms without epibionts, Roscoff Harbor worms with and without epibionts), the apicomplexan fish parasites are very common, bacterial associates and vorticellid ciliates were quite distinct. We did not observe any lethal effects of ciliates on Capitella maintained in the laboratory (unpublished data) by contrast to the enhanced mortality reported for freshwater leeches covered by vorticellid ciliates (Gouda, 2006).

Even though Capitella with and without epibionts were found in the same sediment sample at the Roscoff Harbor, associated bacterial communities from epibiotic animals were quite distinct from non-epibiotic Capitella. Assuming the animals were exposed to the same environmental conditions in the Roscoff Harbor, this observation suggests that the two groups are characterized either by physiological or genetic differences. As previously shown, the barcoding effort revealed that up to three lineages are present in Roscoff, all of which can be the host to the large epibiotic filamentous bacteria. As a consequence, intraspecific genetic differences do not explain the presence of epibiosis and the polymorphic physiological response of the worms seems to represent the best explanation.
Pollution, even at sub-lethal levels can affect the physiology of organisms and affect their relationships with other organisms. Several studies have shown that, when they are not directly lethal, thermal and/or chemical modifications of the environment often induce endocrine and behavioral changes in marine organisms, as well as alterations of their energetic metabolism and immunity (Harvell et al., 1999; Waldichuk, 1979). Cuvillier-Hot et al. (2018) showed that heavy metals and phthalates, even at concentrations below the toxicity index, alter the immune response as well as the trans-generational immune priming of natural populations of the coastal annelid Hediste diversicolor and make them less resistant to an experimental infection by the environmental bacterium Bacillus hwajinpoensis SW-72 isolated from the burrow of the worm (Bernier et al., 2019; Cuvillier-Hot et al., 2018). These observations clearly show the impact of changing environmental conditions on host-bacteria interactions in marine invertebrates.

Worm epibiosis is characterized by a tegumental association with the giant sulfur oxidizing bacterium Thiomargarita sp.

The combined analyses of the RNAseq data, the targeted bacterial 16S amplification results and microscopic observations, allowed the estimation of the abundance and the phylotype diversity of the epibiotic bacteria associated with the Capitella worms in the Roscoff Harbor. Most abundant bacteria fall into three groups: (i) sulfur-oxidizing bacteria (mostly Thiomargarita but also Thiotrix, Thioalkalivibrio, and Sulfuromonas), (ii) mollicutes (including Spiroplasma), typically found in invertebrate guts, and (iii) spirochaetes. We identified the largest and most visible epibiont as being a large gammaproteobacterium belonging to genus Thiomargarita, closely related to Candidatus ‘Thiomargarita nelsonii’.

This is the first report of Thiomargarita in a coastal ecosystem. This giant chemolithotrophic bacterium was often encountered as a free-living species associated
with deep-sea microbial mats. *Thiomargarita* were also found attached to the byssus of a mussel at deep-sea hydrothermal vents (Schulz, 2006), the shell of gastropod *Provanna laevis* at deep-sea methane cold seeps, and on the integument of other seep fauna (Bailey et al., 2011). The ecological behavior of the gastropod *Provanna laevis* was shown to be modified by the presence of *Thiomargarita*, the snail orienting its shell downward to allow its *Thiomargarita* epibionts to be exposed to sulfide-rich water while the animal had access to the oxygen-rich overlaying water, leaving its head partially exposed (Bailey et al., 2011). The fluctuating sulfide-driven chemosynthetic environment appears as an obvious shared characteristic between the *Capitella* and the seep fauna habitats.

Unlike its close relatives *Thioploca* and *Beggiatoa*, *Thiomargarita* are not motile. They store elemental sulfur as granules at the periphery of a very large vacuole that occupies 98% of the cell volume where nitrate is stored (Schulz, 2006). Because of their lack of motility, *Thiomargarita* cells must live in an environment where they will be alternatively exposed to sulfide in the porewater and to nitrate in the overlaying seawater. Compared to previously reported *Thiomargarita* morphologies, the cells attached to *Capitella* are more elongated but the observation of budding structures are similar to those reported in *Provanna laevis* and byssal threads of *Bathymodiolus* mussels from deep-sea cold seeps (Bailey et al., 2011), and suggests that the cells are actively growing. Unlike *Thioploca*, whose populations decline at oxygen concentrations greater than 3 µM, and *Beggiatoa* mats, which thrive with oxygen concentration of 1-2.5 µM, *Thiomargarita* cells can withstand exposure to full atmospheric oxygen concentrations (Schulz, 2006). *Thiomargarita* morphotypes have also been observed attached to various debris while sorting the sediment samples, suggesting their ability to efficiently colonize a wide variety of surfaces, including *Capitella*. The presence of *Thiomargarita* can easily be viewed as a form of biofouling. Their density was, however, higher on the worms, suggesting that these
animals offer a more suitable environment. Moreover, we found that *Thiomargarita* was present on the tegument of the three genetic lineages, cryptic species of *Capitella*, but at a higher prevalence on large worms during the summer period, irrespectively of gender, although more frequently encountered on males and indeterminate individuals.

Is thiothiont epibiosis a facultative mutualistic association to face transient concentrations of sulfide?

The complex of *Capitella* species living in the English Channel is exposed to high concentrations of sulfide in the sediment while pumping overlaying oxygenated water by peristalsis in their burrow. Since *Thiomargarita* is a non-motile, facultative anaerobic sulfur-oxidizing bacterium, the association with the animal could thus represent an opportunistic strategy from the bacterial viewpoint, bridging the oxic-anoxic gap and allowing bacteria access to both electron donors and acceptors. On the other hand, sulfide uptake might be a way to detoxify the environment of *Capitella* and a positive by-product of the bacterium's activity, although this hypothesis needs to be tested. Other sulfur bacteria detected could interact as a consortium of smaller filamentous bacteria working at the surface of *Thiomargarita* cells, as already shown in Namibia sediments (Bailey et al., 2011) but also found in association with the hydrothermal-vent species *Alvinella pompejana* (Le Bris and Gaill, 2006). During the survey of epibiosis over nearly a year, we found a greater abundance of worms with *Thiomargarita* in during the summer on the largest animals from both sampled sites. Summer is the period of the year when temperatures are the highest and thus during which bacterial degradation of organic matter, producing sulfide, is likely to be at its highest in the sediment. The prevalence of the association depends on the presence of free bacteria in the mud what remains to be seasonally surveyed. One might assume that *Thiomargarita* which oxidizes dissolved
sulfide in the pore water grow better during the summer period (Schulz, 2006).

Capitella is a typical member of the ‘sulfide system’. Fenchel & Riedl (Fenchel and Riedl, 1970) coined this term to describe life under these hostile conditions (later called ‘thiobiome’ or ‘thiobios' by Boaden (Boaden, 1975)). Although the thiobiome allows less competitive stress, specific physical and structural adaptations are needed for the survival and thriving of this complex and specific biome. Our observations suggest that at highly “toxic” levels of hydrogen sulfide, physiological adaptations of Capitella alone could not be sufficient to detoxify the reduced sulfur compounds and that a facultative epidermal association with Thiomargarita and other sulfur oxidizing bacteria available in sediment may constitute a vital additional strategy. The tolerance assay provided here evidenced that the observed epibiosis is beneficial to the host when subjected to highly sulfide-rich environments. Besides detoxication, sulfur-oxidizing epibionts may provide nutrients to the host as suggested for deep sea hydrothermal annelids (Desbruyères et al., 1983). Capitella has been shown to feed on free-living autotrophs that use sulfide oxidation to fix CO₂ (Hiroaki et al., 2001). Thiobionts might supply Capitella in nutrients presumably explaining why epibiotic specimens are larger than the non-epibiotic ones.

There is ample empirical evidence of symbioses providing protection against specific natural enemies, e.g. in aphids facing parasitoids and predators (Dion et al., 2011; Oliver et al., 2014; Polin et al., 2014) or pathogens (Clay, 2014; Tasiemski et al., 2015). Such symbioses have also been suggested as potential means to explain the success of some invasive species in new habitats (Amsellem et al., 2017; Chabrerie et al., 2019; Macke et al., 2017). While many of the aforementioned symbioses involved obligatory endosymbionts, the present data bring to light an adaptive advantage of a facultative ectosymbiosis to face changing habitats.

A derived question was to know if this Capitella-Thiomargarita association was species-
specific; to find a specific niche may allow to avoid competition with congeneric species. *Capitella teleta* and *C. capitata* which form a cryptic species complex (Grassle and Grassle, 1976; Nygren, 2014). Even if the populations of *Capitella* inhabiting Roscoff constitute an assemblage of cryptic species (Boidin-Wichlacz et al. under review), barcode analyses performed on the main lineages showed that the epibiotic association is not completely genetically determined (e.g. an intraspecific polymorphism of the immune genes involved in the control of the association might exist). The facultative association is likely due to physiological differences between individuals, more or less correlated to their size and possibly micro-environments at the scale of the worm itself. The observation could also mean that *Thiomargarita* and other epibiotic bacteria correspond to biofouling/parasitic agents capable of colonizing a range of invertebrates, including *Capitella* from different species, when they are under high sulfidic stresses.

**Conclusion**

Our data provide clear evidences of the impact of sediment microgeochemistry on associations between *Capitella* and its surrounding microorganisms with the peculiar development of a transient beneficial epibiosis in worms exposed to high sulfide concentrations. Occurrence and maintenance of an epibiotic community depend on the host’s ability to control the epibiont’s colonization and proliferation through its immune actors. Such defense is probably influenced by variable environmental conditions. Consequently, the next step will be to investigate how and if the immune system of *Capitella* can become permissive to the establishment of this facultative epibiosis as observed for the hydrothermal vent worm, *Alvinella pompejana* and shrimp *Rimicaris exoculata* (Le Bloa et al., 2020; Tasiemski et al., 2014). Regardless of future findings, this emphasizes the importance of investigating symbiotic associations in their proper environmental context.
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**Figure legends**

**Figure 1**: Location of the study (Roscoff, France) with the sampling sites. Sites coordinates are the following: Le Laber: 48°42'47.67"N 4° 0'5.17"O- 48°42'45.92"N 4° 0'3.85"O- 48°42'45.12"N 4° 0'3.60"O, Roscoff Harbor: 48°43'35.46"N 3°58'52.05"O -48°43'34.49"N 3°58'51.64"O- 48°43'34.20"N 3°58'50.53"O and 48°43'34.03"N 3°58'49.15"O

**Figure 2**: (A) Sedimentary AVS and CRS concentration profiles (mgS kg\(^{-1}\) sed) in Roscoff Harbor (blue line) and Le Laber (red line) performed in November 2014 (corresponding to the sampling of the animals for NGS sequencing). (B) Semi-thin sections of *Capitella* sampled for the NGS sequencing: not colonized (in Le Laber, FPKM1 and in the Roscoff Harbor, FPKM2) and colonized by the epibiotic community (in the Roscoff Harbor only, FPKM3).

**Figure 3**: Visible (top) and electron microscopy (bottom) showing non epibiotic (A, C) and epibiotic *Capitella* (B, D). Squares show a zoom on the microbial epibiotic community.

**Figure 4**: Epibionts of *Capitella* spp. (A) Electron microscopy of the *Thiomargarita* like bacteria (C) Notice that *Thiomargarita*-like bacteria are strongly anchored on the tegument and (A, B) themselves host epibiotic communities most likely consisting of bacteria some displaying filamentous morphologies. (D) Several *Thiomargarita*-like structures and other microbial morphotypes. (E) DAPI staining of a *Thiomargarita*-like structure (in the center) attached to the tegument of *Capitella*. (F, G) FISH hybridization on the tegument of an epibiotic *Capitella* specimen using the generalist probe EUB338. Notice the abundance and diversity of bacterial morphologies including rods, cocci and filamentous bacteria.

**Figure 5**: Phylogenetic reconstruction of the position of the *Thiomargarita* sp. sequence obtained from 16rRNA clone libraries obtained from epibiont-covered *Capitella* annelids. See material and methods for detail (FYI: Maximum likelihood using a General Time Reversible Model using MEGA7. Heterogeneity in rates of evolution was accounted by using
Gamma distributed rates (5 categories and invariants). 1140 nucleotide positions were analyzed. Scale bar corresponds to 2 % sequence variation. Bootstrap values at nodes were obtained based on 100 ML replications (>50 shown).

**Figure 6:** Predicted probability of association with epibiotic microorganisms as a function of the time of the year (month, x-axis) and the size of the worm (in mm, y-axis), obtained from model-averaging 166 GLMs linking site, size, date, date² and sex to association with epibiotic microorganisms. Predictions are made for a uniform sampling of worms among the sexes (undetermined, females and males represent 1/3 of the sample each), the sizes (uniform distribution between 0 and 1.8 mm), the sampling dates and the sampling sites. The color of each square on the heatmap indicates the average predicted probability of association of all worms of that size sampled at that date, following the legend on the right.

**Figure 7:** Neighbor-joining tree reconstruction of epibiotic and non-epibiotic *Capitella* spp. individuals barcoded using the mitochondrial marker *Cox-1*. Distances between individuals were calculated according to the substitution model HKY.

**Figure 8:** Tolerance tests to sulfides. Kaplan-Meier plots showing the survivorship of non-epibiotic (red) versus epibiotic (blue) worms sampled from the Roscoff Harbor (2020) experimentally exposed to 0 (solid lines), 1 mM (dotted lines) and 3 mM (dashed lines) concentrations of sodium sulfides. Time in hours.
Table 1: Total and HCl 1M-extracted metals concentrations in the first 5 cm depth sediments of Le Laber and the Roscoff harbor (fraction <63µm). For HCl 1M extraction, an average has been calculated from results obtained between July and December 2015. See table S3 for discrete values and table S4 for a comparison with sediments from other similar North Atlantic French stations (Boulogne, Gravelines and Authie).

<table>
<thead>
<tr>
<th>Concentration (mg kg⁻¹)</th>
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<tbody>
<tr>
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</tr>
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</tr>
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<td><strong>Cu</strong></td>
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<td><strong>Mn</strong></td>
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</tr>
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</tr>
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<td><strong>Pb</strong></td>
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<tr>
<td><strong>Zn</strong></td>
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</tr>
<tr>
<td><strong>Ca</strong></td>
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<td><strong>Fe</strong></td>
<td>27.8</td>
</tr>
<tr>
<td><strong>Mg</strong></td>
<td>51.2</td>
</tr>
<tr>
<td><strong>Al</strong></td>
<td>25.1</td>
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</table>

| Lability (%)            | 76.0 | 4.3  | 17.9| 9.0  | 13.7| 21.3| 26.6|
| Lability (%)            | 39.2 | 20.9 | 18.7| -    |
| **Roscoff**             | 15.1 | 0.1  | 8.7 | 17.9 | 12.1| 11.7| 28.7| 25.1|
| **Harbor**              | 21.2 | 18.1 | 8.5 | -    |
| Ratio of total :Roscoff | 4.93 | 2.08 | 2.49| 1.33|
| Harbor/Laber            | 1.55 | 1.06 | 6.27| 1.60 | 1.93| 1.55| 2.64|

ND: Not detected
Table 2: Dissolved sulfide concentrations (mg L⁻¹). Averaged values for 0-3, 3-15 and 0-15 cm sedimentary horizons from Le Laber and Roscoff Harbor sites (in 2015). In bold, the concentration values where the worms live.

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<th>Concentration (mg/L)</th>
<th>11/8</th>
<th>12/8</th>
<th>18/8</th>
<th>21/8</th>
<th>1/9</th>
<th>9/9</th>
<th>15/9</th>
<th>24/9</th>
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<td></td>
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</tr>
<tr>
<td>0-3 cm</td>
<td>0.15</td>
<td>0.01</td>
<td>&lt;0.005</td>
<td>-</td>
<td>&lt;0.005</td>
<td>0.27</td>
<td></td>
<td></td>
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<tr>
<td>3-15 cm</td>
<td>0.68</td>
<td>3.1</td>
<td>3.1</td>
<td>5.9</td>
<td>6.8</td>
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<td></td>
</tr>
<tr>
<td>0-15 cm</td>
<td>0.58</td>
<td>2.5</td>
<td>2.5</td>
<td>4.8</td>
<td>5.5</td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Roscoff Harbor</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0-3 cm</td>
<td>5.8</td>
<td>1.1</td>
<td>2.8</td>
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<tr>
<td>3-15 cm</td>
<td>13</td>
<td>10</td>
<td>12</td>
<td></td>
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</tr>
<tr>
<td>0-15 cm</td>
<td>12</td>
<td>8.2</td>
<td>9.8</td>
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Table 3: Contigs with intermediate FPKM values (ratios between 30 and 0.03). Le Laber sample (FPKM1), Roscoff Harbor sample without (FPKM2) or with (FPKM3) epibiotic microorganisms. Only hits for FPKM values greater than 100 are represented. Contigs ranked in decreasing order of the greatest FPKM value (shaded in grey).

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<th>FPKM2</th>
<th>FPKM3</th>
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<td>658</td>
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<td><em>Ancora sagittata</em> isolate Ancora2011 external transcribed spacer, partial sequence; 18S rRNA gene, ITS 1, 5.8S rRNA gene, ITS 2, and 28S rRNA gene, complete sequence; and external transcribed spacer, partial sequence</td>
<td>Gregarin of <em>Capitella</em></td>
<td>925</td>
<td>48</td>
<td>1594</td>
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<tr>
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<td><em>Gymnodinium aureolum</em> strain GrAr01 18S ribosomal RNA gene, partial sequence; ITS 1, 5.8S ribosomal RNA gene, ITS 2, and large subunit ribosomal RNA gene, complete sequence; external transcribed spacer, partial sequence</td>
<td>Dinoflagellate algae</td>
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<td>1550</td>
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<td>33</td>
<td>1134</td>
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<td>Coccidian apicomplexa fish parasite</td>
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<td>Uncultured bacterium clone N0004 16S ribosomal RNA gene, partial sequence</td>
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<td>AF185190.1</td>
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Table 4: Contigs found with greater FPKM values in Le Laber sample (FPKM1) compared to the Roscoff harbor without (FPKM2) or with (FPKM3) epibiotic microorganisms' samples. Only hits for FPKM values greater than 100 are represented. Contigs ranked according to decreasing values of FPKM1.

<table>
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<th>FPKM1</th>
<th>FPKM2</th>
<th>FPKM3</th>
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<td>Coral parasite</td>
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<td>Toxoplasma gondii strain CASTELLS chromosome 1a region 5 genomic sequence</td>
<td>Animal parasite</td>
<td>651</td>
<td>95</td>
<td>1</td>
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<td>Bird parasite</td>
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<td>X75453.1</td>
<td>7 10^-99</td>
<td>Toxoplasma gondii (strain P) rDNA for 17s,5.8s,26s, and 5s ribosomal RNA</td>
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<td>Corynebacterium aurimucosum ATCC 700975, complete genome</td>
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<td>Uncultured Glomus clone ZHwq2-227 18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 28S rRNA gene, partial sequence</td>
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**Table 5:** Contigs found with FPKM values at 50 times greater in animals with (FPKM3) and without (FPKM2) epibiotic organisms compared with animals from Le Laber (FPKM1). Only hits for FPKM values greater than 100 are represented. Contigs ranked according to decreasing values of FPKM3.

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<td>Giant sulfur bacterium</td>
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<td>Uncultured bacterium clone Tui57 16S ribosomal RNA gene, partial sequence</td>
<td>Oceanospirillales symbiotic with vent snail <em>Alviniconcha</em></td>
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<td>0</td>
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Figure 2

A

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- **Laber**
- **Harbor**

Depth (cm)

-16

B

**LABER**

- Capitella (100% of the population)

FPKM1

**HARBOR**

- Capitella (80% of the population)
- Epibiotic Capitella (20% of the population)

FPKM2

FPKM3
Figure 3
Figure 4
Figure 5

FR690957 “Candidatus Thiomargarita nelsonii” isolate NAM069
HF954113 Uncultured Thiomargarita sp. clone HYR001
FN811661 “Candidatus Thiomargarita nelsonii” isolate Costa COS008
FR690966 “Candidatus Thiomargarita nelsonii” isolate COS015
FR690959 “Candidatus Thiomargarita nelsonii” isolate NAM071
FR690953 “Candidatus Thiomargarita nelsonii” isolate NAM064
HF954103 Uncultured Thiomargarita sp. clone NAM094
FR690945 “Candidatus Thiomargarita nelsonii” isolate NAM056

00FR690927 “Candidatus Thiomargarita nelsonii” isolate NAM037

MZ053470 “Candidatus Thiomargarita sp.” from body surface of Capitella spp.

FR690879 Thiomargarita namibiensis isolate NAM001
FR690914 Thiomargarita namibiensis isolate COS006

AF532774 “Candidatus Parabeggiatoa communis” isolate Limfjorden L8
AF532772 “Candidatus Parabeggiatoa communis” isolate Limfjorden L22
FJ814745 Uncultured Beggiatoa sp. clone V1994 9A02
FR666858 Uncultured Beggiatoa sp. clone AMV1058
AF035956 Beggiatoa sp. Bay of Concepcion
FR847869 “Candidatus Halobeggiatoa sp.” clone HMW-R907
FR847865 “Candidatus Halobeggiatoa sp.” HMW-S2548
FR847884 Uncultured Beggiatoa sp. clone HMW-S2139

AF110277 Beggiatoa sp. MS-81-6
FR717278 Beggiatoa sp. 35Flor
Figure 6
Figure 7

BioNJ 499 sites HKY 100 repl.

C-Channel 1

C-Channel 2

C-Atlantic

0.021

0.057

0.111

0.122

CA449-Epibiosis
CA406-Epibiosis
CA348-Epibiosis
CA279
CA591
CA499-Epibiosis
CA244-Epibiosis
CA100
CA265
CA640-Epibiosis
CA293
CA245
CA277-Epibiosis
CA81
CA242-Epibiosis
CA656-Epibiosis
CA354-Epibiosis
CA551
CA568
CA618-Epibiosis
CA638-Epibiosis
CA112
CA561
CA537
CA572
CA225
CA420
CA588
CA650-Epibiosis
CA541
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CA662-Epibiosis
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CA406-Epibiosis
CA449-Epibiosis
Credit author statement

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Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: