Photosynthesis in a sewage pond

Chonnada Chandaravithoon

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Environmental Management Technology (International Program)

Prince of Songkla University

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Major Program	Environmental Management Technology (International Program)

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ABSTRACT

Leachate sewage ponds at Phuket Integrated Waste Management (Phuket, Thailand) are typical hypereutrophic red-water ponds found at sewage treatment plants and piggery, feedlot and poultry waste ponds with mixed communities of anoxygenic purple photosynthetic bacteria (PPB) (Bacteriochlorophyll *a*) and Chlorella-type green algae (Chl *a* + *b*). *In vivo* integrating sphere spectrometer scans (Model A&E-S90-2D, A&E Lab (UK)) showed absorbance maxima at 678–680 nm (*in vivo* Chl *a*) and a double peak at 802 and 844 nm (*in vivo* BChl *a*). High Na_2S (8.3 mol m^{-3}) added to PM media selected for the PPB whereas *Chlorella* overwhelmed PPB in PM medium without high H_2S. Photosynthetic electron transport rate (ETR) was measured using a blue-diode-based Junior PAM (Pulse Amplitude Modulation Fluorometer) on sewage pond leachate filtered onto glass fibre disks. Diuron herbicide resistance experiments allowed measurement of both oxygenic and anoxygenic photosynthesis of a mixed population of oxygenic and anoxygenic organisms to be estimated only in special circumstances. In separate culture, the ETR vs. E curves were *Chlorella*-type algae, $E_{opt} \approx 191 \pm 10$ μmol quanta m^{-2} s^{-1}, $ETR_{max} = 184 \pm 6.7$ μmol e^{-} g^{-1} Chl a s^{-1}; PPB, Eopt = 386 ± 15 μmol quanta m^{-2} s^{-1}, $ETR_{max} = 316 \pm 7.3$ μmol e^{-} g^{-1} BChl *a* s^{-1} but in a mixture of *Chlorella* and PPB only the oxygenic photosynthesis could be detected. In sewage pond samples, PAM rapid light curves in the presence and absence of DCMU allowed separate estimates of oxygen and anoxygenic photosynthesis to be made only if the Chl *a* content was very low (Chl $a \approx 0.26$ μg mL^{-1}; BChl $a \approx 1.4$ μg mL^{-1}).

Keywords: Sewage Leachate Pond, oxygenic photosynthesis, anoxygenic photosynthesis, integrating sphere spectrophotometry, PAM fluorometry.

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Thank you, Phuket Municipality, for allowing me to collect samples for the project from the Phuket Municipal Waste treatment facilities and the extensive co-operation of help the staff offered during the course of the project.

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An important part of me successfully completing this study was my family and my friends who helped to collect samples, consulting, support and encouraged me.

Chonnada Chandaravithoon

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LIST OF ABBREVIATIONS

Abt	Absorptance
BChl	Bacteria Chlorophyll
BOD	Biochemical oxygen Demand
Chl	Chlorophyll
DCMU	Diuron Photosynthetic Inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea)
ETR	Electron Transport Rate
mg	Milligram
ml	Milliliter
nm	Nanometer
PAM	Pulse Amplitude Modulation Fluorometry
PS	Photosystems
PSB	Photosynthetic bacteria
μm	Micrometer
μg	Microgram

CHAPTER 1

INTRODUCTION

Sewage lagoons and wastewater ponds from industrialised swine and poultry farms are typically hypereutrophic and dominated by purple non – sulphur bacteria (Ectothiorhodaceae, mainly rhodopseudomonads), some purple sulphur bacteria (Chromatiaceae) and green unicellular algae. The mode of nutrition of the photosynthetic organisms in such habitats is typically photoheterotrophic (Irving and Dromgoole, 1986). They are typically foul smelling obnoxious environments generating public concern and so the metabolic activity going on in such habitats is important for environmental management (Takahashi and Ichimura, 1970; Siefert *et al*., 1978; Koelsch *et al.,* 1997; Kim and Lee, 2000; Zhang *et al.,* 2002; Kim *et al.,* 2004; Belila *et al.,* 2013). BOD of sewage ponds is done routinely but photosynthetic activity, particularly anoxygenic photosynthetic activity, is acknowledged to be occurring in the ponds but is generally not easily measured. In a hypereutrophic anaerobic environment dominated by photoheterotrophy perhaps COD (Chemical Oxygen Demand) is a more realistic measure of the environmental impact of such wastewater (Meng *et al.,* 2018). Photosynthesis of most types of oxygenic photosynthetic organisms can be measured easily using PAM (Pulse Amplitude Modulation Fluorometry) but it has only recently been shown that PAM machines can also be used to measure photosynthetic electron transport in purple non-sulphur bacteria such as rhodopseudomonads (Ritchie, 2013; Ritchie and Runcie, 2013) and also in purple sulphur bacteria (Ritchie and Mekjinda, 2015). Pigment analysis can be a useful guide to the balance of oxygenic and non-oxygenic photosynthetic organisms in a sewage pond (Ritchie, 2018) but to gauge their metabolic activity, it is necessary to measure their photosynthesis. Although their presence in sewage ponds is easily identified by pigment analysis or *in vivo* spectral signature (Siefert *et al.,* 1978; Gitelson *et al.,* 1997, 1999), the role of these photosynthetic bacteria is often overlooked because their photosynthetic activity is not as easily detected. In the case of oxygenic photosynthesis, photosynthetic activity can be measured by oxygen electrode techniques, light-dark bottle experiments or $^{14}CO_2$ There are many habitats

where the presence of anoxygenic photosynthetic organisms is largely unsuspected, for example the open ocean (Kolber *et al.,* 2000) and in stromatolites (Papineau *et al.,* 2005). The photosynthetic activity of aerobic photosynthetic bacteria is largely unquantified in most habitats (Yurkov and Beatty, 1998). A wide range of anoxygenic photosynthetic bacteria is found in sewage ponds and industrialised agriculture wastewater lagoons. Both purple sulphur and purple non-sulphur bacteria may be present as well as green sulphur bacteria (*Chlorobium* -type bacteria containing bacteriochlorophyll *c*, BChl *c*) and so BChl *c* might be present as well as bacteriochlorophyll *a* (BChl *a*) (van Niel 1944, 1971; Siefert *et al.,* 1978; Blankenship *et al.,* 1995; Gitelson *et al.,* 1997, 1999; Koelsch *et al.,* 1997; Kim *et al.,* 2004). The range of photosynthetic bacteria found in sewage ponds is very wide based on genomic evidence (Belila *et al.,* 2013) and are known to metabolise a large range of organic compounds (Meng *et al.,* 2018).

In a previous study, algorithms were developed to estimate Chl *a* and *b* and BChl *a* in solvent in 100% ethanol, 90% acetone and in a 7:2 mixture of acetone and ethanol (Ritchie, 2018). In that study, it was pointed out that the routine use of 750 nm as a zero for in solvent assays of chlorophylls has the inherent effect of masking the presence of BChl *a* and *b* in environmental samples and it is better to use 850 nm as an absorbance zero. An absorbance zero at 850 nm is well outside the absorbance ranges of both chlorophyll and bacteriochlorophylls in solvent and so the presence of BChl *a* and *b* in solvent extracts of wastewater and many types of microbial mats would be readily apparent. In this study, we have attempted to measure oxygenic and anoxygenic photosynthesis in a hypereutrophic sewage pond using PAM fluorometry (Ritchie, 2013; Ritchie and Larkum, 2013; Ritchie and Runcie, 2013; Ritchie and Mekjinda, 2015). It is difficult to find estimates of photosynthetic rates of photosynthetic bacteria in natural habitats because the simple-to-use oxygen-based methods used for measuring oxygenic photosynthesis cannot measure anoxygenic photosynthesis. For example, photosynthetic bacteria are abundant and ubiquitous in oceanic environments (normally thought of as aerobic and hence unsuitable for anoxygenic photosynthesis) but their contribution to global photosynthesis is hard to estimate (Kolber *et al.,* 2000; Goericke, 2002; Falkowski and Raven, 2007). Until recently, $^{14}CO_2$ fixation seemed to be only obvious way to measure photosynthesis of photosynthetic bacteria (Takahashi and Ichimura, 1970; Hubas *et al.,* 2011) but in a sewage pond situation photoheterotrophy rather than photoautotrophy would be expected to be the dominant form of photosynthesis (Irving and Dromgoole, 1986; Koelsch *et al.,* 1997; Kim and Lee, 2000; Kim *et al.,* 2004).

In such a situation $^{14}CO_2$ fixation would underestimate actual photosynthesis by both oxygenic and anoxygenic organisms because much of the harvested light energy would to be used to take up and rearrange organic compounds in the waste water or the sewage pond.

1.1 Statement of the Problem

The leachate from landfills and sewage pond effluent are widespread and important problems. Because they have high sediments, ammonia, high dissolved solids, usually an acid pH and typically high but highly variable levels of heavy metals (Cameron and Kock, 1980; Bull *et al*., 1983; Chueng *et al.,* 1993; Devare and Bahadir, 1994; Xu *et al.,* 2006). Often landfill leachate is pumped into sewage ponds worsening the pollution potential of the sewage pond effluent. Waste is a problem for Phuket because of the large the amount of waste generated by the tourist industry that is more than can be accommodated in the area and the presence of various toxins. Landfill leachates have a negative effect on the environment and are often untreated or inadequately treated. Waste water is also generated from the waste processing as well as the landfill and the storage of garbage before processing. Water that leaches through a landfill typically has an acid pH (that tends to mobilize metals), high levels of dissolved solids and variable levels of metals of varying toxicity depending on the source of the landfill components. Mobilised metals are undesirable in a sewage pond and so separate treatment of landfill leachate is preferable. Leachates contain organic compounds; some pathogens and the organic content allows potential pathogens to grow. The garbage from everywhere in Phuket is stockpiled in a garbage storage pit for one to a few days (which generates a garbage pit leachate) awaiting incineration, the ashes from the incinerator is dumped in landfills near the incinerator. The Solid waste incineration capacity is 250 tonnes / day continuously for 24 hours and can work at least 7,008 hours / year with an electricity production capacity of 2.5 MW, which is more than enough for running the solid waste incineration plant and the sewage treatment works. The surplus electricity can be utilized otherwise and is a source of extra income.

Leachate from landfills is highly polluting and should be treated because of contamination of the environment, health dangers and pollution in the contaminated area and water

supplies. An efficient collection method is needed to collect leachate otherwise the waste water will seep into the area beside the landfill pile. Clay seals and plastic seals are never 100% effective: part of the leachate is absorbed into the ground and will eventually contaminate groundwater creating problems with the health of people who consume the water or use it for cropping.

In addition, solid waste also affects the quality of soils and the use ability of land. Broken glass, remains of fluorescent lamps, batteries and battery scrap and metal scrap cause significant problems. It affects the amount of heavy metals, mercury, cadmium, lead in soil, which would adversely affect the ecology of the soil and its use ability for agriculture. Organic material in garbage also worsens the problems of solid waste because biodegradation of waste increases acidity in the soil and mobilizes metals. The interaction of solid waste and the biological breakdown of organic matter exacerbates pollution problems. Waste water from the solid waste stream contaminates soil in the surrounding areas, causing pollution of the soil in adjacent areas. Misappropriation of abandoned hazardous waste sites can generate severe contamination in local soils. Outdoor burning of waste causes toxic fumes and causes of significant air pollution. Burning of waste in gutters at the sides of roads is familiar throughout SE Asia. It generates significant air pollution and there is great potential for significant pollution from the waste gases or vapours. The burnt residues in the gutters are eventually washed away and cause significant pollution from metals and organic residues from incomplete combustion.

Leakage of effluent from garbage dumps resulting from microbial breakdown of organic matter is a major environmental hazard of garbage pumps leading to pollution of rivers and estuaries and groundwater. It is a worldwide problem, but few studies have been made under tropical conditions (Thongpinyochai and Ritchie, 2014). Pollution of rivers and estuaries and groundwater can lead to heavy metal pollution (lead, cadmium, mercury, copper, zinc) because the redox reactions of anaerobic respiration lead to mobilisation of metals and organic compounds. In the previous study on landfill leachates in Phuket (Thongpinyochai and Ritchie, 2014) it was found that *most* heavy metals did not seem to be a major problem, but leachates contained unacceptably high toxic levels of zinc. Leachates can contaminate groundwater leading to pollution far removed from the garbage dump as well as the more obvious pollution of waterways. This is a problem for Phuket because old water-filled tin mine excavations are used for water supplies. These ponds are filled by both rainwater and groundwater and urban runoff. The ponds are generally permanently

stratified into an aerobic upper layer and anaerobic bottom water. The redox conditions in the bottom of the ponds mobilises metals.

Microbes can produce enzymes to digest fats and oils as an ingredient in the form of Triglyceride production of glycerol and fatty acids that microbes can absorb and metabolize and then use in growth and metabolic activity (Benchapatarapong, 1997). *Rhodopseudomonads* are able to easily digest waste oils (Mekjinda and Ritchie, 2015; Phongjarus *et al.,* 2018). Waste oil seepage is sometimes a very troublesome component of leachate at some landfills.

Biological treatment is environmentally friendly because it does not leave toxic residues and are less costly and allow proper treatment of wastewater, waste oil and fat. One problem with biological treatments is that they may result in the mobilization of toxic metals. Biological treatment can be used in the treatment of waste oil, on both small and large scales. Many kinds of microorganisms can be used in the digestion of fats and oils. *Staphylococcus warneri* is effective in degrading oil and grease and is resistant to salinity (Lanciotti *et al.,* 2005)

Rhodopseudomonas can use organic and inorganic nitrogen (Larimer *et al.,* 2004; Meng *et al.,* 2018) from organic sources of nitrogen including amino acids, peptides, proteins, nucleic acid. Nitrogen sources include inorganic nitrate (NO_3^-) and ammonia (NH_3 + NH_4^+). *Rhodopseudomonas* when grown under anaerobic conditions is capable of N-fixation (conversion of N_2 into NH_3) with H_2 as a by-product but N-fixation only occurs under strict anaerobic conditions. Hydrogen gas is a by-product. A disadvantage of *Rhodopseudomonas* growing under N-fixing conditions is that its growth rate is considerably slower than when organic nitrogen or ammonia are available and so hydrogen gas production is limited to conditions where the cells are not growing very quickly.

1.2 Objective

The objectives of this study were to:

1) Study and compare the growth of photosynthetic bacteria (*Rhodopseudomonas palustris*) on wastewater as a carbon sources using similar experimental protocols as were

previously developed where *Chlorella* was used for bioremediation of landfill leachate (Thongpinyochai and Ritchie, 2014).

2) Measure photosynthesis of the photosynthetic bacterium using a PAM fluorometer and measure bacteriochlorophyll *a* of the photosynthetic bacterium using a Spectrophotometer.

3) Make environmental impact assessments based on laboratory experiments on anaerobic photosynthetic bacteria and using bacteria (*Rhodopseudomonas palustris*) to reduce BOD and COD in sewage pond effluent.

4) Investigate and record heavy metal contamination of leachates from the Garbage Pit Leachate and Landfill Leachate. The separate documentation of the leachate from the holding bays of the Garbage Pit Leach is important. Heavy metal reduction in leachate by using bioassay based on *Rhodopseudomonas palustris*. It was expected that *Rhodopseudomonas palustris* would also efficiently remove ammonia from leachate and wastewater.

5) The main research questions were to study to find estimates of photosynthetic rates of photosynthetic bacteria in natural habitats. In a sewage pond situation photoheterotrophy rather than photoautotrophy would be expected to be the dominant form of photosynthesis.

1.3 Scope

Garbage dump effluent has three other characteristics which are environmentally important. Feeding it into the sewage treatment stream has significant negative effects on the satisfactory treatment of wastewater. The redox reactions occurring in the anaerobic fermentation of the garbage can lead to heavy metal pollution (lead, cadmium, mercury, zinc, copper) because the redox reactions of anaerobic respiration lead to mobilisation of metals (Bull *et al.,* 1983; Devare and Bahadir, 1994). Very high ammonia levels increase the solubility of many toxic metals. Anaerobic fermentation processes also tend to partially break down and mobilize toxic organic compounds, in particular organochlorine compounds. Hence, garbage dump leachate effluent may have a very high organic content having environmental effects similar to raw sewage because of its very high BOD and COD (Bull *et al.,* 1983; Devare and Bahadir, 1994). Much less is known about

the chemical composition of dissolved organic matter in garbage leachate than ammonia and metal content (Xu *et al.,* 2006). The composition of the organic matter also depends on the age of the garbage dump (Xu *et al.,* 2006). High molecular weight (tannin) compounds are a major contributor to the toxicity of leachate and its very long-term toxicity (Cameron and Kock, 1980; Silva *et al.,* 2004; Xu *et al.,* 2006). Photosynthetic bacteria such as *Rhodopseudomonas* have one of the widest known metabolisms of any organisms (Larimer *et al.,* 2004; Meng *et al.,* 2018) and have the able to grow aerobically, anaerobically and photoheterotrophically under anoxic conditions on a wide variety of organic compounds (Ritchie, 2013; Mekjinda and Ritchie, 2015).

1. Measure bacteriochlorophyll *a* and photosynthesis for study the growth of *Rhodopseudomonas palustris.*

2. Measure bacteriochlorophyll *a* of the photosynthetic bacterium using a Spectrophotometer and measure their photosynthesis using a Pulse Amplitude Modulation (PAM). Compare Chlorophyll *a* and bacteriochlorophyll *a* contents of the sewage pond (Ritchie, 2018).

3. Attempt to measure the separate contributions of aerobic green algae (*Chlorella*) and photosynthetic bacteria to the total photosynthesis of hypereutrophic "Red water" photoheterotrophic sewage ponds (Belila *et al.,* 2013).

1.4 Expected Outcome

1.4.1 Development of a method to use *Rhodopseudomonas* as a convenient organism for bioremediation of leachates and wastewater.

1.4.2 It is the policy of the Phuket Municipality to reduce the environmental impact of the wastewater treatment plant and reduce BOD, COD and toxic heavy metal content of effluent from the treatment plant.

CHAPTER 2

LITERATURE REVIEW

2.1 The Leachate

Landfill leachate is the liquid seepage out of landfills. This leachate has a high turbidity, high levels of dissolved solids and BOD and COD and depending on the source of the material dumped in the landfill might also contain hazardous levels of heavy metals. A leachate is any liquid that, during passing through matter, extracts soluble or suspended solids, or any other component of the material through which it has passed. Typically, the leachate is acid from fermentation activity by microbes. The acidity has the undesirable side effect that it tends to dissolve and mobilize otherwise insoluble metal wastes in the landfill. There will be both organic compounds for pathogens and an inorganic heavy metal. A concern of landfill leachates is their polluting effects on groundwater.

2.2 Chemical composition and toxicity of leachate

Garbage dump leachates are a world-wide problem; typically, they have very high salinities, very high ammonia content and high but variable levels of heavy metals (Cameron and Kock, 1980; Bull *et al*., 1983; Chueng *et al.,* 1993; Devare and Bahadir, 1994; Xu *et al.,* 2006). This generalization is not however universal: leachate from some dumps are relatively low in heavy metals (Thongpinyochai and Ritchie, 2014). Cameron and Kock (1980) working in Vancouver, Canada concluded that about 94% of the toxicity of leachates could be attributed to ammonia, pH, copper content and recalcitrant organic materials (tannins). Aerobic and anaerobic treatment

systems have been used to deal with garbage dump leachates and similar wastes such as piggery waste effluent (Bull *et al.,* 1983; Chueng *et al.,* 1993; Kim & Lee, 2000; Kim *et al.,* 2004). Although physic-chemical methods to reduce the toxicity of leachate have been tried (flocculation-precipitation and membrane separation methods) (Silva *et al.,* 2004) they are probably too expensive to be practical on a large scale. Using evaporation towers to remove ammonia are standard practice although very power consuming and the leachate must first be alkalinized to be able to volatilize ammonia (Cameron and Kock, 1980; Bull *et al.,* 1983). Biological methods in general appear more feasible (Bull *et al.,* 1983). Vascular plants (Vetiver grass; Vetiveriazizanioides (L.) Nash.) planted on the top and sides of old dumps and irrigated with leachate from a collection sump can be useful (Roongtanakiat *et al.,* 2003) but microbiological/algal methods are familiar to sewage treatment engineers from experience with sewage treatment and appear to be more practical. The laboratory has experience in growing algae in artificial treated sewage effluent (Ritchie *et al.,* 1997, 2001) as part of a study of the effects of phosphate in sewage treatment plant effluent and using *Chlorella* as a bioremedial agent (Thongpinyochai and Ritchie, 2014) and extensive experience now with photosynthetic bacteria (Ritchie and Gibson, 1987b; Ritchie and Runcie, 2013; Ritchie, 2013; Mekjinda and Ritchie, 2015; Ritchie and Mekjinda, 2015; Phongjarus *et al.,* 2018; Chandaravithoon, Nakphet and Ritchie, 2018).

2.3 Landfill System Design in Phuket

Present and future water problems in Phuket include both a limited water supply and contamination of available water. Garbage dump leachate is probably the worst form of water pollution in Phuket because of its negative effects on water supply because much of the water used in Phuket comes from ponds formed in old tin mines and there is significant groundwater contamination. It is a long-lasting consequence of previous policies and the project is provide useful information for future policy decisions concerning management of legacy environmental problems arising from old garbage dumps and help in the future location of new facilities.

The landfill at the Phuket waste treatment plant pumps its landfill leachate into its sewage ponds. This increases the organic load of the ponds and increases their anaerobic status

such that they are not normally in a green water aerobic state) but in a red-water state (Belila *et al.,* 2013) with a heavy population of anoxygenic photosynthetic bacteria and tolerant green algae.

2.4 Microorganisms

Microorganisms play an important role in biological and mineral cycling both aerobic and anaerobic organisms. Both types of bacteria are very diverse, features and capabilities are different. Microbes have evolved to adapt to life in the ecosystem and the environment. Microbes can spread into various habitats such as air, soil, water, on trees, in the human body, in the areas with a very high temperature such as hot springs or very low temperatures, a very wide range of pH, in the sea, high salinities or the areas that have a high pressure.

2.4.1 Bacteria

Bacteria are microorganisms that are single-celled organisms that are prokaryotic in their cellular organization. Typically, a few micrometers in length, bacteria have relatively few shapes, from spherical, rod and spiral. Prokaryotes come in only a few shapes and sizes, not rivaling eukaryotes in terms of their structural complexity. The bacteria were the first life forms to appear on earth. Bacteria live in soil, water, acidic hot springs, very alkaline and saline conditions, radioactive waste, and in the depths of the earth (several kilometres underground). Bacteria also live in a symbiotic relationship with plants and animals and parasites. Some bacteria can fix nitrogen – convert N_2 into ammonia. However, this is a very energy consuming process and is strictly anaerobic.

2.5 Photosynthetic bacteria (Purple Non – sulfur Bacteria)

Photosynthetic bacteria can be found in natural sources, both freshwater and saltwater. They can be found in the water column of stratified lakes and can be found in waste water and sewage treatment pond as well (Levett, 1990; Imhoff, 1992, Brock, 1994; Belila *et al.,* 2013). Photosynthetic bacteria are important in the process of absorption of carbon dioxide and nitrogen fixation process. Photosynthetic bacteria are important planktonic food sources in the food chain of small animals, fish, shrimp, crabs and snails and particularly larval stages, especially of sea snails and bivalves such as oysters. Purple Non – sulfur Bacteria are prokaryotic cells. Prokaryotes compensate for their limited structural complexity and morphotypes by a seemingly unlimited spectrum of physiological specialization. Prokaryotes are literally everywhere. Photosynthetic bacteria (Purple Non – sulfur Bacteria) such as *Rhodopseudomonas* are noted for their remarkable biochemical versatility (Larimer *et al.,* 2004; KEGG, 2013). Rhodopseudomonads have the largest range of metabolic activity compared to other bacteria. Photosynthetic bacteria use bacteriochlorophylls as their photosynthetic pigments not chlorophylls and do not use water as an electron source in photosynthesis and hence do not produce oxygen. The photosynthetic system of *Rhodopseudomonas* has only one photosystem (RC - 2) which is very similar in structure to the PSII found in oxygen producing photosytheic organisms (Larimer *et al.,* 2004; Ritchie and Runcie, 2013). It is based on bacteriochlorophyll *a* as the primary photosynthetic pigment. While water is not used as an electron source; organic componds, Fe^{2+} and some sulphur compounds (thiosulphate and hydrogen sulphide) are used as electron sources (Ritchie and Runcie, 2013). One of the problems working on photosynthetic bacteria has been that it was difficult to directly measure their photosynthetic rates and growth is not necessarily a good measure of photosynthetic activity. This is not a trivial problem because they are not only capable of CO_2 fixation using the Calvin cycle using RUBISCO (Larimer *et al.,* 2004) but much of their photosynthetic activity is photoheterotrophic. Hence $^{14}CO_2$ fixation in many situations will underestimate their photosynthetic activity because they are using much of the light harvested by their photosynthetic apparatus to take up and metabolise existing organic compounds in their environment. Photoheterotrophy is difficult to estimate because it may be difficult to determine what organic compounds are being used in their natural environment.

The pulse amplitude modulation (PAM) fluorometer can be estimate the light reactions in a *Rhodopseudomonad.* The pulse amplitude modulation (PAM) method was originally developed for use in measuring oxygenic photosynthesis. The reason why PAM technology works on *Rhodopseudomonas* is that chlorophyll *a* and bacteriochloropyll *a* both absorb blue light and produce far red/infrared fluorescent light that is detectable using a PAM machine (Figure 1). The typically configured PAM machine has a simple highpass filter (>700 nm) to stop reflected light from a specimen being measured by the detector diode and only light .700 nm reaches the detector diode. It is therefore an accident of the design of the standard PAM machine that it detects fluorescence from both Chl *a* in PSII and BChl *a* in RC-2. The response of *Rhodopseudomonas* to various organic compounds can be measured experimentally using a PAM machine. Since the light reactions of photosynthesis is being measured using a PAM machine the photosynthetic electron transport rate (ETR) is measured not carbon metabolism.

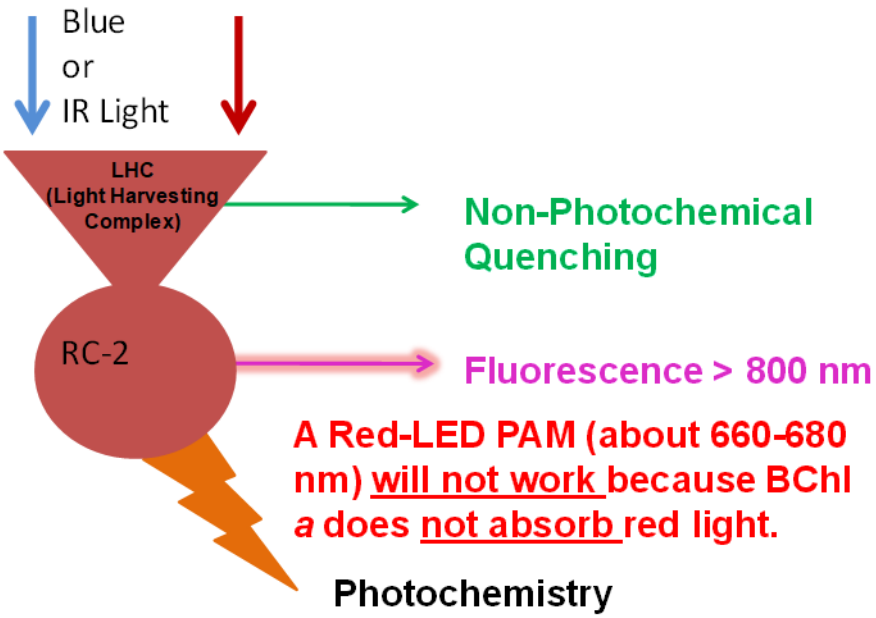


Figure 1 Photosynthesis in *Rhodopseudomonas* and the light reactions measured by PAM technology.

2.6 *Rhodopseudomonas palustris* (Rps. *palustris*)

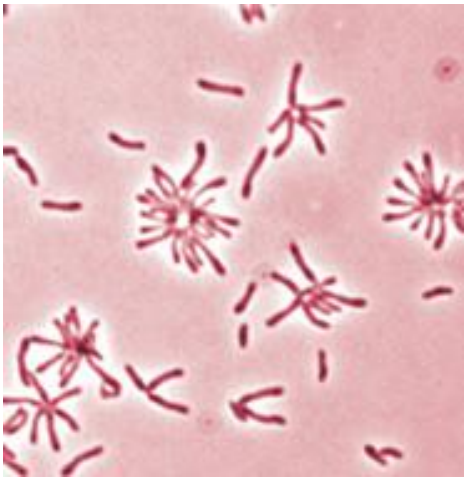


Figure 2 *Rhodopseudomonas palustris* (about 1 μm long, the cells are highly motile) (source: microbewiki.kenyon.edu)

Rhodopseudomonas palustris is a rod-shaped gram-negative non-sulphur purple photosynthetic bacterium. *Rhodopseudomonas palustris* in the family Rhodospirillaceae and it is a rod-shaped highly motile bacterium. It is a Gram-negative bacterium, containing bacteriochlorophyll *a* as the primary photosynthetic pigment, contains no accessory bacteriochlorophylls and does have carotenoids (Larkum *et al.,* 2018). The photosynthetic bacterium has a type of photosynthesis that does not use water as an electron source, it does not produce oxygen and is photosynthetic under anoxygenic and microaerobic conditions. The tolerance of photosynthetic bacteria to oxygen varies greatly. *Rhodopseudomonas palustris* can grow photosynthetically under microaerobic conditions. Experiments on RC-2 type photosynthetic bacteria such as *Rhodopseudomonas*, *Afifella* and *Thermochromatium* filtered onto glass fibre discs and the light reactions measured using a PAM machine in atmospheric oxygen shows that the light reactions of anoxygenic photosynthesis are not very oxygen sensitive (Ritchie and Runcie, 2013; Ritchie, 2013; Ritchie and Mekjinda, 2015; Phongjarus *et al.*, 2018). Anoxic conditions are however required for bacteriochlorophyll synthesis even if the anoxic conditions are only available in microenvironments (Larkum et al., 2018). When growing photosynthetically *Rhodopseudomonas* is a bright red colour but has almost no colour when grown under fully aerobic

conditions. *R. palustris* is usually found as a wad of slimy masses and cultures appear from pale brown to peach-coloured to bright purple. Etymologically, *rhodium* is a Greek noun meaning rose, *pseudes* is the Greek adjective for false and *monas* refers to a unit in Greek. Therefore, *Rhodopseudomonas*, which implies a unit of false rose colour, describes the appearance of the bacteria. *Palustris* is Latin for marshy, and indicates the common habitat of the bacterium. *R. palustris* can grow with or without oxygen, or it can use light, inorganic or organic compounds for energy. It can also acquire carbon from either carbon dioxide fixation or green plant-derived existing compounds. Finally, *R. palustris* is also capable of fixing nitrogen for growth but this only happens under strictly anaerobic conditions. The energy cost of N-fixation are however, very high. Hydrogen production is an incidental by-product of N-fixation and so Rhodopseudomonads can produce hydrogen gas. This metabolic versatility has raised interest in the research community, and it makes this bacterium attractive for potential use in biotechnological applications.

2.7 Spectrophotometer (Shimadzu UV1601 UV-visible)

Figure 3 Spectrophotometer

Spectroscopy is the instrumentally aided study of the interactions between matter (sample being analyzed) and energy (any portion of the electromagnetic spectrum). This is a dual beam UV- Visible Spectrophotometer useful for spectroscopy from 180 nm to 1100 nm.

Compounds that strongly absorb light at a given wavelength can be measured using light absorption (absorbance) as a measure of the amount of substance present.

This is a standard laboratory spectrophotometer which can be used to assay chlorophylls and bacteriochlorophylls. Chlorophylls in organic solvents, have absorption peaks in the blue (400-450 nm) range and in the red part of the spectrum (630-720 nm. Bacteriochlorophylls such as BChl *a* Bacteriochlorophyll *a* (BChl *a*) has absorption peaks in the blue part of the spectrum (at about 400 nm) and in the near infrared (775 nm). Normally only the red/IR peaks are used for chlorophyll and bacteriochlorophyll assays. Algorithms are used to convert absorbance data into chlorophyll and bacteriochlorophyll assay in μg/ml.

1. UV-visible spectrum (400 – 720 nm).

2. IR-range for bacteriochlorophylls (750 – 1100 nm).

There are many different algorithms for the measurement of chlorophylls and bacteriochlorophylls depending on the organic solvent used. Older formulae are less accurate than more recently developed algorithms mainly because of improvements in estimating the quantitative relationship between the absorption peaks of chlorophylls and bacteriochlorophylls and the mass of substance (the extinction coefficient). In the present study the chlorophyll algorithms for 90% acetone and 100% ethanol developed by Ritchie (2006) were used in conjunction with the algorithms for acetone/ethanol (7:2) developed by Ritchie (2018).

2.8 Photosynthesis measurements

2.8.1 Pulse Amplitude Modulated (PAM) fluorometer

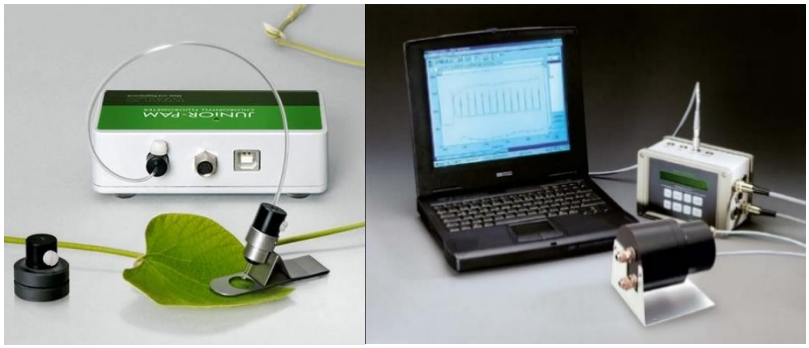


Figure 4 Pulse Amplitude Modulated (PAM) fluorometer

PAM fluorometry is very useful for estimating photosynthetic activity of plants, stress physiology. PAM methods are the most rapid techniques for monitoring photosynthesis. PAM machines are simple to use to measure photosynthesis, is a rapid method and is easy to set up in the field. Alternative methods such as the IRGA are very slow and very expensive and very hard to use in real field situations. PAM directly measures photosynthetic electron transport by measuring PSII fluorescence in higher plants and so measures the light reactions of photosynthesis directly. PAM machines can also be used to measure photosynthesis in some types of photosynthetic bacteria because their photosystem (RC-2) is like the PSII of oxygenic photosynthesis (Ritchie, 2013; Ritchie and Runcie, 2013; Ritchie and Mekjinda, 2015). Thus, PAM technology provides a simple technique for measuring photosynthesis in certain photosynthetic bacteria. There are however, some other types of photosynthetic bacteria which have different photosystems and different fluorescence characteristics which cannot be measured using PAM technology.

Flourometric methods are a very convenient way to measure photosynthesis. Light saturation curve measurements on the algae and photosynthetic bacteria were made using a Junior PAM portable chlorophyll fluorometer (Gademann Instruments GmbH, Wurzburg, Germany) fitted with a 1.5mm diameter optic fibre and a blue diode light source. The Junior PAM uses a magnetic

clamp to hold specimens about 1 mm from the end of light pipe. PAM parameters (effective quantum yield, rETR ,qP, NPQ) were calculated using the WINCONTROL software (2.133/03.00) using standard settings for rapid light curves (Heinz Walz GmbH, Effeltrich, Germany)(Genty *et al.,* 1989). The default absorptance factor of 0.84 and the default value of 0.5 for estimated absorption of light by photosystems (PSI and PSII) and a value of 1.0 for a single photosystem (RC-2) was used on the Junior PAM to calculate the relative Electron Transport Rate or rETR (Ritchie, 2008b). On the standard setting for a rapid light curve, sets of PAM light curve measurements took about 88s to complete with 10 s between actinic flashes of light and each flash of light is set at 0.8 s duration. The flashes were in order of increasing intensity as nine graded irradiance increments from a nominal zero irradiance. The protocols using the Junior – PAM in the present study are described in Ritchie (2008b), Saetae *et al.* (2013), Ritchie and Runcie (2013), Ritchie (2013), Ritchie and Mekjinda (2015), Phongjarus *et al.* (2018) and Chandaravithoon *et al.* (2018).

Walz Junior Blue PAM – works for <u>both</u> Chl *a* and BChl *a* Fluorescence

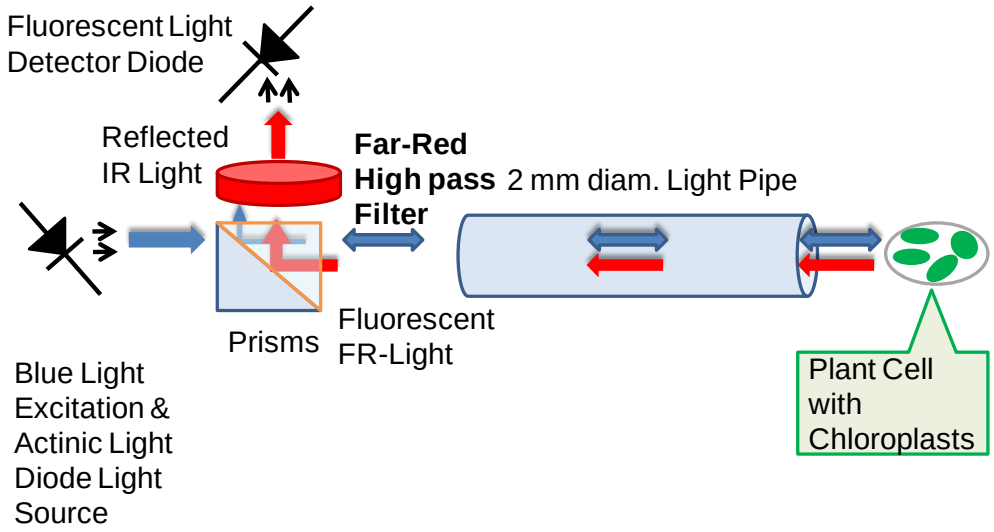


Figure 5 How the Junior PAM works.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Collecting Leachate

Leachate and sewage pond water samples were taken it from Garbage Pit Leachate at the wastewater treatment plants at Saphan Hin in Phuket town. The clarifier ponds total 3 concentrations of waste, depending on the season and number of ponds.

Figure 6 Sewage Ponds at Phuket Waste Management. The Central Pond is at Lat 7.860077, Long 98.391960. The red colour is due to Rhodopseudomonad photosynthetic bacteria although green algae are also present.

3.2 Using bioassays

Using bioassays are a convenient way of monitoring the toxicity of leachates (Chueng *et al.,* 1993; Devare and Bahadir, 1994). *Rhodopseudomonas* is very easy to grow in fully defined media. It has a high growth rate and so experiments can be run quickly. It grows well at relatively low light intensities and is a facultative aerobe and so complex growth facilities are not required, growth on lighted shelves and orbital shakers is all that is required. Its growth can be monitored easily by measuring cell counts and by working out the correlation between cell counts and light scattering at a suitable wavelength (650 nm) (Ritchie and Gibson, 1987a).

Rhodopseudomonas photosynthesis can be measured using a PAM machine (Ritchie, 2008; Ritchie and Runcie, 2013; Ritchie, 2013). We have a saltwater *Rhodopseudomonad* growing in the laboratory that originated from a Thai pearl farm. This organism, *Afifella marina* would be a very suitable alternative candidate organism for monitoring the effects of leachates in estuarine and marine environments and may be useful for other leachate studies because *Rhodopseudomonas palustris* has a limited salinity tolerance. Some leachates have very high salinities, beyond the salinity tolerances of many freshwater organisms. We have found that *Afifella* is very tolerant of salinity differences and will grow equally well in brackish media ($\approx$ 5-10% seawater) and so could serve as a very useful alternative test organism. Our colleague Prof Duangporn Kantachote (PSU Hat Yai) has a wide variety of euryhaline *Rhospseudomonads* from Songkla Lake and saline rice paddy fields that would be useful for further studies on anoxygenic photosynthetic bacteria.

Rhodopseudomonads are typically found in habitats with very high nutrient levels and high amounts of organic matter such as in piggery waste and anaerobic sludge ponds (Kim and Lee, 2000; Kim *et al.,* 2004). They are also common in the sludge ponds of Oil Palm processing plants. Palm Oil Milling Effluent (POME) is basically an emulsion of palm oil and vegetable waste (Takeno *et al.,* 2005, Lam and Lee, 2011). Recently it was shown that *Rhodopseudomonas* readily broke down cooking oil waste (Phongjarus *et al.,* 2018). *Rhodopseudomonads* are able to break down a very large variety of organic compounds including chlorinated hydrocarbons and tannins and can live aerobically or anaerobically. Photosynthetic bacteria are usually only thought to perform bacterial photosynthesis only under anerobic conditions but this is not correct. They will

perform anoxygenic photosynthesis under microaerobic conditions. This metabolic versatility was taken advantage of in the project. Thongpinyochai and Ritchie (2014) showed that *Chlorella* successfully grew in landfill leachate and reduced the BOD and COD but large dilutions of the leachate were necessary to allow the *Chlorella* to grow.

A Rhodopseudomonad has also been enriched from the sewage pond. It has a slightly different bacteriochlorophyll-protein complex spectrum compared to *Rhodopseudomonas palustris*. It grows well on PM medium with acetate, benzoate or acetate + benzoate as carbon sources. High Na_2S (8.3 mol m^{-3}) could be used to eliminate *Chlorella* (van Niel, 1944, 1971). The specific oxygenic photosynthesis inhibitor DCMU (Diuron) 20 mmol m^{-3} was also effective in eliminating oxygenic photosynthetic organisms.

Thermochromatium (Chromatium) *tepidum* was a kind gift from Dr Christopher Sherman (Blankenship Laboratory, Biology Dept, Washington University in St. Louis, St. Louis, Missouri 63130, USA. It is a purple sulphur bacterium containing bacteriochlorophyll *a* (Chromatiaceae) and PAM methods have been successfully used to measure photosynthetic electron transport in the organism (Ritchie and Mekjinda, 2015). In the present study, it was grown in PM medium with acetate as the organic carbon source because it does not grow well on PM medium with benzoate added.

The green alga *Chlorella sp* 'Kik' was originally isolated from water samples from a prawn farm at Tumbon (Commune) Marui, Amphur (District) Thap-Put, Pang-Nga Province, Thailand (Ritchie and Runcie, 2013). The alga grows well in either seawater or freshwater. For consistency, *Chlorella* was grown in freshwater BG-11 medium with 5 mol m-3 NH4Cl as the nitrogen source instead of the more usual nitrate N-source (Nicholls, 1973). No added vitamins were needed. The "Kik" strain of *Chlorella* would also grow quite successfully photoheterotrophically in PM medium with acetate or acetate + benzoate as the organic carbon sources. A *Chlorella*-like green alga was isolated from the sewage pond by culturing in PM medium without thiosulphate or Na2S as potential electron sources. The photosynthetic bacteria were quickly eliminated in PM medium with no thiosulphate, Na_2S or organic carbon source. Photosynthetic bacteria would not grow in BG-11 medium because it contains no organic carbon source.

3.3 Experimental organisms

Rhodopseudomonas palustris (CGA009) was a gift by Prof Carrie Harwood, University of Washington, and is the most well-known strain of the organism (Larimer *et al.,* 2004). It is classified as a purple non-sulphur bacterium and uses BChl *a* as its primary photosynthetic pigment (Ectothiorhodaceae). It was grown in fully defined simplified PM medium (Table 3.1) with 10 mol $^{-3}$ sodium acetate as the carbon sources as described by Kim and Harwood (1991) and Ritchie (2013), using the methods used by Ritchie and Runcie (2013) and Ritchie (2013). The simplified PM medium used citric acid instead of EDTA and NTA (nitrilotriacetic acid) as the chelation agent for the trace element mix. *Rhodopseudomonas palustris* when growing photosynthetically is much more tolerant of oxygen than is generally believed. It will grow very well in a PM medium with acetate, benzoate or acetate + benzoate as a carbon source in a conical flask open to the atmosphere (Ritchie 2013; Ritchie et al., 2017). *Thermochromatium* (Chromatium) *tepidum* was a kind gift from Dr. Christopher Sherman (Blankenship Laboratory, Biology Dept., Washington University in St. Louis, USA). It is a purple sulphur bacterium containing BChl *a* (Chromatiaceae). PAM methods have been successfully used to measure photosynthetic electron transport in the organism (Ritchie and Mekjinda, 2015). In the present study, it was grown in PM medium with acetate as the organic carbon source because it does not grow well on PM medium with benzoate added. The green alga *Chlorella sp.* 'Kik' was originally isolated from water samples from a local prawn farm (Ritchie and Runcie 2013). The alga grows well in either seawater or freshwater. For consistency, *Chlorella* was grown in freshwater BG-11 medium with 5 mol m^{-3} NH_4Cl as the nitrogen source instead of the more usual nitrate N-source (Nicholls, 1973). No added vitamins were needed. The Kik strain of *Chlorella* would also grow quite successfully photoheterotrophically in the PM medium with acetate or acetate + benzoate as the organic carbon sources.

Table 3.1 Medium for *Rhodopseudomonas palustris*

PM component	For 100 ml PM (ml)
Double distilled water	800
0.5M Na_2HPO_4 (MW 142)	25
0.5M KH_2PO_4 (MW 136.1)	25
$CaCl_2 \cdot 2H_2O$ (MW 147)	1
$MgSO_4 \cdot 7H_2O$ (MW 246.5)	1
Concentrated trace element base (Trace elements) (Kim and Harwood, 1991)	1
2 mg/ml p-aminobenzoic acid (PABA)	1
Sodium Acetate (1M)	10
Sodium Benzoate (1M)	10
	Bring volume to 1000 ml

3.4 Isolates from the sewage pond

A *Chlorella*-like green alga was isolated from the sewage pond by culturing in PM medium without thiosulphate or Na_2S as potential electron sources. The photosynthetic bacteria were quickly eliminated in a few days in samples incubated in PM medium with no thiosulphate, Na_2S or organic carbon source. A rhodopseudomonad was also enriched from the sewage pond. It had a slightly different bacteriochlorophyll-protein complex spectrum compared to *Rhodopseudomonas palustris*. It grew well on PM medium with acetate, benzoate or acetate + benzoate as carbon sources. High Na_2S (8.3 mol m^{-3}) could be used to eliminate *Chlorella* (van Niel 1944, 1971). The specific oxygenic photosynthesis inhibitor DCMU (Diuron) 20 mmol m^{-3} was also effective in eliminating oxygenic photosynthetic organisms.

3.5 Diuron Photosynthetic Inhibitor [DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea)]

The Photosystem II inhibitor DCMU was used as a specific inhibitor of oxygenic photosynthesis. DCMU was purchased from Sigma-Aldrich (D2425) (Sigma-Aldrich Corp. St. Louis, MO, USA). Stocks of DCMU (100 mol m^{-3}) were made up in 100% ethanol. In the present study, a dose of 20 mmol m^{-3} completely inhibited oxygenic photosynthesis in the green alga *Chlorella* and so the added ethanol was only 0.2%. This is consistent with PAM fluorescence studies on isoproturon (a DCMU analogue) (*Scenedesmus obliquus*: Dewez *et al.,* 2008) and DCMU (seagrasses: Haynes *et al.,* 2000). *Rhodopseudomonads* are typically highly resistant to DCMU although some mutants are highly sensitive to it (IC50 > 10 mol m^{-3}: Sinning *et al.,* 1989; Sinning, 1992; Sinning *et al.,* 1990), well above the solubility of DCMU in water ($\approx$ 40 ppm). There does not seem to be any documentation on the sensitivity of purple sulphur bacteria such as *Thermochromatium tepidum* to DCMU.

3.6 Culture Conditions

Cultures of all the organisms were kept on shelves fitted with overhead fluorescent lights (Pansonic 36 W—daylight, colour temperature 6500 K: TIS 956-2533) in continuous light at about 27 °C (van Niel 1944, 1971). Stock cultures were routinely grown in capped McCartney bottles. *Rhodopseudomonas* could be grown in capped 250 mL bottles, which were mixed once a day, but would also grow quite well photosynthetically microaerobically in unshaken conical flasks with cotton plugs. They did not require strict anoxic conditions to grow photoshterotrophically. *Chlorella* was grown in 250 or 500 mL conical flasks with cotton plugs. The cultures were mixed by inversion or swirling daily. The light intensity in the culture room was approximately 100–200 μmol photons m^{-2} s^{-1} (PPFD 400–700 nm), measured using a Li-Cor photon flux meter Model LI-189 (Li-CorCorp, USA) and a MQ-200 photon flux meter from Apogee Instruments (USA). No

special lighting was needed to grow the *Rhodopseudomonas palustris*, *Thermochromatium tepidum* or the sewage pond rhodopseudomonad.

3.7 Preparation of Solvent Extracts for Chlorophyll and Bacteriochlorophyll determinations

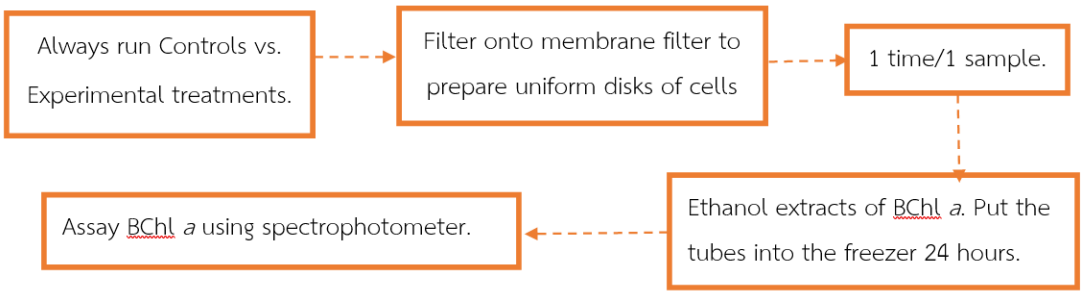
Sewage pond water samples, *Chlorella* and *Rhodopseudomonas* cells could be filtered onto Whatman GF/C glass fibre disks (Whatman International, Maidstone, England, U.K.) using a Millipore apparatus designed for 25 mm filters (Ritchie, 2013; Ritchie and Runcie, 2013). The photosynthetic pigments on the filtrates were then extracted in the appropriate solvent, in the present study in 100% ethanol or a 7:2 mixture of acetone and ethanol (Ritchie, 2018). Cells could also be collected by centrifuging at 5000 rpm (RCF = 9000g) in a swing-bucket centrifuge (Hermle Z323K, Hermle Labortechnik, Wehingen, D-78564, Germany), the supernatant poured off, the pellets were then resuspended using a vortex and then the photosynthetic pigments were extracted in solvent.

3.8 Analysis of cell concentration during growth

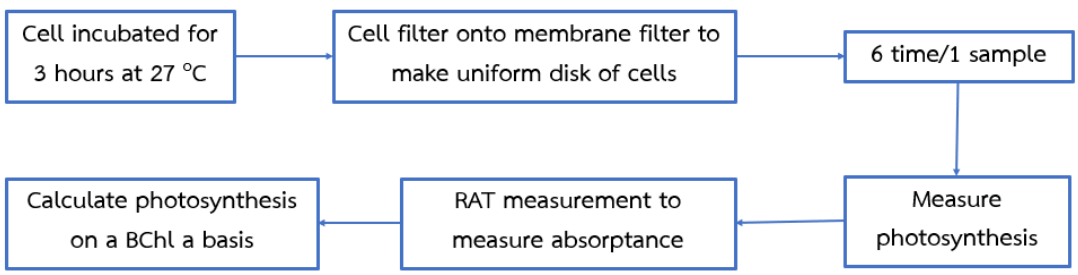
We have routinely used bacteriochlorophyll *a* to monitor the number of cells used in experiments (Ritchie and Runcie, 2013; Ritchie, 2013; Ritchie and Mekjinda, 2015; Chandaravithoon *et al.,* 2018). This is appropriate for a photosynthetic bacterium. The relationship between bacteria chlorophyll a (BChl *a*) present and cell absorbance at 650 nm was measured using the Shimadzu double-beam spectrophotometer Optical density of the cells was measured at 650 nm as described (Ritchie and Runcie, 2013). For assay of the bacteriochlorophyll *a*, 5 ml samples of cell suspension were centrifuged at 5000 rpm for 10 min. After centrifugation, the liquid was decanted off as much as possible. 3 ml of acetone: ethanol (7:2 ratio) was added and then put into a freezer for 1 hour. After 1 hour samples were then centrifuged at 5000 rpm for 10 min and the

supernatant was used for BChl *a* determination using the spectrophotometer at 772 nm and bacteriochlorophyll *a* estimated as described by Ritchie and Runcie (2013) and Ritchie (2018). Absorbance at 850 nm was used as a blank. Numbers of cells (as measured by absorbance at 650 nm) was directly proportional to BChl *a* content up to an absorbance at 650 nm of at least 1.2. The original formulae for assay of BChl *a* were based on a 7:2 mixture of acetone and methanol. The methanol in the extractant was replaced by ethanol for safety reasons (7:2, Acetone Ethanol) (Absorption max at 775 nm) and extinction coefficients for BChl *a* in both ethanol and acetone/ethanol solvents and BChl *a* formulae are now available (Ritchie, 2018).

3.8.1 Measuring bacteriochlorophyll using a Spectrophotometer (Adapted from Phongjarus *et al.*, 2016 and Ritchie, 2018).



3.8.2 Measuring photosynthesis using a PAM machine (Adapted from Phongjarus *et al.*, 2016 and Ritchie, 2018). Photosynthetic rates were calculated as mol e- g^{-1} BChl *a* s^{-1}.



3.9 Bacteriochlorophyll *a* Determinations

Bacteriochlorophyll *a* was extracted in a Mg carbonate-neutralized (7:2) mixture of acetone and methanol (Ritchie, 2018) or 100% ethanol. No heat treatment was necessary for the effective extraction of the BChl *a*: there seemed to be no especial difficulties in extracting it from the photosynthetic bacterial cells. A 60 ^{o}C heat treatment was used on the *Chlorella* cells and the sewage pond samples to ensure effective extractions of both BChl *a* and Chl *a* + *b*. Spectrophotometric measurements were made using a Shimadzu UV-1601 UV-Visible double-beam spectrophotometer (Shimadzu Corp., Kyoto, Japan) at 649, 665, 774 & 850 nm in ethanol and 647, 663, 774 & 850 nm in 7:2 mixtures of acetone and ethanol, the 850 reading served as the blank (Ritchie, 2018) based on Ritchie (2006), Ritchie (2013), Ritchie and Runcie (2013) and Ritchie and Mekjinda (2015). Chl *a, b* and BChl *a* were calculated using the algorithms of Ritchie (2018). Integrating sphere scans on the sewage pond water samples did not show any evidence for significant amounts of BChl *c* or BChl *b in vivo* (Siefert *et al.,* 1978; Ritchie *et al.,* 2017) and neither did the *in solvent* scans. BChl *a* was calculated as μg ml^{-1} of solvent and total BChl *a* in the total volume of the extract.

3.10 Chlorophyll *a & b* Determinations

To ensure proper extraction of pigments from *Chlorella* and the sewage pond material in the material used in the present study a routine 60 ^{o}C treatment in a beaker of hot water in the dark was used (Ritchie, 2018). Pellets in solvent extracts need to be checked for effective extraction of pigments. Absorbances at 850 nm, not 750 nm, were used as a blank. Chl *a* and *b* were calculated as μg ml^{-1} of solvent using the algorithms developed by Ritchie (2018) and also calculated as total Chl *a* and *b* in the total volume of the extract.

3.11 Chlorophylls and Bacteriochlorophyll Assay procedure.

Cells were filtered onto 0.45 μm millipore-nitrocellulose filter disks (MF 0.45 μm HA, Merck-Millipore, Tullagree, Republic of Ireland) or Whatman GF/C glass fibre disks (GE Healthcare, UK Ltd, Amersham Place, Little Chalfont, UK) using a Millipore filter setup. The disk of filtrate cells was 16.2 mm in diameter (206.12×10^{-6} m^2) and so the Chl *a* and/or BChl *a* extracted from the cells on the disk could be calculated as mg/m^2.

3.12 Integrating Sphere Spectrophotometry

A spectrophotometer fitted with a Taylor sphere can measure the spectral properties of cells in cell suspensions which scatter light and can be used to measure the Transmission (Tλ%), Absorbance ($A\lambda_{\%}$) and Reflectance (Rλ%) of cells and hence the Absorptance (Abtλ%) can be calculated if both transmission and reflectance measurements are made (Ritchie, 2013; Ritchie and Runcie, 2013; Ritchie, 2017). A UV-VIS Spectrophotometer fitted with a Taylor Sphere (Model A&E-S90-2D, A&E Lab (UK) Co. Ltd, London N14 5BP, U.K.) was used to compare the spectral properties of *Rhodopseudomonas* and *Chlorella* cell suspensions and mixtures of the two. Scans were also made of sewage pond samples from the local sewage treatment plant as an example of a naturally occurring mixed population of oxygenic and anoxygenic photosynthetic organisms.

3.13 PAM fluorometry

Cultured cells were centrifuged and resuspended in fresh culture media and control and DCMU treated cells were routinely incubated for 1h before experiments in 40 ml culture

tubes. The freshly collected sewage pond material was incubated in the light overnight and placed in 40 ml culture tubes for experiments. Light saturation curve measurements were made on cells filtered onto glass fibre disks or 0.45 µm nitrocellulose filter disks using a Junior PAM portable chlorophyll fluorometer (Gademann Instruments, Würzburg, Germany) fitted with a 1.5 mm-diameter optic fibre and a blue diode light source (465 ± 40 nm). Each light saturation curve used nine irradiances and light curves were routinely generated for six or eight disks. The filter disks were kept in Petri dishes with filter paper moistened (0.5 ml) with the experimental medium used to incubate the cells experimentally.

The PAM parameters (Y, rETR, qN and NPQ) were calculated by the WinControl software ver. 2.13 (Heinz Walz; Effeltrich, Germany), using the standard settings for rapid light curves (default absorptance (Abt_F) = 0.84, PSI/PSII allocation factor PSII/PSI = 0.5) to calculate the rETR (Genty *et al.,* 1989; Brestic and Zivcak, 2013; Ralph and Gademann, 2005; Rascher *et al.,* 2000). The fluorescence yield is part of the WinControl software output as the effective quantum yield (Y or ΦPSII). Effective quantum yields have ranges from 0 to 1 (maximum usually no higher than ~0.85 in oxygenic organisms, typically about 0.4 in anoxygenic organisms) (Ritchie, 2013; Ritchie and Runcie, 2013; Ritchie and Mekjinda, 2015).

Yield is calculated by the Walz software as:

$$Y = 1 - Fo/Fm' \qquad \text{Equation 1}$$

where, Fo is the fluorescence in the modulated measuring light and Fm' is the fluorescence in the light acclimated state after a flash of actinic light (Genty *et al.,* 1989; Brestic and Zivcak, 2013). Yield is defined from $1 \longrightarrow 0$. It is found experimentally that if Y is plotted against irradiance (*E*), it follows a simple exponential decay function of the form $y = e^{-kx}$ for both oxygenic and anoxygenic photosynthetic organisms (Ritchie, 2008; Ritchie, 2013). In the presence of DCMU the measured Fo is sometimes greater than the measured Fm' resulting in a calculated Y value slightly less than zero but this is not apparent from the WinControl spreadsheet. Equation (1) is the same for both oxygenic and anoxygenic photosynthesis.

The photosynthetic electron transport rate (ETR) is proportional to the product of the yield (Y) × Irradiance (E). The actual ETR has to be corrected for the proportion of light actually absorbed by the organism (the Absorptance, Abt) and whether or not the photosynthetic organism has a single photosystem (photosynthetic bacteria) and has two photosystems (PSII & PSI) arranged in a Z-scheme in the case of oxygenic organisms. In the case of oxygenic

photosynthesis, the PSI/PSII allocation factor was taken as 0.5 as the default by the Walz software (Ritchie, 2008; Ritchie and Larkum, 2013), for anoxygenic organisms the rETR calculated by the WinControl software was multiplied by two (2) to give the anoxygenic rETR value (Ritchie, 2013; Ritchie and Runcie, 2013; Ritchie and Mekjinda, 2015). Actual measurements of the Absorptance of the filter disks impregnated with cells were made at 465 nm ($Abt_{465\ nm}$) using the Reflectance-Absorptance-Transmission (RAT) machine (Ritchie and Runcie, 2014) were used to correct rETR to actual ETR.

rETR

$$rETR = Y \times E \times (PSII/PSI = 0.5) \times (Abt_F = 0.84) \quad \text{Equation 2}$$

Oxygenic Photosynthesis

$$ETR = Y \times E \times 0.5 \times Abt_{465nm}/0.84 \quad \text{Equation 3}$$

$$ETR = rETR \times Abt_{465nm}/0.84$$

In the case of oxygenic photosynthesis the electron source is water: $2H_2O \longrightarrow 4H^+ + 4e^- + O_2$ and so the Photosynthetic Oxygen Evolution Rate (POER) from the light reactions of photosynthesis is an estimate of gross photosynthesis (Pg) but it does not take photorespiration into account (1 μmol O_2 g^{-1} Chl *a* $s^{-1} \equiv 4$μmol e^- g^{-1} Chl *a* s^{-1}) (Apichatmeta *et al.,* 2017; Quinnell *et al.,* 2017**).**

Anoxygenic Photosynthesis

$$ETR = Y \times E \times 1 \times Abt_{465nm}/0.84 \quad \text{Equation 4}$$

$$ETR = rETR \times 2 \times Abt_{465nm}/0.84$$

where, rETR is the relative Photosynthetic Electron Transport rate calculated by the WinControl software in default mode, *Y* is the Yield calculated by the WinControl software, *E* is the irradiance (μmol photons m^{-2} s^{-1}), $Abt_F = 0.84$ is the default absorptance value used by the WinControl Software, $Abs_{465\ nm}$ is the experimentally measured absorptance measured at 465 nm, PSII/PSI = 0.5 is the default PSII/PSI allocation factor of the WinControl software, which for anoxygenic photosynthesis is corrected for by multiplying by 2 (Equation 4).

Since yield (Y) *vs.* Irradiance is of the form $y = e^{-x}$, and since photosynthesis is proportional to the product of the yield and irradiance (Equations 2, 3 and 4) then an appropriate model for photosynthesis is of the form $y = x.e^{-x}$ (Ritchie, 2008). The equation $y = x.e^{-x}$ has a maximum at $x = 1$ and the slope of the line at $x = 0$ is 1 and there is a point of inflection ($d^2y/d^2x = 0$) at $x = 2$. A form suitable for modelling photosynthesis with experimentally determinable

constants that are easily recognisable on a graphical representation of the data (Ritchie, 2015; Quinnell *et al.,* 2017) is

$$\mathrm{ETR} = \frac{\mathrm{ETR}_{\max} \times E}{E_{\mathrm{opt}}} \times \mathrm{e}^{1-E/E_{\mathrm{opt}}} \qquad \text{Equation 5}$$

where, ETR is electron transport rate as a measure of the photosynthetic electron transport rate (μmol e^- m^{-2} s^{-1}), E is the irradiance (μmol photon m^{-2} s^{-1} 400–700 nm PPFD), E_{opt} is the optimum irradiance, ETR_{max} is the maximum photosynthetic electron transport rate.

The maximum photosynthetic efficiency (α_0) is the initial slope of the curve at E = 0 ($\alpha = ETR_{max} \times e/E_{opt}$). It can be shown by analysis of Equation 5 that the half-maximum photosynthesis ($ETR_{half\text{-}max}$) occurs at 0.231961 × E_{opt} and that photosynthesis is also inhibited by 50% at 2.67341 × E_{opt}. Hence, good rates of photosynthesis (>50% of maximum) are found under irradiances ranging from 0.25 to 2.5 times the optimum irradiance. The asymptotic photosynthetic efficiency at zero irradiance (α_0) is theoretically useful but perhaps a more informative expression for productivity studies is the photosynthetic efficiency at optimum irradiance (α_{Eopt}). It can be shown that $\alpha \times E_{opt}$ is equivalent to $\alpha_{Eopt} = \alpha_0/e$.

The ETR is initially calculated on a surface area basis (mol e^- m^{-2} s^{-1}). In this, as in previous studies, cells were filtered onto disks to form a uniform layer of cells suitable for PAM fluorometry. Since the Chl *a* and/or the BChl *a* content of the disks was known it was possible to express ETR as mol e^- g^{-1} Chl *a* s^{-1} or mol e^- g^{-1} BChl *a* s^{-1} (Ritchie, 2008; Ritchie, 2013; Ritchie and Larkum, 2013; Ritchie and Runcie, 2013; Ritchie and Mekjinda, 2015).

3.14 Statistics and Excel Routines

The standard statistical text used in the present study is Cochran and Snedecor (1989). The Microsoft-EXCEL non-linear least squares fit routines (Microsoft-EXCEL) used for curve fitting in the present paper are available on the INTERNET (Ritchie, 2015).

CHAPTER 4

RESULTS AND DISCUSSION

This study is on the use by PAM (Pulse Amplitude Modulation) fluorometry to measure photosynthesis in a sewage pond. PAM estimates the photosynthetic electron transport rate (ETR) by measuring fluorescence of chlorophyll (Chl) *a* (> 700 nm) induced by blue (Soret, QX) band or red light (QY) band.

4.1 The inhibition of photosynthesis by Diuron Photosynthetic Inhibitor [DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea)]

To test cultures of all the organisms were kept on shelves fitted with overhead fluorescent lights (Pansonic 36 W daylight, colour temperature 6500 K: TIS 956-2533) in continuous light at about 27 ^{o}C (van Niel, 1944, 1971). The light intensity in the culture room was approximately 100 – 200 μmol m^{-2} s^{-1} (PPFD 400 – 700 nm), measured using a Li-Cor photon flux meter Model LI-189 (Li-Cor Corp, Lincoln, Nebraska, USA) and a MQ-200 photon flux meter from Apogee Instruments, Logan, UT,USA.

Figure 7 shows plots of Yield of *Chlorella* (Chl *a* + *b*) *vs*. Irradiance for control cells and cells incubated 1h in 20 μM DCMU. Yield *vs.* Irradiance fits a simple exponential decay curve for the control cells (r = 0.9564, n = 72, see Table 4.1) but DCMU completely eliminates oxygenic Photochemical Yield. Figure 8 shows photosynthetic Electron Transport Rate (ETR) *vs.* Irradiance for *Chlorella* in the presence and absence of DCMU. The ETR *vs.* Irradiance curves for the control *Chlorella* cells fits the waiting-in-line equation very well (r = 0.9743, n = 72, see Table

4.1). Figure 7 and 8 shows that DCMU eliminates oxygenic photosynthetic electron transport. However, it is not apparent from Figure 7 and 8 that DCMU has little or no effect on Fm' in Equation 1 and Yield drops to zero because Fo becomes $\approx$ Fm' (Table 4.1). The fluorescence of PSII in the presence of DCMU is still very high.

Figure 9 shows the results of a similar experiment on the photosynthetic bacterium *Rhodopseudomonas* in an XYY graph format. Figure 9 shows the plots of Yield of *Rhodopseudomonas* (BChl *a*: a purple non-sulphur photosynthetic bacterium) *vs.* Irradiance for control cells and for cells incubated 1h in 20 μM DCMU. Yield *vs.* Irradiance of both the control and DCMU-treated cells both fit a simple exponential curve for the control cells (Control: r = 0.9648, n = 72; + DCMU: 0.9602, n = 72; see Table 4.1) and there is no significant difference. The ETR *vs.* Irradiance for *Rhodopseudomonas* in the presence and absence of DCMU was calculated on a BChl *a* basis as μmol e^{-} g^{-1} BChl *a* s^{-1}) The ETR *vs.* Irradiance curves for both the control and DCMU treated cells of *Rhodopseudomonas* fit the waiting-in-line equation very well (Control: r = 0.9232, n = 72; + DCMU: 0.8797, n = 72; see Table 4.1). There is no significant effect of DCMU on the anoxygenic photosynthetic electron transport of *Rhodopseudomonas*.

Figure 10 shows the Yield and ETR of *Thermochromatium* (BChl *a*: a purple sulphur photosynthetic bacterium) *vs.* Irradiance for control cells and cells incubated 1h in 20 μM DCMU presented on a XYY graph format. Yield *vs.* Irradiance of both the control and DCMU-treated cells both fit a simple exponential curve for the control cells (Control: r = 0.9931, n = 72; + DCMU: 0.9941, n = 72; see Table 4.1). There is no effect of DCMU on Yield of the purple sulphur bacterium. The ETR *vs.* Irradiance curves for both the control and DCMU treated cells of *Thermochromatium* fit the waiting-in-line equation (Control: r = 0.8504, n = 72; + DCMU: 0.9174, n = 72; see Table 4.1). There is no significant effect of DCMU on the anoxygenic photosynthetic electron transport of *Thermochromatium.*

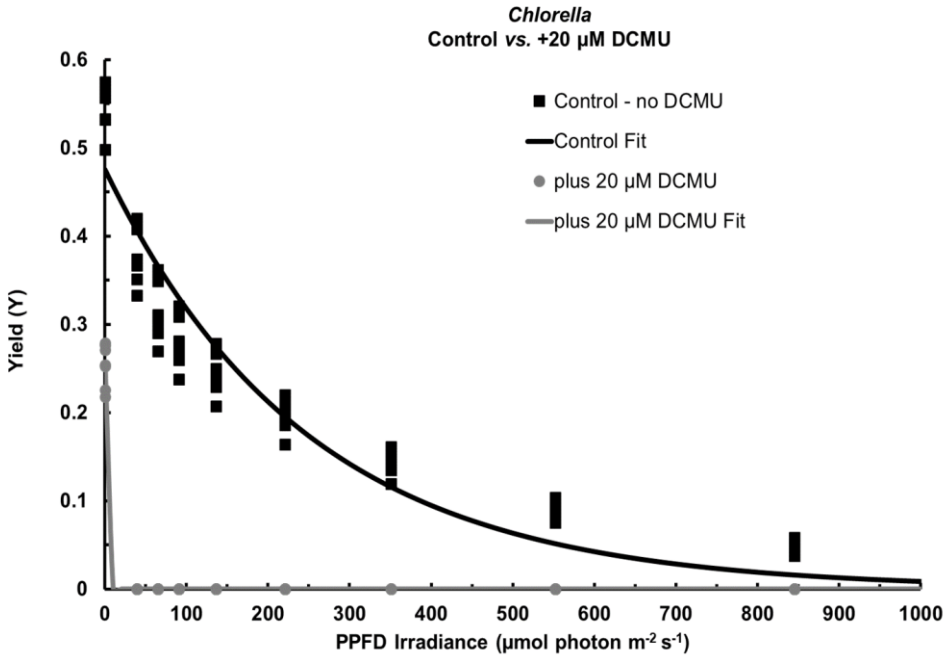


Figure 7 Plots of yield of *Chlorella* (Chl *a* + *b*) vs. irradiance for control cells and cells incubated 1 h in 20 µM DCMU. Yield vs. irradiance fits a simple exponential curve for the control cells ($r = 0.9564$, $n = 72$, see Table 4.1) but DCMU completely eliminates photochemical yield.

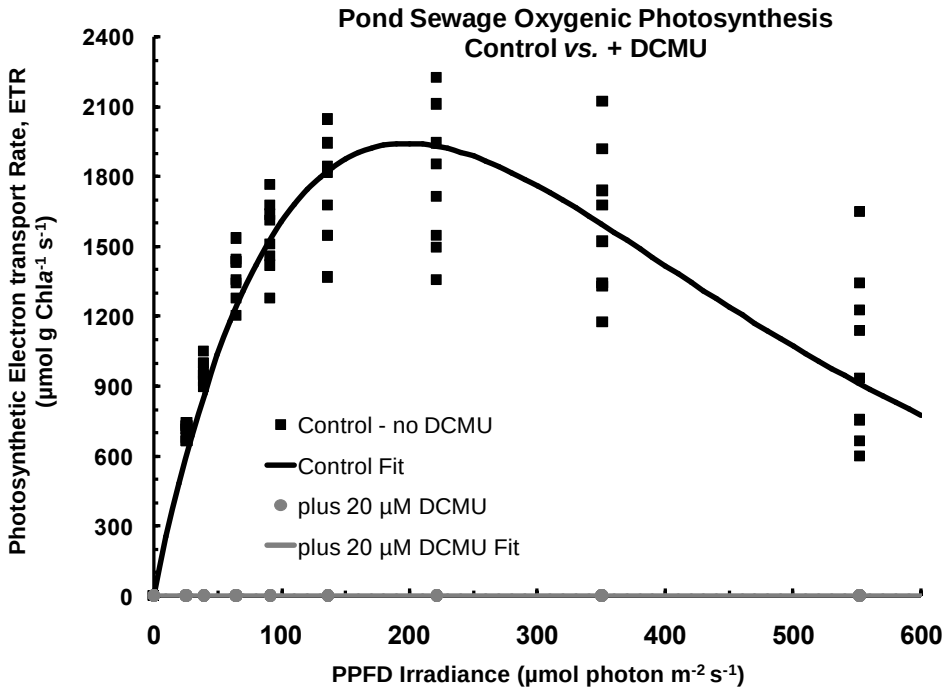


Figure 8 Photosynthetic electron transport rate (ETR) vs. irradiance for *Chlorella* in the presence and absence of DCMU. The ETR vs. irradiance curves for the control Chlorella cells fits the waiting-inline equation very well (r =0.9743, n = 72, see Table 4.1). DCMU completely eliminated oxygenic photosynthetic electron transport.

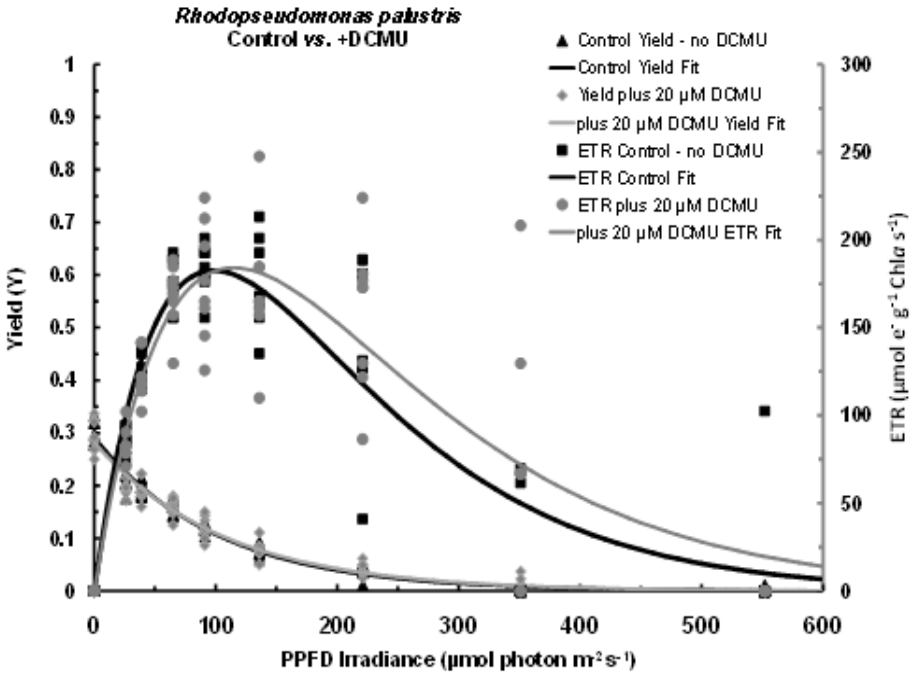


Figure 9 Plots of yield and ETR of *Rhodopseudomonas* vs. irradiance for control cells and cells incubated 1 h in 20 µM DCMU presented in a XYY graph format. Yield vs. irradiance curves of both the control and DCMU-treated cells both fit a simple exponential curve for the control cells (Control: r = 0.9648, n = 72; +DCMU 0.9602, n = 72; see Table 4.1).

Table 4.1 Statistics on PAM Yield and ETR Curves

	Experiment	Disks	Fm'	Y_{max}	Y_k	Irradiance $Y_{0.5}$	r & P	E_{opt}	P_{max}	Alpha α_0	r & P
Fig. 7	*Chlorella* Y	n = 8	1915	0.476	0.004038	172	0.9564	433	122	0.767	0.9743
	Control ± DCMU		±89.3	±0.0234	±0.000495	21.0	2.1E-102	±20.6	±3.25	±0.0418	4.61E-47
Fig. 8	*Chlorella* ETR	n = 8	1387	0.257	8.112	0	0	0	0	0	0
	Control ± DCMU		±67.9	±0.0053	∞	∞	0	0	0	0	0
Fig. 9	*Rhodopseudomonas*	n = 8	93.3	0.284	0.003517	197	0.9648	382	146	1.04	0.9232
	Control		±3.25	±0.0118	±0.000375	21.0	7.6E-106	0.000201	6.735777	0.0929	8.73E-31
	Rhodopseudomonas	n = 8	93.0	0.279	0.003498	198	0.9602	385	143	1.01	0.8797
	+DCMU		±7.70	±0.0123	±0.000397	22.5	7.1E-104	38.3	8.54	0.117	2.72E-24
Fig. 10	*Thermochromatium*	n = 8	90.4	0.402	0.00694	99.9	0.9931	110	332	8.22	0.8504
	Control		±4.53	±0.0082	±0.000337	4.86	3E-131	10.8	21.4	0.965	3.32E-21
	Thermochromatium	n = 8	88.3	0.402	0.006439	108	0.9941	114	355	8.48	0.9174
	+DCMU		±3.76	±0.0073	±0.000286	4.78	8.9E-134	8.12	16.6	0.723	1.01E-29

Fig. 12	Fresh Sewage Pond	n = 8	184.8	0.273	0.005337	130.0	0.9772	429	2143	13.57	0.9624
	control						9.9E-				
			±7.31	±0.0110	±0.000503	12.2	113	51.9	143	1.88	2.32E-41
	Fresh Sewage Pond	n = 8	184.4	0.138	0.001635	424	0.9639	590	1048	4.827	0
	+DCMU						1.9E-				
			±6.88	±0.0037	±0.000145	37.5	105	41.1	34.5	0.372	0
Fig 13	1 Week in Light										
	Sewage	n = 8	683.8	0.507	0.01704	40.7	0.9912	198	1944	26.64	0.9317
	control						1.8E-				
			±32.6	±0.0145	±0.000935	2.23	127	12.4	80.0	2.00	1.67E-32
	1 Week in Light										
	Sewage	n = 8	595.4	0.074	4.370	0.159	0.9413	0	0	0	0.8922
	+DCMU						1.55E-				
			±17.3	±0.0060	∞	∞	97	0	0	0	1.6E-25

Caption for Table 4.1 Fitted Yield and ETR parameters for the rapid light curves in the study (means ± 95% confidence limits fitted to a simple exponential decay curve for the Yield data and the Waiting-in-Line Equation (Equation 5). Six or eight disks were used and since the rapid light curves each had 9 data points the total n = 54 or 72. Fm' is the fluorescence in the light acclimated state after a flash of actinic light (Equation 1). Fm' is very high for experiments with a large amount of Chl a in the specimen but very low in the case of samples with only BChl a. DCMU, although it eliminates oxygenic electron transport, it does not greatly decrease Fm'. Ymax is the asymptotic maximum Yield at zero irradiance of the Yield vs. Irradiance curves and Yk is the exponential decay constant of yield in increasing irradiance. Irradiance Y0.5 is the irradiance that decreases Yield by 50% calculated from the exponential decay constant. All the Yield vs. Irradiance curves had very high correlations close to 1.0 with $p <<< 0.001$. Eopt is the point on the Waiting-in-Line where photosynthetic electron transport is maximal (ETRmax), Alpha $\alpha 0$ is the photosynthetic efficiency which is the slope of the ETR vs. Irradiance curves at zero irradiance (Ritchie, 2008; Ritchie, 2015; Quinnell et al., 2017).

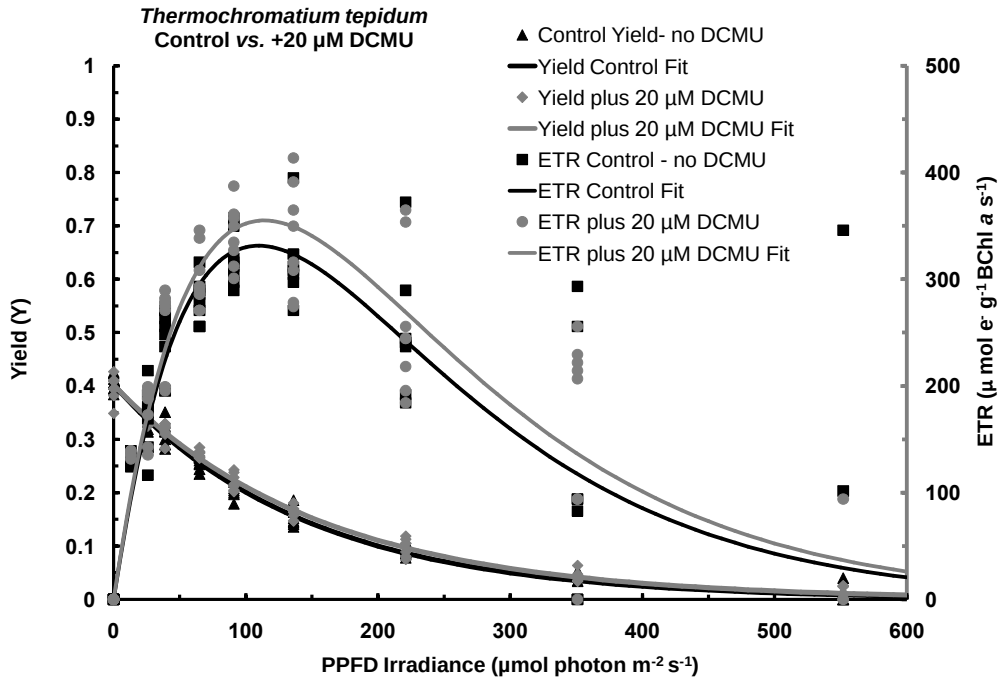


Figure 10 Yield and ETR of *Thermochromatium vs.* irradiance for control cells and cells incubated 1 h in 20 µM DCMU presented in a XYY graph format. Yield vs. irradiance of both the control and DCMU-treated cells both fit a simple exponential curve for the control cells (Control: r = 0.9931, n = 72; + DCMU: 0.9941, n = 72; see Table 4.1). There is no effect of DCMU on Yield of *Thermochromatium*. The ETR *vs.* Irradiance curves for both the control and DCMU treated cells of *Thermochromatium* fit the waiting-in-line equation (Control: r = 0.8504, n = 72; + DCMU: 0.9174, n = 72; see Table 4.1). There is no significant effect of DCMU on the anoxygenic photosynthetic electron transport of *Thermochromatium*.

Comparing the results of the *Chlorella* (Figure 7 and 8), the *Rhodopseudomonas* experiments (Figure 9) and the *Thermochromatium* experiments (Figure 10) it would seem that the use of DCMU on a mixture of anoxygenic photosynthetic bacteria and an oxygen-evolving photosynthetic organism would easily resolve oxygenic from anoxygenic photosynthesis. However, there is a large difference in the fluorescence behaviour of BChl *a* compared to Chl *a*. The Fm' fluorescence of BChl *a* in RC-2 is quite low compared to that found in the case of Chl *a* (Table 4.1) and DCMU does not decrease the overwhelming fluorescence of PSII.

Scans often in approximately equal amounts (Chl *a* and BChl *a* basis). The of sewage pond samples and solvent extracts from them had previously shown that both Chl *a* containing and BChl *a* containing organisms were present in the Phuket sewage ponds (Ritchie, 2018), photosynthetic characteristics of pure cultures of *Chlorella* and the two purple photosynthetic bacteria (*Thermochromatium, Rhodopseudomonas*) were very successfully measured (Figures7-10).

4.2 Model A&E-S90-2D Taylor sphere spectrophotometer

The next logical step in the project was to attempt to measure photosynthesis of a synthetic mixture of *Chlorella* and *Rhodopseudomonas* cultures. Figure 11 shows the in vivo absorbance of cell suspensions of *Chlorella*, *Rhodopseudomonas* and a 1:1 mixture of *Chlorella* and *Rhodopseudomonas* using the Model A&E-S90-2D Taylor sphere spectrophotometer in transmission (T%)/absorbance mode (Abs). The *in vivo Chlorella* Chl *a* peak is at 677 nm (0.2605) and the blue peak is at 440 nm (0.3672). The twin NIR (Qx) peaks at 797 nm (0.2530) and 866 nm (0.2512) are characteristic of Rhodopseudomonads *in vivo*. The blue peak of *Chlorella* is at 436 nm (0.3744) and the *Rhodopseudomonas* peak is at 373 nm (0.2950): these blue peaks are comparable to the Qx peaks of Chl *a* and BChl *a*. There is a conspicuous minor absorption peak (Qx-Qy) attributable to BChl *a* at 592 nm (0.1183). *Rhodopseudomonas* has two in vivo peaks in the blue part of the spectrum. The BChl *a in vivo* peak is at 373 nm (0.2950) and the carotenoid peak is at 503 nm (0.1914). The 1:1 mixture of the two cultures is very closely the sum of the two cultures measured separately. A 3 ml suspension of *Chlorella* only cells filtered onto a glass fibre

disk had 37.0 ± 5.1 mg Chl *a* m^{-2}. A similarly prepared disk of the *Rhodopseudomonas* cells had 31.4 ± 1.6 mg BChl *a* m^{-2}. The 1:1 *Chlorella/Rhodopseudomonas* cell mixture filtered onto a glass fibre disk would have had 37.0 ± 5.1 mg Chl *a* m^{-2} and 31.4 ± 1.6 mg BChl *a* m^{-2}.

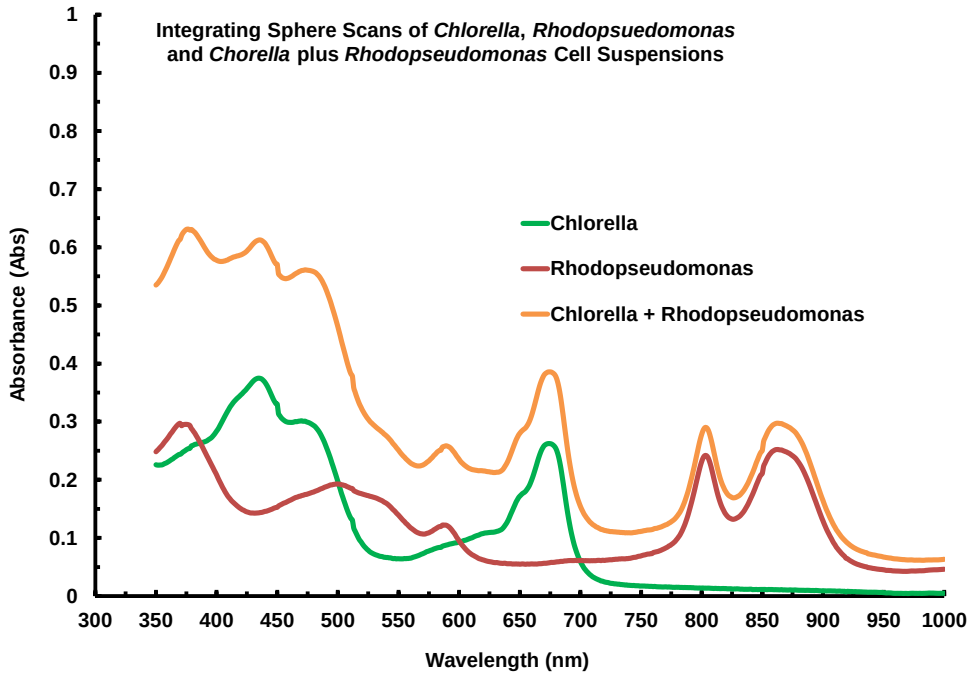


Figure 11 Absorbance of cell suspensions of *Chlorella*, *Rhodopseudomonas* and a 1:1 mixture of *Chlorella* and *Rhodopseudomonas* using the Model A&E-S90-2D Taylor sphere spectrophotometer in transmission (T%)/absorbance mode (Abs). The *in vivo Chlorella* Chl *a* peak at 677 nm (0.2605) and the blue peak is at 440 nm (0.3672). The twin NIR (Qx) peaks at 797 nm (0.2530) and 866 nm (0.2512) are characteristic of Rhodopseudomonads *in vivo*. There is a conspicuous minor absorption peak (Qy) attributable to BChl *a* at 592 nm (0.1183). *Rhodopseudomonas* has two in vivo peaks in the blue part of the spectrum. The BChl *a in vivo* peak is at 373 nm (0.2950) and the carotenoid peak is at 503 nm (0.1914). The 1:1 mixture of the two cultures is very closely the sum of the two cultures measured separately.

The results of the PAM ± DCMU experiments on the *Chlorella*-only and *Rhodopseudomonas*-only cell suspensions shown in Figure 11 (Analysis in Table 1). The results are similar to those shown in Figure 7 and 8 for *Chlorella* and Figure 9 for *Rhodopseudomonas* (The statistical analyses of the data used to prepare Figures 7, 8 & 9, Table 4.1). It was reasoned

that a control *vs.* DCMU treatment of a mixture of oxygenically photosynthetic *Chlorella* and *Rhodopseudomonas* (Data for Figure 7) should have been able to distinguish oxygenic from non-oxygenic photosynthesis in the mixture of the two types of cells.

4.3 Volume treatment

The proportion of mixing had different effects on the result. The result should have been able to distinguish oxygenic from non-oxygenic photosynthesis in the mixture of the two types of cells, but it shows a different result to what was expected.

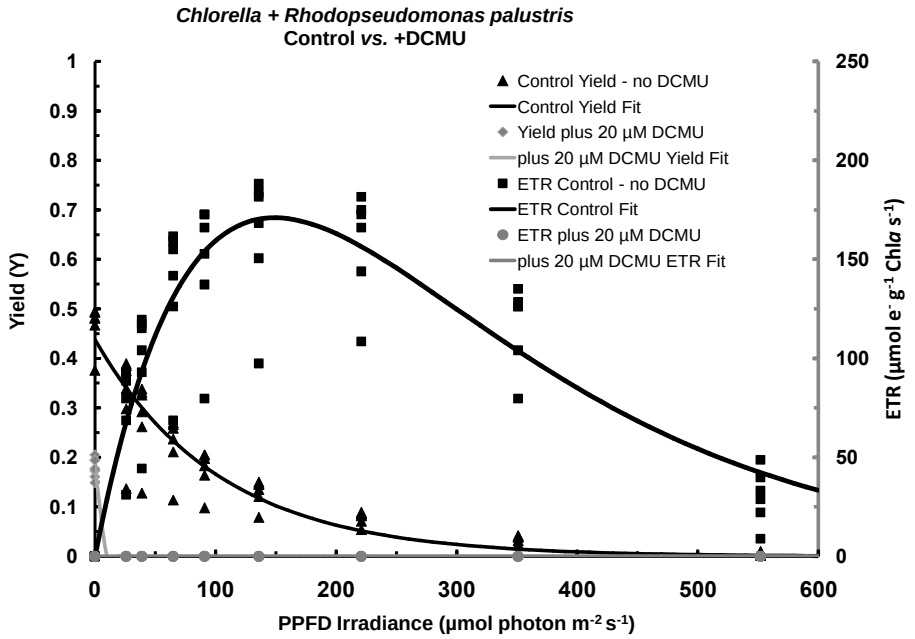


Figure 12 Shows plots of Yield and ETR of *a* 1:1 mixture of *Chlorella and Rhodopseudomonas* cultures *vs.* Irradiance for control cells and cells incubated 1h in 20 µM DCMU presented in a XYY graph format. The mixed cell suspension had similar levels of Chl *a* and BChl *a* (2.54 ± 0.350 µg/ml Chl *a*; 2.16 ± 0.110 µg/ml BChl *a*). Yield *vs.* Irradiance curves of the control *Chlorella* + *Rhodopseudomonas* mixture fitted a simple exponential decay curve as would be expected from Figure 7 and Figure 9 and Table 4.1). The DCMU-treated *Chlorella* + *Rhodopseudomonas* cell mixture showed no apparent yield, as if the *Rhodopseudomonad* cells did not exist in the culture. Hence, only the photosynthetic electron transport rate of the *Chlorella* could be calculated (on a µmol e^- g^{-1} Chl *a* s^{-1} basis). The E_{opt} was 149 ± 13.5 µmol quanta m^{-2} s^{-1}. The calculated result was similar to that measured on the *Chlorella*-only culture. From Figures 7, 8 and 9 one would have expected a residual yield curve due to the presence of the *Rhodopseudomonas* cells would appear when the activity of PS-11 in *Chlorella* was suppressed by DCMU. Analysis in the Table 4.1 shows that the fluorescence of the *Chlorella* cells was still very high in the presence of DCMU and the fluorescence of the *Rhodopseudomonas* was very low. The fluorescence of PS-11 of *Chlorella* even in the presence of DCMU drowned out that of RS-2 from the *Rhodopseudomonas* cells.

4.4 Sewage pond water

Sewage pond water was collected from the Phuket Integrated Waste Management (03 August 2017) and stored overnight in the dark at 4 ^{o}C. Next day after 3h in the light in the culture room (30 ^{o}C) the cells were set up in tubes with control cells in sewage pond water and DCMU treated cells with 20 μM DCMU added. The control and experimental treatments were incubated for 1h before being filtered onto glass fibre disks and photosynthetic electron transport measured using the PAM machine. The results are shown in Figure 12 in XYY graph format. The sewage pondwater was a red brown colour and contained very little Chl *a* and was dominated by BChl *a* (BChl *a*/Chl *a* = 5.25 ± 0.28). Such a sewage pond would be classed as "red water" phase (Belila *et al.,* 2013). 2 ml of the sewage pond water had 0.268 ± 0.0067 μg/ml Chl *a* and 1.408 ± 0.075 μg/ml BChl *a* and hence 2.28 ± 0.062 mg Chl *a* m^{-2} and 13.03 ± 0.696 mg Chl *a* m^{-2} on the glass fibre disks. Chlorophyll and Bacteriochlorophyll were extracted using 7:2 mixture of acetone and ethanol and assayed using the equations of Ritchie (2018). Photosynthesis by the photosynthetic bacteria was detectable as Yield in the presence of DCMU and so *both* oxygenic and anoxygenic photosynthesis could be measured in the sewage pond water (unlike the synthetic sewage example in Figure 11) because the Chl *a* content was so low. The fluorescence of Chl *a* in the presence of DCMU did not drown out the fluorescence of BChl *a*. Since Yield in the absence and presence of DCMU were both measureable it was possible to calculate the Yield of the oxygenic and anoxygenic organisms separately (Figure 12). Both curves were simple exponential decay curves and ETR could be calculated for oxygenic and anoxygenic photosynthesis (Equations 3 and 4). Oxygenic photosynthesis could be calculated as μmol e^{-} g^{-1} Chl *a* s^{-1} and anoxygenic photosynthesis as μmol e^{-} g^{-1} BChl *a* s^{-1}. ETR *vs.* Irradiance curves could be fitted to the Waiting-in-Line equation (Equation 5). The curves for the control was like the *Chlorella* cells shown in Figure 8 and in the presence of DCMU the curves were similar to that for *Rhodopseudomonas* (Figure 9, purple non sulphur bacterium) and for *Thermochromatium* (Figure 10, purple sulphur bacterium). Photosynthetic ETR rates were very high compared to the laboratory-grown cultures and the optimum irradiance was also high as would be expected from photosynthetic organisms

growing in full sunlight: Control, E_{opt} = 429 ± 52 µmol quanta m^{-2} s^{-1}; ETR_{max} = 2143 ± 143 µmol e^- g^{-1} Chl *a* s^{-1}; + DCMU, E_{opt} = 590 ± 41 µmol quanta m^{-2} s^{-1}; ETR_{max} = 1048 ± 34.5 µmol e^- g^{-1} BChl *a* s^{-1}.

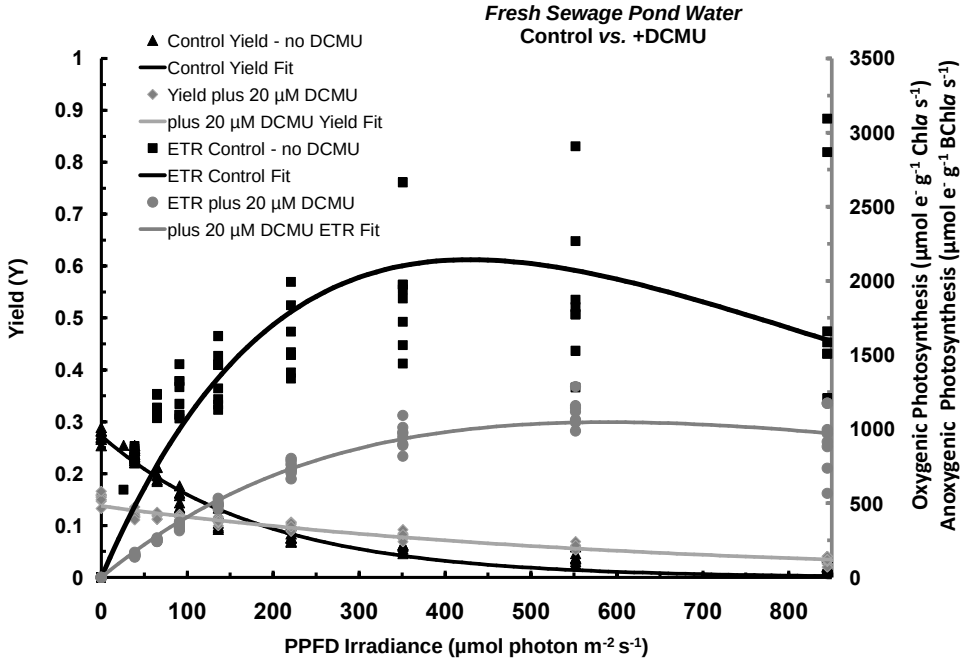


Figure 13 are plots of Yield and ETR of a freshly collected sewage pondwater sample from the Phuket Waste Treatment plant. Yield and ETR for Control cells and for cells incubated 1h in 20 µM DCMU presented in a XYY graph format. 2 ml of the sewage pond water had 0.268 ± 0.0067 µg/ml Chl *a* and 1.408 ± 0.075 µg/ml BChl *a* and hence 2.28 ± 0.062 mg Chl *a*/m^2 and 13.03 ± 0.696 mg Chl *a*/m^2 on the glass fibre disks. The cells suspension was dominated by BChl *a* (BChl *a*/Chl *a* = 5.25 ± 0.28). Photosynthesis by the photosynthetic bacteria was detectable as Yield in the presence of DCMU and *both* oxygenic and anoxygenic photosynthesis could be measured in the sewage pond water (unlike the synthetic sewage example in Figure 12) because the Chl *a* content was low.

4.5 Spectrophotometric scans

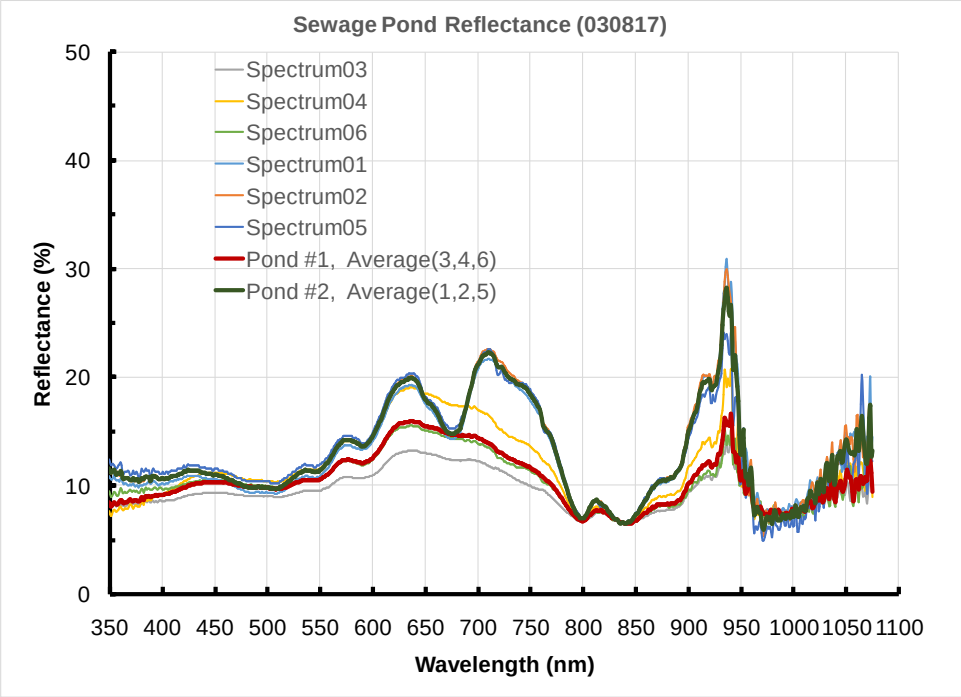


Figure 14 shows the means of three spectrophotometric scans made on the sewage ponds #1 and #2 at the Phuket waste treatment plant on the 03 August 2017 collecting trip using the ASC FieldSpec Handheld 2 Spectroradiometer. In pond #1 the spectral signature of BChl *a* is evident but there was almost no sign of Chl *a*. This is consistent with the Chl *a* and BChl *a* content of the freshly collected wastewater (BChl *a*/Chl *a* = 5.25 ± 0.28). Chl *a* containing organisms were much more apparent in pond #2 and the scan was comparable to the *Chlorella* / *Rhodopseudomonas* mixture shown in Figure 11.

DISCUSSION

Figures 7 to 12 show that it is difficult to distinguish oxygenic from anoxygenic photosynthesis using a blue-diode based PAM machine when both types of photosynthetic organisms are present. Figures 7 and 8 show that photosynthesis in *Chlorella* (Y and ETR) are completely inhibited by 20 mmol m^{-3} DCMU which is a very specific PSII inhibitor. However, the Fm' fluorescence of Chl *a* is very high in both the presence and absence of DCMU (Table 4.1: Fm' control, 1915 ± 89.3 (8); +20 mmol m^{-3} DCMU, 1387 ± 67.9). Figures 9 and 10 show that DCMU had no effect of Y or ETR of the two anoxygenic photosynthetic bacteria used in the present study, *Rhodopseudomonas palustris* (purple non-sulphur bacterium) and *Thermochromatium tepidum* (purple sulphur bacterium). A critical difference between the PAM results for *Chlorella* compared to *Rhodopseudomonas* and *Thermochromatium* was that the fluorescence of BChl *a* was very low compared to that of *Chlorella* above (Fm' *Rhodopseudomonas*: control, 93.3 ± 3.25 (8); +20 mmol m^{-3} DCMU, 93 ± 7.70 (8); *Thermochromatium*: control, 93.4 ± 4.53 (8); +20 mmol m^{-3} DCMU, 88.3 ± 3.76 (8)) (Table 4.1). The Y and ETR curves of anoxygenic photosynthetic bacteria look much the same as those of oxygenic photosynthetic organisms (Figures 7 – 10, this study; Ritchie, 2008; Ritchie, 2013; Ritchie and Larkum, 2013; Ritchie and Runcie, 2013; Ritchie and Mekjinda, 2015). Lack of sensitivity to DCMU of photosynthetic electron transport measured using a PAM machine is clear evidence of anoxygenic photosynthesis by RC-2 type photosynthetic bacteria.

From such results it might appear that in a mixture of oxygenic and anoxygenic photosynthetic organisms distinguishing the two type of photosynthesis would be a simple matter of comparing Y and ETR in the presence and absence of DCMU. The Y and ETR of the DCMU-poisoned cells would give an estimate of anoxygenic photosynthesis and the oxygenic component of the Y and ETR observed in the control mixture of cells could be deduced. Unfortunately, this was not found to be possible. Y and ETR of separate *Chlorella* and *Rhodopseudomonas* cell suspensions could be measured easily (Figures 8 and 9) and the oxygenic photosynthesis of *Chlorella* was sensitive to DCMU whereas that of *Rhodopseudomonas* was not. But in the case of a 1:1 mixture of the two types of cells (Figure 11) the apparent Y and ETR was completely inhibited by DCMU as if no *Rhodopseudomonas* was present (Figures 12 and 13, analyses in Table 4.1).

The reason for this was that the fluorescence of Chl *a* in both the presence and absence of DCMU overwhelms BChl *a* fluorescence (Figure 12 and 13, analyses in Table 4.1).

CHAPTER 5

CONCLUSIONS

5.1 Conclusions

Photosynthetic Bacteria are ubiquitous in fresh and marine water, soil, wastewater, and activated sludge. They are anaerobically photoautotrophic and photoheterotrophic in the light and aerobically chemoheterotrophic in the dark, so they can use a broad range of organic compounds as carbon and energy sources (Larimer *et al.,* 2004).

For swine wastewater treatment (Kim *et al.,* 2004) and are microbiologically they are similar to organic carbon overloaded red-water sewage ponds (Belila *et al.,* 2013). In this study, purple, non-sulfur bacteria degrading organic acids were isolated from eutrophic ponds and identified.

Rhodopseudomonas palustris and *Chlorella* was grown routinely in PM medium with acetate and benzoate as carbon sources and thiosulphate as electron source but grew successfully on wastewater from the Leachate ponds. This leachate pond wastewater provides a source of organic carbon, useable electron sources, vitamins and minerals for growth. Thus, growing *Rhodopseudomonas palustris* on wastewater from the Leachate sewage ponds in a simple bioreactor is a viable proposition.

A typical sewage pond contains both aerobic and anaerobic organism either living together or stratified along a redox gradient (Chandaravithoon *et al.,* 2018). Sewage ponds are not the only habitat where substantial anoxygenic photosynthesis occurs in the presence of oxygenic photosynthesis and more effort is needed in developing methods to quantitatively measure it. Common examples are mudflats and microbial mats and stromatolite communities (Hubas *et al.*, 2011; Papineau *et al.,* 2005; Díez *et al.,* 2007). This study has shown that it is possible to estimate oxygenic and anoxygenic photosynthesis in field-collected material under certain circumstances

using DCMU as a selective inhibitor of PSII electron flow. It has also shown its limitations: fluorescence in the presence of DCMU in oxygenic photosynthesis is still very high whereas fluorescence by BChl *a* is low and tends to be drowned out by that of Chl *a* in a field sample containing both types of photosynthetic organisms.

Sewage ponds are not the only habitat where substantial anoxygenic photosynthesis occurs in the presence of oxygenic photosynthesis. Knowing how much bacteriochlorophyll is present in a habitat has important consequences for understanding the nitrogen economy of the habitat because anoxygenic photobacteria use their photosynthetic electron transport to drive production of H_2 and also nitrogen fixation (Larimer *et al.,* 2004; Falkowski and Raven, 2007).

In the present study a simple blue-diode Junior PAM machine was mainly used (Chandaravithoon *et al.,* 2018). The blue light ($\approx$ 465 nm) can be used by both photosynthetic bacteria and oxygenic photosynthesis. The junior PAM can detect fluorescence from both Chl *a* and BChl *a* because a simple high pass filter (> 700 nm) is used to block reflected blue light from the detector diode of the machine.

Gitelson *et al.* (1997, 1999) proposed that remote sensing could be used to monitor sewage ponds. The absorptance profiles of the sewage ponds used in their study closely resembled the scans of the sewage pond water in the present study in the lab and the relectance due to Chl *a* and BChl *a* was readily identifiable on field spectroradiometric scans (Chandarayithoon *et al.,* 2018).

The contribution of anoxygenic photobacteria to global photosynthesis is not clear and only rough estimates of the magnitude of anoxygenic photosynthesis are available (Blankenship *et al.,* 1995; Falkowski and Raven, 2007). As pointed out by Ritchie (2018) substantial activity of non-oxygenic photosynthetic organisms is often unsuspected and so bacteriochlorophyll is not even specifically looked for. Another reason why their importance is largely unsuspected is that contrary to the impression gained from microbiology textbooks many photosynthetic bacteria tolerate aerobic or microaerobic conditions to a much greater degree than is usually suspected (Siefert *et al.,* 1978; Blankenship *et al.,* 1995; Kim and Lee, 2000; Kolber *et al.,* 2000; Kim *et al.,* 2004; Hubas *et al.,* 2011; Ritchie, 2013; Ritchie and Mekjinda, 2015; Ritchie and Runcie, 2013; Chandaravithoon *et al.,* 2018; Chandaravithoon, Ritchie and Runcie, 2019). Anoxic conditions are needed for the synthesis of BChl but RC-2 will function in the presence of

oxygen (Blankenship *et al.,* 1995; Ritchie, 2013; Ritchie and Runcie, 2013; Ritchie and Mekjinda, 2015). It is sometimes incorrectly assumed that although photosynthetic bacteria are present, they are not performing photosynthesis because O_2 is present (Chandaravithoon *et al.,* 2018). BChl a synthesis is inhibited by O2 but the photosynthetic mechanism of RC-2 type photosynthetic bacteria will function under aerobic conditions, at least in purple sulphur and purple non-sulphur bacteria (Ritchie, 2013; Ritchie and Mekjinda, 2015; Ritchie and Runcie, 2013; Chandaravithoon *et al.,* 2018). In addition, there is a whole class of photosynthetic bacteria that are obligate aerobes but nevertheless have a functional RC-2 type photosynthetic system that are known as Aerobic Phototrophic Bacteria (APB) (Yurkov and Beatty, 1998; Koblizek, 2015). These APB organisms could well have been present in the Phuket Sewage Ponds but were not specifically looked for in the present study and the culture conditions used for isolating the *Chlorella* and the *Rhodopseudomonas* from the sewage pond might not have been favourable for them to grow well. The photosynthetic electron transport rate of APB bacteria does not appear to have been measured experimentally.

Commonly used oxygen-based techniques for measuring photosynthesis such as light/dark bottle methods and oxygen electrodes will not detect photosynthetic activity of photosynthetic bacteria although it will detect their aerobic metabolism in the presence of oxygen as most are facultatively aerobic. The importance of anoxygenic photosynthetic bacteria in ecosystems is often missed. Both Chl *a* and BChl *a* absorb blue light and so a blue-light diode is needed to detect anoxygenic photosynthesis using PAM methods.Kolber *et al.* (2000) used a blue-diode based FRRF technique to estimate photosynthetic electron flow from variable fluorescence in oceanic phytoplankton and estimated that anoxygenic photosynthesis was equivalent to about 2-5% of oxygenic photosynthesis in open ocean environments, a conclusion also drawn independently by Goericke (2002) based on the abundance of BChl *a*. Much of these oceanic photosynthetic bacterium are APB-type bacteria. Ritchie and Runcie (2013) found that a blue-PAM could be used to measure photosynthetic electron transport in the marine photosynthetic bacterium *Afifella marina* by accident and later confirmed it using the classic non-sulphur photosynthetic bacterium *Rhodopseudomonas palustris* (Ritchie, 2013) and the purple sulphur bacterium *Thermochromatium tepidum*(RitchieandMekjinda,2015).

Oxygenic (oxygen-producing) photosynthetic algae and photosynthetic bacteria live together in the sewage ponds: this can be readily demonstrated spectrophotometrically

(Chandaravithoon *et al.,* 2018; Ritchie 2018). The photosynthetic organisms are living photoheterotrophically: they are using photosynthetic energy to power the take up and metabolism of organic compounds from the sewage does not fix CO_2. Photosynthesis by the green algae can be measured using a PAM machine but PAM machines do not measure respiration. Oxygenic photosynthesis is easily measured using the PAM and water-based photosynthetic electron transport is completely inhibited by DCMU. Unfortunately, because the florescence of Chl *a* is so high with an ordinary blue-PAM it is not possible to distinguish oxygenic and anoxygenic photosynthesis in the sewage pond although you can measure oxygenic photosynthesis in green alga isolated from a sewage pond and anoxygenic photosynthesis from pure cultures of the photosynthetic bacteria isolated from the pond. Research on how to resolve oxygenic and antioxygenic photosynthesis are ongoing and a combination of better fluorescence spectral resolution and DCMU treatment protocols have been successful in separately estimating oxygenic and anoxygenic photosynthesis in cell suspensions containing both types of organisms (Chandaravithoon *et al.,* 2019 and Chandaravithoon *et al.,* 2019, unpublished).

When we have developed a means of measuring oxygenic and anoxygenic photosynthesis in complex mixtures of photosynthetic organisms, the methods developed here can be applied to hypereutrophic heavily polluted water "black water" and the ecologically very interesting microbial mat communities (Hubas *et al.,* 2011; Papineau *et al.,* 2005; Díez *et al.,* 2007).

5.2 Suggestions for future work

5.2.1 Logistics and funding restricted the scope of the present study and a more complete collection of data would facilitate better statistical analysis. Sampling protocols need to be more rigorous than used in the present study to achieve more homogeneous and representative samples. The results of this study show the samples from sewage ponds have high densities of *Chlorella and Rhodopseudomonas palustris* and includes hazardous material

5.2.2 The Millipore and glass fibre filters should be especially carefully selected because the different sizes of the cells in the leachate can lead to biased results and the cell density makes the filling process very slow.

5.2.3 A Spectroradiometer was used to investigate the spectral properties of the sewage ponds (Chandaravithoon *et al.,* 2018). We recognised the spectrum of green algae and photosynthetic bacteria quite easily. This means the ponds could be monitored by satellite imaging. But It will be good when used in conjunction with the laboratory results.

5.2.4 The PAM experiments did show the toxicity of the leachates. The results confirmed that the toxicity of the leachates was an immediate effect and did not show a cumulative effect.

5.2.5 Results so far on developing PAM machines that can specifically measure oxygenic and anoxygenic photosynthesis without interferences from the other type of photosynthesis are very promising. Effective spectral separation is under further development ((Chandaravithoon *et al.,* 2019 and Chandaravithoon *et al., 2019*, unpublished).

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