In 1995 a strain of *Ectocarpus* was isolated from Hopkins River Falls, Victoria, Australia, constituting one of few available freshwater or nearly freshwater brown algae, and the only one belonging to the genus *Ectocarpus*. It has since been used as a model to study acclimation and adaptation to low salinities and the role of its microbiota in these processes. To provide more background information on this model, we assessed if *Ectocarpus* was still present in the Hopkins River 22 years after the original finding, estimated its present distribution, described its abiotic environment, and determined its in situ microbial composition. We sampled for *Ectocarpus* at 15 sites along the Hopkins River as well as 10 neighboring sites and found individuals with ITS and cox1 sequences identical to the original isolate at three sites upstream of Hopkins River Falls. The salinity of the water at these sites ranged from 3.1 to 6.9, and it was rich in sulfate (1–5 mM). The diversity of bacteria associated with the algae in situ (1312 operational taxonomic units) was one order of magnitude higher than in previous studies of the original laboratory culture, and 95 alga-associated bacterial strains were isolated from algal filaments on site. In particular, species of *Planctomycetes* were abundant in situ but rare in laboratory cultures. Our results confirmed that *Ectocarpus* was still present in the Hopkins River, and the newly isolated algal and bacterial strains offer new possibilities to study the adaptation of *Ectocarpus* to low salinity and its interactions with its microbiome.

Key index words: distribution; *Ectocarpus subulatus*; freshwater colonization; low salinity adaptation; microbiota

**Abbreviations:** ANOSIM, analysis of similarity; BLAST, Basic Local Alignment Search Tool; ENA, European Nucleotide Archive; HPAEC, high-performance anion exchange chromatography; NCBI, National Center for Biotechnology Information; NMDS, non-metric multidimensional scaling; OTU, operational taxonomic unit; R2A agar, Reasoner's 2A agar

Brown algae (Phaeophyceae) are widespread in the tidal and subtidal zone of rocky shores in temperate marine environments, but they are rarely...
found in fresh water (Dittami et al. 2017). While there are ca. 2,000 known species of marine brown algae, covering a large range of morphologies from small filamentous algae to large and morphologically complex kelp species, there is only a handful of known freshwater brown algae, all of them small and with simple morphology (crust forming or filamentous). Among these freshwater brown algae the genus *Ectocarpus* has a unique position because it corresponds to a predominantly marine genus, which, on two occasions, has been recorded also in rivers: one occurrence of *Ectocarpus crouaniorum* in a highly salt-contaminated section of the Werra river in Germany (Geissler 1983); and one occurrence of *Ectocarpus subulatus* in a nearly freshwater habitat (salinity 1) in the Hopkins River, Victoria, Australia (West and Kraft 1996, Peters et al. 2015).

The isolate from the latter site (Culture Collection of Algae and Protozoa accession 1310/196) constitutes a potential model system to study marine–freshwater transitions in brown algae. The species *Ectocarpus subulatus* (Peters et al. 2015) is related to the genomic model species *Ectocarpus siliculosus* (Cock et al. 2010) and has previously been found in highly variable environments, including environments with high levels of abiotic stressors. Its occurrence was reported, for instance, at Port Aransas, Texas, USA, where monthly average water temperatures reach 30°C in July (Bolton 1983). More recently, the nuclear genome of *E. subulatus* has been sequenced, revealing that *E. subulatus*, in comparison to *Ectocarpus siliculosus*, has lost members of gene families down-regulated in low salinities, and conserved those that were up-regulated (Dittami et al. 2020). The *E. subulatus* strain from Hopkins River Falls has further been used for physiological experiments: it can grow in both seawater and fresh water and its transcriptomic and metabolic acclimation to these conditions has been examined (Dittami et al. 2012) along with the composition of its cell wall with regard to sulfated polysaccharides (Torode et al. 2015). Moreover, the capacity of the freshwater strain to grow in low salinities has been shown to depend on its associated microbial community, although the nature of this dependence is still unknown (Dittami et al. 2016). Extensive efforts have been made to develop a collection of cultivable bacteria to study this phenomenon (KleinJan et al. 2017).

Despite this increasing quantity of data on the physiology of the Hopkins River Falls strain of *Ectocarpus subulatus*, we currently know little about its abiotic environment in situ. The original paper describing its isolation (West and Kraft 1996) states that it was isolated on March 24th, 1995 from cracks between the basalt rock of the Hopkins River, just above the Hopkins River Falls. Water temperature was 16°C, salinity was ~1, and conductivity ~3 mS·s⁻¹. However, it remains unknown if *E. subulatus* is still present at Hopkins River Falls, and if so what its current distribution is. Furthermore, the culture has undergone >20 years of cultivation in different laboratories, potentially having a strong impact on its associated microbiota.

In this study, we address both of these knowledge gaps by returning to the Hopkins River and searching for this alga for the first time since its discovery 20 years ago. We found *Ectocarpus subulatus* individuals at three locations along the Hopkins River, examined its associated microbiome in situ, and isolated several novel alga-associated bacterial strains from these samples. These data provide important background information for the use of *E. subulatus* as a model to study low salinity acclimation/adaptation and the role of microbes in these processes.

**MATERIALS AND METHODS**

*Biological samples.* The sampling campaign was carried out from March 21st to March 27th, 2017 and covered several locations along the Hopkins River between Warrnambool and Ararat (sites 1–15; selected due to their accessibility and to cover the entire length of the river), as well as 10 sites selected arbitrarily along the Southern Australian Coastline between Port Fairy and Avalon (Fig. 1, Table 1). At each sampling site, we manually searched for filamentous algae resembling a member of the *Ectocarpales* within a range of ca. 50 m and for at least 30 min. If filaments were found, small amounts of live samples were taken and rinsed three times in sterile 50 mL Falcon tubes with 0.2-µm-filtered local water (three replicates). A small piece of each sample was stored at max. 20°C in sterile 2 mL Eppendorf tubes filled with the surrounding water for live algal cultures. The second part of the samples was ground on-site according to Tapia et al. (2016), with 50 µL of 0.2-µm-filtered local water in a sterile mortar and the proximity of a Bunsen burner. One, 7, and 35 µL of the ground alga were diluted with 0.2-µm-filtered local water to a final volume of 50 µL and spread immediately onto prepreared Reasoner’s 2A (R2A) agar plates (Sigma-Aldrich, St. Louis, MO, USA) for isolation of cultivable bacteria. These plates were kept at ambient temperature (max. 25°C) and were monitored for 2 weeks. Newly emerging colonies were purified once more on fresh R2A plates and then put into culture in liquid Zobell medium (Zobell 1941) with 8-fold reduced salt concentration, identified by 16S rRNA gene sequencing (see below), and put into stock at −80°C in 40% glycerol. The remaining sample was dried using silica gel for downstream analysis of the microbial community composition, and frozen at −20°C after the sampling campaign.

For all sites, we also collected samples for germling emergence experiments to detect the presence of *Ectocarpus* spores: three to seven sediment samples including small pieces of solid substrate (shells, pebbles, and branches) if present. Approximately 0.1 mL of sediment was kept as live samples in sterile 2 mL Eppendorf tubes. After 2 weeks these samples were transferred to fresh Provasoli-enriched (Starr and Zeikus 1993) medium based on 5, 25, or 100% seawater, depending on the salinity of the water at the sampling site. Seawater for culture media was collected in Roscoff (48°46'40" N, 3°56'15" W), 0.45 µm filtered, and autoclaved at 120°C for 20 min prior to use. The sediment samples were then kept at 13°C in a 14:10 h light/dark cycle at an irradiance level of 25 µmol PAR m⁻² s⁻¹, and the emergence of *Ectocarpus*-like germlings was monitored over 4 months.

Both live algae collected in situ and those recovered from germling emergence experiments were cleaned by rigorous
pipetting with a Pasteur pipette and several transfers to fresh, sterile, medium. Any diatoms that remained attached to the algal filaments were removed via treatment with 3 mg L\(^{-1}\) GeO\(_2\) for 3 weeks.

**Water samples.** Approximately 100 mL of water was taken from each site, immediately filtered with 0.45 \(\mu\)M syringe filters to remove particulate matter, and then pasteurized for 1 h at 95–100°C to remove any remaining bacterial activity. Filtered samples were stored at ambient temperature until the end of the sampling campaign (max. 2 weeks) and then frozen at −20°C until analysis. The conductivity of water samples was determined using an AA3 auto-analyzer (SEAL Analytical, Southampton, UK) following the method of Aminot and Kérouel (2007) with an accuracy of 0.02 \(\mu\)mol L\(^{-1}\), 0.01 \(\mu\)mol L\(^{-1}\), and 0.01 \(\mu\)mol L\(^{-1}\) for NO\(_3\)\(^{-}\), NO\(_2\)\(^{-}\), and PO\(_4\)\(^{3-}\), respectively. Sulfate concentrations were determined by high-performance anion-exchange chromatography (HPAEC), according to a protocol adapted from Préchoux et al. (2016). After suitable dilution, water samples were injected onto an IonPac\textsuperscript{SM} AS11-HC column (4 × 250 mm) equipped with an AG11-HC guard column (4 × 50 mm), using an ICS-5000 Dionex system (SP-5 & Analytical CD Detector; Thermo Fisher Scientific, Waltham, MA, USA). Elution was performed with isocratic 12 mM NaOH at a flow rate of 1 mL min\(^{-1}\), and sulfate ions were detected in conductimetry mode (ASRS 500, 4 mm) and quantified using a standard calibration curve. To
### Table 1. Overview of samples taken and species identified. The numbers in the location column correspond to site numbers in Figure 1.

| Date       | Location                  | Material sampled          | Ectocarpus tissue found | GPS coordinates              | Germinating emergence | Sequence accession(s) |
|------------|---------------------------|----------------------------|-------------------------|-----------------------------|-----------------------|------------------------|
| 2017-03-22 | 1. Merri Island           | Pebbles/mussels in tide pool | No                      | -38.402116, 142.471548     |                       |                        |
| 2017-03-22 | 2. Point Ritchie          | Sand/sandstone             | No                      | -38.401275, 142.509318     | Ectocarpus siliculosus | LR735221 (cox1) LR735414 (ITS) |
| 2017-03-22 | 3. Mahoney Road           | Sand, wood/ plastic        | No                      | -38.392103, 142.531644     |                       |                        |
| 2017-03-22 | 4. Smith Lane             | Mud, wood                  | No                      | -38.397984, 142.578286     |                       |                        |
| 2017-03-22 | 5. Allan’s Ford           | Rock (granite)             | No                      | -38.585925, 142.587398     |                       |                        |
| 2017-03-22 | 6. Donovan’s Lodge        | Granite, clay              | No                      | -38.356110, 142.509290     |                       |                        |
| 2017-03-22 | 7. Hopkins River Falls    | Volcanic rock              | No                      | -38.335509, 142.621352     |                       |                        |
| 2017-03-23 | 8. Framingham Forest      | Rock, pebbles              | E. subulatus Au8 (abundant) | -38.297064, 142.668291     |                       | LR735222 (cox1)       |
| 2017-03-23 | 9. Kent’s Ford            | Rock, pebbles              | E. subulatus Au9 (abundant) | -38.191574, 142.698058     |                       | LR735415 (ITS)        |
| 2017-03-23 | 10. Hexham                | Mud, wood                  | E. subulatus Au10 (rare) | -37.995732, 142.689141     |                       | LR735224 (cox1)       |
| 2017-03-23 | 11. Chatsworth            | Mud, sand, detritus        | No                      | -38.856357, 142.650644     |                       | LR735417 (ITS)        |
| 2017-03-23 | 12. Wickliffe              | Sand, detritus             | No                      | -38.694348, 142.726074     |                       | LR735416 (ITS)        |
| 2017-03-23 | 13. Rossbridge            | Rock, pebbles              | No                      | -38.480217, 142.849465     |                       |                       |
| 2017-03-23 | 14. Ararat                | Sand                       | No                      | -38.500211, 142.979379     |                       |                       |
| 2017-03-24 | 15. Green Hill Lake       | Pebbles                    | No                      | -38.295320, 142.979170     |                       |                       |
| 2017-03-24 | 16. Merri River, Warmabool River | Concrete              | No                      | -38.362077, 142.484414     |                       |                       |
| 2017-03-24 | 17. Killarney beach airport | Sand, lava               | No                      | -38.357966, 142.306880     | *Kuckuckia* sp.       | LR735225 (cox1) LR735418 (ITS) |
| 2017-03-24 | 18. Belfast Lough          | Pebbles, shells            | No                      | -38.361889, 142.262045     |                       | LR735418 (ITS)        |
| 2017-03-25 | 19. Curdies River         | Rock                       | No                      | -38.519965, 142.833558     |                       | LR735417 (ITS)        |
| 2017-03-25 | 20. Port Campbell         | Tidal pool, sand          | No                      | -38.620681, 142.992981     | *Feldmannia* sp.      | LR735226 (cox1)       |
| 2017-03-25 | 21. Gellibrand River      | Mud, detritus              | No                      | -38.727482, 143.250932     |                       |                       |
| 2017-03-25 | 22. Aire River            | Mud, pebbles               | No                      | -38.763797, 143.474727     |                       |                       |
| 2017-03-25 | 23. Wild Dog Creek        | Sand                       | No                      | -38.735911, 143.683545     |                       |                       |
| 2017-03-25 | 24. Smythe’s Creek        | Pebbles, biofilm           | No                      | -38.704648, 143.762856     |                       |                       |
| 2017-03-26 | 25. Lorne                 | Tidal pool, sand shells    | No                      | -38.531281, 143.980994     | *Arinetospora* sp.    | LR735227 (cox1)       |
| 2017-03-26 | 26. Hovell’s Creek        | Clay                       | No                      | -38.018825, 144.402156     |                       |                       |

Bacterial cultures were identified by partial sequencing of their 16S rRNA gene. Fifty µL of dense bacterial culture was heated to 95°C for 15 min, spun down for 1 min, and 1 µL of supernatant was used as a template in a PCR reaction with the 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R 5′-GGTTACCTTGTTACGACTT-3′ (Weisburg et al. 1991) at a final concentration of 0.5 µM. Except for the annealing temperature (53°C here), the same PCR protocol as above was employed.

All PCR products were purified using ExoStar (Thermo Fisher Scientific) and the purified 16S rRNA gene amplicons were sequenced with Sanger technology at the GENOMER platform (FR2424; Roscoff Biological Station), using the BigDye Xterminator v3.1 cycle sequencing kit (Applied Biosystems, Waltham, MA, USA). For bacterial strains, sequencing was carried out only in one direction using the 8F primer, and for algal sequences both the forward and the reverse strand were sequenced and manually assembled. Sequence identification was carried out using RDP classifier (Wang et al. 2007) for bacterial 16S rRNA gene sequences, and Basic
Local Alignment Search Tool (BLAST) searches against the National Center for Biotechnology Information (NCBI) nt database (July 2017) for algal sequences. They were further aligned together with reference sequences from the NCBI nt database using the MAFFT server (Katoh et al. 2002) and the G-INS-i algorithm. All positions with less than 95% site coverage were eliminated. Phylogenetic analyses were carried out with MEGA 7 (Kumar et al. 2016) using the Maximum Likelihood method based on the GTR+G+I model and 1,000 bootstrap replicates.

**Ampli-**
**con sequencing of in situ bacterial communities.** Amplification sequencing of bacterial communities was carried out to assess the in situ composition of the *Ectocarpus subulatus* microbiome. Sufficient material for these analyses was obtained at two of the three sites with *Ectocarpus* individuals: sites 8 and 9 (Fig. 1). Approximately 20 mg dry weight for each of the three replicate samples for each site were ground twice for 45 s at 30 Hz in a TissueLyser II (Qiagen, Hilden, Germany). DNA was then extracted using the Qiagen DNeasy Plant mini kit according to the manufacturer’s instructions. Approximately 50 ng of DNA (as estimated using a NanodropONE; Thermo Fisher Scientific) was then used to amplify the V3-V4 region of the 16S rRNA gene. Furthermore, a mock community comprising a mix of DNA from 26 bacterial genera was cultivated in our laboratory (see Thomas et al. 2020 for details), as well as a negative control was added alongside the samples. PCR amplification, indexing, and library construction were carried out following the standard “16S Metagenomic Sequencing Library Preparation” protocol (Part # 15044223 Rev. B). Final library concentrations were measured using a BioAnalyzer (Agilent, Santa Clara, CA, USA) before pooling. Libraries for each sample were then pooled in an equimolar way, diluted to 5 nM final concentration and supplemented with 20% PhiX to add sufficient diversity for sequencing on an Illumina MiSeq using a 2 × 300 bp cartridge. Raw data were deposited at the European Nucleotide Archive (ENA) under project accession number PRJEB34906 (https://www.ebi.ac.uk/ena/data/search?query=PRJEB34906).

Raw reads were first trimmed and filtered using the fastx_quality_trimmer script (http://hamonlab.cshl.edu/ftx_toolkit/), assembled using Pandaseq 2.11 (Masella et al. 2012), and further processed with mothur according to the MiSeq SOP (version April 4th, 2018; Kozich et al. 2013). Sequences were aligned to the non-redundant SSU ref database version 132, chimeric sequences removed using Vsearch (Rognes et al. 2016), and operational taxonomic units (OTUs) defined based on a 97% identity threshold (Stackebrandt and Goebel 1994). Rare sequences (<5 reads across all samples) were removed from the final analyses. Taxonomic assignments were generated for both the raw reads and the final OTUs using the RDP classifier method (Wang et al. 2007). Non-metric multidimensional scaling (NMDS) of the OTU matrix was carried out in R 3.5.1 using the isoMDS function of the Vegan package and Bray-Curtis dissimilarity as a distance measure. An Analysis of Similarity (ANOSIM) was used to test for differences in the overall community composition between the two sites (3 replicates each, 719 permutations). Statistical differences between the two sites at the level of individual OTUs were assessed by multiple two-sided t-tests (one test per OTU) on log-transformed abundance data with subsequent correction for multiple testing according to Benjamini and Hochberg (1995). Alpha diversity was estimated using the Shannon index with e as a base and the diversity() function of the VEGAN package. A two-sided t-test was used to compare these indexes obtained for the replicate samples of both sites. Differences between sites were considered significant if the Type I error rate was below 0.05.

**RESULTS**

**Distribution of Ectocarpus subulatus along the Hopkins River.** We found live *Ectocarpus subulatus* at 3 of the 15 sampled sites along the Hopkins River, and germlings of other Ectocarpales emerged from four additional marine sites along the Victorian coastline, including at the mouth of Hopkins River (Table 1). Despite extensive searches, no traces of *Ectocarpus* were found at the original isolation site of *E. subulatus* at Hopkins River Falls (site 7; Fig. 1). *Ectocarpus* was, however, abundant at two sites (Framlingham Forest reserve and Kent’s Ford, sites 8 and 9; Fig. 1), which were ~12 km and 37 km upstream of Hopkins River Falls. The third finding of *Ectocarpus* was registered 83 km upstream (site 10), although only a few filaments were found at this site. The coxl and ITS sequences obtained from *Ectocarpus* cultures from all three sites were identical to those of the strain isolated from Hopkins River Falls in 1995 (Fig. 2). We found no *E. subulatus* individuals in other sampled rivers, along the coastline, or in germling emergence experiments.

**Water chemistry.** The salinity of the Hopkins River was highest close to the source (8.4; ~3/4 that of sea-water), and then gradually decreased toward the mouth of the river, where it dropped to ca. 1, before re-spiking due to the influence of seawater (Fig. 3). This decrease corresponded to an increase in the flow of water masses toward the mouth river. Sulfate concentrations followed the same pattern as salinity (Pearson correlation $r = 0.995$, df = 12, $P < 0.001$) and decreased from nearly 7 mM to ~0.4 mM close to the river mouth. Finally, phosphate and nitrite/nitrate concentrations were variable along the river. They were highest at the Chatsworth site (site 11), reaching 3.3 and 22.8 μM, respectively, and then strongly decreased at sites where *Ectocarpus* was found (PO$_4^{3-}$ 0.5–0.8 μM, NO$_2^-$/NO$_3^-$ 0.8–1.7 μM; Fig. 3).

**Bacterial communities associated with algae.** In situ bacterial community composition was determined by 16S rRNA gene amplicon sequencing for field samples taken at Framlingham Forest reserve (site 8) and Kent’s Ford (site 9; Fig. 4A). We detected 1312 OTUs across the three sampled individuals from both sites (Table S1 in the Supporting Information). The bacterial communities of both sites were dominated by *Alphaproteobacteria* (25% of reads), *Bacteroidetes* (20%), *Gammaproteobacteria* (8%), *Planctomycetes* (8%), and *Actinobacteria* (8%) (Figure 4A), and there was a significant difference in the community structure between the two sites (ANOSIM, $R_{1,4} = 1$, $P = 0.001$; Fig. 4B). Examining the OTUs individually, we identified 86 OTUs that were specific to Framlington Forest reserve (including 31 *Proteobacteria* and 22 *Planctomycetes*), and 60 more had a higher relative abundance there. At Kent’s Ford, 27 OTUs were site specific (including
13 Proteobacteria and 6 Bacteroidetes), and 13 more exhibited higher relative abundance. In three cases, site-specific OTUs from both sites were found to belong to the same genera: Rickettsiales of the SM2D12 group, Flavobacterium, and Luteolibacter. A detailed list of these OTUs is provided in Table S1. Alpha-diversity (Shannon index) was also slightly higher at the Framlingham Forest reserve ($t_9 = 3.1802$, $P = 0.03$; Fig. 4C). Amplicon sequencing analyses of bacterial communities were complemented by in situ isolation of bacterial strains from the algae after thorough rinsing with sterile river water (Fig. 5). They comprise Gamma-proteobacteria (48 isolates, including 28 Pseudomonas), Firmicutes (27 isolates), Actinobacteria (8 isolates), Alphaproteobacteria (7 isolates), and Bacteroidetes (5 isolates). No members of the Planctomycetes were isolated.

**DISCUSSION**

The data presented in this study confirm that the original finding of Ectocarpus subulatus by West and Kraft was not the result of a transient “contamination”, but that algae with identical ITS sequences are present in the river at three sites upstream of the original location. At the time of sampling, the water at these sites was saline and contained high levels of sulfate for a river, but low levels of nitrite/nitrate and phosphate compared to other upstream and downstream sites. Furthermore, the bacterial community associated with the algae in situ comprised over 1300 OTUs, which is highly diverse compared to laboratory cultures. It also included a high diversity of Planctomycetes. Each of these findings, discussed in more detail below, provides valuable background information when using E. subulatus cultures as a model system to study acclimation, adaptation, or interactions with their associated microbiome.

Based on these observations, it seems likely that Ectocarpus subulatus has persisted in the Hopkins River for over 20 years, maintaining a population despite the water currents. Ectocarpus spores and gametes are motile, but swimming speeds reported are only in the range of 150–270 μm·s⁻¹ (Müller 1978). This implies that E. subulatus in Hopkins River either (1) does not rely on gamete releases for reproduction, (2) that its gametes are able to remain close to the substratum as has been suggested for male gametes (Müller 1978) and direct their movement upstream, or (3) that gametes rely on zoochory, as has been proposed in the case of red algae (Žuljević et al. 2016). Our findings thus open interesting perspectives for population genetics studies as well as more detailed studies of the reproductive biology of Ectocarpus in this area. Furthermore, the fact that no traces of E. subulatus were found in nearby rivers or along the coastline suggests that it may be restricted to the Hopkins River, although the range of colonization within the river may have been subject to variation, notably because individuals of E. subulatus were no longer found at the original isolation site.

Although limited to a single point in time, our sampling campaign also provides novel information on the chemical parameters in the Hopkins River at the time of sampling. Notably, the observed salinity at sites with Ectocarpus subulatus between 3.1 and 6.9 leads us to classify the water in the Hopkins River at the sites with E. subulatus at the time of sampling as low salinity brackish water rather than fresh water (usually defined by a salinity <0.5, International Symposium for the Classification of Brackish Waters 1958). This may be one of the factors enabling E. subulatus to be competitive in this environment, a hypothesis which is supported by the fact that no individuals were found in the lower portions of the river with lower salinity. High salinity in our samples also positively correlated with high sulfate concentrations between 1 and 5 mM – average sulfate concentrations in fresh water are 0.12 mM (vs. 28 on average in the ocean; Wetzel 2001). Sulfated polysaccharides are typical components of the cell walls of marine plants and algae (Popper et al. 2011) and require sulfate for their synthesis, but their importance for Ectocarpus remains to be explored. In the same vein the question remains open to what extent the low nitrate concentrations at sites with E. subulatus compared to upstream and downstream sites are related to the presence of the algae, either as a cause or as an effect. It should be noted, though, that a direct metabolomic comparison of E. subulatus and the marine E. siliculosus revealed markers for high nitrogen status (total amino acids, ratio of glutamine to glutamate) in E. subulatus (Dittami et al. 2012). Regardless of the physiological implications of the composition of the Hopkins River water, we argue that it may be more appropriate to refer to the E. subulatus strains isolated from the Hopkins River as “fluvialite” (i.e., “river” strains rather than freshwater strains) despite their capacity to grow in fresh water in laboratory conditions (Dittami et al. 2012).

In addition to these facts about the distribution and environment of E. subulatus, this study provides insights into its associated microbiome – a component likely connected to the capacity of this species to grow in low salinity (Dittami et al. 2016). The number of OTUs associated with E. subulatus in our in vivo study was one order of magnitude higher than in a previous study of the laboratory strain after 20 years of cultivation (1312 OTUs for six samples from two sites vs. 84 OTUs for six samples in two conditions; Dittami et al. 2016). Moreover, a direct taxonomic comparison of these two studies at the genus level revealed only five genera (Acinetobacter, Phycisphaera, Maribacter, Mariniscillium, and Gaiella) that were found in both studies. All of them were rare, that is, supported by <0.01% of reads in our study; Table S1). Both studies were based on sequencing runs with similar depth and employed...
Fig. 2. Maximum-likelihood tree of *Ectocarpus* isolates from Hopkins River and related strains. Panel A displays a tree based on the COX1 gene (alignment of 677 bp after curation), and Panel B on the ITS region (860 bp after curation). Bold names indicates isolates from this study. Support values correspond to the percentage of support using 1,000 bootstrap replicates. [Color figure can be viewed at wileyonlinelibrary.com]
similar analysis pipelines, yet many technical factors could contribute to such differences: the sampling protocol, the primers used, library preparation, the sequencing platform, and chemistry (Illumina MiSeq V2 vs. V3), etc. Nevertheless, the profoundness of the observed differences suggests that either the microbiome of *E. subulatus* in the Hopkins River has evolved and diversified over time or that the cultivation of algae in the laboratory has impacted its microbiome, leading to a reduction in diversity and a change in composition. In a context of the development of new laboratory models for the study of marine holobionts (Dittami et al. 2019), a targeted examination of these potential changes (e.g., by following the evolution of alga-associated microbiomes in the field as well as over several cultivation cycles) may yield important insights on possible limitations of laboratory model systems. If confirmed, such biases would underline the necessity of devising targeted experiments to test the validity of laboratory findings in the field.

The availability of parallel amplicon sequencing data of bacterial communities and untargeted cultivation efforts further allows us to identify undersampled lineages in cultivation experiments. In this study, particularly *Planctomycetes* stand out, as

**Fig. 3.** Water chemistry at the different sampling sites along the Hopkins River (see Fig. 1). Panel A displays salinity (gray bars) and nutrient concentrations (solid and dashed lines), and Panel B shows sulfate concentrations. Each measurement corresponds to a single sample collected between 2017-03-22 and 2017-03-24. [Color figure can be viewed at wileyonlinelibrary.com]
they constituted 176 OTUs and 8% relative abundance of all algae-associated reads but did not have a single associated culture. *Planctomycetes* are notoriously difficult to cultivate, partially due to their long doubling time of up to 1 month. They require low organic content in media, physical separation from fast-growing competitors (e.g., via dilution to extinction experiments, and they may benefit from the use of fungicides; Lage and Bondoso 2012). In contrast to the present study, previous barcoding data (Dittami et al. 2016) on cultivated *Ectocarpus subulatus* revealed the presence of very few *Planctomycetes* (0.1% of reads), implying that these culturing techniques would need to be put into place using freshly collected material. In contrast, the high abundance of *Firmicutes* in the isolation experiments although they account for only 1% of the reads in the amplicon sequencing data may be because these bacteria were particularly amenable to the culture condition.

This study enhances our knowledge on *Ectocarpus subulatus* from the Hopkins River and its associated microbiome. It furthermore provides a new set of microbes for coculture experiments and thus strengthens the use of *E. subulatus* both as a model for the study of acclimation and adaptation to low salinity and of algal–bacterial interactions.
This work was funded partially by ANR project IDEALG (ANR-10-BTBR-04) "Investissements d’Avenir, Biotechnologies-Bioressources", the European Union’s Horizon 2020 research and innovation Programme under the Marie Skłodowska-Curie grant agreement number 624575 (ALFF), and an internal call for proposals from the UMR8227 (CNRS, Sorbonne University). We thank Cécile Hervé, Amandine Simeon, and Agnieszka P. Lipinska for helpful discussions; Gwenn Tanguy and Erwan Legeay from the GENOMER platform; Roscoff for support during the library construction and sequencing; and the ABIMS platform for providing the computational facilities for the amplicon sequencing analyses.

CONFLICT OF INTEREST STATEMENT
The authors declare no conflict of interest.

AUTHOR’S CONTRIBUTIONS
SD, AFP, HK, SE, JW, and CB planned the study; TC performed nutrient analyses; AP measured sulfate concentrations; BBD performed amplicon sequencing analyses of bacterial communities; SD performed sampling, culturing, in silico analyses,
and wrote the manuscript; All authors corrected the manuscript and approved the final draft.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web site:

Table S1. Amplicon sequencing results of bacterial communities.