2 Background

Since the advent of medicine and pesticides, microbial pathogens have been targeted with chemicals that have had the unintended and devastating effect of decimating cohabiting and often beneficial taxa. Over the last century this has led to the rise of drug-resistant strains and exacerbated the consequences these pathogens posed[1,2,3]. As the pharmaceutical industry ramps up their search for the next great compounds in this fight, microbiome research is beginning to produce successful alternatives to compound synthesis[4]. Current state-of-the-art microbiome therapies function by overcrowding pathogenic strains with a surplus of transplanted 'good' microbial (primarily bacterial) taxa sourced from healthy donors[5,6,7]. In plants, the foliar transplantation of fungal symbionts from related species has been shown to confer disease resistance to a population of the nearly extinct and fungal-treatment dependent plant species *P. kaalaensis*[8]. Panama disease, caused by the fungus *F. oxysporum* and correlated to soils with high fungal content and low bacterial diversity, has been shown to respond positively to the treatment with microbes from healthy soils [9]. To further understand the protective effect of microbiota transplants against pathogens encroaching on the commercially important crops, it is necessary to study the fundamental principles behind the establishment of microbiomes, their stability and dynamics, are necessary[4,10,15].

2.1 Microbiomes

A microbiome is defined as the collective genomes of the totality of microorganisms interacting with and within a defined environment ultimately forming complex ecosystems[11]. These systems are not inconsequential and, in the case of soil and ocean microbiomes, contribute significantly to biogeochemical cycles like the nitrogen and oxygen cycles[12,13]. Microbiomes, however, are not limited to open environments where conditions are in constant flux, such as those found in lakes and oceans, and are often described in conjunction with a host organism[13,14,15]. Of particular interest in contemporary microbiome research are those microbiomes associated with humans and crops, and the impact they have on the overall fitness of the host[8,10,16]. In humans, disturbed microbiomes have been linked to digestive disorders, cancers, Multiple Sclerosis and

Alzheimer's among other afflictions [17,18]. In plants, dysbiosis of their microbiomes permit opportunistic pathogens to settle and disrupt their normal processes [4,19].

2.1.1 The Rhizosphere and Phycosphere`

Each of the plant's organ harbors its own unique microbiome, but the rhizosphere, defined at the root-soil interface, boasts the most taxonomically diverse among them[20]. Rhizosphere microbiota are comprised of a diverse but specific set of members at specific relative abundances [4,21]. Analogously, the phycosphere is the nutrient rich area surrounding phytoplankton. These autotrophs, like plants, convert sunlight to chemical energy and provide their immediate surroundings with chemical currency to exchange with their adjacent bacterial community[22]. Both of these microbiomes are known to alter their immediate environment through their secretions[22]. The compounds exuded into the soil by plants are believed to initiate and support microbial colonization and stability [23]. Similar mechanisms are presumably at play in the phycosphere. Members of these communities in turn confer defense on their host through the release of compounds into their respective environments, which can adversely affect opportunistic pathogens [4,24]. The similarity of the phycosphere to the rhizosphere makes it a great tool to study the fundamental properties that drive colonization and stability of the microbiota from photosynthetic communities.
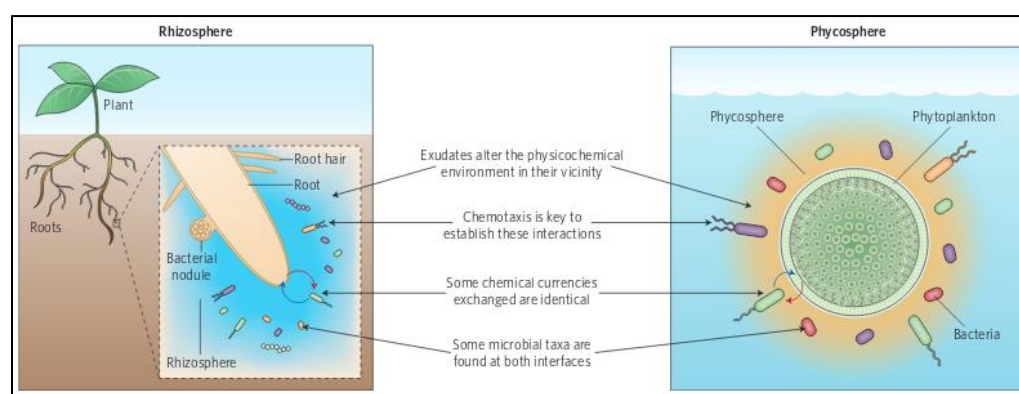

Figure 2.1: The physical similarities between the Rhizosphere and Phycosphere. Adapted from [22]

2.2 Metagenomics & 16S rRNA Profiling

The current state and ever-growing body of knowledge cementing the rhizosphere's beneficial roles in plant health would not be possible without the advent of next-generation sequencing (NGS) technologies and techniques, especially Metagenomics and 16S profiling. This field of study and technique are defined as the study of genomes from environmental and community samples using shotgun whole-genome sequencing [25]. Depending on the environment under

study, there are detailed guidelines for sample collection and preparation, but beyond this, typical metagenomic pipelines are similar to most other NGS pipelines and consist of the following general steps: DNA extraction, library preparation, sequencing, assembly, annotation and analysis [26]. Metagenomic studies are not limited to determining 'who' is present in the sample; rather its main goal is to determine the functional genome composition in the sample [25]. These functional genomes, or metagenomes, allow investigators to extract probable biosynthetic pathways in that environment, which highlight possible metabolic inter-dependencies between present taxa. Determining these metabolic dependencies and probable biosynthetic pathways that exist in these communities provides a rich set of information from which fundamental insights may be obtained[24]. While 16S rRNA profiling, on the other hand, is concerned with determining the presence and relative abundance of the taxa in the natural or synthetic communities[24,27].

2.2.1 Metagenomics Costs & Possible Solution

General Metagenomic studies document and contrast community compositions across space while others aim to document a specific community's evolution across time during environmental change [4,26,27]. While these environmental studies are important for a myriad of reasons, they only provide snapshots of the metagenomes and community composition during its collection. In order to glean more fundamental information, which can be subsequently exploited for plant therapeutic use, additional snapshots across shorter time intervals are required. While the price of NGS studies has gone down dramatically, studying microbiomes in the current paradigm is an expensive endeavor for most laboratories. In order to reduce cost and increase productivity, a high-throughput system and research method is currently under development in the Garrido-Oter lab at the Max Planck Institute for Plant Breeding Research (MPIPZ; Cologne, Germany). The system, currently in the prototyping phase, is meant to allow investigators to quickly iterate over hypotheses and experiments that require sequencing only sparingly. The system relies on the bridging of the rhizosphere and phycosphere, and the use of spectrophotometry to determine the abundances of the host and its microbiota. In the current phase of the project, the group first aims to obtain a qualitative sense of the system's composition and dynamics rather than quantitative information. However, as development progresses, the use of Metagenomics techniques will allow researchers to correlate the qualitative observations to quantitative metabolic information and ultimately help answer fundamental questions about the formation and dynamics of microbiomes.

2.3 Bridging the Microbiomes

The rhizosphere's analogous aquatic counterpart, the phycosphere, has as its most appealing attribute to plant research a relative facility through which it lends itself for scientific study. Unlike rhizosphere samples, these already aqueous samples are quickly prepared for DNA extraction [14]. In addition, rhizosphere samples also require more involved DNA extraction steps including the meticulous selection of root-interface soil and filtration steps to separate microbes from it, resulting in time-costly overheads [24,26,27]. In order to benefit plant research, the rhizosphere needs to be transformed into a synthetic version of a phycosphere with reduced complexity. To achieve this, the system under development employs a collection of representative soil bacteria from which to engineer simplified synthetic communities to present to the chosen host, *Chlamydomonas reinhardtii* (henceforth, Chlamydomonas), a soil and freshwater microalgae. Bridging the microbiomes and extracting insights for use in the plant research community is made possible by the overlap of the bacterial taxa that congregate with Chlamydomonas and the rhizospheres of most plants (Table 2.1) [10,27,28].

Phylum	Order	Core root microbiota (Yeoh *et al.*, 2017) [27]	Chlamydomonas-enriched (both soils and media)
Actinobacteria	Actinomycetales	YES	YES
Proteobacteria	Caulobacterales	YES	YES
	Rhizobiales	YES	YES
	Sphingomonadales	YES	YES
	Burkholderiales	YES	YES
	Xanthomonadales	YES	YES
	Rhodospirillales	YES	NO
Armatimonadetes	Fimbriimonadales	YES	NO
Tenericutes	Anaeroplasmatales	YES	NO

Table 2.1. The ICL study shows significant overlap between Chlamy-enriched taxa and core root microbiota. Adapted from [27]

2.3.1 Chlamydomonas and the ICL

Chlamydomonas has long been a model organism in biology and has been extensively used to understand chemo- and phototaxis as well as the mechanisms behind circadian rhythms [29,30,31]. Given its presence in soils and aquatic environments all over the world, the extensive body of research detailing its many features, as well as the amount of commercially available wild and genetically modified strains, *C. reinhardtii CC-1690* [32] was chosen as host organism in the development of the synthetic phycosphere model**.** Furthermore,

preliminary results from the research group also indicate that Chlamydomonas is capable of recruiting a distinctive soil microbial community when co-cultured with soil samples, further providing evidence for its use as host[33]
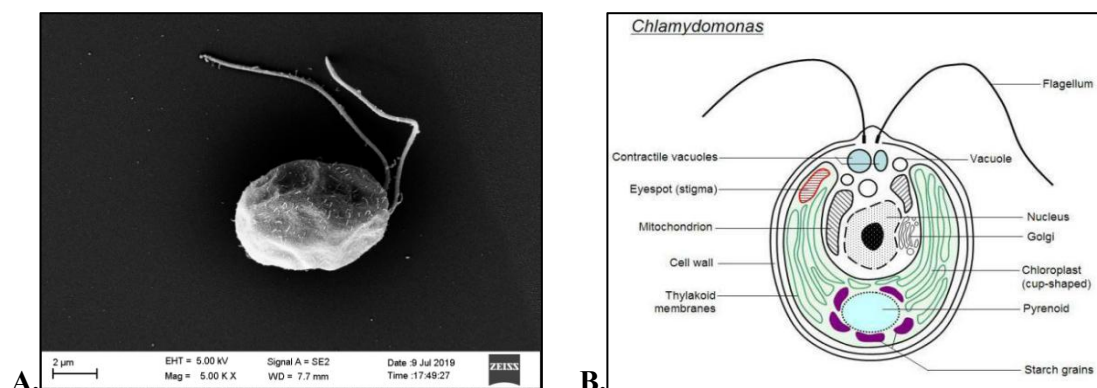

Figure 2.2: A. Electron Microscopy of Chlamydomonas from ongoing experiments, Courtesy, Rainer Franzen(MPIPZ). **B.** Components of a Chlamydomonas cell, Google search: Chlamydomonas schematic.

The Indexed Chlamydomonas-associated bacterial Library (ICL) was isolated from co-cultures of Chlamydomonas and Cologne agricultural soil samples using methodologies developed in Paul Schulze-Lefert's Department of Plant Microbe Interactions at MPIPZ[34]. From these co-cultures 3,800 bacterial strains were isolated and 200 of them had their genomes sequenced at the MPIPZ genome center. Genomic analysis revealed that the ICL covers most of the soil bacterial diversity associated with Chlamydomonas during the co-cultivation experiment[33]. The phycosphere modeling system employs Chlamydomonas and strains from the ICL in liquid cultures from which samples are taken and analyzed using spectrophotometry.

2.4 Spectroscopy

Spectroscopy is the general study of the interaction between light energy and matter with many sub-fields taking advantage of particular bandwidths of the electromagnetic spectrum. Matter can either absorb, transmit, or itself emit (fluoresce or phosphoresce) light of different wavelengths. These interactions can be measured thus allowing researchers to infer the matter's intrinsic properties [35, 36]. The schematic in Figure 2.3 highlights a typical experimental set-up. Light at a specific wavelength of radiation is passed through a sample in a vessel of known dimensions, and a detector measures the resulting light.

Figure 2.3: Schematic of a typical spectroscopic measurement.

2.4.1 Spectrophotometry & the Beer-Lambert Law

Spectrophotometry is a type of spectroscopy that measures the amount of light absorbed by matter. As light passes through a solution, the various particles which comprise the sample, including the media, become energized and thus subtract from the original intensity of the incident light (I_o)[36,37]. The amount of light measured at the detector is the amount of light which was not absorbed by the system and is known as the transmitted light (I_t). The ratio between these two known quantities (transmitted vs. incident light) is known as transmittance (T) and is highlighted in Equation 1[37].

$$Transmittance \ (T) = \left(\frac{I_t}{I_o}\right)$$

Equation 1

The Beer-Lambert law is based on the transmittance property and states that the absorbance of light by a substance is proportional to the concentration (c) of the sample and the path length (l) through which the light travels. This relationship is derived as follows:

$$-\frac{dI}{dl} \propto c \bullet I$$

Equation 2

$$-\frac{dI}{dl} = K \bullet c \bullet I$$

Equation 3

$$-\int_{I_o}^{I_t} \frac{dI}{I} = K \bullet c \int_0^l dl$$

Equation 4

$$-log\left(\frac{I_t}{I_o}\right) = K \bullet c \bullet l$$

Equation 5

$$Absorbance \ (A) = -log(T) = K \bullet c \bullet l$$

Equation 6

$$A_\lambda = \epsilon_\lambda \bullet c \bullet l$$

Equation 7

Equation 2 states that the infinitesimal decrease in light intensity with respect to path length is proportional to the product of the sample's concentration and incident light intensity (I). A proportionality constant (K) makes this relationship a differentiable equation, and is subsequently integrated in Equation 4. The integration results in the inverse log of the transmittance (i.e. absorbance), being equal to the product of the proportionality constant, the concentration, and the path length (Equation 5) [36].
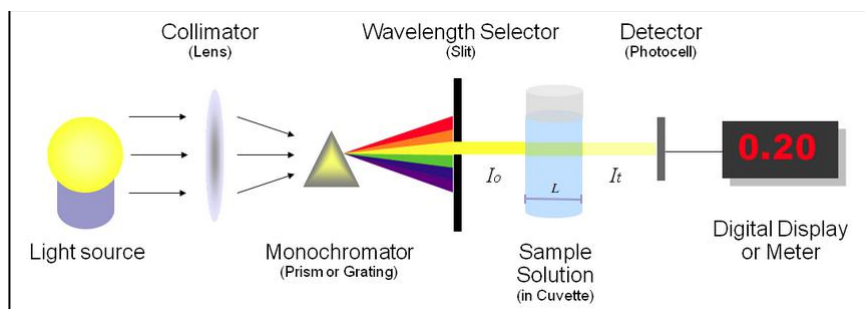
Figure 2.4: Matter absorbs light as it passes through; the longer the path (L) the more light is absorbed. Adapted from[37]

As noted in Figure 2.4**,** the incident light usually monochromatic and is shone at the sample at a specific energy or wavelength (λ) of radiation. Some compounds tend to absorb light more strongly at specific wavelengths, but that same compound is also able to absorbs light at any other wavelength. Monochromatic light absorption for a given compound can be represented as in Equation 7, where the proportionality constant (K) is rewritten to symbolize the molar extinction coefficient (ϵ_λ). These coefficients are specific for any given compound and wavelength, and represent how well that compound absorbs that particular wavelength of radiation[36]. This equation allows researchers to create linear calibration curves and estimate the unknown concentration of a sample. This technique is further explained and employed in the methodology.

2.4.2 Fluorescence Spectroscopy

This type of spectroscopy is based on the observation that certain compounds emit visible light, i.e. fluoresce when subjected to high energy wavelengths of radiation, typically in the ultraviolet range. The emitted light's intensity can be measured and is proportional to the concentration of substance in the system at low concentrations (OD < 0.1) of the sample as measured by optical density [35]. The derivation of the fluorescence linear equation utilizes Beer-Lambert's law at key steps. However, the determination of the coefficients is much more complex for any given compound and involves knowing specific details about its physico-chemical properties. Since the project involves measuring concentrations of cells rather than compounds, derivation details are not provided; it suffices to know, however, that the end result is an equation similar to Equation 7 from which simple calibration curves can be produced[38].

$$F_\lambda = \varphi_F \bullet f_\lambda \bullet j \bullet ln(10) \bullet I_o \bullet \varepsilon_\lambda \bullet c \bullet l \qquad \text{Equation 8}$$
$$F_\lambda = k_\lambda \bullet c \bullet l$$