Revisiting Australian Ectocarpus subulatus (Phaeophyceae) from the Hopkins River: distribution, abiotic environment, and associated microbiota

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REVISITING AUSTRALIAN *ECTOCARPUS SUBULATUS* (PHAEOPHYCEAE) FROM THE HOPKINS RIVER: DISTRIBUTION, ABIOTIC ENVIRONMENT, AND ASSOCIATED MICROBIOTA[1]

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Abstract

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 twenty-two 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 *cox*1 sequences identical to the original isolate at three sites upstream of Hopkins River Falls. The salinity of the water at these sites ranged from 3.1-6.9 psu, 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.

Keywords: *Ectocarpus subulatus*; distribution; low salinity adaptation; freshwater colonization; microbiota.

Introduction

Brown algae (Phaeophyceae) are widespread in the tidal and sub-tidal 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* Thuret in a highly salt-contaminated section of the Werra river in Germany (Geissler 1983), and one occurrence of *Ectocarpus subulatus* Kützing in a nearly freshwater habitat (salinity 1ppt) 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 *E. subulatus* (Peters *et al.* 2015) is related to the genomic model species *Ectocarpus siliculosus* (Dillwyn) Lyngbye (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. subulatu*s, 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.* 2018, preprint). 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 *E. 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 approximately 1 ppt, and conductivity approximately 3 $mS \cdot s^{-1}$. However, it is 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 *E. 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 ten sites selected arbitrarily along the Southern Australian Coastline between Port Fairy and Avalon (Figure 1, Table 1). At each sampling site, we manually searched for filamentous algae resembling a member of the Ectocarpales within a range of ca. 50m 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 0.2µm-filtered local water (3 replicates). A small piece of each sample was stored at max. 20°C in sterile 2mL 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, seven, and 35 µL of the ground alga were diluted with 0.2µm-filtered local water to a final volume of 50 µL and spread immediately onto pre-prepared R2A agar plates (Sigma-Aldrich, St. Louis, MO, USA) for isolation of culturable bacteria. These plates were kept at ambient temperature (max. 25°C) and were monitored for two 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, branches) if present. Approximately 0.1 mL of sediment were kept as live samples in sterile 2 mL Eppendorf tubes. After two 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 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 four 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 3mg·L^{-1} GeO_2 for 3 weeks.

Water samples

Approximately 100 mL of water were taken from each site, immediately filtered with 0.45 µM syringe filters to remove particulate matter, and then pasteurized for 1h 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 a Type CD78 conductivity meter (Tacussel Electronique, Villeurbanne, France) and converted to practical salinity units (psu) according to Fofonoff and Millard (1983). Phosphate, nitrite, and nitrate concentrations were determined using an AA3 auto-analyser (SEAL Analytical, Southampton, UK) following the

method of Aminot and Kérouel (2007) with an accuracy of 0.02 µmol·L^{-1}, 0.01 µmol·L^{-1}, and 0.01 µmol L^{-1} for NO_3^-, NO_2^-, 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™ AS11-HC column (4 x 250 mm) equipped with an AG11-HC guard column (4 x 50 mm), using an ICS-5000 Dionex system (SP-5 & Analytical CD Detector, Thermo Fisher Scientific, Waltham, MA, USA*)*. Elution was performed with isocratic 12mM 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 test if variations in sulfate concentration merely mirrored variations in the overall salinity, a Pearson correlation coefficient was calculated between both variables.

Barcoding of algal and bacterial isolates

Algal isolates were identified to a species level using the mitochondrial *cox*1 and the nuclear ITS1+2 markers. Algal DNA was extracted from the cleaned cultures using the Macherey Nagel (Düren, Germany) NucleoSpin Plant II kit according to the manufacturer's instructions (PL1 protocol with two 25 µL elutions), and 1µL of DNA (10-30 ng) was used in subsequent PCRs. For the ITS region, we used the AFP4LF (3'- CAATTATTGATCTTGAACGAGG-5') and LSU38R (5'-CGCTTATTGATATGCTTA-3') primers (Lundholm *et al.* 2003; Peters *et al.* 2004), and for the 5'*cox*1gene the GAZF2 (3'-CCAACCAYAAAGATATWGGTAC-5') and GAZR2 (3'-GGATGACCAAARAACCAAAA-5') primers (Lane *et al.* 2007), each at a final concentration of 0.5 µM. PCRs were carried out using a GoTaq polymerase and the following program: 2 min. 95 °C followed by 30 cycles [1 min 95 °C; 30 sec. 50°C for ITS or 55°C for *cox*1; 3 min 72 °C] and a final extension of 5 min 72 °C.

157 Bacterial cultures were identified by partial sequencing of their 16S rRNA gene. Fifty µL of
158 dense bacterial culture were heated to 95°C for 15 min, spun down for 1 min, and 1 µL of
159 supernatant was used as a template in a PCR reaction with the 8F (5'-
160 AGAGTTTGATCCTGGCTCAG-3') and 1492R 5'-GGTTACCTTGTTACGACTT-3')
161 (Weisburg *et al.* 1991) at a final concentration of 0.5 µM. Except for the annealing temperature
162 (53°C here), the same PCR protocol as above was employed.

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

175 ### *Amplicon sequencing of* in situ *bacterial communities*

176 Amplicon sequencing of bacterial communities was carried out to assess the *in situ* composition
177 of the *E. subulatus* microbiome. Sufficient material for these analyses was obtained at two of the
178 three sites with *Ectocarpus* individuals: sites 8 and 9 (Figure 1). Approximately 20mg dry weight
179 for each of the three replicate samples for each site were ground twice for 45 sec. 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), were 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 cultivated in our laboratory (see Thomas *et al.* 2019 for details) as well as a negative control, were 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 5nM final concentration and supplemented with 20%PhiX to add sufficient diversity for sequencing on an Illumina MiSeq using a 2x300bp 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://hannonlab.cshl.edu/fastx_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 *E. subulatus* at three 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, Figure 1). *Ectocarpus* was, however, abundant at two sites (Framlingham Forest reserve and Kent's Ford, sites 8 and 9, Figure 1), which were approximately 12km 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 *cox*1 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 (Figure 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 psu; approximately ¼ that of seawater), and then gradually decreased towards the mouth of the river, where it dropped to ca. 1 psu, before re-spiking due to the influence of seawater (Figure 3). This decrease corresponded to an increase in the flow of water masses towards the mouth river. Sulfate concentrations followed the same pattern as salinity (Pearson correlation $r=0.995$, $p<0.001$) and decreased from nearly 7 mM to approximately 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; Figure 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) (Figure 4A). We detected 1312 OTUs across the three sampled individuals from both sites (Supporting Information File S1). The bacterial communities of both sites were dominated by *Alphaproteobacteria* (25% of reads), *Bacteriodetes* (20%), *Gammaproteobacteria* (8%), *Planctomycetes* (8%), and *Actinobacteria* (8%) (Figure 4a,b), and there was a significant difference in the community structure between the two sites (ANOSIM $p=0.001$, Figure 4B). Examining the OTUs individually, we identified 86 OTUs that were specific to Framlingham 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 Supporting Information File S1. Alpha-diversity (Shannon index) was also slightly higher at the Framlingham Forest reserve (t-test p= 0.03; Figure 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 (Figure 5). They comprise *Gammaproteobacteria* (48 isolates, including 28 *Pseudomonas*), *Firmicutes* (27 isolates), *Actinobacteria* (8 isolates), *Alphaproteobacteria* (7 isolates), and *Bacteriodetes* (5 isolates). No members of the *Planctomycetes* were isolated.

Discussion

The data presented in this paper confirm that the original finding of *E. 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, it 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 *E. 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 $\mu m \cdot s^{-1}$ (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 *E. subulatus* between 3.1 and 6.9 psu 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 psu, 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;

291 Wetzel 2001). Sulfated polysaccharides are typical components of the cell walls of marine plants
292 and algae (Popper *et al.* 2011) and require sulfate for their synthesis, but their importance for
293 *Ectocarpus* remains to be explored. In the same vein the question remains open to what extent the
294 low nitrate concentrations at sites with *E. subulatus* compared to upstream and downstream sites,
295 are related to the presence of the algae, either as a cause or as an effect. It should be noted,
296 though, that a direct metabolomic comparison of *E. subulatus* and the marine *E. siliculosus*
297 revealed markers for high nitrogen status (total amino acids, ratio of glutamine to glutamate) in *E.*
298 *subulatus* (Dittami *et al.* 2012). Regardless of the physiological implications of the composition
299 of the Hopkins River water, we argue that it may be more appropriate to refer to the *E. subulatus*
300 strains isolated from the Hopkins River as "fluviatile", *i.e.* "river" strains rather than freshwater
301 strains, despite their capacity to grow in fresh water in laboratory conditions (Dittami *et al.*
302 2012).

303 In addition to these facts about the distribution and environment of *E. subulatus*, the present
304 study provides insights into its associated microbiome – a component likely connected to the
305 capacity of this species to grow in low salinity (Dittami *et al.* 2016). The number of OTUs
306 associated with *E. subulatus* in our *in vivo* study was one order of magnitude higher than in a
307 previous study of the laboratory strain after 20 years of cultivation (1312 OTUs for six samples
308 from two sites vs 84 OTUs for six samples in two conditions) (Dittami *et al.* 2016). Moreover, a
309 direct taxonomic comparison of these two studies at the genus level revealed only 5 genera
310 (*Acinetobacter*, *Phycisphaera*, *Maribacter*, *Marinoscillum*, and *Gaiella*) that were found in both
311 studies. All of them were rare *i.e.* supported by < 0.01% of reads in our study; Supporting
312 Information File S1). Both studies were based on sequencing runs with similar depth and
313 employed 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 of 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, preprint), 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 under-sampled lineages in cultivation experiments. In this study particularly *Planctomycetes* stand out, as 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 one 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 *E. 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.

The present study enhances our knowledge on *E. 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.

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Conflict of interest statement

The authors declare no conflict of interest.

Author's contributions

SD, AFP, HK, SE, JW, 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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Tables

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	Germling 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 (*cox*1) 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	-38.385325, 142.587398	-	-
2017-03-22	6. Donovans Lodge	granit, clay	no	-38.355110, 142.599250	-	-
2017-03-22	7. Hopkins River Falls	volcanic rock	no	-38.333509, 142.621352	-	-
2017-03-23	8. Framlingham Forest Reserve	rock, pebbles	*E. subulatus* Au8 (abundant)	-38.297064, 142.668291	-	LR735222 (*cox*1) LR735415 (ITS)
2017-03-23	9. Kent's Ford	rock, pebbles	*E. subulatus* Au9 (abundant)	-38.191574, 142.698058	-	LR735223 (*cox*1) LR735416 (ITS)
2017-03-23	10. Hexham	mud, wood	*E. subulatus* Au10 (rare)	-37.995732, 142.689141	-	LR735224 (*cox*1) LR735417 (ITS)
2017-03-23	11. Chatsworth	mud, sand, detrirus	no	-37.856357, 142.650644	-	-
2017-03-23	12. Wickliffe	sand, detritus	no	-37.694348, 142.726074	-	-
2017-03-24	13. Rossbridge	rock, pebbles	no	-37.480217, 142.849465	-	-
2017-03-23	14. Ararat	sand	no	-37.300211, 142.973979	-	-
2017-03-24	15. Green Hill Lake	pebbles	no	-37.295320, 142.979170	-	-
2017-03-24	16. Merri River, Warnambool	concrete	no	-38.362077, 142.484414	-	-
2017-03-24	17. Killarney beach	sand, lava pebbles, shells	no	-38.357966, 142.306880	*Kuckuckia* sp.	LR735225 (*cox*1) LR735418 (ITS)
2017-03-25	18. Belfast Lough airport	sand, wood pollar	no	-38.361889, 142.262045	-	-
2017-03-25	19. Curdies River	rock	no	-38.519965, 142.833558	-	-
2017-03-25	20. Port Campbell	tide pool. sand	no	-38.620681, 142.992981	*Feldmannia* sp.	LR735226 (*cox*1)
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	tide pool, sand shells	no	-38.531281, 143.980994	*Acinetospora* sp.	LR735227 (*cox*1)
2017-03-26	26. Hovell's Creek	clay	no	-38.018825, 144.402156	-	-

Figure legends

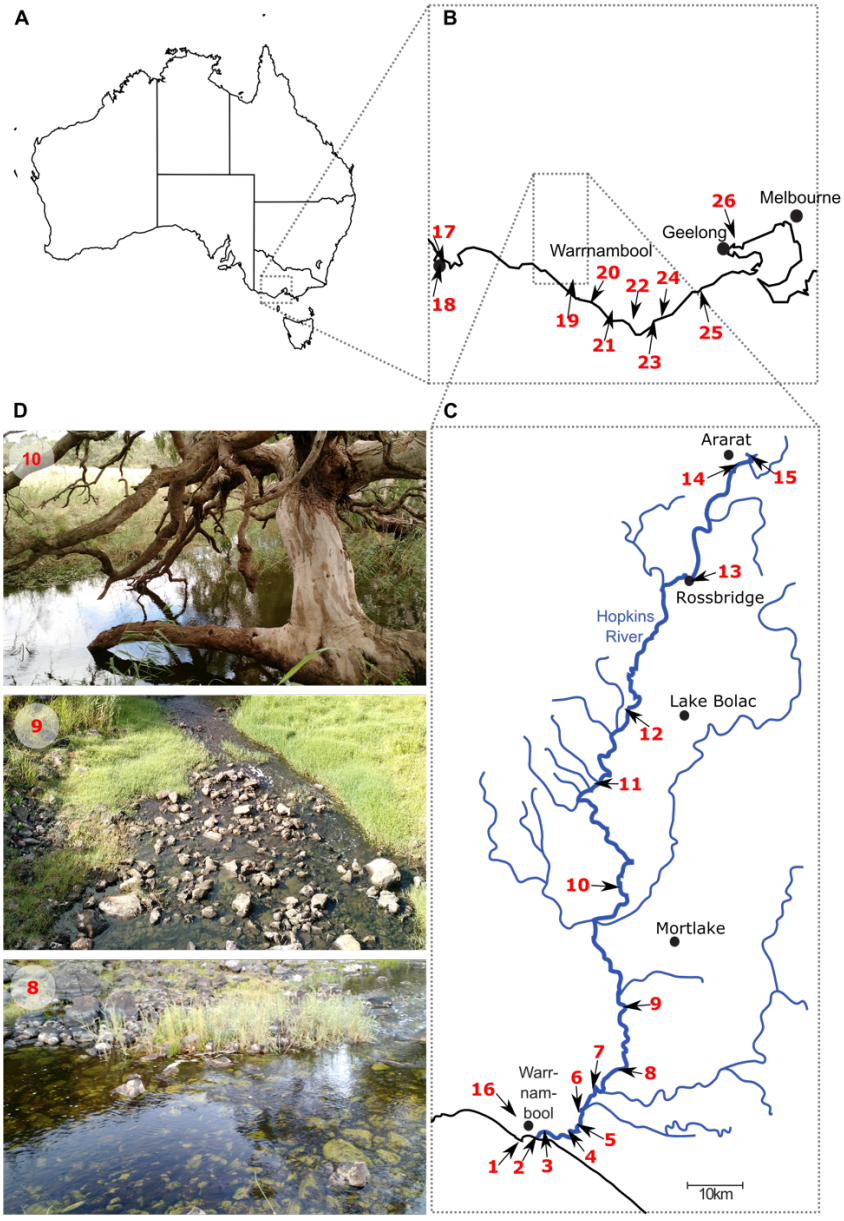
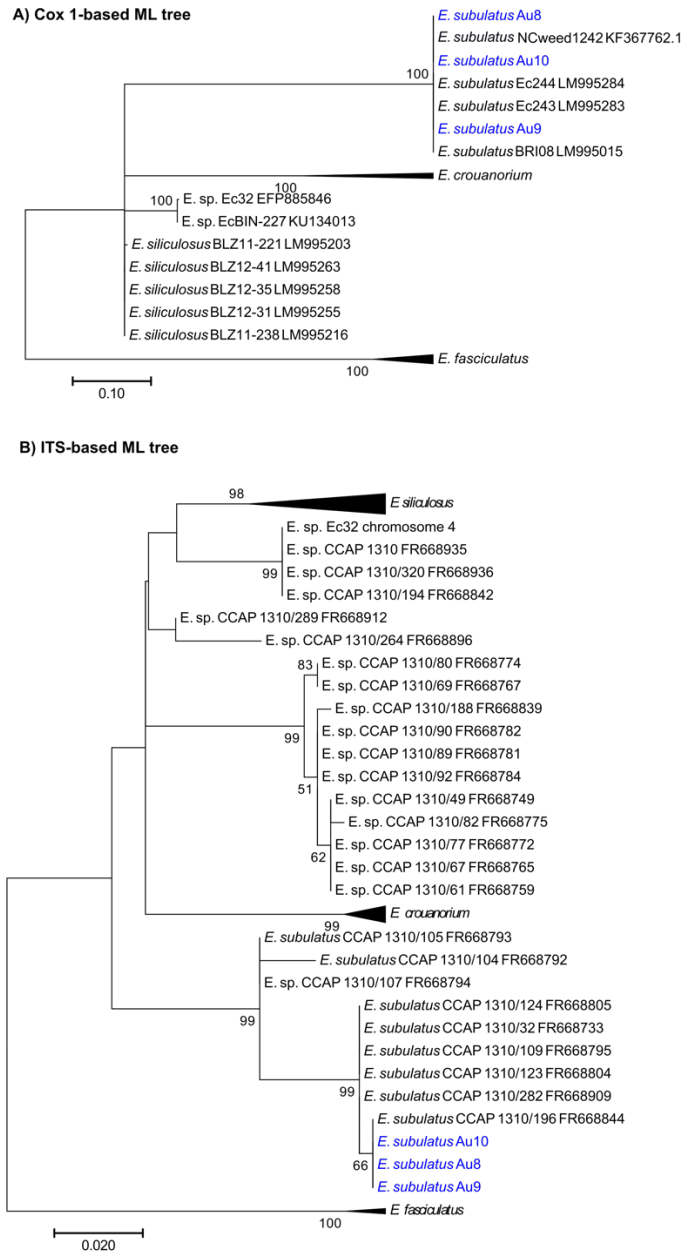
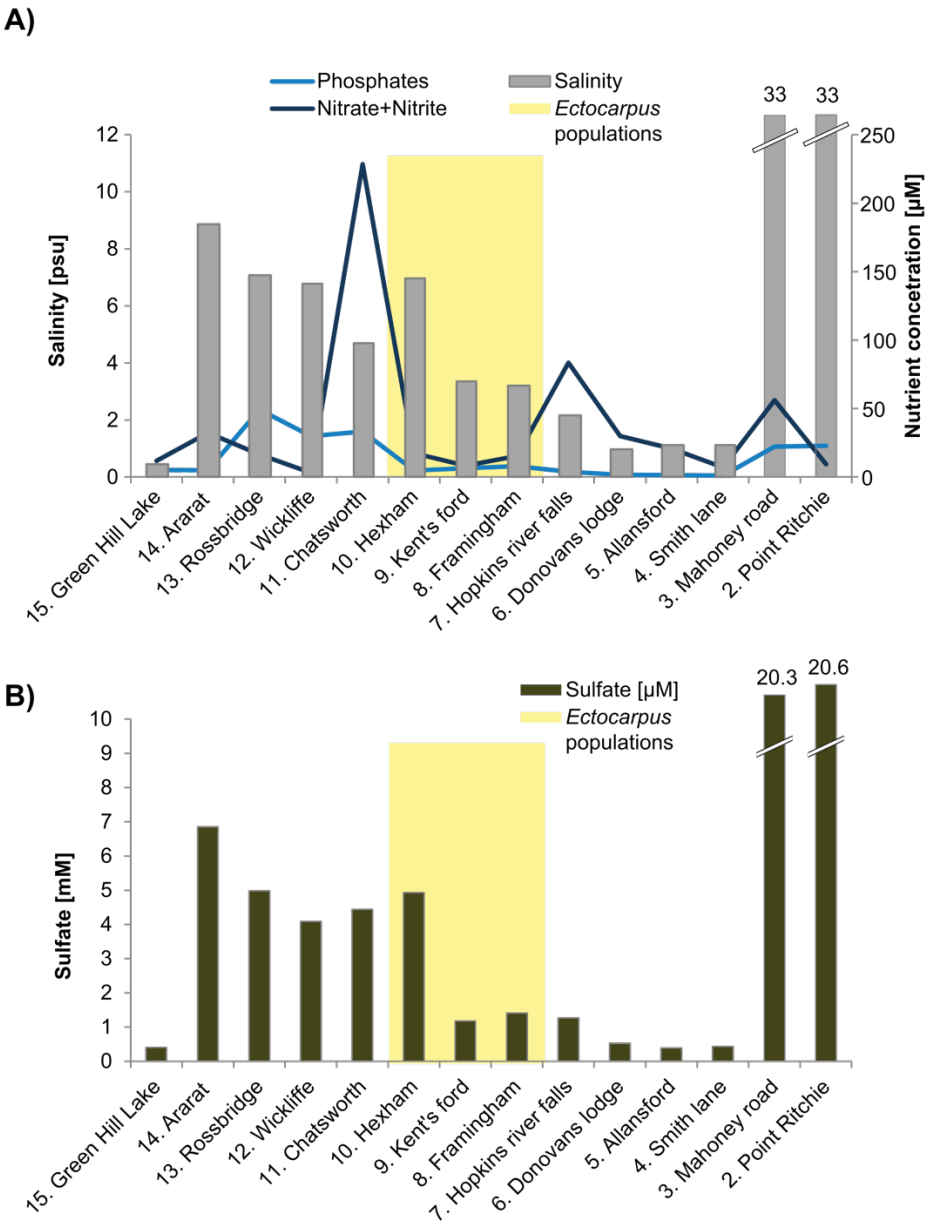


Figure 1: Sites sampled. Panels A-C: Map of sampling sites at an increasing scale. Each sampling site is numbered (1-26, see Table 1). *Ectocarpus* individuals were found at (sites 8, 9, 10), and Panel D contains photos taken at these sites. Hopkins River Falls, the original site of isolation, corresponds to site 7.



458

459 **Figure 2: Maximum-likelihood tree of *Ectocarpus* isolates from Hopkins River and related**
460 **strains.** Panel A displays a tree based on the *COX1* gene (alignment of 677 bp after curation),
461 and Panel B on the ITS region (860bp after curation). Blue color indicates isolates from this
462 study. Support values correspond to the percentage of support using 1000 bootstrap replicates.



463

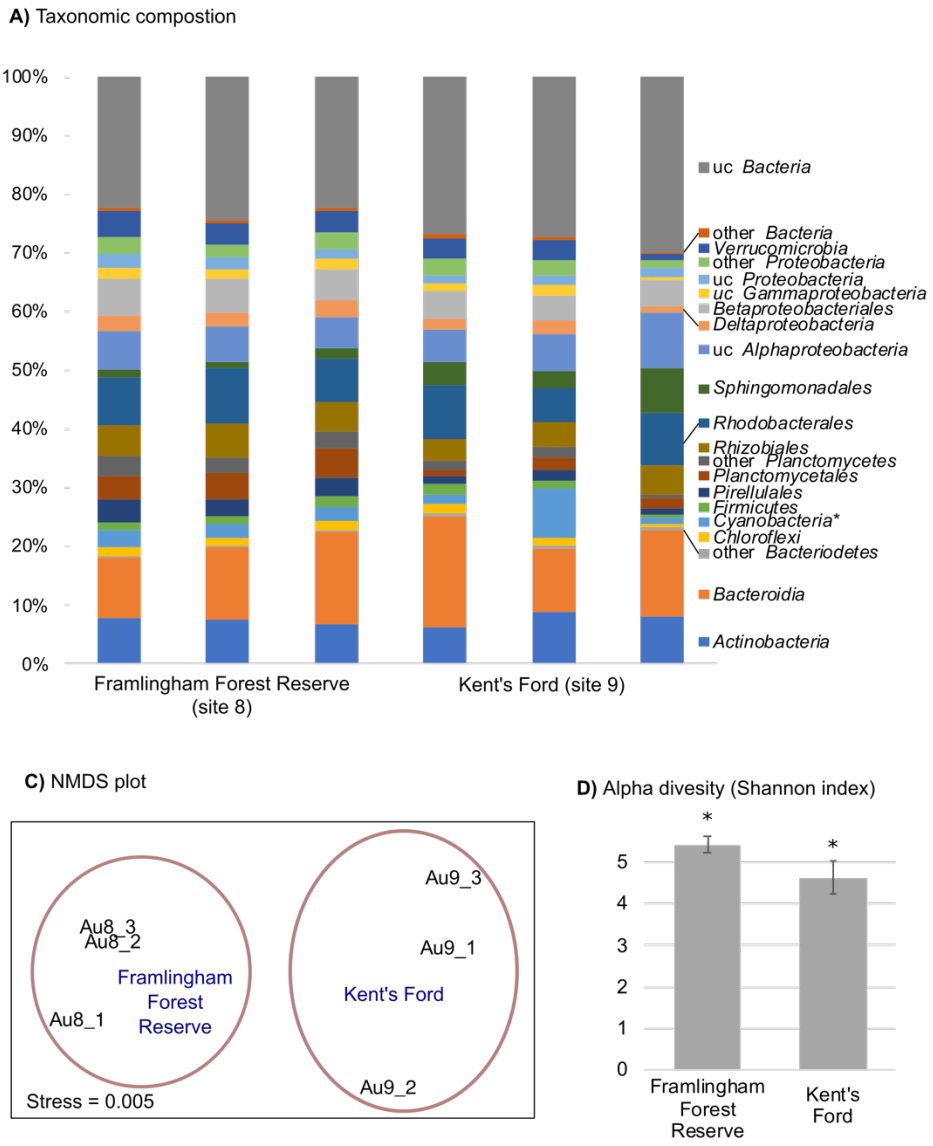
464 **Figure 3: Water chemistry at the different sampling sites along the Hopkins River** (see

465 Figure 1). Panel A displays salinity (gray bars) and nutrient concentrations (blue lines), and Panel

466 B shows sulfate concentrations. Yellow background indicates sites with the occurrence of

467 *Ectocarpus*. Each measurement corresponds to a single sample collected between 2017-03-22

468 and 2017-03-24.



469

470 **Figure 4: Bacterial community at isolation sites determined by 16S rDNA amplicon**
471 **sequencing of bacterial communities.** Panel A shows the taxonomic distribution of the bacterial
472 communities associated with each replicate at the two sampling sites with sufficient material, uc
473 = unclassified. Panel B shows the distances between the communities (NMDS plot based on
474 Bray-Curtis dissimilarity matrix); communities at both sites differed significantly (ANOSIM
475 p=0.001). Panel C shows the alpha-diversity of bacterial communities in the two sites (mean of
476 three replicates ± SD; * indicates a significant difference, two-sided t-test, p=0.03).

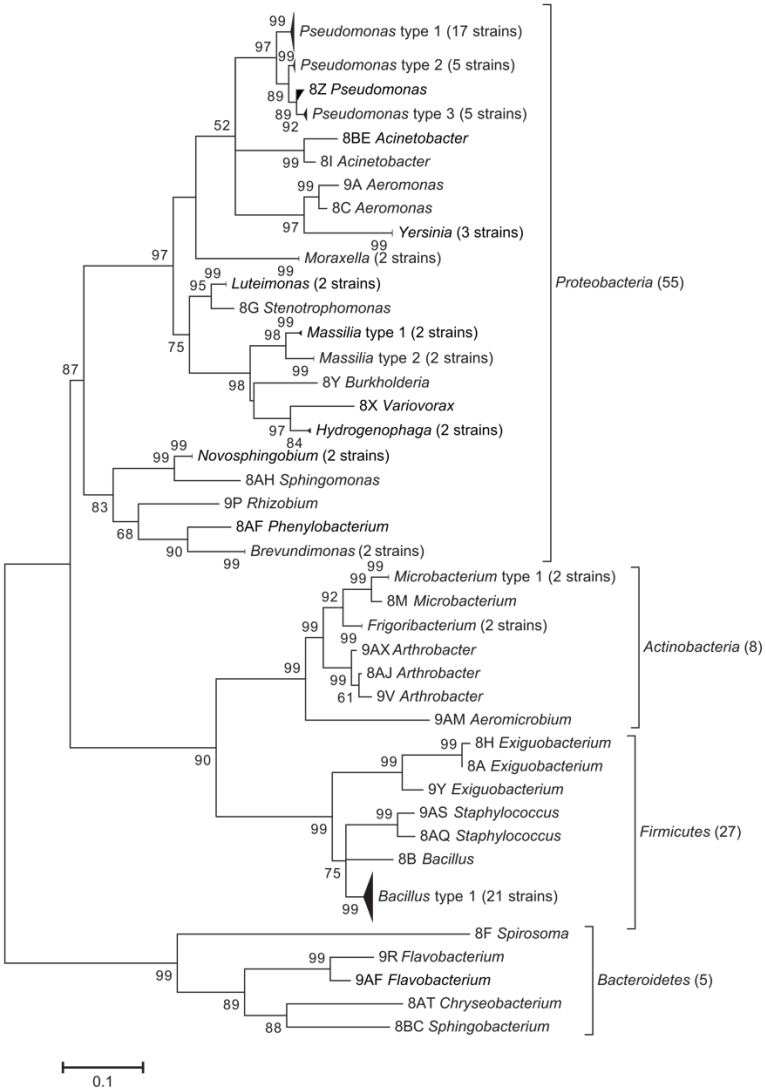


Figure 5: Maximum-likelihood tree of bacterial isolates obtained from *E. subulatus* in situ. The tree is based on an alignment of 16 rRNA gene sequences of all isolated strains and comprised 598 bases after cleaning. Support values correspond to the percentage of support using 1000 bootstrap replicates. Sequence accession numbers are ENA:LR735444-LR735537.

Supporting Information

Supporting Information File S1 – Amplicon sequencing results of bacterial communities.