A commmercial seaweed extract strongly structured microbial communities associated with tomato and pepper roots and significantly increased crop yield

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Seaweeds have been used as a source of natural fertilizer and biostimulant in agriculture for centuries. However, their effects on soil and crop roots microbiota remain unclear. Here, we used a commercially available *Ascophyllum nodosum* extract (ANE) to test its effect on bacterial and fungal communities of rhizospheric soils and roots of pepper and tomato plants in greenhouse trials. Two independent trials were conducted in a split block design to test to effect of ANE amendment. We used amplicon sequencing targeting fungal ITS and bacterial 16S rRNA gene to determine microbial community structure changes. We find that productivity parameters of root, shoot and fruit biomass were positively and significantly incluenced by the ANE amendment. In addition, *a*-diversity differed significantly between amended and control plants, but only in some of the experimental conditions. Species composition among sites (*b*-diversity) differed according to the admentent treatment in all four communities (fungal-root, fungal-soil, bacterial-root and bacterial-soil). Finally, we identified a number of candidate taxa most strongly correlated with crop yield increases. Further studies on isolation and characterization of these microbial taxa linked to the application of liquid seaweed extract may help to enhance crop yield and sustain agro-ecosystems.

Keywords: Stella Maris®, 16S, ITS, soil microbial diversity, Illumina MiSeq, ANE, Amplicon Sequence Variants, OTU

RunningTitle: Seaweed extracts affect microbiomal communities

INTRODUCTION

Seaweeds (also known as marine macroalgae) have been used as a source of organic matter and mineral nutrients for centuries, especially in coastal areas (Khan *et al.*, 2009; Craigie, 2011). Liquid seaweed extracts, developed in the 1950s in order to concentrate plant growth-stimulating compounds, facilitate their usage (Milton, 1952). Today, most commercially available extracts are made from the brown algae *Ascophyllum nodosum*, *Ecklonia maxima* or *Laminaria spp*. Unlike modern chemical fertilizers, seaweed extracts are biodegradable, non-toxic and come from a renewable resource (Dhargalkar and Pereira, 2005). Industry-funded bodies such as the European Biostimulant Industry Coalition and the United States Biostimulant Coalition have been working to accommodate biostimulants into mainstream legal architecture. These organizations extoll benefits arising from modes-of-action research, agricultural applications and positive effects on yield and quality of many commercial species (i.e. fruits, vegetables, turf, ornamentals and woody species). Legal recognition will further allow a fluid integration of various biostimulants, including *Ascophyllum nodosum* Extracts (ANE) into sustainable long-term crop management programs (Craigie, 2011; Jardin, 2015).

Several comprehensive reviews have described the effects of seaweed extracts on agricultural plant productivity (Khan *et al.*, 2009; Craigie, 2010, 2011; Battacharyya *et al.*, 2015). The science points to wide-ranging effects from biotic to abiotic resistance, effects on growth and development, and ultimately, to their impact on plant establishment, crop yield and/or quality, and shelf life. At the physiological level, these extracts have been found to influence hormonal changes that in turn, influence physiological processes even at very low concentrations (Wally *et al.*, 2013).

Starting in the 1990's, high quality ANE was developed and let to an increased usage

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by farmers, in addition to an increase in cause-effect research, especially on plant diseases (Jayaraj and Ali, 2015). Noted increases in the activity of superoxide dismutase, glutathione peroxidase and ascorbate peroxidase helped support the argument that ANE improve plant tolerance to oxidative stress (Ayad *et al.*, 1997; Schmidt and Zhang, 1997; Ayad, 1998; Allen *et al.*, 2001). Positive effects were also found on phytoalexin production suggesting that ANE may increase activity of these protective enzymes that target oxidizing toxins naturally emitted by disease pathogens (Lizzi *et al.*, 1998; Jayaraj *et al.*, 2008; Jayaraman *et al.*, 2010).

Improved plant stress resistance and tolerance to foliar and soil treatments is attributed to a cascade of various physiological reactions. ANE can impact plant-signalling mechanisms through a multitude of plant processes and cellular modifications including osmotic/oxidative stresses such as salinity, freezing and drought stress (Jithesh *et al.*, 2012). ANE can also impart drought-stress tolerance to plants by reducing stomatal conductance and cellular electrolyte leakage (Shotton and Martynenko, unpublished data; Spann and Little, 2011). These results indicate that ANE can influence cellular membrane maintenance leading to a higher tolerance for various osmotic stresses and can mitigate oxidative damage.

Although there is an abundance of published evidence detailing systemic plant effects from ANE, outstanding questions remain as to the effects of ANE on the rhizosphere biology. Various microbes, small arthropods, nematodes and insects thrive in the soil rhizosphere. This microbial biodiversity then contributes to the aggregation of soil particles, enhances nutrient cycling and delivery to plants, degrades toxic substances, allows better soil water retention and plays a role in plant disease management. It has been suggested that the plant immune system is composed of inherent surveillance systems that perceive several general microbial elicitors, which allow plants to switch from growth

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and development into a defense mode (Newman *et al.*, 2013). This may allow the plant to avoid infection from potentially harmful microbes. An examination of sustainable products that can positively influence microbial interactions between plant roots and soil biota will in turn help to further understand soil borne plant-pathogens competition dynamics. The effect of ANE on the bacterial profile suggests that ANE applications increased strawberry root and shoot growth, berry yield, and rhizosphere microbial diversity and physiological activity (Alam *et al.*, 2013). Similar results were found in sandy loam soils as Alam and colleagues (2014) showed a strong relationship between carrot growth, soil microbial populations and activity.

The recent development of culture-independent molecular techniques and high throughput sequencing should permit to circumvent the inherent biases of culture-based approaches by targeting the ubiquitous component of life, DNA. In turn, this will help to identify a larger proportion of the microbial diversity and lead to a better understanding of the soil microbial response to seaweed extract. DNA barcoding targeting specific regions of the genome (e.g. ITS: fungi, 16s ribosomal genes: bacteria) are now regarded as a prerequisite procedure to comprehensively document the diversity and ecology of microbial organisms (Toju *et al.*, 2012; Klindworth *et al.*, 2013).

Here the general objective was to quantify the impact of a commercial seaweed extract on plant growth and test how the bacterial and fungal communities responded to the addition of these extracts. We also aimed to identify specific taxon positively correlated with increases in plant productivity following ANE amendments. We hypothesized that the addition of liquid seaweed extracts would improve productivity and alter significantly the bacterial and fungal communities. We used a commercially available ANE, Stella Maris®, developed by Acadian Seaplants Ltd (NS, Canada). Stella Maris® is derived from the marine algae *A. nodosum*, and harvested from the nutrient-laden waters of the

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North Atlantic off the Eastern Coast of Canada. We tested the effect of ANE on two agricultural plants commonly grown in greenhouse conditions (tomato and pepper). Several traits related to plant productivity were measured and soil and root bacterial and fungal diversity were quantified using High Throughput Illumina (San Diego, CA, USA) Miseq sequencing.

MATERIAL AND METHOD

Experimental design

Greenhouse trials were set up in large trays (60x30x18 cm LxWxH) using two different crops: tomato (*Solanum lycopersicum* L.) and pepper (*Capsicum annuum* L.). Tomato cultivar Totem Hybrid#A371 was planted in November 16th 2015 and pepper cultivar Ace Hybrid#318 was planted in December 9th 2015. Tomato and pepper seeds were purchased from William Dam Seeds Ltd (ON, Canada). These cultivars were selected for greenhouse production. Soil was collected from an agricultural field under organic regime at the IRDA research station in St-Bruno (Qc, Canada, 45º32'59.6"N, 73º21'08.0"W) on October 7th 2015. The soil was a loamy sand and was collected from the 15 cm top layer. Natural soil was homogenized and put into trays, filled to 15 cm in height. Soil analysis was done using a commercial service provided by EnvironeX (formerly AgriDirect, Longueuil, QC) and soil characteristics are shown in Table S1. Eight seeds per tray were planted and after germination, only four seedlings per tray were kept.

For each plant species, a randomized split block design (Table S2) was used with four trays set up per block and eight blocks for each trial. Half of the trays were amended with ANE, as described below. Half of the trays were also planted (planting effect) with four plants per tray, while the other trays were not planted. This allowed a direct comparison of fungal and bacteria soil communities with respect to the ANE amendment and planting effects.

Two different amendment regimes were used according to the plant species. For tomatoes, plants were amended using multipurpose organic fertilizer (pure hen manure, 18 g per tray repeated every 4 weeks, 5-3-2) from Acti-sol (Notre-Dame-du-Bon-Conseil, QC) in addition to Stella Maris® (3.5 ml per 1L, each tray received 250 ml, repeated every

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2 weeks) for the duration of the experiment. The other half were not treated, but watered with 250ml per tray instead. The physico-chemical composition of Stella Maris® is shown in Table S3. For the pepper experiment, the amendment treatment consisted solely of Stella Maris® (3.5 ml per 1L, each tray received 250 ml, repeated every 2 weeks) for the duration of the experiment. The other half was not amended, but watered with 250 ml per tray instead. Both experiments were managed under organic farming practices. Thrips were controlled using *Neoseiulus cucumeris* (syn. *Amblyseius cucumeris*) (1 bag per plant), Fungus gnats were also controlled using predatory mite *Gaeolaelaps gillespiei* (1L; Natural Insect Control, ON). Plants were treated once a week with Milstop, a Potassium Bicarbonate-based foliar fungicide to control the powdery mildew on both crops.

Plant productivity

Tomato and pepper experiments were harvested on March 29th 2016. The following traits assessed plant productivity: fruit number, fruit weight, shoots fresh weight and roots fresh weight. Together, these traits are expected to represent well the plant overall productivity. Traits were measured on three plants chosen randomly per tray for each amended / control plant, crop (tomato / pepper) and block (eight blocks) for a total of 96 samples. In addition, both shoot and root samples were dried in a 70 degrees drying oven, and dry weights were quantified after 48 hours.

Sample preparation, DNA extraction and High throughput sequencing

Soil and root samples were taken for both experiments. Soil DNA was extracted using NucleoSpin® Soil DNA extraction kit (Macherey-Nagel, BioLinx, ON) on 250 mg of soil, following the manufacturer's protocol. Roots were first washed with tap water and rinsed with sterile water. Chopped roots sub-samples (100 mg) were subjected to DNA extraction using DNeasy Plant Mini kit (Qiagen Inc - Canada, ON), following the manufacturer's recommendations. Amplicon sequencing targeting bacterial 16S rRNA gene and

fungal ITS was performed on both root and soil samples.

For fungal ITS, we used the following primers with the universal CS1 and CS2 adapters: CS1_ITS3_KYO2 (5′-ACA CTGA CGA CAT GGT TCT ACA GAT GAA GAA CGY AGY RAA-3′) and CS2_ITS4_KYO3 (5′-TAC GGT AGC AGA GAC TTG GTC TCT BTT VCC KCT TCA CTC G-3′) to produce a final amplicon size of approximately 430bp including adapters (Toju *et al.*, 2012).

For bacterial 16S, we used the following primers with CS1 and CS2 universal adapters: 341F (5′-CCT ACG GGN GGC WGC AG-3′) and 805R (5′-GAC TACC AGG GTA TCT AAT C-3′) to produce a final amplicon size of approximately 460 bp and targeting specifically the bacterial V3-V4 region of the 16S ribosomal gene (Klindworth *et al.*, 2013).

DNA samples were then barcoded, pooled and sequenced (2X300bp, paired-end) using an Illumina MiSeq sequencer through a commercial service provided by the Genome Quebec Innovation Centre (Montreal, QC). Sequences were demultiplexed by the sequencing facility and further processed as described below.

Bioinformatics

All bioinformatics, statistical, and graphical analyses further described were performed in R 3.5.1 (R Core Team, 2018) and detailed scripts are available here (https://github. com/seb951/Acadian_Seaplants).

We used the R package DADA2 (Callahan *et al.*, 2016) to infer *Amplicon Sequence Variants* (ASV). DADA2 offers accurate sample inference from amplicon data with single-nucleotide resolution in an open source environment. Unlike the Operational Taxonomic Unit (OTU) approach (e.g. Schloss *et al.*, 2009; Caporaso *et al.*, 2010), ASV are not treated as cluster of

sequences defined with an *ad hoc* sequence similarity threshold. Instead, after sequences are quality trimmed and error-corrected, `DADA2` reveals the unique members of the sequenced community, thus allowing sequences and abundance counts to be comparable among studies (Callahan *et al.*, 2016).

First, sequences were trimmed following strict quality thresholds (removing primers and low quality nucleotides, see parameter details in the accompanying `R` scripts). Following this, we applied the error model algorithm of `DADA2`, which incorporates quality information after filtering, unlike other OTU based methods. Then dereplication, sample inference, merging of paired end reads and removal of chimera were performed in order to obtain a sequence (ASV) table of abundance per sample. Taxonomy was assigned through the `DADA2` pipeline using the Ribosomal Database Project (RDP) Naive Bayesian Classifier algorithm from Wang *et al.* (2007). Depending on support (minimum bootstrap support of 80), we assigned taxonomy from Kingdom to species. We used the silva database formatted for `DADA2` to infer bacterial taxa (Callahan, 2018). We used the Unite (Community, 2018) fasta release (including singletons) to infer fungal taxa after formatting it to the `DADA2` format using a custom `R` script. The pipeline was run on a multithreaded (48 CPUs) computer infrastructure provided by Westgrid (https://www.westgrid.ca/support/systems/cedar) and Compute Canada (www.computecanada.ca). Note that the pipeline was run separately for fungal-root, fungal-soil, bacteria-soil and bacteria-root samples given that these were sequenced separately and therefore a specific error model for each dataset was calculated.

Statistical analyses - plant productivity

Each plant species (tomato and pepper) were analysed seperately. We tested for the amendment effect (tomato: hen manure + ANE, pepper: ANE) on six plant productivity measures (fruit number, average fruit weight, shoots fresh weight, roots fresh weight,

shoots dry weight, roots dry weight). We used Linear Mixed effect Models (LMM) in the R package NLME (Pinheiro *et al.*, 2017), which are more appropriate than an Analysis of Variance (ANOVA) given the current block design (blocks and replicates were treated as random variables). All six plant productivity measures were either square root or log transformed in order to help satisfy the assumption of normality of the residuals in the LMM statistical framework. For the variables *fruit number* and *average fruit weight*, we also verified statistical significance using a permutation-based 2-way ANOVA (Anderson and Legendre, 1999) given that the residuals of the LMM were not normally distributed. Results were similar according to the 2-way ANOVA.

Statistical analyses - microbial and fungal diversity

For each fungal-root, fungal-soil, bacterial-root and bacterial-soil datasets seperately, we removed samples that showed poor sequencing output and contained few ASV. In order to do this, we summed the abundance of all ASV for each sample ($\sum_{i=1}^{n} ASV$) and eliminated samples that had fewer that the mean sum minus four standard deviations ($\overline{\sum_{i=1}^{n} ASV} - 4\sigma$). In addition, we removed ASV from our dataset that were present in fewer than 5% of the samples (less than ten individuals in the soil samples or less than five in the root samples). This was done to remove very rare ASV unique to a block or replicate, but not found in the majority of samples.

We then conducted community-based analyses looking at the amendment effect on ASV abundance in the tomato and pepper experiments seperately. To reduce the complexity of the datasets, relative abundance of all taxa was calculated per family using the R package DPLYR (Wickham *et al.*, 2015). Barplots were drawn using GGPLOT2 (Wickham, 2016) to visualize communities. ASV alpha (*a*)-diversity was calculated based on all ASV (excluding rare ASV, see paragraph above) for each sample using the inverse Simpson diversity index in VEGAN (Oksanen *et al.*, 2013). The effect of the amendment and planting for soil

communities were assessed using a Linear Mixed effect Model (LMM) model in the R package NLME (Pinheiro *et al.*, 2017), given the unbalanced, replicated block design. Alpha diversity was *log* transformed in order to help satisfy the assumption of normality of the residuals in the LMM statistical framework.

Using the community matrix data of ASV abundance, we performed PERmutational Multivariate ANalysis Of VAriance tests (PERMANOVA; Anderson, 2001) to identify relationships between the communities according to the experimental design. Data were analyzed separately for fungal-root, fungal-soil, bacterial-root and bacterial-soil in tomatoes and peppers. The ASV abundance matrix was Hellinger-transformed and significance was assessed using 10,000 permutations in vegan (Oksanen *et al.*, 2013). Blocks and replicates were factored as strata in the model.

We also performed redundancy analyses (RDAs) using the Hellinger-transformed ASV abundance matrix in vegan (Oksanen *et al.*, 2013) to visually assess the grouping of samples, ASV and their association with productivity variables (*species* scaling based on ASV matrix). Data were analyzed separately for fungal-root, fungal-soil, bacterial-root and bacterial-soil in tomatoes and peppers. This gave a total of eight RDAs. Data were constrained based on four productivity measures (fruit number, average fruits weight, shoots fresh weight, roots fresh weight). We excluded the shoots & roots dry weights as constraints to simplify the model. In addition, these were highly correlated with the fresh weight already included as constraints (r^2=0.98 and 0.76 for shoot dry/fresh weights and root dry/fresh weights, respectively).

Finally, we attempted to identify candidate ASV positively associated with productivity. As such, we identified the ten ASV most positively associated with the measures of fruit number, shoots fresh weight and roots fresh weight from each redundancy analysis for a

total of 40 fungal and 40 bacterial candidate ASV. We aligned candidate sequences from these candidates ASV using the Bioconductor `R` package `DECIPHER` (Wright, 2016) and build pairwise distances matrices using a JC69 substitution models of DNA sequence evolution (equal base frequencies, Jukes and Cantor, 1969) in `PHANGORN` (Schliep, 2010). Phylogenetic trees (neighbour-joining) for bacteria and fungi were plotted using `APE` (Paradis *et al.*, 2004). This permitted to identify if similar candidate ASV were found under different experimental conditions (soil/root, pepper/tomato), thus reinforcing their role in productivity increase, and decreasing the rate of false positives.

RESULTS

Effects of the amendment treatment on productivity The effects of the amendment treatment on tomato (hen manure + ANE) and pepper (ANE) were determined by measuring six agronomic parameters (fruit number, average fruit weight, shoots fresh weight, shoots dry weight, roots fresh weight, roots dry weight). We observed a significant increase of almost all these agronomic parameters (LMM, p-value<0.005, Figure 1) for amended plants except for the average fruit fresh weight for tomato that did not differ between amended and control plants (LMM, $F_{(1,23)}$ = 1.81, p-value=0.19, Figure 1 and Figure S1). The amendment effect was stronger in the tomato plants (fold changes between amemded and control plants shown in Figure 1), likely due to the fact that these plants were fertilized with both hen manure and ANE.

Amplicon Sequencing

A total of 2.7 million paired-end raw reads were obtained for all samples combined (976,000 for fungi-soil, 920,000 for fungi-root, 309,000 for bacteria-soil and 535,000 for bacteria-root, Table S4). On average, 47,664 paired-end reads were obtained per sample. After quality filters were applied, including removing chimeras, and paired-end reads were merged, an average of 19,690 sequences remained per sample. From 192 soil samples for fungi and bacteria, and 96 root samples for fungi and bacteria sequenced, seven fungi-soil samples, 15 fungi-root samples and one bacteria-root samples were removed because they had to few reads based on our strict quality thresholds.

The `DADA2` pipeline inferred, on average, 170 Amplicon Sequence Variants (ASV) per sample (average of 176 fungal-soil ASV, 37 fungal-root ASV, 269 bacterial-soil ASV and 92 bacterial-root ASV). Many of these were unique to one or a few samples (total number of 6,112 fungal-soil, 845 fungal-root, 9,352 bacterial-soil and 2,023 bacterial-roots ASV).

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After quality filtering, we retained 413, 106, 811 and 325 ASV respectively for fungal-soil, fungal-root, bacterial-soil and bacterial-roots. These retained ASV comprised 94%, 95%, 89% and 98% of all reads in the fungal-soil, fungal-root, bacterial-soil and bacterial-root samples, respectively.

Fungal and bacterial diversity in root and soil biotopes

The microbial community structures of soil and root samples were analyzed and the relative abundance of their taxa was determined at the family level (Figures 2 & 3). Fungal communities were dominated by Nectriaceae, both in the root and soil samples, while the bacterial family Bacilaceae dominated to a lesser extent the soil samples. Bacterial root communities were largely dominated by Cyanobacteria (identified as *chloroplast* in the silva database according to the RDP Bayesian Classifier).

Local (a-diversity)

The *a*-diversity was calculated separately for each sample, under each experimental condition (fungi-soil, fungi-root, bacteria-soil and bacteria-root for both tomato and pepper, Figure 4). Linear mixed effects models showed that the *a*-diversity (Inverse Simpson Index) was significantly higher in the soil biotope that in the roots for both fungi (mean *a*-diversity soil-fungi = 2.88 vs mean *a*-diversity root-fungi = 27.3, $F_{(1,239)}$=899.5, *p*-value<0.0001) and bacteria (mean *a*-diversity soil-fungi = 1.3 vs mean *a*-diversity root-fungi = 69.3, $F_{(1,256)}$=2048.4, *p*-value<0.0001).

In soil samples, fungal *a*-diversity was not significantly different in amended vs control plants for neither tomato ($F_{(1,66)}$=1.6, *p*-value=0.21) nor pepper ($F_{(1,69)}$=1.2, *p*-value=0.05). In root samples, fungal *a*-diversity was significantly different in amended versus control plants for tomato ($F_{(1,21)}$=10.2, *p*-value=0.004), but not pepper ($F_{(1,56)}$=3.1, *p*-value=0.10).

In soil samples, bacterial a-diversity was significantly different in amended vs control plants for pepper ($F_{(1,66)}$=31.2, p-value<0.0001), but not tomato ($F_{(1,69)}$=1.9, p-value=0.17). In root samples, bacterial a-diversity was significantly different in amended versus control plants for tomato ($F_{(1,23)}$=19.5, p-value<0.0001), but not pepper ($F_{(1,22)}$=0.01, p-value=0.92).

Differences in species composition among sites

Using a PERMANOVA, we identified that the ANE amendment treatment had a highly significant effect on both fungal and bacterial community structures (Table 1). This effect was stronger in the root (9-30% of variance explained in the models) than in the soil (3-6% of variance explained in the models). Planting also had a significant effect on fungal and bacterial community structures in both tomato and pepper plants (12-24% of variance explained in the models).

Redundancy analyses (RDAs, Figures 5 for fungi and Figure 6 for bacteria) illustrated that roots fresh weight, shoots fresh weight and fruit number responded similarly, while average fruit weight behaved differentially as noted previously (in fact nearly orthogonally to the other three parameters in most ordinations). In addition, it showed that fertilized samples clustered together and were positively correlated with increases in these four productivity measures.

Next, we identified, for each RDA, the ten ASV most closely related to the three constraints of the model (roots fresh weight, shoots fresh weight and fruit number). These ASV were considered as putative candidate taxa most positively impacted by increases in productivity due to the ANE amendment treatment. We further analyzed the corresponding sequences for these eighty candidate ASV (ten candidates * eight ordinations) in two separate alignments (one for fungi and one for bacterial ASV) and their accompanying phylogenetic trees.

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In fungi, we identified a number of different ASVs positively associated with productivity (Figure S2). Notably, five different ASVs taxonomically assigned to the family Microascacea (phylum Ascomycota) in all conditions except the pepper-root were positively associated to productivity. In addition, two ASV assigned to *Mortierella* spp (soil saprotrophs in the phylum Mucoromycota), and a cluster of six different fungal closely related ASV in tomato-soil (ASV67 & ASV132), tomato-root (ASV10, ASV1017, ASV1018, ASV1019) and pepper-soil (ASV67) were positively associated to productivity in both tomato and pepper roots. Given that no taxonomy was assigned to these sequences through the DADA2 RDP bootstrap approach, we used a BLASTn (Altschul *et al.*, 1997) approach to identify the most closely related sequences against NCBI nr. The most closely related fungal reference sequences were from an *uncultured fungus clone* (BLASTn, 86% identity, e-value=9e-58, sequence ID: EU517002.1). Similarly, two unknown ASV (ASV61 & ASV81) also matched an *uncultured fungus clone* (BLASTn, 94% identity, e-value=4e-165, sequence ID: DQ900965.1). Finally, another cluster of ASVs in the pepper-root was assigned to *Olpidium brassicae*, a fungal parasite belonging to flagellate fungi (Lay *et al.*, 2018).

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In bacteria-roots, we identified a large diversity of ASV positively correlated (increased abundance of these ASV) with the four measures of productivity (Figure S3). Some of them are well known soil bacteria such as *Rhizobium*, or *Sphingomonas* and were identified in both biotopes and species.

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DISCUSSION

In the current study, we investigated the effects of *Ascophyllum nodosum* extracts (ANE) on root, shoot and fruit biomass in addition to bacterial and fungal communities in tomato and pepper. Overall parameters related to plant growth (root, shoot and fruit weights) significantly increased in both plant species in response to ANE amendment. These results corroborate previous studies documenting the impact of ANE on productivity in strawberries (Alam *et al.*, 2013) and carrots (Alam *et al.*, 2014).

In the tomato experimental set up, the effect of the amendment treatment was especially high, likely due to the fact that plants were also fertilized with hen manure in addition to ANE (Figure 1). This was not the case for the pepper plants and the increase in productivity was solely due to the addition of ANE. The commercial extract used in this investigation contained about 0.1% nitrogen, 0.2% phosphorus, 5% potassium, along with several micronutrients (Table S3). In the current experimental setup, ANE was diluted to 3.5 ml/L prior to application (250 ml per tray every two weeks). In fact, in the tomato plants the amounts of N, P and K supplied via the application of ANE were 200-1000 times less than from the hen manure itself. As such, these nutrients were given at very low concentrations relative to the crop requirements and are not expected to significantly impact growth relative to a regular agricultural fertility program (Bruulsema *et al.*, 2012; Alam *et al.*, 2013). Instead, organic molecules such as betaines, polyamines, cytokinins, auxins, oligosaccharides, amino acids and vitamins present in ANE have been found to have overall beneficial productivity effects on plant growth (Khan *et al.*, 2009; Craigie, 2010, 2011; Battacharyya *et al.*, 2015).

One of primary goal of the study was to document how bacterial and fungal communities responded to the addition of ANE. We used Illumina MiSeq amplicon sequencing target-

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ing DNA regions specific to fungi (ITS) and bacteria (16S). Then, we identified bacterial and fungal taxa present in the samples using a relatively novel bioinformatics approach developed by (2016). The approach, based on the widely used programming language R (R Core Team, 2018), identifies unique, non-clustered sequences (ASV) that are then comparable among studies. In addition, the current analytical pipeline uses a bayesian classifier for taxonomy rather than the widely used BLAST approach, thus providing more conservative, but more accurate taxonomic identifications (Wang *et al.*, 2007).

In the current experimental set up, most ASV identified were rare and unique to one or a few sample. In fact, approximately 90% of all ASV were discarded given that they were found in singletons or present in very few samples and were thus not representative of a particular experimental treatment. These 'rare' ASV comprised a small minority of all sequencing reads (approximately 5% of all sequences), a pattern reminiscent of the early species abundance models showing that in most ecological communities, few species are exceptionally abundant whereas most are rare (Fisher *et al.*, 1943).

Nectriaceae, a family of fungi in the order Hypocreales and often encountered as saprotrophs on decaying organic matter comprised most of the diversity both in the soil and plant roots (between 25-70% of the total number of sequencing reads, Figure 2). With respect to bacterial communities of the soil, theses were much more diverse and comprised many different families (Figure 3). Surprisingly, most sequencing reads in the bacterial communities of roots likely originate from the plants themselves (identified as chloroplastic or mitochondrial in origin in Figure 3), despite the fact that the DNA primers pair used should have primarily targeted the bacterial V3-V4 region of the 16S ribosomal gene. The ANE amendment treatment had a significant effect on both fungal and bacterial *a*-diversity (total richness of ASV) in the root biotope, except for bacteria in pepper plants. In the soil biotope, it only had a significant effect for bacteria in the tomato plants (Figure

439 4).

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441 The ANE amendment treatment significantly influenced fungal and bacterial community
442 composition (*b*-diversity) among root and soil biotopes. This effect was relatively small
443 (3-30% of variance explained in the models, Table 1) but significant, implying that the
444 addition of ANE (pepper) or ANE and hen manure (tomato) is responsible for shaping
445 microbial communities. In addition, a significant proportion of the variance in soil com-
446 munities (12-24%) was explained by the planting effect, showing how plants can alter
447 their microbiome. We also tested the effect of plant species identity on community struc-
448 ture on a combined dataset comprised of both the tomato and pepper plants. In the root
449 biotope, we find that this effect is relatively strong (24 and 52% of variance explained in
450 the models for fungal and bacterial communities, respectively, Table S5) and in line with
451 numerous studies reporting how plants select their microbial communities (Chaparro *et*
452 *al.*, 2014; Reinhold-Hurek *et al.*, 2015). Nevertheless, we recognize that the current exper-
453 imental setup precludes any strong conclusion regarding the plant species' effect of com-
454 munity structure, as it does not allow to explicitly distentangle the species effect from the
455 "addition of hen manure" effect.

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457 Finally, we also aimed to identify candidate taxa positively correlated with increased
458 plant productivity in response to ANE application. In fungi, one cluster of ASV taxo-
459 nomically assigned to *Mortierella* (soil saprotrophs in the phylum Mucoromycota) was
460 positively correlated to productivity in both tomato and pepper roots. Interestingly, Li *et*
461 *al.* (2018) found that a closely related species (*M. elongata*) can defend against soil degra-
462 dation, improve soil health, and stimulate production of plant growth hormones. In their
463 study, Chung *et al.* (2007) showed how higher plant species richness and increases in
464 productivity led to greater microbial biomass and greater number of saprophytic and ar-
465 buscular mycorrhizal fungi. Perhaps, this can be explained by the fact that microbial

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communities experienced greater substrate availability, potentially increasing their activity, and the activity of saprophytic fungi feeding on organic matter.

Several other fungal ASV in tomato (soil) and pepper (root) were linked to increases in productivity. Surprisingly, a putative plant pathogenic fungus (*Olpidium brassicaceae*, Figure S2) was also positively associated with increased productivity. However, *O. brassicae* is likely a species complex and has been shown to constitute a large proportion of the plant roots or rhizosphere fungal community in many different systems, particularly in Brassicaceae crops (Lay *et al.*, 2018). In addition, current databases usually confuse the *O. brassicae* with the virus-carrier *O. virulentus* (Lay *et al.*, 2018). As such, this may explain its presence in the soil and association with plant productivity.

In bacteria roots samples, a diverse number of ASV were positively impacted by the amendment treatment (Figure S3). The specific role of those taxa on crop productivity will need further investigations.

It is now well established that seaweed extracts have a positive effect on agricultural plant productivity. Concurrently, DNA barcoding permits a more comprehensive understanding of the diversity and ecology of microbial organisms and how they interact. In fact, plants and microbes should likely be redefined as *holobionts*, an assemblage of different species that form an ecological unit (Margulis and Fester, 1991). In this study, we showed that the addition of ANE increased plant productivity. It also increased, by a small, but significant margin, the fungal and bacterial (only in the rhizosphere) biodiversity and changed the microbial community structure in the roots and in the rhizosphere of both tomato and pepper plants. Finally, we identified bacterial and fungal taxa, especially saprotroph, that were positivity associated with plant productivity. Further studies, for example using inoculum of the candidate microbial species linked to increases in produc-

tivity that we identified, may help to identify a causative link between liquid seaweed extracts, microbes and productivity.

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REFERENCES

Alam, M.Z., Braun, G., Norrie, J., and Hodges, D.M. (2014) Ascophyllum extract application can promote plant growth and root yield in carrot associated with increased root-zone soil microbial activity. *Canadian Journal of Plant Science* **94**: 337–348.

Alam, M.Z., Braun, G., Norrie, J., and Hodges, D.M. (2013) Effect of ascophyllum extract application on plant growth, fruit yield and soil microbial communities of strawberry. *Canadian Journal of Plant Science* **93**: 23–36.

Allen, V., Pond, K., Saker, K., Fontenot, J., Bagley, C., Ivy, R., et al. (2001) Tasco: Influence of a brown seaweed on antioxidants in forages and livestock—a review 1. *Journal of Animal Science* **79**: E21–E31.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped blast and psi-blast: A new generation of protein database search programs. *Nucleic acids research* **25**: 3389–3402.

Anderson, M.J. (2001) A new method for non-parametric multivariate analysis of variance. *Austral ecology* **26**: 32–46.

Anderson, M.J. and Legendre, P. (1999) An empirical comparison of permutation methods for tests of partial regression coefficients in a linear model. *Journal of statistical computation and simulation* **62**: 271–303.

Ayad, J. (1998) The effect of seaweed extract (ascophyllum nodosum) on antioxidant activities and drought tolerance of tall fescue (festuca arundinacea schreb). *Ph D Thesis, Texas Tech University*.

Ayad, J., Mahan, J., Allen, V., and Brown, C. (1997) Effect of seaweed extract and the endophyte in tall fescue on superoxide dismutase, glutathione reductase and ascorbate peroxidase under varying levels of moisture stress. In, *American forage and grassland council conference proceedings*.

Battacharyya, D., Babgohari, M.Z., Rathor, P., and Prithiviraj, B. (2015) Seaweed ex-

tracts as biostimulants in horticulture. *Scientia Horticulturae* **196**: 39–48.

Bruulsema, T.W., Heffer, P., Welch, R., Cakmak, I., Moran, K., and others (2012) Fertilizing crops to improve human health: A scientific review. *Better Crops* **2**: 96.

Callahan, B. (2018) Silva for dada2: Silva taxonomic training data formatted for dada2 (silva version 132). *Zenodo*.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., and Holmes, S.P. (2016) DADA2: High-resolution sample inference from illumina amplicon data. *Nature methods* **13**: 581.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., et al. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature methods* **7**: 335.

Chaparro, J.M., Badri, D.V., and Vivanco, J.M. (2014) Rhizosphere microbiome assemblage is affected by plant development. *The ISME journal* **8**: 790.

Chung, H., Zak, D.R., Reich, P.B., and Ellsworth, D.S. (2007) Plant species richness, elevated co2, and atmospheric nitrogen deposition alter soil microbial community composition and function. *Global Change Biology* **13**: 980–989.

Community, U. (2018) UNITE general fasta release. Version 01.12.2017.

Craigie, J.S. (2011) Seaweed extract stimuli in plant science and agriculture. *Journal of Applied Phycology* **23**: 371–393.

Craigie, J.S. (2010) Seaweed extract stimuli in plant science and agriculture. *Journal of Applied Phycology* **23**: 371–393.

Dhargalkar, V. and Pereira, N. (2005) Seaweed: Promising plant of the millennium.

Fisher, R.A., Corbet, A.S., and Williams, C.B. (1943) The relation between the number of species and the number of individuals in a random sample of an animal population. *The Journal of Animal Ecology* 42–58.

Jardin, P. du (2015) Plant biostimulants: Definition, concept, main categories and regulation. *Scientia Horticulturae* **196**: 3–14.

23

Jayaraj, J. and Ali, N. (2015) Use of seaweed extracts for disease management of vegetable crops. In, Ganesan,S., Vadivel,K., and Jayaraman,J. (eds), *Sustainable crop disease management using natural products*. CAB International, pp. 160–183.

Jayaraj, J., Wan, A., Rahman, M., and Punja, Z. (2008) Seaweed extract reduces foliar fungal diseases on carrot. *Crop Protection* **27**: 1360–1366.

Jayaraman, J., Norrie, J., and Punja, Z.K. (2010) Commercial extract from the brown seaweed ascophyllum nodosum reduces fungal diseases in greenhouse cucumber. *Journal of Applied Phycology* **23**: 353–361.

Jithesh, M.N., Wally, O.S., Manfield, I., Critchley, A.T., Hiltz, D., and Prithiviraj, B. (2012) Analysis of seaweed extract-induced transcriptome leads to identification of a negative regulator of salt tolerance in arabidopsis. *HortScience* **47**: 704–709.

Jukes, T. and Cantor, C. (1969) Evolution of protein molecules, pp. 21–132 in mammalian protein metabolism, edited by munro hn Academic Press, New York.

Khan, W., Rayirath, U.P., Subramanian, S., Jithesh, M.N., Rayorath, P., Hodges, D.M., et al. (2009) Seaweed extracts as biostimulants of plant growth and development. *Journal of Plant Growth Regulation* **28**: 386–399.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., and Glöckner, F.O. (2013) Evaluation of general 16S ribosomal rna gene pcr primers for classical and next-generation sequencing-based diversity studies. *Nucleic acids research* **41**: e1–e1.

Lay, C.-Y., Hamel, C., and St-Arnaud, M. (2018) Taxonomy and pathogenicity of olpidium brassicae and its allied species. *Fungal biology*.

Li, F., Chen, L., Redmile-Gordon, M., Zhang, J., Zhang, C., Ning, Q., and Li, W. (2018) Mortierella elongata's roles in organic agriculture and crop growth promotion in a mineral soil. *Land Degradation & Development* **29**: 1642–1651.

Lizzi, Y., Coulomb, C., Polian, C., Coulomb, P., and Coulomb, P. (1998) Seaweed and mildew: What does the future hold? *Phytoma La Defense des Vegetaux (France)*.

Margulis, L. and Fester, R. (1991) Symbiosis as a source of evolutionary innovation:

Speciation and morphogenesis Mit Press.

Milton, R. (1952) Improvements in or relating to horticultural and agricultural fertilizers. *British Patent* **664989**:

Newman, M.-A., Sundelin, T., Nielsen, J.T., and Erbs, G. (2013) MAMP (microbe-associated molecular pattern) triggered immunity in plants. *Frontiers in Plant Science* **4**:

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'hara, R., et al. (2013) Vegan: Community ecology package. R package version 1.17.2. *R software*.

Paradis, E., Claude, J., and Strimmer, K. (2004) APE: Analyses of phylogenetics and evolution in r language. *Bioinformatics* **20**: 289–290.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., and Team, R.C. (2017) Nlme: Linear and nonlinear mixedeffects models. R package version 3.1-128. *R software*.

R Core Team (2018) R: A language and environment for statistical computing.

Reinhold-Hurek, B., Bünger, W., Burbano, C.S., Sabale, M., and Hurek, T. (2015) Roots shaping their microbiome: Global hotspots for microbial activity. *Annual review of phytopathology* **53**: 403–424.

Schliep, K.P. (2010) Phangorn: Phylogenetic analysis in r. *Bioinformatics* **27**: 592–593.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., et al. (2009) Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and environmental microbiology* **75**: 7537–7541.

Schmidt, R. and Zhang, X. (1997) Influence of seaweed on growth and stress tolerance of grasses. In, *American forage and grassland council conference proceedings*. Ft. Worth, TX, pp. 158–162.

Spann, T.M. and Little, H.A. (2011) Applications of a commercial extract of the brown seaweed ascophyllum nodosum increases drought tolerance in container-grown 'hamlin'sweet orange nursery trees. *HortScience* **46**: 577–582.

Toju, H., Tanabe, A.S., Yamamoto, S., and Sato, H. (2012) High-coverage its primers for

the dna-based identification of ascomycetes and basidiomycetes in environmental samples. *PloS one* **7**: e40863.

Wally, O.S., Critchley, A.T., Hiltz, D., Craigie, J.S., Han, X., Zaharia, L.I., et al. (2013) Regulation of phytohormone biosynthesis and accumulation in arabidopsis following treatment with commercial extract from the marine macroalga ascophyllum nodosum. *Journal of plant growth regulation* **32**: 324–339.

Wang, Q., Garrity, G.M., Tiedje, J.M., and Cole, J.R. (2007) Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and environmental microbiology* **73**: 5261–5267.

Wickham, H. (2016) Ggplot2: Elegant graphics for data analysis Springer.

Wickham, H., Francois, R., Henry, L., and Müller, K. (2015) Dplyr: A grammar of data manipulation. *R package version 0.4* **3**:

Wright, E.S. (2016) Using decipher v2.0 to analyze big biological sequence data in r. *R Journal* **8**: 352–359.