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The effect of seagrass components on the associated bacterial diversity and antimicrobial activity in *Zostera marina*

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Abstract

Seagrasses are marine angiosperms located in intertidal and sub-tidal waters. They are important ecosystem engineers that play a crucial role in the structure of habitats, allowing them to support a comprehensive diversity of life. Through fundamental ecological processes and the generation of niches, each seagrass species has aggregated an associated microbiome that lives among the various seagrass components and exhibit symbiotic relations. There have been studies investigating the diversity of the associated bacteria and elements of the resulting microbial activity, however these have primarily focused on tropical seagrass species and have failed to examine the extent of this bioactivity. This study explored the relative colony diversity and bioactivity associated with the root matrix, bare sediment, and leaf components of *Zostera marina* beds in the Salcombe Estuary, UK. Basic morphological characterisation and antimicrobial screening of seagrass isolates against *E. coli* and *S. aureus* showed how the location of the seagrass bed itself has no significant effect on the colony diversity and bioactivity present. The individual seagrass components were found to significantly influence these communities, with the root matrix being found to harbour the highest diversity and antimicrobial activity of all the components. Amplicon sequencing of 16S rRNA genes demonstrated how three bacterial genera (*Thalassomonas, Tenacibaculum* and *Vibrio* sp.) are the greatest contributors to this activity. These results suggest that there is a degree of habitat dependence influencing the aggregations of microbes in the proximal seagrass beds. There is also a potential interaction between microbial diversity and bioactivity levels in the different seagrass components. The antimicrobial activity displayed against human pathogenic organisms expresses a potential for the exploitation of these bacteria, following further research into the extent of their possible medical value.

Keywords: *Zostera marina,* seagrass microbiome, microbial diversity, antimicrobial activity, colony diversity, bioactivity, seagrass component, *Thalassomonas haliotis Tenacibaculum gallaicum, Tenacibaculum gallaicum, Vibrio metschnikovii*

Introduction

Seagrasses are derived from terrestrial angiosperms that inhabited the sea through a number of colonization events starting approximately 100 million years ago (Olsen et al., 2016). These modern aquatic angiosperms are phylogenetically diverse, having diverged into four or five families (dependent on the informative source) that differ from other aquatic plants due to their ability to form pollen (Waycott et al., 2006; Kuo and Hartog, 2006). Despite the extensive research, the total coverage of seagrass meadows has been poorly established so far. All continents harbour seagrass beds, with the exception of Antarctica (Green, 2003), since seagrass growth requires a high amount of light to penetrate the water column in coastal aquatic ecosystems (Schubert and Demes, 2017). While there are a total of 72 species of seagrass found in coastal waters around the globe (Larkum et al., 2006), only 4 of these species are native to different coasts around Europe: Cymodocea nodosa is found in the Mediterranean and South Atlantic; Posidonia oceanica is endemic to the Mediterranean Sea; and both Zostera marina and Zostera noltii are located along all provincial coasts around Europe, particularly in sublittoral and intertidal UK waters (de los Santos et al., 2019).

Seagrass beds are well-known ecosystem engineers (Wright and Jones, 2006) that are responsible for the establishment of stable and structured habitats that act as the infrastructure for complex environments (Conte *et al.,* 2021) and support an extensive diversity of life. These ecosystems host a diverse array of macrofaunal species at various stages of their life cycles, through providing a suitable habitat that allows these organisms to thrive. They provide nursery and foraging grounds for many herbivorous grazers and invertebrate species while also contributing as corridors between neighbouring habitats (Beck *et al,* 2001; Lilley and Unsworth*,* 2014). They also harbour a complex benthic invertebrate community and grant coastal protection through the sediment stabilisation provided by root structures (Hemminga and Duarte, 2000; Alfaro, 2006). They are additionally a hive of coastal productivity (Bay, 1984) through the conduction of ecological processes like carbon fixation and nutrient cycling with the aid of associated microbes (Donnelly and Herbert, 2010).

The seagrass plant itself is home to many microbial organisms that display symbiotic relations with the host and, resultingly, play a vital role in biological, physiological, and ecological function (Conte *et al.,* 2021). Rich epiphytic communities are present on the physical substrata provided by the seagrass tissues above ground (with the leaves of the seagrass otherwise referred to as the phyllosphere), as well as below ground in the root matrix, rhizomes, and surrounding sediment (known as the rhizosphere – Ugarelli *et al.,* 2017; Tarquinio *et al.,* 2019). Each species of seagrass has derived an associated microbiome throughout its evolutionary history - the associated bacteria, fungi and viruses are thought to have been accumulated based on the influence of the surrounding environment (Zhang *et al.,* 2020). This implies that two seagrass species subject to similar environmental parameters would consequently possess similar microbiomes. The seagrass host and the associated microbial interactions are better viewed as an intricate interactive network (Wilkins *et al.,* 2019) that serves as a complex, yet singular, functional unit known as the holobiont (Zilber-Rosenberg and Rosenberg, 2008). The microbial communities present within the various constituents of the seagrass holobiont can promptly respond to any environmental changes and disturbances, consequently meaning the monitoring of their configuration could act as an early sign of environmental stress

(García-Martínez *et al.,* 2008; Mejia *et al.,* 2016; Martin *et al.,* 2020). A recent series of studies described by Conte *et al.* (2021) have examined just that, with the aspirations of establishing whether the host and associated organisms could be used as an ecological indicator; so far the results have been promising, although further clarification is still needed.

There is a continual growth of knowledge accruing around the globe relating to seagrass-microbial interactions and their roles in fundamental ecological processes, including that of carbon cycling and nitrogen fixation. Ugarelli *et al.* (2017) provides a detailed overview on all studies examining the microbiome of seagrasses. There is a notable lack of research surrounding the seagrass beds in Northwest Europe, particularly in coastal waters around the UK. There has previously been a heavy focus on the symbiotic relationships alongside the biological and physiological functions of organisms within the microbiome in different parts of the seagrass plant; there is yet to be comprehensive research addressing other active potentials of bacteria in different seagrass species. Antimicrobial activity in seagrass has previously been examined under several different contexts: their antipathogenic ability against phytopathogens (Arumugam *et al.,* 2010) and human pathogens (Kannan *et al.,* 2010); their antibacterial and antifungal properties (Bernard and Pesando, 1989); and their natural antioxidant and anti-inflammatory properties (Yuvaraj *et al.,* 2011). These studies found encouraging signs and resulting benefits of antimicrobial activity in the seagrass holobiont - the most bioactive and sensitive bacteria have previously been detected in the root and rhizome of the seagrass when tested against various pathogens (Kannan *et al.,* 2010; Ravikumar *et al.,* 2011). However, the majority of these studies were conducted in more tropical climates with no current research being undertaken in UK regions, which then queries the application of the results to other seagrass species in temperate locations, like that of the subtidal *Zostera marina* (*Z. marina*). Consequently, this poses the question of whether bacteria within European seagrass, particularly that of Northwest Europe, have any antimicrobial properties, and if so which part of the seagrass ecosystem harbours the most bioactivity.

The aims of this present study were to: a) establish where the greatest diversity of bacterial colonies is present within the different components of the seagrass plant, b) identify which seagrass component harbours the highest antimicrobial activity levels and c) determine which microbes are the biggest contributors to this activity. Hypotheses relative to these aims were made as follows:

- The location of the seagrass bed will have no effect on the colony diversity or antimicrobial activity.
- The root matrix will contain the highest colony diversity of all the seagrass components.
- The root matrix will also accommodate the highest levels of antimicrobial activity found in comparison to the bare sediment and leaf seagrass components.

Both the colony diversity and antimicrobial activity were predicted to be higher in the root matrix/rhizosphere samples based on the analogy that the highest bioactivity within seagrass has been previously recorded in the roots and rhizomes (Kannan *et al.,* 2010; Ravikumar *et al.,* 2011). The seagrass bed location was proposed to have

no effect on either diversity or antimicrobial activity since the microbiomes associated with the rhizosphere have been formerly classified to be habitat dependent in tropical species (Zhang *et al.,* 2020) - with the two *Z. marina* beds being situated near each other within the estuary, they are likely subject to the same environmental conditions and therefore similar bacteria in their relative microbiomes. The hypotheses were tested through the isolation of bacteria from the leaf, root matrix and bare sediment within two *Zostera marina* beds in Salcombe estuary, before then making comparisons to test for differences in the colony diversity and bioactivity between the two locations and three treatments sites. Bacteria displaying bioactivity that could be of therapeutic interest were profiled further via the use of 16s rRNA sequencing.

Materials and Methods

Sample Collection

A total of 30 samples were collected from two *Zostera marina* seagrass beds in Salcombe Estuary, SW England, on 5th November 2021. The study sites were situated at either side of Castle Rock (Fig.1); 15 samples were collected from the upper bed (50.23301°N, 3.77076°W) a further 15 from the lower bed (50.23157°N, 3.77264°W). Leaf (LM), root matrix (RM) and bare sediment (BS) samples were obtained from five areas within each of the seagrass beds and stored in appropriately labelled sample pots – i.e. 1-1 RM, 2-4 BS etc.

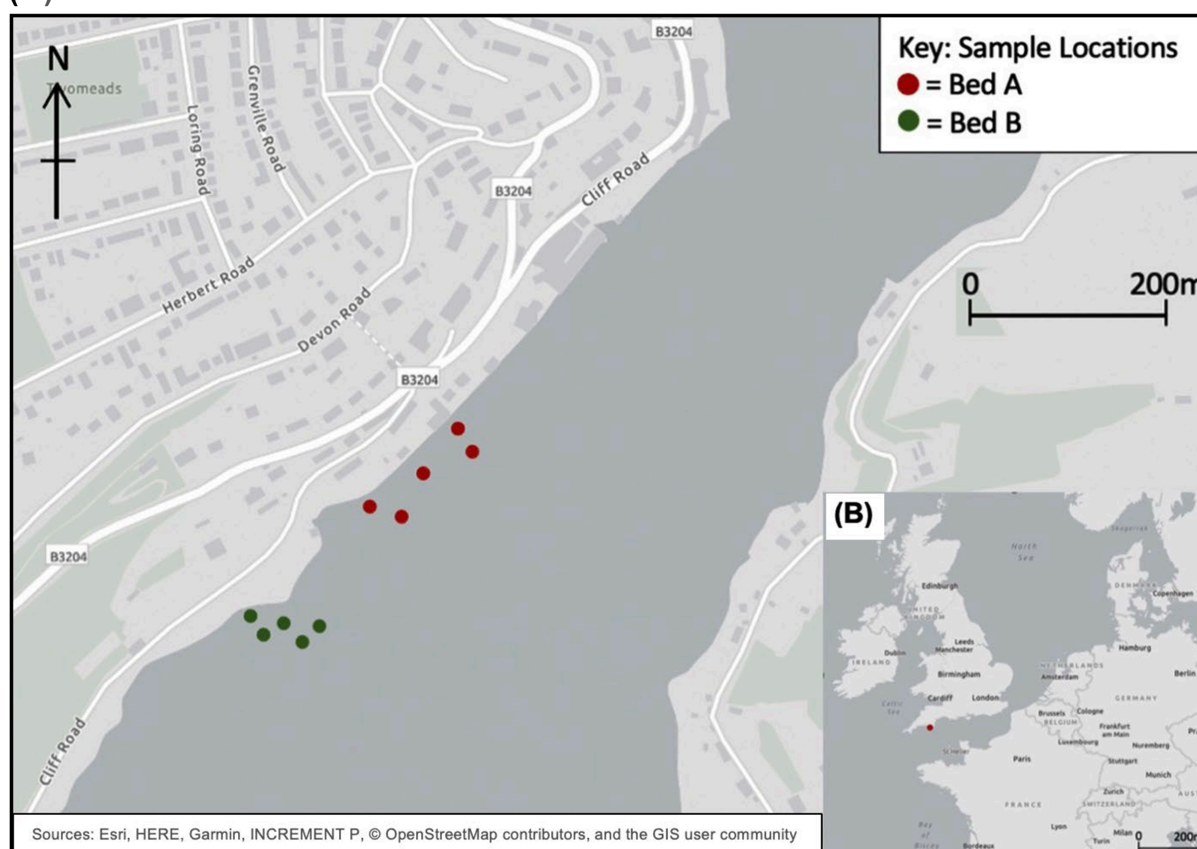

Figure 1: The location of the two seagrass beds and associated sample sites in Salcombe Estuary(A), alongside their relative location in Northwest Europe (B).

The leaf samples contained sections of seagrass leaves that had been removed from a single plant and the root matrix samples involved removing a small volume of sediment from the rhizosphere of the same plant using a trowel. Bare sediment samples were taken in the same manner from the centre of the nearest barren area that measured a minimum of 1 m^2. The samples were stored in a temperature-controlled room at 15 °C until lab analysis commenced.

Media Preparation

R2A Agar

R2A agar plates were made using 85% artificial seawater (AS). 1000 ml distilled water was measured alongside 33.3 g Tetra salt before mixing the components using a magnetic stirrer at 500 RPM. Once dissolved, 850 ml of the artificial seawater solution was remeasured and 150 ml distilled water was added to the solution, resulting in an 85% solution. Dehydrated R2A agar (18.1 g) was mixed into the solution before being heated in the microwave at 800 W for 7 minutes with frequent stirring. The medium was heated for a further 2 minutes until the dehydrated agar was thoroughly mixed with the artificial seawater. Once completely dissolved, the medium was transferred to a 1 l glass beaker and autoclaved at 121 °C for 15 minutes and then cooled to 50 °C. 2 ml of the R2A agar was then pipetted into individual petri dishes and left to solidify, resulting in 50 R2A agar plates being poured. The volumes and measurements were changed accordingly when preparing the agar based on the number of agar plates required at different stages of the research.

Mueller-Hinton Agar (MHA)

MHA agar was prepared for the antimicrobial screening stage of the research using 85% artificial seawater as seen with the R2A agar. For 500ml of agar, 425 ml of 100% artificial seawater (500 ml distilled water and 16.6 g Tetra salt) and 75 ml distilled water were mixed with 19 g dehydrated Mueller-Hinton agar. The medium was heated at 800 W with frequent agitation until the agar was brought to boil and had completely dissolved. Sterilisation then occurred by autoclaving the agar at 121 °C for 15 minutes before cooling the medium to pour. 2 ml of the MHA was transferred into each of the 25 petri dishes using a pipette and left to cool for 30 minutes. Once solidified, the plates were ready to use.

Agarose Gel (for Gel Electrophoresis)

0.45 g SeaKem agarose and 30 ml 0.5 ml TAE buffer were mixed and heated for 30-second intervals until boiling. The medium was then placed in a water bath and cooled to 65 °C before adding 5 µl SYBR safe DNA gel stain. Once the casting tray was assembled, the gel was poured into a tray and left to harden for approximately 20 minutes.

Initial Isolation, Processing and Characterisation of Bacteria

The initial laboratory analysis began on the 11th of November 2021 and was conducted until 26th January 2022; in this time various methods were followed to process the bacteria found in the marine samples. The samples were first diluted by degenerating 2 g of the sample material before then suspending in 18 ml of artificial seawater (at 33 ppt), creating an initial 10^{-1} dilution for each of the sample types and study sites. Each of the suspensions were vortexed before then creating serial dilutions 10^{-2}, 10^{-3} and 10^{-4} 1000 µl of each 10^{-1} dilution was transferred into accordingly labelled microcentrifuge tubes, with 100 µl of the supernatant from 10^{-1} being transferred into the next microcentrifuge tube with 900 µl of artificial seawater

to create the 10^{-2} dilution. The suspension was then centrifuged before transferring 100 µl of the supernatant across to create the 10^{-3} serial dilution, and then repeating this process to create the 10^{-4} dilution for each of the 30 samples – creating a total of 120 dilutions. 100 µl of each serial dilution was then transferred onto a plate containing R2A agar and spread, starting from the most diluted solution of each sample, and working towards the least diluted. All plates were than incubated at 15 °C for 9 days minimum.

Basic Characterisation
The colonies on all plates were analysed after the incubation period for diversity and growth; all plates within the 30-300 colony threshold were counted. Differing individual colonies underwent basic characterisation that described various aspects of their morphology including shape, edge, surface texture, and elevation. They were then coded using the initial sample type, sample number and dilution before then being assigned a correlating letter and number. The colonies were extracted and aseptically streaked onto R2A 85% AS plates and isolated. The resulting isolates were incubated at 15 °C for 7 days. Each of the isolates then underwent gram staining to further comprehend the character and nature of the bacteria (Coico, 2005). A small amount of each colony was transferred onto a drop of deionised water on an appropriately labelled microscope slide, the bacteria were then emulsified in the water, smeared, and fixed onto the slide by passing through a Bunsen flame. The smears were then subject to various stains including crystal violet, iodine, iodine in acetone and the counterstain safranin. Each staining stage except for that of decolourisation were conducted for a fixed period of 30 seconds and were rinsed with tap water between stages, decolourisation involved the bacteria being subject to iodine in acetone for 5 seconds before being rinsed with tap water. After staining the slides were blotted dry and transferred to a microscope. All isolates were observed at progressive magnification intervals starting at the x10 objective, moving to x40 objective before being examined at x100 magnification with the use of immersion oil allowing the a.

Antimicrobial Screening
Two solutions containing naïve strains of *Escherichia coli* and *Staphylococcus aureus* from the 1950's were created using 3.5 ml of AS and a corresponding number of colonies of each bacterium – one *E. coli* colony and two *S. aureus* colonies. The cultures were then vortexed, and the transparency measured against a premixed turbidity measure. Once all solutions measured the same, both the *E. coli* and *S. aureus* solutions were swabbed onto premade Mueller-Hinton agar made with 85% artificial seawater. The solution was swabbed from the top of the plate downwards in a sideward sweeping motion until it reached a halfway point on the plate, the plate was then rotated 90° and the motion proceeded again, continuing until the plate had been covered from four angles. A total of 22 Muller-Hinton plates were created this way, 11 *E. coli* and 11 *S. aureus*, and each plate was labelled with the corresponding bacteria. Nine points were marked in a 3x3 manner on the underside of the plates, each numbered 1-8 with a C marked next to the final point to indicate the location of a positive Amoxycillin control disc. Isolates B1-B8 were each transferred aseptically onto the first *E. coli* and *S. aureus* culture using a sterile pipette tip and placed in the associated location before then transferring a 2 µg Amoxycillin disc into the final location. This process was repeated until all isolates were subject to the *E. coli* and *S. aureus* cultures across 22 plates and then were once again incubated at 15 °C for 48 hours before measuring the antimicrobial

activity of the isolates and the inhibition zone growth. Isolates that developed an inhibition zone of 10mm≤ were re-streaked in preparation for biochemical testing.

Further Characterisation and Identification of Isolates

Phenotypic and Biochemical Analysis

Isolates that showed higher activity levels were tested further for their possible characterisation through several phenotypic tests. Oxidase, catalase, indole, and motility tests were conducted following various methods (Kovács, 1956; McFaddin, 2000; Miller and Wright, 1981; Aygan and Arikan, 2006) each using a positive control measure to determine the reaction and characterisation of the isolates.

DNA Extraction

The isolates showing the most activity underwent further processing to prepare for 16S sequencing. DNA was extracted from the desired isolates and a positive *E.coli* control using the HotSHOT method of DNA preparation by Truett *et al.* (2000). 100 µl of lysis buffer was transferred into microcentrifuge tubes, appropriately labelled with the isolates. Colonies of each desired isolate were then added to the according tube, followed by the addition of 100µl of the Hotshot A Alkaline solution. The mixtures were vortexed, and the transparency approved against a pre-made turbidity measure. All tubes were placed in a hot plate and heated at 95 °C for 5 minutes before standing for 10 minutes to allow them to cool. Once cooled, 100 µl of the neutralising Hotshot B solution was added to each isolate solution and all tubes were placed in the centrifuge, spinning at 123 bpm for 1 minute. The supernatant was then transferred into clean microcentrifuge tubes in preparation for PCR amplification.

PCR Amplification and Purification

PCR reagents were pipetted into a PCR tube to create a 10 R^x master mix of the reaction mixture (Lorenz, 2012). The following reagents and volumes were measured: 150 µl 2x PCR buffer with DNA Polymerase, 10 µl forward primer (10 µM), 10 µl reverse primer (10 µM) and 120 µl sterile H_2O. The master mix was divided into nine 0.2 ml thin-walled PCR tubes, with 29 µl being transferred into each tube. 1 µl of the DNA template from each isolate was transferred into the corresponding tube (1-7 and +) and in the final PCR tube, 1 µl of sterile H_2O was added to the solution to act as a negative control. All PCR tubes were then capped and placed into a thermal cycler and programmed to run 30 cycles at 94 °C for 2 hours with denaturation at 94 °C, annealing at 65 °C and amplification at 72 °C occurring during this time frame.

The PCR tubes were removed from thermal cylinder and 1µl of the amplified DNA for each isolate and the two controls was pipetted into wells in the agarose gel made with SYBR Safe DNA stain. The gel ran for 30 minutes at 100 v and was next transferred into a gel documentation imaging system to detect the products of PCR and their migration in addition to capturing an image of the gel. The amplified DNA underwent enzymatic PCR purification following the methodology of Werle *et al.* (1994) whereby 5 µl of each PCR product was transferred into a new tube with 5 µl exonuclease I and 1 µl shrimp alkaline phosphate (SAP). Each mix was incubated at 37 °C for 15 minutes allowing the enzymes to remove the primers and DNTPs from the PCR cycles, the mixtures were then heated to 80 °C for a further 15 minutes to deactivate and denature the enzymes. The purified DNA were transferred onto barcoding strips and transported to an external facility to conduct 16S rRNA sequencing. The resulting nucleotide sequences were then analysed in Geneious

Prime (Geneious Prime, 2022) and the subsequent species extracted from a database.

Statistical Analysis

Data analysis was conducted in R (R Core Team, 2019) to establish the effects of the location of the seagrass bed and the sample site treatments on measures of the bacterial assemblage present. Preceding the analysis, the datasets were tested for normality using the Shapiro-Wilks test and homogeneity using Levene's Test; the data for both the accumulate colony diversity and mean colony diversity were both normally distributed and homogenous. Following the success of the assumption tests, two-way ANOVA tests (location and seagrass component) were performed on both the accumulative colony diversity and mean colony diversity. After the completion of both ANOVA analysis, a post-hoc Tukey Honest Significant Difference test was used to compare and establish the relationships between the seagrass beds and treatments, in conjunction with their individual effects on bacterial colony diversity.

The bioactivity data failed the normality and homogeneity tests and was also unable to be transformed, resulting in a non-parametric test being required. Chi-squared tests (McHugh, 2013; Gergely *et al.,* 2014) were conducted to examine the antimicrobial activity associations between the seagrass bed locations and the study sites, as well as between the study sites themselves. Once complete, the relative contributions of the bioactive colonies in each study site were calculated.

Various packages within R were used for different stages of the analysis: 'car' to conduct the assumption of homogeneity tests (Fox and Weisberg, 2019); 'dplyr' to efficiently manipulate the data frames (Wickham *et al.*, 2022); 'ggpubr' to visualise data prior to and post analysis (Kassambara, 2020); and 'multcomp' to generate a compact letter display of all pair-wise comparisons in the data sets, establishing significant comparisons (Hothorn *et al.,* 2008).

Results

Location and Seagrass Component

The location of the seagrass bed had no significant effect on the accumulative colony diversity and mean colony diversity in Salcombe estuary (depicted by the results of the Two-Way ANOVA analysis displayed in Table 1; Figure 2A). The individual seagrass components however significantly influenced the colony diversity present from both an accumulative and average perspective (Table 1; Figure 2B); the highest diversity was present in the root matrix, followed by the bare sediment and lastly the leaf (displayed in Figure 2). Additionally, there was no significant interaction between the seagrass beds and seagrass components (Table 1).

Table 1: A summary of results from Two-Way ANOVA analysis on the accumulative (increasing) and mean morphological colony diversity at two seagrass bed locations and three seagrass components in Salcombe estuary, plus the interaction between the two factors.

Colony Diversity (*Accumulative & Mean*)	**df**	**F**	**P**
Bed (Accumulative)	1	1.959	0.174
Component (Accumulative)	2	44.346	<0.001
Bed:Component (Accumulative)	2	2.203	0.132
Bed (Mean)	1	0.439	0.514
Component (Mean)	2	30.829	<0.001
Bed:Component (Mean)	2	0.948	0.402

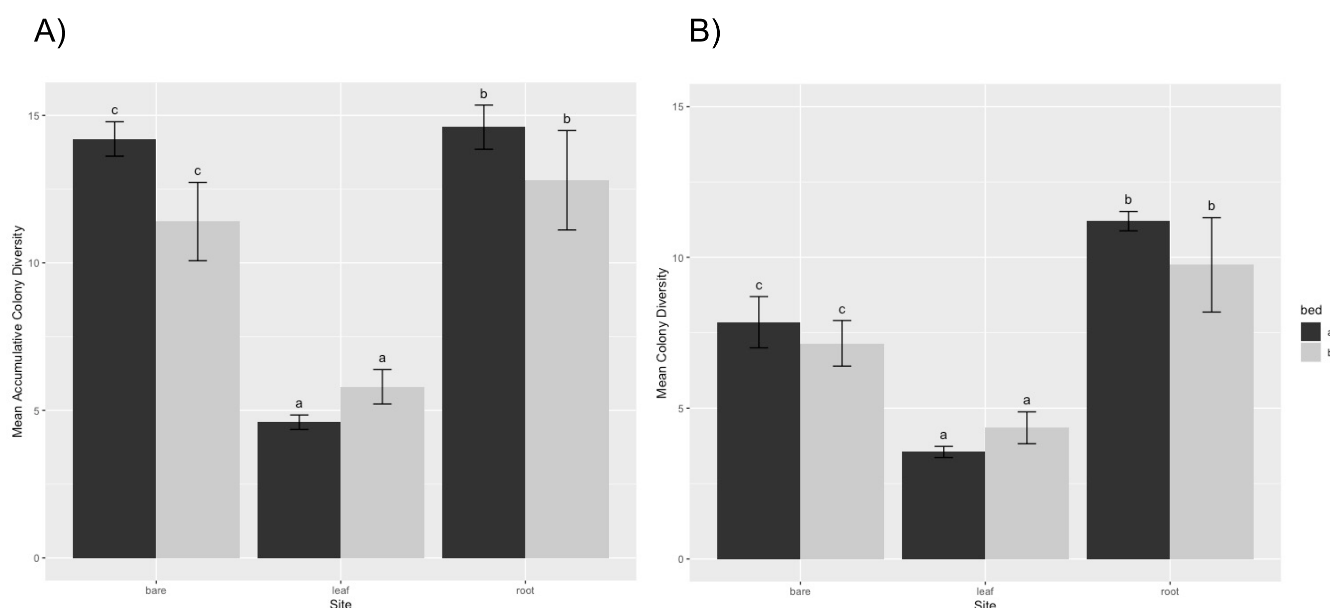

Figure 2: The accumulative (A) and mean (B) bacterial colony diversities at each of the seagrass bed locations and study sites in Salcombe Estuary. The error bars depict the variability in colony diversities for each bed location and study site, letters indicate significant differences between sites at a 95% confidence interval.

There were no significant associations present between the seagrass beds and seagrass components when analysing the antimicrobial activity of the colonies at a 95% confidence interval (x^2 = 2.230, df = 2, p-value = 0.328; displayed in Figure 3A) yet there were bioactive associations between the bare sediment, leaf, and particularly the root matrix study sites (x^2 = 9.125, df = 2, p-value = 0.010; Figure 3B). Finally, the root matrix was found to have the greatest contribution to the overall bioactivity (65.982%), followed by the leaf colonies (22.831%) and the bare sediment colonies (11.187%) – presented in Figure 3C.

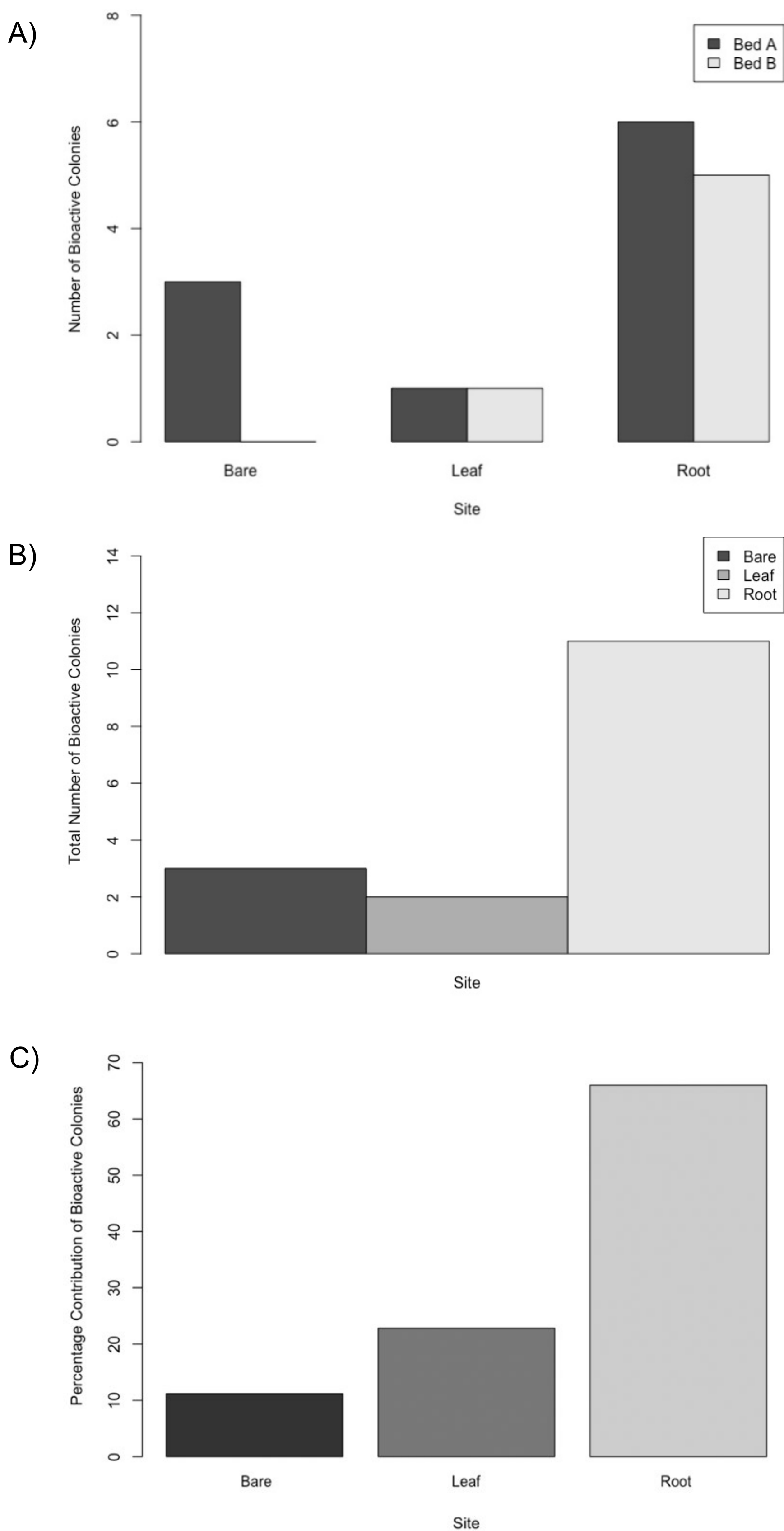

Figure 3: The number of bioactive colonies in relation to: (A) the seagrass bed locations in Salcombe estuary; (B) the total bioactive colonies across the seagrass components; and (C) the relative colony contributions of each seagrass component to total bioactivity.

Phenotypic Characterisation and Bacterial Identification

A total of 84 different bacterial colonies were isolated from the 30 seagrass samples: 31 from the bare sediment samples, 20 from leaf samples and the remaining 33 from root matrix. All 84 isolates underwent basic characterisation based on morphological attributes (Table 2). The 16 isolates that displayed any sign of antimicrobial activity were characterised further using several phenotypical and biochemical parameters (results displayed in Table 3), with the majority of bioactive bacteria (68.8%) being isolated from the root. Seven of these isolates that presented higher levels of activity (Table 4) were subject to 16S rRNA sequencing to reveal their identity. A total of 3 genera and 3 species were identified amongst the 5 sedimentary isolates that were able to be sequenced: *Thalassomonas haliotis* was identified amongst the bare sediment samples (B24) and both *Tenacibaculum gallaicum* and *Vibrio metschnikovii* were identified amongst the root matrix isolates (R20, R25 and R33). Although the R8 isolate was sequenced, the sequence received was not long enough to identify the organism.

Table 3: Biochemical test results for isolates presenting signs of antimicrobial activity. Seagrass components identified by constituent letters: B = Bare Sediment, L = Leaf and R = Root Matrix.

Isolate	Oxidase	Catalase	Indole	Motility
B14	-	+	-	-
B24	-	-	-	-
B27	+	+	-	+
L5	-	+	-	+
L11	-	+	-	+
R3	-	+	-	+
R8	-	-	-	+
R11	-	-	-	+
R13	+	-	-	+
R16	-	+	-	-
R18	-	-	-	+
R20	+	+	-	+
R21	-	-	-	+
R23	+	-	-	+
R25	+	+	-	+
R33	+	-	-	-

Table 4: A summary of all the biochemical reactions and antimicrobial activity displayed by the seven most bioactive isolates alongside their identity following 16s rRNA sequencing. Seagrass components identified by constituent letters: B = Bare Sediment, L = Leaf and R = Root Matrix.

Isolate	Gram Stain	Description	E. coli (mm)	S. aureus (mm)	Oxidase	Catalase	Indole	Motility	Species
B24	-	Stumped rods	10	16	-	-	-	-	*Thalassomonas haliotis*
B27	-	Stumped rods - clusters	13	10	+	+	-	+	N/A
L11	-	Rods - clusters	0	20	-	+	-	+	N/A
R8	-	Rods - clusters	12	24	-	-	-	+	N/A
R20	-	Serpentine + rods - chains	11	0	+	+	-	+	*Tenacibaculum gallaicum*
R25	-	Rods - chains	12	0	+	+	-	+	*Tenacibaculum gallaicum*
R33	-	Rods - clusters	11	16	+	-	-	-	*Vibrio metschnikovii*

Discussion

This research aimed to establish where the greatest diversity of bacterial colonies and bioactivity is situated within the seagrass plant and to also identify the greatest bacterial contributors to this activity. The effects of location and seagrass component on the bacterial composition and antimicrobial activity of the relative microbial populations were established, alongside the scope for the potential influence of alternative parameters on these populations. Additionally, the bacteria displaying the highest activity within the seagrass were characterised to species level.

The results of this study indicate that location of the seagrass beds has no effect on the colony diversity or bioactivity levels, in regard to the Salcombe Estuary. These findings support those reported by Zhang *et al.* (2020) that illustrate how the associated microbiomes found in the seagrass plant are deemed to be habitat dependent, particularly within the rhizosphere. The accumulation of the relative microbiomes is determined by multiple sedimentary parameters within an environment including chemical composition, nutrient status, and texture (Ettinger *et al.,* 2017; Zhang *et al.,* 2020), which can be seen to override the species-specific microbial accumulations. Therefore, although both beds studied within Salcombe Estuary were that of *Zostera marina* within a close proximity to one another, it is likely that the similarities in colony diversity and bioactivity found in the two locations are driven by the environmental parameters in the estuarine habitat itself rather the beds being that of the same species. Within this field of research, previous studies have focused on the associated microbial populations of various seagrass beds in more tropical coastal systems. In this study, there was a degree of habitat dependence observed in the *Z. marina* beds that was linked to the involvement of the accumulation of the associated bacteria. Through these observations, the collective preceding knowledge regarding the mechanisms surrounding microbe accumulation is expanded. The addition of this research conducted within a more temperate region allows for inferences to be made about the extent of the

aggregatory effects that occur. Results show the effects to operate on a much larger geographical scale than previously anticipated. However, further research into additional temperate regions would be highly beneficial to make definitive conclusions on whether the influences of environmental parameters are observed in seagrass beds throughout the globe, and whether similar bacteria could be observed in analogous habitats as a result.

The root matrix was the seagrass component found to harbour both the greatest colony diversity and highest antimicrobial activity. This was followed by the bare sediment and then the leaf components. These results support observations made by Ettinger *et al.* (2017) that describe there being distinct microbial communities associated with the various seagrass components. The differences in diversity between the rhizosphere and bare sediment communities have previously been accounted for by the presence of absence of the host plant itself (Ettinger *et al.,* 2017). With seagrass acting as an ecosystem engineer, it has the ability the actively modify the chemistry of the sediment and the topography of an environment (Orth *et al.,* 2006; Bos *et al.,* 2007) and therefore can influence the composition of micro-organisms found in the sedimentary perimeter. The presence of the seagrass plant increases the microbial biodiversity in the sediment surrounding the root matrix. This is through the provision of fundamental ecological processes that increase habitat complexity and create ecological niches (Alsaffar *et al.,* 2020).

These results also support further observations made by Ettinger *et al.* (2017) in that there were significant differences in the colony diversity between all seagrass components found in this present study. There were significant differences previously noted between the sediment and root/leaf diversity in the seagrass plant. This is comparative to some terrestrial systems in which the surrounding soil harbours an increased diversity against the host-sample types (Edwards *et al.,* 2015) which may be a result of the important ecological roles played by the host flora in their relative environments (Palmer *et al.,* 2000). However, since there is no true representation of diversity in this study, there are limitations to this measurement due to lack of characterisation measures. It is possible for different colonies to be various strains of the same microbial species, in order to counteract this, colony diversity was used as a measure of comparative diversity. To discover true bacterial diversity within the seagrass, it would be beneficial to conduct 16S rRNA sequencing on all isolates to identify all species present followed by alpha diversity indices. Further sequencing of all isolates would also allow for a measure of bacterial population composition, increasing the comprehensive scope of the study.

The bioactivity in the seagrass plant was found to be significantly higher in the root than the bare sediment and leaf components. This reinforces conclusions made in past studies that have described the root and rhizomes of the seagrass as encompassing the highest bioactivity of all seagrass components (Kannen *et al.*,2010; Ravikumar *et al.* 2011). The abundance of microbial activity in the rhizosphere of the seagrass can be explained by the interactions between microbial diversity and ecosystem function. Finlay *et al.* (1997) describes how microbial diversity plays a role within ecosystem function through reciprocal interactions including that of new microbial niches. When filled, the microbial niches have a higher microbial diversity and subsequently higher microbial activity in a progressive cycle pathway. This interaction pathway implies that with an increase in microbial diversity, there will also be an increase in microbial activity in an environment that

will consequently drive ecosystem function. The root of the seagrass and all surrounding structures were found to harbour the greatest diversity of all seagrass components. The root structure itself assists in the generation of niches and are the sites of major ecological processes within the seagrass ecosystem (Alsaffar *et al.,* 2020). The niches are filled with diverse microbial populations which subsequently then increases the bioactivity present within the rhizosphere. This would also account for the patterns seen in the bare sediment and leaf components in relation to the colony diversity. It does, however, raise the question of the extent of the interactions present between microbial diversity and activity in relation to overall seagrass ecosystem function and whether they are more directly linked than originally believed.

The previous research surrounding microbial activity and antimicrobial potential in seagrass components has been predominantly isolated to tropical beds which has queried the worldwide scale reliability and application of the findings. The findings of this study agree with those formerly observed by both Kannan *et al.* (2010) and Ravikumar *et al.* (2011) in that the greatest microbial activity can be detected in the root and the rhizomes of the seagrass. However, both studies investigated the activity within tropical host species. The inclusion of *Zostera marina* as a host species from temperate origins in this investigation widens the application of the results to colder regions like those found in Northwest Europe and the UK. The collective results depict how the roots and surrounding components of all species studied host to the highest microbial activity levels in both temperate and tropical environments. Studies that have investigated the activity present in the roots and rhizomes of *Z. marina* and have assessed the organic productivity and biomass-density patterns (Iizumi and Hattori, 1982; Kenworthy and Thayer*,* 1984). However, they failed to assess the antimicrobial activity present. The findings of this study add to the continually growing knowledge surrounding seagrass in that the bioactivity levels proven to exist in the roots and rhizomes are consistent throughout various species from temperate and tropical origins. Additionally, the presence of antimicrobial properties within the root component have now been shown to exist in temperate regions, extending the known distribution of the bioactive bacteria.

Vibrio and *Photobacterium* have previously been described as abundant bacterial genera in the sediments surrounding seagrass (Zhang *et al.,* 2020), with various *Vibrio* and *Photobacterium* strains being identified as diazotrophs in the rhizosphere of *Zostera marina* (Shieh *et al.,* 1987). *Vibrio* was one of the genera displaying high levels of antimicrobial activity identified in this study, alongside *Tenacibaculum* and *Thalassomonas.* All three genera found in the seagrass are commonly found in the marine environment and contain pathogenic species that have been identified in and isolated from aquatic organisms (Pruzzo *et al.,* 2005; Thompson *et al.,* 2006; Avendaño-Herrera *et al.,* 2006; Hosoya *et al.,* 2009). The different genera are responsible for causing various diseases in humans and marine organisms: both *Tenacibaculum* sp. and *Vibrio* sp. are known to cause disease in humans and various fish species (Colwell and Grimes, 1984; Fernández-Álvarez and Santos, 2018) while *Thalassomonas* sp. have been shown to cause white plague in hard coral reefs (Thompson *et al.*, 2006). Each genus identified in this study have previously been associated with various components of seagrass beds around the globe (Hassenrück *et al.,* 2015; Webb, 2018; Reusch *et al.,* 2021), yet the antimicrobial potential of these bacteria has rarely been examined.

Various *Thalassomonas* sp. have been discovered to contain antimicrobial properties including that of proteins (Adams, 2019; Pheiffer, 2020) and polysaccharides (Wang *et al.*, 2022). However, the work surrounding *Tenacibaculum* and *Vibrio* sp. examining the antimicrobial properties of the bacteria is scarce (Heindl *et al.,* 2010) and has instead often focused on the antimicrobial activity against the individual pathogenic species. In this study, all genera were found to show antibiotic potential against *Escherichia coli* through antimicrobial screening, and both *Vibrio* and *Thalassomonas* also displayed this potential against *Staphylococcus aureus.* These findings support those previously described regarding the active potential of *Thalassomonas* sp. and provide larger scope to the extent of the antimicrobial activity displayed against indicator pathogenic organisms. The active potential demonstrated in *Vibrio* sp. has previously been displayed against indicator organisms by bacterial species located in the microbiome associated with coral mucus (Shnit-Orland and Kushmaro, 2009). The addition of these findings to previous research provides confirmation of the antimicrobial properties demonstrated by *Vibrio* species against various human pathogens. This largely emphasises and provides further scope for the potential of *Vibrio* species to be exploited for these active properties, while also demonstrating how analogous activity is displayed in a number of marine ecosystems. Future research examining these properties in alternative marine systems may be beneficial to disclose the distribution of *Vibrio* activity and further understand it's exploitation potential.

Similar antimicrobial properties have also been displayed by *Tenacibaculum* sp. associated with bryozoans, yet the findings of this study oppose those observed by Heindl *et al.* (2010) in that *Tenacibaculum* were not seen to display antimicrobial activity against *E.coli*. There is notable variation in the antimicrobial potential between different species within the *Tenacibaculum* genus, which has previously not been exploited for the active antimicrobial potentials demonstrated (Heindl *et al.*, 2010). This raises the question as to the extent of the antimicrobial divergence between species and whether there are some *Tenacibaculum* species that may show great active potential against pathogenic species and could therefore be exploited.

Conclusions

There are many implications of this research going forward. Opportunities have emerged for a wide scope of progressive studies to be conducted that address the various ecological influences on microbial population composition in seagrass beds. Potential investigations may include the extent of which environmental parameters influence microbe aggregation to determine whether analogous compositions are displayed in similar habitats. Additionally, further research examining the population diversity in relative seagrass components would be profitable. This would allow an understanding of the true microbial diversity situated within each seagrass component in various species around the globe, which would also provide further clarity on the mechanisms effecting their composition.

There is also scope for major advances in the comprehensive knowledge surrounding the antimicrobial activity in these populations. The root and rhizosphere have been shown on multiple occasions to harbour the highest antimicrobial potential of all seagrass components, but the true extent of their active capacity remains to be seen. The bacteria displaying the highest antimicrobial activity in the *Z. marina* beds in Salcombe Estuary were found to be of three genera – *Thalassomonas, Vibrio and Tenacibaculum.* These genera previously had limited to

no research investigating their antimicrobial potentials, and while more research is still required, there is now a baseline understanding regarding their antimicrobial abilities against indicator organisms that can be built from. With additional research and a more developed understanding, there is the possibility for bacterial species within these genera to be sustainably exploited from a variety of marine ecosystems due to their pharmaceutical value and ability to overcome human pathogens. Additionally, with the expanded knowledge encompassing the medical value of the seagrass associated micro-organisms, opportunities may arise to implement further protections and conservation efforts on various seagrass species - protecting not only the plant itself, but the cohort of micro-organisms that reside within its boundaries.

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