Sustainable Production of Biofuel from Microalgae Grown in Wastewater

Thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

2014

Olumayowa Osundeko
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Abstract

Algae have been the centre of recent research as a sustainable feedstock for fuel because of their higher oil yield in comparison to other plant sources. However, algae biofuel still performs poorly from an economic and environmental perspective due to the high reliance on freshwater and nutrients for cultivation, among other challenges. The use of wastewater has been suggested as a sustainable way of overcoming these challenges because wastewater can provide a source of water and nutrients for the algae. Moreover, the ability of the algae to remove contaminants from wastewater also enhances the total economic output from the cultivation. However, the success of this strategy still depends greatly on efficient strain selection, cultivation and harvesting. Therefore, this PhD thesis has focussed on strain isolation, characterisation, optimisation and cultivation in open pond systems. Five algae strains were isolated from wastewater treatment tanks at a municipal water treatment plant in North West England. The isolated strains were morphologically and genetically characterised as green single-celled microalgae: *Chlamydomonas debaryana*, *Hindakia tetrachotoma*, *Chlorella luteoviridis*, *Parachlorella hussii* and *Desmodesmus subspicatus*. An initial screening of these strains concluded that *C. luteoviridis* and *P. hussii* were outstanding in all comparisons and better than some of the strains previously reported in the literature. Further tests carried out to elucidate the underlying tolerance mechanisms possessed by these strains were based on stress tolerance and acclimation hypotheses. In the following experiments, *C. luteoviridis* and *P. hussii* were found to have higher anti-oxidant enzyme activity that helps in scavenging reactive oxygen species produced as a result of exposure to wastewater. This result provides a new argument for screening microalgal strains for wastewater cultivation on the basis of anti-oxidant activity. In addition, the two strains could grow heterotrophically and are better adapted to nutrient deficiency stress than the other three isolates. In order to understand the role of microalgae acclimation in wastewater cultivation, strains identical or equivalent to the wastewater treatment tank isolates were obtained from an algae culture collection. These strains had not been previously exposed to wastewater secondary effluent. The initial growth of these strains in wastewater secondary effluent was very poor. However, after two months of acclimation to increasing concentrations of secondary wastewater effluent, it was observed that growth, biomass and lipid productivities of most of the strains were significantly improved, although still not as high as the indigenous strains. Therefore, it was concluded that continuous acclimation is an additional factor to the successful growth of algae in wastewater. Furthermore, addition of 25% activated sludge centrate liquor to the secondary effluent was found to increase algal growth and biomass productivity significantly. Further tests to examine the continuous cultivation of *C. luteoviridis* and *P. hussii* in wastewater showed that a biomass productivity of 1.78 and 1.83 g L$^{-1}$ d$^{-1}$ can be achieved on a continual basis. Finally, the capability of *C. luteoviridis* and *P. hussii* for full seasonal cultivation in a 150 L open pond in a temperate climate was studied, using the optimised secondary wastewater +25% liquor medium. Each strain was capable of growth all year including in autumn and winter but with strongest growth, productivity and remediation characteristics in the summer and spring. They could maintain monoculture growth with no significant contamination or culture crash, demonstrating the robustness of these strains for wastewater cultivation in a northern European climate.
Declaration

No part of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of the University of Manchester or any other university or other institute of learning.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ACCase</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ACP</td>
<td>Acyl carrier protein</td>
</tr>
<tr>
<td>APX</td>
<td>Ascorbate peroxidise</td>
</tr>
<tr>
<td>BOD</td>
<td>Biochemical oxygen demand</td>
</tr>
<tr>
<td>BODIPY 505/515</td>
<td>4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacen</td>
</tr>
<tr>
<td>CCAP</td>
<td>Culture Collection of Algae and Protozoa</td>
</tr>
<tr>
<td>CN</td>
<td>Cetane number</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical oxygen demand</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacyl glycerol</td>
</tr>
<tr>
<td>DAGAT</td>
<td>Diacylglycerol acyltransferase</td>
</tr>
<tr>
<td>DCFH-DA</td>
<td>2′,7′-dichlorofluorescein diacetate</td>
</tr>
<tr>
<td>DGDG</td>
<td>Digalactosyldiacylglycerol</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty acid methyl ester</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>G-3-P</td>
<td>Glycerol-3-phosphate</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography–mass spectrometry</td>
</tr>
<tr>
<td>GHG</td>
<td>Green house gases</td>
</tr>
<tr>
<td>GPAT</td>
<td>Glycerol-3-phosphate acyltransferase</td>
</tr>
<tr>
<td>IV</td>
<td>Iodine value</td>
</tr>
<tr>
<td>KASIII</td>
<td>3-ketoacyl ACP synthase III</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography–mass spectrometry</td>
</tr>
<tr>
<td>LPAT</td>
<td>Lysophosphatidyl acyltransferases</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>N</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>OD&lt;sub&gt;680&lt;/sub&gt;</td>
<td>Optical density at 680nm</td>
</tr>
<tr>
<td>P</td>
<td>Phosphorus</td>
</tr>
<tr>
<td>PAP</td>
<td>Phosphatidic acid phosphatise</td>
</tr>
<tr>
<td>PUFA</td>
<td>Poly unsaturated fatty acid</td>
</tr>
<tr>
<td>R&amp;D</td>
<td>Research and development</td>
</tr>
<tr>
<td>RMWSE</td>
<td>Raw municipal wastewater secondary effluent</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SV</td>
<td>Saponification value</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TAP</td>
<td>Tris-Acetate-Phosphate</td>
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</table>
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This thesis is dedicated to the memory of my late parents Mr Jacob Osundeko and Mrs Alice Osundeko. Although you departed this world while I was so young, but your memories are forever alive with me.
This introductory chapter comprises of two sections. The first section provides a general knowledge of microalgae, its lipid biosynthesis and relevance to biofuel research. The second section provides a review on algae cultivation in wastewater, and has been accepted for publication as a book chapter (Olumayowa Osundeko, Amit K. Bajhaiya, Jon K. Pittman, M. R. Suseela (in press) Promises and challenges of growing microalgae in wastewater for biofuel production. In: Biotechnological Applications for Environmental Protection, P. C. Abhilash (Editor), published by Springer Books). My contribution in this review was 95%. Amit K. Bajhaiya contributed in the introductory part and the review was edited by Jon K. Pittman and M. R. Suseela.
General introduction

1.1 Introduction

Currently, the world depends mainly on petroleum as a source of fuel. The increasing demand for energy has led to a price spike of petroleum oil coupled to increasing emission of green house gases (GHG). Recent study has found that the peak fossil fuel risk will start to be critical by 2020 (Leggett and Ball, 2012). Therefore, there is increasing global interest for sustainable biofuel production in order to mitigate GHG emissions and reduce reliance on fossil fuels.

Biofuel, which refers to solid, liquid or gaseous fuel that is produced from biomass (Berktay and Nas, 2006; Chhetri and Islam, 2008), has been in existence long before the discovery of fossil fuel. Recent awareness about fossil oil depletion and its contribution to GHG emissions has heightened interest in alternative forms of energy including biofuel. Biofuel can be produced from plant oil, animal oil, sugar or starch and food waste (Berktay and Nas, 2006; Christian et al., 2007; Demirbas, 2007; Chhetri and Islam, 2008; Mata et al., 2010).

Many sources of biofuel feedstock like wheat, corn, sugarcane, sugar beets or molasses have been criticised because of their competition with food crops for agricultural land, water and nutrients (Doornbosch and Steenblik, 2007; Chisti, 2007, 2008; Duer and Ovre, 2010; Ewing and Msangi, 2009; Hoekman, 2009; Subhadra, 2010). The use of some non-food crops like Buffalo Grass (*Buchloe dactyloides*), *Jatropha curcas* and *Switchgrass* (Sujatha et al., 2008; Robins, 2010) for biofuel production has also been examined, but these may still not fully solve the issue of not using agricultural resources. Recent advancements in biofuel feedstock
have been focusing on fast-growing microalgae, enzymatic hydrolysis of forest waste, thermal depolymerization of organic waste to form “biocrude”, and direct biological synthesis of more complex biofuels (Chisti, 2007; Subhadra, 2010; Chandra et al., 2012).

Microalgae have been proposed for a long time as a potential renewable fuel source (Benemann et al., 1977). They constitute an efficient system that utilizes solar energy to synthesize products of high value such as lipids and starch (Li et al., 2008a; Khan et al., 2009). Biofuel products from microalgae include biodiesel, biomethane and bioethanol which can be obtained by transesterification, anaerobic digestion and fermentation respectively. Microalgae have the potential to generate greater quantities of oil suitable for biofuel production than most known plant-based feedstocks, they require less cultivated land area and do not have direct impact on food supplies (Chisti, 2007). Over the last 50 years, there have been continuous efforts to examine the possibility of extracting important products of high economic value from microalgae (Metting and Pyne, 1986; Murakami et al., 1988; Mata et al., 2010). The use of microalgae for generating renewable energy provoked heightened interest during the energy crisis in the 1970s (Spolaore et al., 2006). In 1978, the USA National Renewable Energy Laboratory through the Aquatic Species Program, launched a specific R&D programme which lasted up to 1996, dedicated to finding alternative renewable fuels, including biofuel from microalgae (Sheehan et al., 1998). The recommendation from this research was that the use of microalgae for the low-cost production of biofuel is technically feasible especially when combined with wastewater treatment strategy, but still needs considerable long term R&D to achieve commercial-scale production.
1.2 Algae

Algae are a versatile and ubiquitous group of photosynthetic organisms ranging from unicellular to multicellular forms. They are naturally found in the ocean, freshwater bodies, on rock, soils and on vegetation (Chen, 2008). They are oxygenic and possess significant diversity in terms of morphological, cytological, molecular and reproductive characteristics. Algae are broadly divided into two groups; macroalgae and microalgae. The macroalgae include the large, multi-cellular seaweeds and they are generally classified into three groups: Green (Chlorophyta), Red (Rhodophyta), and Brown-Kelps (Phaeophyta - related to Chromista) (Fitzgerald et al., 2011). On the other hand, microalgae are small (+/- 1 to 50 μm), unicellular algae, which normally grow in marine or fresh water bodies, but are also found on soil or vegetation (Aresta et al., 2005).

Microalgae include prokaryotic cyanobacteria and eukaryotic algae. Their simple cellular structure makes them efficient converters of solar energy; they grow photoautotrophically and produce approximately half of the global atmospheric oxygen by fixing carbon dioxide. Biologists have categorized microalgae into different classes, mainly on the basis of their pigmentation, life cycle and basic cellular structures. Most microalgae belong to the Archaeplastida group in the phylogeny of major eukaryotes (Fig. 1). Archaeplastida group comprise of photosynthetic eukaryotes including Rhodophyta, Glaucophyta and Chloroplastida (green algae and land plants) (Baldauf, 2008). The four important forms of microalgae are blue-green algae (Cyanophyceae), green algae (Chlorophyceae), diatoms (Bacillariophyceae), and golden algae (Chrysophyceae) (Baldauf, 2008).
These tiny biological factories harness nature’s energy using photosynthesis and can double their biomass every few hours during their exponential growth period (Chisti, 2007 and Louime et al., 2012). The fact that they can grow so quickly and produce valuable biomass makes them a promising crop for human use.

General characteristics of microalgae include fast growth and high doubling rate and a capability of accumulating lipid up to 50% of their biomass (Chisti, 2007 and Metting, 1996). Calculations made based on the theoretical potential of microalgae for oil production shows that 100 times more oil per unit area of land can be
achieved than using soybean, which is currently in use for biodiesel production in USA (Chisti, 2007 and Hu et al., 2008). Photosynthetic carbon can be converted into large amounts of lipids in many microalgal species; for example, some microalgae can generate up to 50% oil by weight, giving an estimated yield per unit area of oil between 5,000 to 20,000 gallons per acre, per year; which is 7 to 31 times greater than the next best crop like palm oil (Zhu and Lee, 1997; Chisti, 2007; Wang et al., 2008; Demirbas, 2009). Microalgae are known to be very adaptive and can be grown in salt-water, freshwater or even on contaminated industrial effluents / wastewater without any extra requirements for nutrients or prime agricultural land.

1.3 Biosynthesis of lipids in microalgae

The understanding of lipid biosynthesis in algae is still fairly fragmentary (Merchant et al., 2012) unlike for higher plants (Harwood, 1998) and bacteria (Rock and Jackowski, 2002), but knowledge of lipid metabolism in species like Chlamydomonas reinhardtii with sequenced genomes is growing quickly (La Russa et al., 2012; Nguyen et al., 2013). Based on sequence homology of genes that are involved in lipid metabolism between algae and higher plants such as Arabidopsis (Hu et al., 2008), it is generally assumed that algae has an equivalent lipid metabolic pathway as that of higher plants (Blatti et al., 2013). The main carbon donor for fatty acid biosynthesis is from acetyl-CoA which is a product of multiple pathways including glycolysis. In general, fatty acid biosynthesis utilizes acetyl-CoA and malonyl-ACP as starting substrates and acetyl unit donors (Baba and Shiraiwa, 2013). The first step in the biosynthesis of fatty acid is the irreversible formation of malonyl-CoA from acetyl-CoA by acetyl-CoA carboxylase (ACCase). There are two stages involved in this reaction: the first is the transfer of CO₂ (HCO₃⁻) by the biotin
carboxylase portion of ACCase to a biotin prosthetic group that is attached to the E-amino group of a lysine residue. The second reaction involves the transfer of activated CO\textsubscript{2} from biotin to acetyl-CoA. ACCase is considered as the main enzyme that catalyses the first reaction step in lipid biosynthesis and is thought to be important in fatty acid synthesis regulation. It has three main functional sites that promote the formation of malonyl–CoA, the main carbon donor for fatty acid biosynthesis.

**Fig. 2.** The reactions of saturated fatty acid biosynthesis. Acetyl-CoA is the basic building block of the fatty acid chain and enters the pathway both as a substrate for acetyl-CoA carboxylase (ACCase; reaction 1) and as a primer for the initial condensation reaction (reaction 3). Reaction 2, catalyzed by malonyl-CoA:ACP transacylase, transfers malonyl from CoA to form malonyl-ACP, which is the carbon donor for all subsequent elongation reactions. After each condensation, the 3-ketoacyl-ACP product is reduced (reaction 4), dehydrated (reaction 5), and reduced again (reaction 6), by 3-ketoacylACP reductase, 3-hydroxyacyl-ACP dehydrase, and enoyl-ACP reductase, respectively (Adapted from Hu et al., 2008)
The malonyl group is first transferred from CoA to a protein cofactor called acyl carrier protein (ACP), which is then involved in the whole process up to the formation of a 16-18-carbon product ready to be transferred to glycerolipids or exported from the plastid, as shown in Figure 2.

The next step after the formation of malonyl-ACP is a condensation reaction. At this stage, the malonyl group of malonyl-ACP undergoes a series of condensational reactions with acetyl-CoA releasing CO₂ that helps to drive the reaction forward. As described by Hu et al. (2008), at least three groups of condensing enzyme (commonly called 3-ketoacyl-ACP synthases) are involved in the synthesis of 18-carbon fatty acids. The initial condensational reaction leads to the formation of a four carbon product, 3-ketoacyl-ACP, catalysed by 3-ketoacyl ACP synthase III (KASIII). Other condensational enzymes are KASI which is thought to be involved in the production of 6-16 carbon chain products and KASII that is required for the elongation of 16-carbon ACP to stearoyl-ACP. This process is followed by a reduction process where 3-ketoacyl-ACP is reduced at the carbonyl group by the enzyme 3-ketoacyl-ACP reductase and then dehydration by the enzyme called hydroxyacyl-ACP dehydratase. The reaction cycle is completed by reduction reaction catalysed by enoyl-ACP reductase. Each cycle of these four reaction steps lengthen the fatty acid precursor chain by 2-carbons while still attached to ACP as a thioester (a process known as elongation) leading to the formation of saturated 16:0- and 18:0-ACP.

Desaturation of fatty acid is form when a double bond is introduced by the enzyme ACP desaturase. Elongation of a fatty acid up to 18-carbon fatty acid takes place in the plastid and continues until the acyl group is removed from ACP either as a result
of hydrolysis of acyl-ACP releasing free fatty acid, or by the transfer of fatty acid from ACP to glycerol-3-phosphate or to monoacylglycerol-3-phosphate by one of the two acyltransferases in the plastid. Further elongation is thought to occur by an elongase complex at the endoplasmic reticulum (ER) membrane (Kunst and Samuels, 2009). Poly unsaturated fatty acids (PUFA) are elongated fatty acids commonly found in algae (e.g. Arachidonic acid, Eicosapentaenoic acid, and Docosahexaenoic acid). PUFA elongases are known to be structurally similar to the ELO family of enzymes that catalyze the condensation step of fatty acid elongation in animals and fungi (Tonont et al 2005; Khozin-Goldberg and Cohen 2011).

1.3.1 Biosynthesis of triacylglycerol

Like other higher plants and animals, microalgae are capable of biosynthesising the glycerolipid triacylglycerol (TAG) as a stored form of energy. Usually, glycerol-3-phosphate (G-3-P) and acyl-CoA are two major primers in the biosynthesis of triglycerides (Huang et al., 2010). The acyl-CoA is generated in the plastid from malonyl-CoA by the activity of fatty acid synthase (FAS). There are two pathways for glycerolipid synthesis in photosynthetic organisms; prokaryotic and eukaryotic pathways. The prokaryotic pathway, otherwise known as the plastid pathway, involves conversion of free FAs to glycerolipids such as phosphatidic acid and diacyl glycerol (DAG). These are often precursors for membrane and signalling lipids. The eukaryotic pathway involves conversion of DAG to TAG. The plastid DAG that is converted to TAG is stored in lipid droplets in both the chloroplast and the cytosol (Fan et al., 2011).

The initial step in the biosynthesis of TAG is two acylation reactions that transfer fatty acid to G-3-P to form phosphatidic acid catalysed by glycerol-3-phosphate
acyltransferase (GPAT) and lysophosphatidyl acyltransferases (LPAT) (Fig. 3). Activity of phosphatidic acid phosphatase (PAP) produces diacetylglycerol (DAG). Synthesis of TAG is completed by transferring a fatty acid to the vacant position 3 of DAG in a unique enzymatic reaction catalysed by diacetylglycerol acyltransferase (DAGAT). The acyltransferases involved in TAG synthesis may exhibit preferences for specific acyl-CoA molecules which could be an important factor in determining the acyl composition of TAG (Roessler et al., 1994). Algae are capable of accumulating significant quantities of TAG and most are composed of saturated or monounsaturated C_{16}-C_{20} fatty (Harwood, 1998).

![Fig. 3. TAG metabolism in algae. Acetyl-CoA carboxylase generates the substrate for fatty acid synthesis. Acetyl-CoA is carboxylated to malonyl-CoA regulated by ACCase which formed the base for acyl-CoA synthesis by enzyme FAS. The synthesis of DAG from glycerol-3-phosphate and acyl-CoA involved two acyltransferases and a phosphatase. Subsequence acetylation of DAG through enzyme DAGAT formed TAG.](image-url)
Information based on bioinformatics analyses have indicated that regulatory pathways of lipid biosynthesis and import of fatty acids to the ER for synthesis of glycerolipid are relatively similar to those in higher plants (Riekhof et al., 2005; Moellering and Benning, 2010). These regulatory pathways could be a target for engineering high lipid biosynthesis in algae. Modification of fatty acid chain length and saturation levels to produce monounsaturated and polyunsaturated TAG, the ideal form for biodiesel, can be further achieved via expression of thioesterases and desaturases (Khozin-Goldberg and Cohen, 2011).

Although, lipid biosynthesis in algae has been assumed to share a similar pathway to that of higher plants, recent studies have highlighted some differences. For example, a study has shown that in addition to TAG assembly in the ER like in higher plants, *Chlamydomonas reinhardtii* also has the ability to assemble TAG in the chloroplast (Fan et al., 2011). The mechanism underlying TAG accumulation in algae is now of a great interest towards improving algae biofuel technology.

1.3.2 *Lipid quantification in microalgae*

One of the important aspects of algae biofuel research is the ability to rapidly measure lipid content of microalgae species. The conventional methods of lipid detection in microalgae include solvent extraction and gravimetric method (Bligh and Dyer 1959; Folch et al., 1957) and chromatographic-based methods (Krank et al., 2007). Lipids can be quantified directly by liquid chromatography-mass spectrometry (LC-MS) or following transesterification by gas chromatography–mass spectrometry (GC-MS) (Indarti et al., 2005). They can accurately determine absolute concentrations of total lipids and specific characteristics of fatty acids. A major drawback of these conventional methods can be that they are time consuming and
expensive, making it difficult to screen large numbers of microalgae (Chen et al., 2009). Another frequently used method is the use of Nile Red, a lipid-soluble fluorescent dye which has been used to measure intracellular lipid content of microalgae (Cooksey et al., 1987; Dean et al., 2010). However, accuracy of Nile Red methods can be adversely affected by the fact that it cannot stain dead microalgae cells (Elsey et al., 2007) and the accumulation of Nile Red varies amongst microalgae species making it difficult to rely on as a sole quantification method for lipid.

In addition to the Nile red dye, another dye used for broad detection of intracellular lipid in algae is 4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacen (BODIPY 505/515). BODIPY 505/515 is a lipophilic dye that produces a green fluorescence signal and has been used for detection of lipid in algae (Govender et al., 2012). BODIPY 505/515 has been evaluated as a potential substitute for Nile Red and has been reported to be more effective for vital staining of intracellular lipid bodies and single cell sorting than Nile Red (De la Hoz Siegler et al., 2012; Velmurugan et al., 2013). Whilst Nile red and BODIPY 505/515 are good for rapid semi-quantitative detection of lipid in algae, however, they are best when combined with other quantitative methods such as mass spectrometry, gravimetry and Fourier transform infrared (FTIR) spectroscopy (Dean et al., 2010; Velmurugan et al., 2013).

1.3.3 Optimising lipid production in algae

The economic attraction of algal biofuel, especially for biodiesel production, is likely to be favoured by increasing lipid content in an algal cell. There have been continual research efforts in physical and genetic modification of algae to achieve higher lipid productivity. However, lipid accumulation in algae is often associated with a
reduction in biomass productivity (Rodolfi et al., 2009). Balancing growth, biomass productivity and high lipid productivity in algae remains a key challenge to the field.

1.3.3.1 Optimisation of lipid biosynthesis in algae by nutrient stresses

Algal cell lipid content has been shown to increase when essential nutrients are completely removed or reduced below stoichiometric requirements for growth, or if an environmental stress condition is imposed (Griffiths and Harrison, 2009; Courchesne et al., 2009; Dean et al., 2010, Sharma et al., 2012, Adams et al., 2013; Bartley et al., 2013). Examples of such stress conditions are nutrient starvation for nitrogen (N), phosphorus (P), silicon, and citrate, and changes to the physical condition of the culture medium such as temperature, pH, and salinity. Increase in lipid content of algae as a result of nutrient stress may not be economically advantageous for liquid fuel production from mass-cultured algae if the conditions that induce lipid accumulation also result in a significant drop in total biomass, and thus in total lipid productivity. For most nutrient stresses, photosynthesis is down-regulated and carbon is reallocated such that lipid and starch metabolism is increased (Courchesne, et al., 2009; Griffiths and Harrison, 2009; Khozin-Goldberg and Cohen, 2011; Merchant et al., 2012). Therefore elucidating the cellular physiological, metabolic changes and sensors that enable microalgae to perceive nutrient level in an environment during this stressed condition can help understand the mechanisms by which microalgae regulate this change.

A detailed review on the amount of lipid yield achieved when some stress conditions were applied in algae culture is discussed by Griffiths and Harrison (2009). A brief summary of some of this research is given below.
1.3.3.1.1 Nitrogen limitation stress

Nitrogen (N) is one of the essential nutrients for growth of algae and increase in its concentration in the growth medium can enhance or prolong cell growth (Silva et al., 2009). Research has shown that N-starvation significantly increases cell lipid content of algae (Griffiths and Harrison, 2009; Courchesne et al., 2009; Breuer et al., 2013; Simionato et al., 2013). *Chlorella* sp. cultivated in N-depleted medium showed increased lipid productivity significantly greater than when cultivated in non-stress medium (Praveenkumar et al., 2012). Microalgae accumulate large quantities of lipid in the form of TAG when nutrient deprived (Simionato et al., 2013). However, biomass productivity and cell growth decrease under such stress. This is due to inhibition of chlorophyll biosynthesis, downgrade of photosystem II, and thus reduction in photosynthetic activity, and reduction in cell division. A recent transcriptomic study by Miller et al. (2010) has provided more understanding to this and indicated that N deprivation activates a subset of control genes involved in gametogenesis while down-regulating protein biosynthesis. In addition, most of the genes for components of photosynthesis were also down-regulated.

1.3.3.1.2 Phosphorus limitation stress

P limitation, like N limitation, can also cause changes in fatty acid and lipid composition in algae. The total cellular lipid and TAG content of P starved *Chlorella vulgaris* cells were reported to increase (Jiang et al., 2011; Chia et al., 2013). Under P deprivation, the galactolipid digalactosyldiacylglycerol (DGDG) accumulates, compensating for the decrease in phosphatidylcholine caused by absent or low phosphate (Dormann and Benning, 2002; Hartel et al., 2000). DGDG over-accumulation is localized to the extraplastidic membrane just like
phosphatidylcholine (Khozin-Goldberg and Cohen, 2006). Courchesne et al. (2009) reported that decreasing P availability from 175 μM to 52.5, 17.5 and 0 μM (K₂HPO₄) resulted in increased cellular total lipid content of *Monodus subterraneus* (a freshwater alga), while the proportion of phospholipids was reduced from 8.3% to 1.4% of total lipids and the proportion of TAG increased from 6.5% up to 39.3% of total lipids. Similar findings were also reported in *C. reinhardtii* when grown in P depleted medium (Lind et al., 2004; Dean et al., 2010). However, like in N stress conditions, P limitation results in low cell biomass of the algae culture.

1.3.3.2 Other abiotic stresses

Some algae possess the ability to survive in extreme saline conditions and they are referred to as halophytic. Such species produce metabolites to protect their cell from salt injury and balance their osmotic pressure (Richmond, 2004). Algae differ in their ability to tolerate and adapt to saline conditions and some are notable salt tolerant species such as *Dunaliella* sp., particularly *Dunaliella salina*. Increased NaCl concentration from 0.5 to 1.0 M (equal to sea water) has been reported to cause increase in *D. salina* intracellular lipid content to 67% of its weight (Mutsumi et al., 2006). In another study, salt stress has been reported to induce TAG accumulation in freshwater *C. reinhardtii* (Siaut et al., 2011).

Excess carbon dioxide (CO₂) has also been reported to increase lipid biosynthesis in algae. Increase in CO₂ led to an increase in lipid productivity and composition in *C. vulgaris* (Widjaja et al., 2009). A shift in temperature has been reported to affect the lipid content of algae. For example, *Nannochloropsis oculata* lipid content doubled when cultivation temperature increased from 20°C to 25°C (Converti et al., 2009). In
the same report, a further temperature increase to 30°C increased *C. vulgaris* lipid content from 7.90 to 14.92%.

Although abiotic stresses have potential of increasing the lipid biosynthetic in microalgae, they may also slow down photosynthetic efficiency of microalgae and bring about a decrease in biomass and subsequently lower overall lipid productivity. Possible ways to ameliorate this is by carrying out a two-stage cultivation strategy where the algae will be grown in the nutrient rich medium (first stage) until the end of exponential growth phase where growth would have been established, and then transferred into a nutrient deficient medium (second stage). This has been proven to be good in improving the overall lipid productivity of algae in comparison to growing in nutrient deficient medium (Courchesne et al., 2009; Zhou et al., 2012; Fernandes et al., 2013).
1.4 Selection of microalgae for biofuel production

Successful algal biofuel biotechnology is likely to rely on choosing the right algal strain with relevant properties (Pulz and Gross, 2004). This is an important aspect in commercial production of algal biodiesel because it determines not only the amount of possible lipid that can be extracted but also the quality of the lipid and its suitability for fuel. Factors to consider in alga strain selection for mass culture include consistency in growth, resilience to biotic and abiotic stresses, community stability and resistance to predators present in its given habitat. Environmental conditions and cultivation methods also play important roles in determining the type of strain that can be cultivated. For example, in an open cultivation system where marine water has been used, the rate of evaporation can affect the salinity concentration and therefore could require some extreme halophilic strain (Griffiths and Harrison, 2009). Selection of fast-growing, productive strains, optimised for the local climatic conditions is of fundamental importance to the success of the mass cultivation of algae species. A high growth rate can reduce the contamination risk in a continuous culture system because it can out-compete a strain which grows slower (Griffiths and Harrison, 2009; Lundquist et al., 2010). Autoflocculation is also desirable and could bring about cheaper extraction procedure for algal lipid (González-Fernández, 2012). Table 1 below summarises the important characteristics that could be crucial to a strain selection for mass cultivation. However, specific adaptation of a species in a stressful condition is crucial to successful cultivation of microalgae either in an open or close system.
Table 1. Desirable characteristics to be considered in the choice of algae for the open pond cultivation for biofuel production.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Advantages</th>
</tr>
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<tbody>
<tr>
<td>Rapid growth rate</td>
<td>Competitive advantage over competing species and reduces culture area required.</td>
</tr>
<tr>
<td>Maximum biomass concentration</td>
<td>Higher biofuel productivity</td>
</tr>
<tr>
<td>High product content</td>
<td>Higher value of biomass</td>
</tr>
<tr>
<td>Growth in extreme environment</td>
<td>Reduces contamination and predation (Note: Limited number of species can grow in extreme environment and can be difficult to maintain conditions)</td>
</tr>
<tr>
<td>Autoflocculation</td>
<td>Reduce harvesting and downstream processing costs</td>
</tr>
<tr>
<td>Wide tolerance of environmental conditions</td>
<td>Less control of culture conditions required. Growth over range of seasons and ambient weather conditions</td>
</tr>
<tr>
<td>CO₂ tolerance and uptake</td>
<td>Greater potential for CO₂ sequestration and use of waste CO₂</td>
</tr>
<tr>
<td>Tolerance of shear force</td>
<td>Allows cheaper pumping and mixing methods to be used</td>
</tr>
<tr>
<td>Tolerance of contaminants</td>
<td>Potential growth in polluted water and on flue gases containing high CO₂, NOₓ and SO₃</td>
</tr>
<tr>
<td>No excretion of autoinhibitors</td>
<td>Reduces autoinhibition of growth at high biomass densities</td>
</tr>
</tbody>
</table>
1.5 Algae cultivation in waste water

Microalgae are known to be very adaptive and can be grown in salt-water, freshwater or even on contaminated industrial effluents / wastewater without any extra requirements of nutrients and minimal land requirement. Wastewaters are rich in N, P and metals, with concentrations depending on the source (agricultural, municipal, or industrial). Microalgae can therefore potentially perform a dual role for remediation of nutrient pollutants and biomass production for biofuel generation when grown in wastewater, which may improve the cost-efficiency of algal biofuel production.

The ability of microalgae to grow in wastewater varies among species. Many Chlorophytes have been shown to grow in wastewater conditions and accumulate high amount of nutrients. For example, *Chlorella* and *Scenedesmus* have been reported to remove more than 80% of ammonia and phosphate from a secondary treated wastewater and primary settled sewage wastewater (Zhang et al., 2008; Ruiz-Marín et al., 2010).

Despite these promising features of microalgae, there are huge challenges to overcome before this route can be exploited in an economically and environmentally sustainable manner. These include the presence of abiotic and biotic factors. Abiotic factors may be in the form of toxic concentrations of heavy metals such cadmium, or organic chemicals from industrial sources which may cause oxidative damage to algae. Biotic factors could include other microorganisms in the wastewater which can out-compete the microalgae for nutrients. However, the choice of wastewater type and selection of suitable microalgae species can help toward overcoming these
setbacks. Therefore, section 1.5.1 to 1.5.7 of this chapter will focus on the potential and challenges of growing microalgae in wastewater and future implications.

1.5.1 Wastewater treatment processes

Wastewater is a consequence of human activities, which include domestic, agricultural and industrial activities. Animal-derived agricultural waste will likely have high N/P content. Most wastewater emanating from domestic sources contains nutrients, suspended solids, pathogens, oxygen demanding material, dissolved inorganic and refractory organic materials. Industrial wastewater could contain some xenobiotic materials in addition to those in domestic wastewater and some require statutory pre-treatment before discharge into wastewater treatment streams (Ternes, 1998; Badawy et al., 2009). These waters are toxic and hazardous to human health and the environment, and treating them before discharge to the river or land is a regulatory obligation throughout the world.

The treatment of wastewater usually involves a preliminary treatment, then primary and secondary treatment stages (Fig. 4). The preliminary stage requires removing of large and coarse solids. The primary treatment is designed to remove solids containing organic N, P and heavy metals as well as reducing biochemical oxygen demand (BOD) by sedimentation and floatation processes (Sonune and Ghate, 2004; Abdel-Raouf et al., 2012). BOD and COD are used as indicators for measuring the amount of organic compound in wastewater. While BOD measures the amount of dissolved oxygen needed by aerobic biological organisms, COD measures the amount of chemically oxidise organic compounds in wastewater. The effluent from primary treatment stage are characterised by high N, metal, BOD, chemical oxygen demand (COD) and organic substances.
Fig. 4. The schematic diagram of wastewater treatment process.

The secondary treatments require removal of nutrients and reduction of BOD and COD. Most common methods include biological methods. This involves the use of microbes, which perform aerobic and anaerobic biodegradation processes. The aerobic process relies mainly on microorganisms using the dissolved oxygen for conversion of biodegradable substances to biomass and CO₂. In anaerobic processes, microorganisms convert organic substances in the wastewater into methane, CO₂ and water via three basic steps described as hydrolysis and acidogenesis including acetogenesis and methanogenesis in the absent of oxygen (Chan et al., 2009). Whilst biological treatments through activated sludge or biofilm systems are still the popular method in secondary treatment of wastewater, there are challenges in terms of disposal of sludge in relation to regulation, economic and environmental hurdles (Wei et al., 2003). For example, in studies carried out in four European countries (France, Germany, Italy and the UK) which account for 84% of the sludge disposed on the continent, sludge disposed on a per capita basis was in the range 35-119 g dry solid per head per day (Davis and Hall, 1997). The majority of the sludge ends up landfill, is incinerated, or is used in agriculture. The growing interest in the use of
biosolids might help in the sustainable disposal of the sludge and possible revenue generation.

The secondary effluents are usually passed through a clarifier and then disposed into freshwater or marine waters. In some cases they are reused. The current water scarcity in many parts of the world is a growing concern and recycling wastewater has become an attractive option in sustainable water supply. For example, the Middle East and North African region, which is home to about 5% of the world’s population, contains less than 1% of the world’s annual freshwater resource (Abdel-Raouf et al., 2012). To prevent adverse environmental impacts, it is important that the final effluent meets required statutory regulation before discharge. Removing nutrients from final effluent to a safe limit for reuse or discharge has been one of the expensive tasks in wastewater treatment. Most final effluent has been characterised with potentially high concentrations of N and P and other inorganic and organic compounds, which when disposed into rivers can cause eutrophication or environmental damage (Al-jasser, 2011; Abdel-Raouf et al., 2012; Chen et al., 2012). Wastewater treatment plants in many developed countries have incorporated tertiary treatment. This is advanced biological and chemical treatment with the aim to remove N, P and metals. The chemical treatment process works by filtration, ozonation, reverse osmosis and activated carbon absorption but is often limited by its high implementation cost and secondary pollution contribution (Oswald, 1988; Hoffmann, 1998; Wilde and Benemann, 1993; Sonune and Ghate, 2004; Mohamed et al., 2011; Abdel-Raouf et al., 2012). These treatment systems are often capital intensive and result in negative economic return (Chan et al., 2009). Also some of these chemical treatment methods are reaching the limits of their ability to reduce N
and P levels further which may be required by harsher legislation. Biological methods are potentially more attractive for tertiary treatment because of the low cost of implementation and value added by-products.

Microalgae have been well known for decades as capable of removing contaminants from wastewater effluents (Oswald et al., 1957; McGriff and McKinney, 1972). Integrating microalgae into wastewater treatment can potentially offer a sustainable, cheap and efficient process without contributing polluting substances (Hoffmann, 1998). Despite these advantages, there are many challenges to overcome, which include the cost and efficiency of microalgae harvesting. Immobilizing or co-immobilizing microalgae on a matrix and the use of non-suspending microalgae species are some of the methods that can potentially offset these challenges (Hoffmann, 1998; de-Bashan and Bashan, 2010).

1.5.2 Microalgae growth in wastewater – a sustainable approach

The use of microalgae as a sustainable means of remediating wastewater and producing valuable sub-products has received considerable research attention for decades (Oswald et al., 1959; Oswald, 1988; Ruiz-Marin et al., 2010; Rawat et al., 2011; Park et al., 2011; Pittman et al., 2011; Lohrey and Kochergin, 2012). Microalgae growth in wastewater may offer a source of biomass for biofuel and other potential applications such as biomass production for animal feeds in addition to its remediation potential.
1.5.2.1 Remediation opportunity

Studies have shown that microalgae are efficient in removing contaminants such as N, P and metals from wastewater effluents (Ip et al., 1982; González et al., 1997; Martinez, 2000; An et al., 2003; Aslan and Kapdan, 2006). The use of microalgae for wastewater treatment offers an opportunity for efficient recycling of nutrients, and economic benefits by avoiding high-cost chemical treatment, especially at the tertiary stage.

Early research has focussed on the use of microalgae to remove contaminants from secondary effluent as a form of tertiary treatment before discharge into rivers, to prevent eutrophication (Tam and Wong, 1989). However, a recent report has shown that microalgae can also effectively remove contaminants during secondary treatment stage (Wang et al., 2010). In this study, it was reported that \textit{Chlorella} sp. grew well in wastewater activated sludge centrate liquor with a specific growth rate of 0.948 d$^{-1}$ and removed 78.3%, 85.6% and 83.0% of NH$_4^+$, P and COD, respectively within a few days. An integrated approach where two or more organisms are used simultaneously is considered as an alternative process of improving nutrient/contaminant removal compared to a single species pond system. For example, an integrated system involving \textit{C. vulgaris} and water hyacinth \textit{Eichhornia crassipes} resulted in 23% more N removal compared to the high rate algal pond dominated by \textit{C. vulgaris} alone (Bich et al., 1999).

Microalgae can grow in wide varieties of wastewater such as municipal, industrial, artificial and agricultural types (Bajhaiya et al., 2010). \textit{C. vulgaris} and \textit{Scenedesmus dimorphus} was shown to remove about 95% of NH$_4^+$ and 55% of P in secondary effluent of agro-industrial wastewater which emanated from diary and pig farming.
Similarly, *Chlamydomonas* sp. have been reported to remove 100% of NH$_4^+$ and NO$_3^-$ and 33% of PO$_4^{3-}$ when grown in raw industrial wastewater containing 38.4 mg L$^{-1}$ NH$_4^+$, 3.1 mg L$^{-1}$ NO$_3^-$ and 44.7 mg L$^{-1}$ PO$_4^{3-}$ (Wu et al., 2012).

Other benefits of using microalgae for remediation include their disinfection capability by increasing the pH of wastewater as a result of their photosynthetic activity (Abdel-Raouf et al., 2012). This helps in reducing BOD and coliform bacteria in wastewater. Furthermore, microalgae can potentially reduce the energy input associated with oxygen supply in the conventional activated sludge system and emission of CO$_2$ due to autotrophic metabolism (Riaño et al., 2011). Therefore a microalgal-based process helps wastewater industry to meet their emission targets. Growing microalgae in wastewater for remediation also offers additional advantage in terms of biomass production for biofuel. This dual approach has been well discussed as a possible ways of enhance the sustainability and economy of microalgae biofuel strategy (Pittman et al., 2011; Olguin, 2012). Wastewaters contain essential nutrients for microalgae growth such as nitrogen, phosphorus, trace metals, carbon. The concentrations of these nutrients vary in wastewater depending on the source (Metcalf and Eddy, 1991).

**1.5.2.2 Biofuel feedstock opportunity**

Microalgae capability of producing biomass and lipid for biofuel has received research and political attention in many parts of the world. The biofuel potential from algae has been theoretically projected as capable of meeting global demand for transport fuel (Chisti, 2007 and 2008; Amin, 2009; Khan et al., 2009; Bajhaiya et al., 2010; Brennan and Owende, 2010; Demirbas, 2010; Mata et al., 2010; Ahmad et al.,
However, this projection is still far from reality based on its economic and sustainability evaluations (Kovacevic and Wesseler, 2010; Razon and Tan, 2011). The high requirements for fertilizer and freshwater, high cost of production and negative net energy have been major setbacks (Razon and Tan 2011; Acién et al., 2012; Olguín, 2012). Also, P is at risk from depletion in the world (U.S.DOE, 2010). A dual purpose of growing microalgae in wastewater has been proposed as a way to minimise these concerns and improve the economy and sustainability of algae biofuel strategy (Pittman et al., 2011). A net energy life cycle analysis of growing Haematococcus pluvialis and Nannochloropsis sp. in synthetic medium for biofuel has shown large energy deficit even with very optimistic assumptions regarding the performance of processing units (Razon and Tan, 2011). These authors suggested that some energy can be saved when microalgae are grown in wastewater eliminating the use of fertilizer. Similarly, Lundquist et al. (2010) comprehensively analysed different scenarios of algae-based wastewater treatment coupled with biofuel production and concluded that only those scenarios where wastewater were used are able to produce a cost competitive biofuel. In one of the scenarios, the wastewater treatment credit can potentially reduce unit cost of oil or electricity from microalgae by 20-25% in addition to eliminating the cost of nutrient and water.

With fast increasing global population, water usages per year have been estimated to reach 2440 billion m$^3$ by year 2025 (Shiklomanov, 1999). About 70% of this water is expected to be used for agriculture and 22% for industries and 8% for domestic use. Using projections made in Chinnasamy et al. (2010), if 50% of this water usage end up in sewerage and used for growing algae, it could generate approximately 609
40 million tons of biomass and 91 million tons of oil with the assumption that the algae lipid productivity is 15% of its biomass.

As discussed in Section 1.2, lipid can be extracted from microalgae biomass for biodiesel production. In addition, or alternatively, biochemical conversion of algae biomass can yield bioethanol, biogas and biohydrogen (Brennan et al., 2010). Energy recovery from microalgae can be further optimised by anaerobic digestion to produce methane. Sialve et al. (2009) describe some factors that could impede the optimisation of microalgae digestion, including the biochemical composition of the microalgae cell wall and protein content. High protein content in microalgae can lead to excessive release of ammonia that can be potentially toxic. Other factors include the presence of sodium salt which can affect digester performance. Most of these concerns can be overcome by pre-treating microalgae biomass before digestion.

1.5.2.3 Non-fuel applications of wastewater grown algae

Microalgae growth in wastewater has other application in addition to remediation and biofuel. Alginate from microalgae has long been used in pharmaceutical products and as a food additive (Chapman and Chapman, 1980). The biomass produced by microalgae also has potential as animal feed and for the production of chemical raw materials.

1.5.2.3.1 Production of biomass for animal feed

Some species of microalgae such as Chlorella sp., Dunaliella sp. and Spirulina sp. have been known to contain long chain fatty acids and protein that have nutritional benefit for animals (Kumar et al., 2010 and Spolaore et al., 2006). Algae are capable
of producing vitamins and minerals required for healthy growth, better weight control and healthy skin of pets such as cats, dogs and ornamental birds (Certik and Shimizu, 1999 and Spolaore et al., 2006).

A research study has examined biomass and lipid profiling of *C. vulgaris* grown in industrial and agricultural wastewater as an aquaculture feed (Bertoldi et al., 2006). From this study, it was found that the use of wastewater as an alternative culture medium can actually help in generating quality lipid, fatty acid and carotenoid production from *C. vulgaris*.

### 1.5.2.3.2 Fertilizer application

The use of algae for fertilizer has long been a practise in many parts of the world (Booth, 1966). Algae have the ability to improve soil fertility by enhancing the water retention capacity of soil and macronutrient composition. For example, a study on the effects of algal fibre on germination and growth of barley found that it enhanced the soil physical and chemical qualities (Riley, 2002). In the same report, algae fibre waste from an alginate extraction plant in Norway was studied in respect to its potential for soil improvement and as a nutrient source for potato crop.

Algae fibre was shown to have similar effects to pure perlite on soil aeration and water retention capability. Plant-available water was reported to increase by 3.6% by volume when 10% by volume of algae fibre was incorporated, as against 1.2% by volume with the same volume of pure perlite. Furthermore, potato yield also increased with the use of algae fibre.
1.5.3 *Integrating microalgae cultivation in wastewater works*

One of the more efficient ways to fast track large scale production of microalgal biofuel is by incorporating it into the existing wastewater works with little adaptation (Fig. 5). This can potentially reduce the cost of infrastructure (Lundquist et al., 2010). The choice of microalgae to be grown is very important and finding species that can grow efficiently for high biofuel productivity in wastewater is a key to the success of this strategy (Singh and Gu, 2010). Some microalgae have high oil content but are slow growers. For example, *Botryococcus braunii* is well known to have a high percentage of lipid content but grows slower than *Chlorella* sp. for example (Orpez et al., 2009; Kong et al., 2010). Algae that grow faster even with little lipid content will be ideal because it will reduce the production time and energy demands.

Microalgae can either be cultivated by an open or closed system at the secondary or tertiary wastewater treatment stage. This can produce a cheap way of remediating wastewater. Microalgal biomass can be integrated into wastewater digestion sludge for methane and CO$_2$ production (Kumar et al., 2010). The CO$_2$ can be recycled into the algae pond providing a source of carbon. High productivities of microalgae grown in wastewater in terms of biomass and lipids reported in many studies needs to be translated to the large industrial scale for cost effective and sustainable biofuel production. However, there are some challenges to be overcome before this can be realistically achieved.
Fig. 5. A flow-diagram showing how microalgae cultivation can be incorporated in wastewater works for dual role of remediation and biofuel production.

1.5.4 Challenges associated with growing microalgae in wastewater

The uses of microalgae in the wastewater industry either as a remediation agent or for biofuel feedstock are still relatively limited. Some of the set-backs include the presence of growth inhibiting factors and difficult harvesting processes. Some of these inhibitors include biotic and abiotic factors. Biotic factors can be in the form of viruses, bacteria, zooplankton, grazers, phytoplankton and fungi (Kagami et al., 2007; Park et al., 2011), which will impede or significantly inhibit the growth of microalgae. These factors will depend on the source of wastewater effluent being used for cultivation. Abiotic contaminants in wastewater such as CO₂, NOₓ, SOₓ, O₂ and NH₃⁺ and heavy metals can also inhibit microalgae growth (Kumar et al., 2010). In contrast, when essential nutrient concentrations in the wastewater are low, in particular trace mineral nutrients, it can result in poor growth, low biomass and low
lipid productivity (Christenson and Sims, 2011). In such cases there is a need to supplement these nutrients in wastewater to achieve high productivity. In an open pond system, carbon can be limited due to poor mass transfer. Some studies have suggested that bubbling of CO$_2$ can improve algae growth. For example, *Nannochloropsis oculata* growth and biomass productivity were improved when aerated with 2% CO$_2$ in a semi-continuous cultivation system (Chiu et al., 2009). Also, high concentration of oxygen in wastewater can induce oxidative damage to microalgae cell and inhibit photosynthesis (Christenson and Sims, 2011). Therefore more research needs to focus on how to optimise nutrients in wastewater for microalgae growth.

The cost and energy demand of harvesting microalgae from wastewater either by flocculation or centrifugation are still very high (Razon and Tan, 2011; Acién et al., 2012). This can be as high as 20-30% of the total energy and cost required for biofuel production (Rösch et al., 2009). Most of the current techniques for harvesting are yet to be demonstrated on a large scale. There is therefore the need for technological advancement in this area to make microalgae biotechnology more economically attractive.

Research in the use of wastewater for cultivating microalgae is still very limited as compared to research using synthetic inorganic medium. Lam and Lee (2012) found that only ~30% of published research on microalgae cultivation are based on wastewater as a growth medium with the rest using synthetic media, probably because synthetic media are readily available, less contaminated and yields promising results. However the use of synthetic media might be unsustainable in commercial terms.
1.5.5 Wastewater effluent supporting high microalgae productivity

Wastewater usually contains high concentration of nutrients in terms of N, P, carbon and metals which are an essential requirement for microalgae growth in the presence of sunlight, CO₂, O₂, optimal temperature and pH. Much of the N is in the form of NH₄⁺ which is an available form of N for microalgae uptake, although this can potentially become toxic and inhibit growth at higher concentrations (Ip et al., 1982; König et al., 1987; Wrigley and Toerien, 1990). Tolerance towards NH₄⁺ is a very important criterion for selection of microalgae to be grown in wastewater. For example, a comparative study of three green microalgae S. obliquus, Scenedesmus platensis and Chlorella sorokiniana grown in piggery wastewater showed that C. sorokiniana has a high tolerance to high NH₄⁺ compared to the other species (de Godos et al., 2010). Furthermore, in a combined cultivation of these species, C. sorokiniana was able to outgrow the other species and become the dominant species in the continuous photobioreactor. The high concentration of N, P and metals in wastewater sludge centrate liquor effluent especially those from municipal and agriculture sources can potentially inhibit microalgae and in some cases will require dilution before inoculation. As shown in Table 2, a study reported that the use of a biocoil helps in increasing biomass and lipid productivity of C. reinhardtii achieving 2000 mg L⁻¹d⁻¹ of biomass and 505 mg L⁻¹d⁻¹ of lipid when grown in municipal liquor (Kong et al., 2010). The high productivity was attributed to greater light exposure and intensity in the polyvinyl tubing because when the same microalgae species was grown in a flask, its biomass and lipid productivities were less than the half of that in biocoil. Another study reported that Chlorella grown in raw municipal liquor produced 1060 mg L⁻¹d⁻¹ of biomass (Li et al., 2011). However, when the
liquor was treated by autoclave, the productivity increased significantly, providing a biodiesel yield of 0.12 g L⁻¹.

In secondary treated wastewater, which is generally lower in nutrient concentration as compared to liquor or primary effluents, biomass and lipid productivities are usually lower. For example, *B. braunii* grown in municipal secondary treated wastewater in a bioreactor yielded 345.6 mg L⁻¹d⁻¹ and lipid of 62 mg L⁻¹d⁻¹ (Orpez et al., 2009). In agricultural wastewater from piggery manure that contains high concentration of nutrients especially nitrogen, biomass and lipid productivities were recorded to be 700 mg L⁻¹d⁻¹ and 69 mg L⁻¹d⁻¹, respectively (An et al., 2003). Agriculture wastewater from dairy manure has also been shown to promote high biomass and lipid productivities of *Chlorella* sp. attached to supporting polystyrene foam. Biomass productivity based on the algae biomass attached to the foam was 2.6 g m⁻² d⁻¹ and total fatty acid content was 230 mg m⁻² d⁻¹ (Johnson and Wen, 2010).
Table 2. Microalgae strains grown in various wastewaters and their biomass, lipid productivity, growth rate and N and P removal efficiency.

<table>
<thead>
<tr>
<th>Wastewater Type</th>
<th>Microalgae species</th>
<th>N (mgL⁻¹)</th>
<th>P (mgL⁻¹)</th>
<th>Growth rate (d⁻¹)</th>
<th>Biomass (mg L⁻¹.d⁻¹)</th>
<th>Lipid %</th>
<th>Lipid Productivity (mg L⁻¹.d⁻¹)</th>
<th>N removal</th>
<th>P removal</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Municipal settle sewage effluent</td>
<td>nd</td>
<td>40-50</td>
<td>7.8</td>
<td>nd</td>
<td>25³</td>
<td>nd</td>
<td></td>
<td></td>
<td></td>
<td>Ip et al. (1982)</td>
</tr>
<tr>
<td>Municipal wastewater centrate</td>
<td>C. reinhardtii</td>
<td>128.6</td>
<td>120.6</td>
<td>0.564</td>
<td>2000</td>
<td>25.25</td>
<td>505</td>
<td>83</td>
<td>14.45</td>
<td>Kong et al. (2010)</td>
</tr>
<tr>
<td>Municipal wastewater centrate</td>
<td>C. reinhardtii</td>
<td>128.6</td>
<td>120.6</td>
<td>0.346</td>
<td>820</td>
<td>16.59</td>
<td>136</td>
<td>55</td>
<td>15.4</td>
<td>Kong et al. (2010)</td>
</tr>
<tr>
<td>Municipal (secondary treated)</td>
<td>S. obliquus</td>
<td>27.4⁴</td>
<td>11.8</td>
<td>0.0286⁵</td>
<td>26⁶</td>
<td>31.4⁶</td>
<td></td>
<td>8⁶</td>
<td>94% *</td>
<td>98% *</td>
</tr>
<tr>
<td>Municipal (secondary treated)</td>
<td>B. braunii</td>
<td>11.9</td>
<td>11.5</td>
<td>0.14</td>
<td>345.6⁷</td>
<td>17.85</td>
<td>62</td>
<td>nd</td>
<td>nd</td>
<td>Orpez et al. (2009)</td>
</tr>
<tr>
<td>Municipal (primary treated + CO2)</td>
<td>Mix of Chlorella sp., Microcystis spp., Actinastrum sp.</td>
<td>51</td>
<td>2.1</td>
<td>nd</td>
<td>270.7³</td>
<td>9</td>
<td>24.4</td>
<td>99</td>
<td>99</td>
<td>Weertz et al. (2009)</td>
</tr>
<tr>
<td>(autocalved centrate)</td>
<td>Chlorella sp</td>
<td>91</td>
<td>212</td>
<td>0.466</td>
<td>241.7³</td>
<td>30.9</td>
<td>74.7</td>
<td>nd</td>
<td>nd</td>
<td>Zhou et al. (2011)</td>
</tr>
<tr>
<td>(autocalved centrate)</td>
<td>Hinadka sp</td>
<td>91</td>
<td>212</td>
<td>0.498</td>
<td>275</td>
<td>28.3</td>
<td>77.8</td>
<td>nd</td>
<td>nd</td>
<td>Zhou et al. (2011)</td>
</tr>
<tr>
<td>municipal (autocalved centrate)</td>
<td>Scenedesmus sp</td>
<td>91</td>
<td>212</td>
<td>0.472</td>
<td>247.5</td>
<td>30.1</td>
<td>74.5</td>
<td>nd</td>
<td>nd</td>
<td>Zhou et al. (2011)</td>
</tr>
<tr>
<td>Municipal (pre-treated)³</td>
<td>Chlorella sp</td>
<td>18.9</td>
<td>1.7</td>
<td>nd</td>
<td>74</td>
<td>31</td>
<td>22.9</td>
<td>92</td>
<td>86</td>
<td>Cho et al. (2011)</td>
</tr>
<tr>
<td>Agricultural (piggy manure with high NO3-N)</td>
<td>B. braunii</td>
<td>836</td>
<td>40</td>
<td>0.033</td>
<td>700¹</td>
<td>nd</td>
<td>69</td>
<td>80</td>
<td>82.5</td>
<td>An et al. (2003)</td>
</tr>
<tr>
<td>Agricultural (fermented swine urine)</td>
<td>Scenedesmus sp</td>
<td>864</td>
<td>20.2</td>
<td>0.12*</td>
<td>6*</td>
<td>0.9 **</td>
<td>0.54*</td>
<td>nd</td>
<td>nd</td>
<td>Kim et al. (2007)</td>
</tr>
<tr>
<td>Agricultural (anaerobically digested dairy manure)</td>
<td>Mix of Microspora willeana, Ulothrix sp.</td>
<td>225</td>
<td>24.7</td>
<td>nd</td>
<td>5.5</td>
<td>nd</td>
<td>nd</td>
<td>39</td>
<td>51</td>
<td>Wilkie and Mudhr (2002)</td>
</tr>
<tr>
<td>Agricultural (digested dairy manure, 20 s dilution)</td>
<td>Chlorella sp.</td>
<td>2232²</td>
<td>249.7</td>
<td>0.407</td>
<td>81.4 *</td>
<td>13.6 *</td>
<td>11*</td>
<td>100</td>
<td>71.6</td>
<td>Wang et al. (2010)</td>
</tr>
<tr>
<td>Agricultural (dairy wastewater, 25% dilution)</td>
<td>Mix of Chlorella sp., Microcystis spp., Actinastrum sp.</td>
<td>30.5¹</td>
<td>2.6</td>
<td>nd</td>
<td>59</td>
<td>29</td>
<td>17</td>
<td>96</td>
<td>&gt;99</td>
<td>Woertz et al. (2009)</td>
</tr>
</tbody>
</table>

nd = not determined; ¹ measured in form of Ammonium; ² Estimated from biomass value of 1000 mg L⁻¹; after 40 days; ³ Estimated from biomass value of 1.1 mg L⁻¹; ⁴ Fatty acid content and productivity determined rather than total lipid; ⁵ At a temperature of 20°C without culture stirring; ⁶ measured per hour; ⁷ estimated from biomass value of 4.4 mg L⁻¹; after 3 days. ⁸ 4 days growth with CO2 supplement; ¹ represent the highest value recorded; ² Pre-treated by filtration through 0.2 μm-pore size filter; ³ Estimated from biomass value of 7 g L⁻¹ after 10 days; ⁴ Fatty acid content and productivity determined rather than total lipid; ⁵ Pretreated by adding fermented swine urine (3%); ⁶ (v/v) a control medium; ⁷ Estimated from biomass value of 1.71 g L⁻¹ after 21 days; ⁸ The actual value before dilution; ⁹ Estimated from lipid productivity and lipid content value; ¹² Treated by bubbling with ambient air and incubated at 25°C.
In general, microalgae growth rates are lower in many industrial wastewaters, due to low nutrient and high toxin concentrations (Pittman et al. 2011). However, this will vary greatly depending on the industry. Industrial wastewater rich in N and P can be considered for the cultivation of microalgae. Biofuel productivity of microalgae varies greatly in different wastewaters. Therefore, determining the best source of wastewater for microalgae cultivation will be governed by nutrient availability.

1.5.6 Why algal biofuel is not currently produced on a large-scale in wastewater industries?

Scaling up microalgae biofuel production from laboratory scale to commercial scale remains a challenge. Integrating microalgae biofuel in an existing wastewater treatment plant offers an inexpensive and economically feasible way of commercialisation. However, this comes with some major technical and economic problems that still need to be improved.

1.5.6.1 Culture instability

Wastewater provides a cheap source of growing microalgae, however, it comes with some challenges in terms of contamination and difficulty in maintaining a monoculture system. Using a natural strain that is indigenous to the cultivation pond might be a solution to this. For example, in a previous report, consortia of natural strains grown in municipal wastewater has shown to produce lipid of up to 29% of its biomass and lipid productivity reaching 2800 mg l$^{-1}$d$^{-1}$ (Woertz et al., 2009). Fungi, viruses and bacteria are common pathogens. They may out-compete microalgae and disrupt the culture stability in large scale cultivation. Hoffman et al. (2008) reported fungi found in cultures of *Haematococcus pluvialis* that grew
epibiotically on the algal cells and caused the destruction of the host culture. So far little is known about pond speciation, ecological dynamics in wastewater, host specificity and resistance mechanisms. In a US DOE (2010) report, three important facts that should be considered in large scale cultivation of microalgae in wastewater were described. These include determining the extent of algae pathogen and predators in the wastewater pond. Macroalgae and other phytoplankton can dominate the pond or bioreactor. Furthermore, a dynamic pond monitoring technique and preventive measures need to be put in place to limit take-over by a foreign organism.

1.5.6.2 Cultivation and land requirement

Techno-economically viable cultivation of microalgae by open or closed systems in an industrial scale is difficult to predict. Recent life cycle analysis of the production of biodiesel and biogas from *Haematococcus pluvialis* and *Nannochloropsis* has reported that a photobioreactor consumed about 56.5 MJ of electricity as compared to a raceway open pond that required 26.6 MJ (Razon and Tan, 2011). The higher energy requirement can be as a result of culture mixing, gas exchange and temperature maintenance. Bioreactors support higher growth and microalga cell density than open cultivation systems and reduced evaporation which is suffered in an open pond system (Chisti, 2007). In addition, monoculture systems can be achieved in closed systems than in an open system. Land requirement for facilities is also a potential problem. Most wastewater treatment plants are usually sited close to the metropolitan areas with high land price and limited availability (US DOE, 2010). It will be expensive to transport wastewater over a long distance. Therefore integrating wastewater into existing plants with little modification is highly encouraged.
1.5.6.3 Microalgae harvesting challenges

Whilst microalgae are capable of producing lipid and biomass suitable for biofuel, recovering and processing microalgae from the cultivation medium is still a challenge (de-Bashan and Bashan, 2010). The effective harvesting method is the key to integrating this system in the wastewater industry. In older microalgal cultures, flocculation can occur but in some cases flocculating agent such as alum $\text{Al}_2(\text{SO}_4)_3$ can be added to the culture. Flocculation can also be triggered by manipulating culture pH or bioflocculation with other microorganism that promote sedimentation (Sukenik and Shelaf, 1984; Lavoie and de la Noue, 1985). Flocculation increases the particle size and promotes sedimentation. The use of chemically-induced flocculation is currently expensive and unsustainable for harvesting microalgae in a commercial scale. Bioflocculation seemed to be a cheap option but it is slower and not always reliable (Schenk et al., 2008).

Another method that is widely used in the wastewater industry is centrifugation and in some cases has been shown to be effective (Molina et al., 2003). Biomass recovery by centrifugation will depend on the characteristics of the microalgae species such as size, settling rate and speed of the centrifugation system. Molina et al. (2003) reported that cell harvest efficiency of >95% was obtained only at 13,000 g and the harvest efficiency declined to 60% at 6000 g and 40% at 1300 g. Centrifugation can be rapid but consume more energy and can increase the cost of biofuel production. Another method is by filtration using pressure vacuum. This might not be suitable for some microalgae like Scenedesmus and Chlorella that are small in size (Brennan and Owende, 2010). Filtrative recovery of microalgae may be
unsatisfactory because filtration can be relatively slow and comes with the cost of replacing the filter membrane and energy cost of pumping (Pittman et al., 2011).

An alternative method of harvesting microalgae is by immobilization. It has been shown that immobilization is one of the feasible ways to achieve recovery of microalgae from wastewater (Moreno-Garrido, 2008; de-Bashan and Bashan, 2010). An immobilization technique offers a solution to the problems encountered during harvesting in a suspended growth system. This technique involves the absorption of microalgae when passed through the immobilized matrix system (Travieso et al., 1992; Zhang et al., 2008). Although this system is simple and allows the possibility to re-use the immobilised matrix system after recovery but there are still some constraints in term of the cost of the polymer, effectiveness in removing some contaminants, toxicity of some immobilizing techniques.

1.5.6.4 Climatic factors

One of the major parameters limiting commercialization of algae biofuel production is the climate in terms of temperature and sunlight especially in an open cultivation system. Lower temperature below the optimum can reduce cell metabolism and result in low biomass and lipid productivities. The average suitable temperature for algae growth has been described to be in the region of 15°C to 35°C (Lundquist et al., 2010). Some regions of the earth in the upper hemisphere (Fig. 6) where average temperature could be 15°C or less at night, and also shorter daytime in the autumn and winter might be considered unsuitable region for maximum algae growth yield. One of the ways to ameliorate the impact of low temperature for example is by covering the pond with some plastic materials. Alternatively, in the night time, microalgae culture can be transferred into a settling pond that are normally deep and
retain heat unlike the shallow growth pond. The culture can then be circulated back to the growth pond during the day. However, this can potentially add to the cost and energy requirement.

Fig. 6. Regions in the rectangle are those that have average annual temperature of 15°C and above (adapted from Harmelen and Oonk, 2006).

1.5.7 Future perspective of bioenergy from microalgae

Based on current knowledge, it is unlikely that microalgae will produce biofuel which is cost competitive with fossil fuels without major advances in technology. Most significant improvements are expected to be in the area of strain selection, cultivation, harvesting and oil extraction (Pittman et al., 2011). Integrating microalgae cultivation in wastewater for the dual purpose of remediation and biofuel production can potentially accelerate the commercialisation of algae biofuel in near term (Fig. 7). However there are needs for better control of zooplankton, phytoplankton and development of lower cost ponds, clarifiers and digesters (U.S. DOE, 2010).
Fig. 7. Futuristic projection on algae biofuel based on the existing knowledge and technology.

With fast growing technology advancement in harvesting of microalgae, strain improvement and selection, it is possible to break even in incorporating microalgae biofuel in wastewater in the near future. Co-products which are already being produced can also be improved for combined production with biofuel. However, most of these co-products require monocultures and high sterility which can be achieved only in a closed system. A single purpose commercial biofuel/biodiesel production from microalgae is likely to be long term goal owing to the current cost and energy demand for production (Harmelen and Oonk, 2006). Research in algae biofuel needs to start focusing on the pond system rather than small scale laboratory cultures to provide a robust data, minimize edge effects and allow for extrapolating to commercial scale.
1.6 Aims and Objectives

This PhD research was aimed at understanding the requirements and characteristics of microalgae cultivation in wastewater for sustainable biofuel production. An initial aim was to identify suitable microalgae strains that can grow effectively in raw municipal wastewater secondary effluent (RMWSE). Studies have shown that RMWSE contain various biotic and abiotic factors which often impair microalgae growth either through biotic competition or toxic stress induction. Therefore it is essential that microalgae strains utilised for wastewater cultivation have the capabilities to overcome these challenges in order to thrive in this environment. Apart from the ability of microalgae to survive and grow in wastewater, they must also be able to produce high lipid and biomass yield and their lipid must be suitable for biodiesel production.

Experimentally, these aims were to be achieved by the following experimental objectives:

1. To isolate microalgae strains from a wastewater treatment tank and screen them for growth, biomass and lipid productivity characteristics in RMWSE.

2. To study the role that acclimation has played in growth and survival of microalgae in RMWSE.

3. To improve growth and productivity of the new strains in RMWSE by nutrient amendments.

4. To perform a pilot pond cultivation experiment of the new strains in an open cultivation system.
The search for suitable algae species was focused on indigenous species from wastewater treatment tanks. The hypothesis was that strains from this environment might have adapted or developed mechanisms for survival unlike culture collection strains that had not been previously exposed to wastewater.

The first objective (addressed in Chapter 2) was to isolate microalgae species from the RMWSE and screen them for suitability for cultivation and biofuel production. The algae were tested for their ability to grow effectively, tolerance to stress, biomass and lipid productivities and remediation ability. As a result, new strains with promising potential for remediation and biofuel can be discovered. Wastewater has been known to contain toxic compounds which can induce oxidative stress on microalgae. Such stress induces generation of reactive oxygen species (ROS) which causes cell death. For microalgae to survive in RMWSE, they have to produce antioxidant enzyme like ascorbate peroxidase to scavenge induced ROS. An experiment was conducted to identify the level of oxidative stress RMWSE can induce on microalgae. Furthermore, their stress tolerances were quantified in terms of tolerance to nutrient and oxidative stress.

The second objective (addressed in Chapter 3) was to establish a new approach of screening microalgae for wastewater cultivation. An understanding of acclimation of the strains to RMWSE is likely to give insight into the establishment of a better approach of growing microalgae in RMWSE especially non-indigenous strains that will otherwise grow poorly in RMWSE. To do this, similar strains to the indigenous species and some well known algae strains that are less likely to grow in wastewater were obtained from culture collection. They were subjected to a stepwise acclimation
process and examined improvement in their growth and productivities. As a result, a new way of growing culture collection algae strain in wastewater was established.

The third objective (part of chapter 4) was to understand how sludge liquor (derived from dewatering of activated sludge) can be used to optimise microalgae growth and productivity in RMWSE. The addition of nutrients to cultivation medium like P and N are likely to increase the cost of cultivation and have a direct effect on biofuel economy. The use of alternative waste sources of nutrients such as liquor, may be a sustainable alternative to conventional fertilisers that can help in improving productivity and reducing cultivation cost. Liquors are rich in nutrients but its high nutrient concentration and other contaminants can be lethal to microalgae if undiluted. Further tests were to examine the continuous cultivation output of the best performed strains among the isolates in order to understand their real-time commercial viabilities.

The fourth objective (part of chapter 4) was to examine the outlook of non-lab microalgae scale-up cultivation in wastewater in the content of a full UK growth season (summer, autumn, winter and spring). Many projections of microalgae biofuel potential have been based on laboratory-scale experimental results because only few experimental data are available on larger scale cultivation, especially for an open pond system. Projections based on large and pilot scale are likely to be more accurate that those based on laboratory experiments which currently dominate published research studies. For example, in the UK, no known data is available on seasonal productivities of microalgae cultivation in wastewater in an open pond system. Such information will be useful for decision making and projection by water industries looking to enact this approach.
Chapter 2

This Chapter covers the isolation of indigenous algae strains from wastewater as a biofuel feedstock and remediation agent. The underlying mechanisms for growth in wastewater were studied. The Chapter comprises of a thesis-version of a manuscript published in the Biomass and Bioenergy journal (Osundeko O. et al. 2013, *Biomass & Bioenergy* 56: 284-294).
Oxidative stress-tolerant microalgae strains are highly efficient for biofuel feedstock production on wastewater

Olumayowa Osundeko\textsuperscript{1,2}, Helena Davies\textsuperscript{1} and Jon K. Pittman\textsuperscript{1}

\textsuperscript{1}Faculty of Life Sciences, The University of Manchester, Michael Smith Building, Oxford Road, Manchester M13 9PT, UK
\textsuperscript{2}Sustainable Consumption Institute, The University of Manchester, 188 Waterloo Place, Oxford Road, Manchester M13 9PL, UK

Running title: Oxidative stress-tolerant microalgae strains are highly efficient for biofuel feedstock production on wastewater

Key words: Biofuel; Lipid; Microalgae; Oxidative stress; Wastewater bioremediation.

Authors’ Contributions

Olumayowa Osundeko performed all of the experimental work in this manuscript except for the ROS and APX experiments that he performed jointly with Helena Davies. The manuscript was written by Olumayowa Osundeko and edited by Jon Pittman.
Abstract

Nutrient-rich wastewater may provide a sustainable means to cultivate microalgal biomass for biofuel use, yet many microalgal strains are very sensitive to wastewater due to toxicity caused by abiotic and biotic stresses. Naturally adapted strains that can efficiently grow in wastewater effluent are therefore of interest, however, the mechanisms by which such strains tolerate wastewater conditions are unknown. This study isolated indigenous chlorophyte microalgae strains from a municipal secondary wastewater effluent tank. The strains were identified by molecular phylogenetics and characterised by their ability to utilise exogenous organic carbon sources for mixotrophic growth and on the basis of oxidative stress tolerance, in order to elucidate the mechanisms of wastewater adaptation. Two of the strains, identified as *Chlorella luteoviridis* and *Parachlorella hussii*, could grow very well in raw wastewater due to their substantial tolerance to oxidative stress, which is highly induced by the wastewater environment. These strains exhibited high ascorbate peroxidase activity allowing increased scavenging of reactive oxygen species compared to strains that are not well adapted to the wastewater conditions. Both strains displayed high biomass and lipid productivity values in wastewater effluent. The accumulated lipids were suitable for biodiesel usage with characteristics equivalent to palm oil- and sunflower oil-derived biodiesel. The strains were also efficient in nutrient remediation from the wastewater. These results demonstrate the potential of these two strains for future biofuel applications coupled to wastewater remediation and highlight the importance of oxidative stress tolerance as a key indicator of efficient wastewater growth.
2.1. Introduction

The increasing demand for fossil fuel and its contribution to greenhouse gas emission has created more interest in alternative forms of energy including biofuel. Microalgae have the potential to generate high quantities of biomass and oil that can be utilized for biological or chemical conversion to bioethanol, biodiesel or biomethane fuels (Brennan and Owende, 2010). However, there are still challenges in terms of high requirements for fertilizer and freshwater, and high cost of production that need to be overcome in order to improve the economic viability and sustainability of algal-based biofuel (Kovacevic and Wesseler, 2010; Acién et al., 2012). Microalgal cultivation coupled to wastewater treatment is one approach to minimise these concerns and improve the economy and sustainability of algae biofuel production (Pittman et al., 2011; Park et al., 2011). Microalgae have long been known to have potential for wastewater treatment such as in waste stabilisation pond systems (Oswald et al., 1957), as they can efficiently remove contaminating nitrogen (N) and phosphorus (P) nutrients and toxic metal pollutants from wastewaters (Hoffmann, 1998, Ruiz-Marín et al., 2010; de-Bashan and Bashan, 2010). The nutrient-rich wastewater could also be utilised as a sustainable means to cultivate microalgal biomass for downstream applications such as biofuel production, and thus provide economic viability of both algal-based remediation and biofuel generation strategies through dual usage of the biomass. For example, the wastewater treatment credit can potentially reduce unit cost energy from microalgae by 20-25% in addition to eliminating the cost of nutrient and water (Craggs et al., 2011).
Although wastewater may provide a cheap source of nutrients for microalgae cultivation, there are a number of challenges to be overcome, including the difficulty in maintaining the chosen algal strain due to contamination by other algal species, and the toxicity to microalgae caused by various abiotic and biotic stresses. These include organic and metal pollutants, and fungal, viral or bacterial pathogens that can disrupt the culture stability (Hoffman, 1998; de la Noue et al., 1992). In photosynthetic organisms like algae, many environmental stresses trigger the excessive formation and accumulation of intracellular reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and the hydroxyl radical, which induce oxidative stress and cause damage through the oxidation of cellular components (Suzuki et al., 2012). The impact can be exacerbated by high light and UV exposure (Emani et al., 2003). Cells, including algal cells, are able to mediate anti-oxidative defence through the activities of various ROS scavenging enzymes such as ascorbate peroxidase (APX) (Ishikawa and Shigeoka, 2008; Tanaka et al., 2011). However, the importance of oxidative stress tolerance by microalgae growing in wastewater environments as an indicator for selection has not been studied.

Although previous studies have evaluated the wastewater cultivation potential of microalgae strains obtained from culture collections with some success (Kong et al., 2010; Li et al., 2011), many of these strains can be very sensitive to the wastewater conditions (Cho et al., 2011). A recent study has demonstrated a large-scale screening and acclimation procedure of multiple algae strains which successfully identified strains with the ability to grow well and produce high amounts of lipid on wastewater conditions (Zhou et al., 2011). However, such screening can be laborious and time-consuming. Using natural strains that are indigenous to a wastewater
cultivation pond and have therefore adapted to this more stressful environment is an alternative approach. For example, some previous studies have isolated consortia or individual natural strains that grow well in municipal wastewater but also yield high lipid productivity characteristics that may be suitable for biofuel applications (Woertz et al., 2011; Zhou et al., 2012). Such naturally adapted strains also have the potential for efficient wastewater pollutant remediation.

The mechanisms of wastewater tolerance by such strains are unknown and we hypothesise that increased tolerance to oxidative stress by adapted microalgae allows efficient growth in wastewater conditions. This in vitro algal assay has potentially important applications for selection of putative strain for wastewater cultivation as a biofuel feedstock or remediation agent. In addition, it can be used as an ecological indicator for wastewater final effluent quality. The aim of this study was therefore to screen, identify and characterize high-performing indigenous microalgae that are adapted to wastewater secondary effluent, to elucidate the mechanisms of this adaptation, including an analysis of oxidative stress tolerance, and to perform biofuel and wastewater remediation evaluation.

2.2. Material and methods

2.2.1. Isolation of microalgae strains

Raw municipal wastewater secondary effluent (RMWSE) samples were collected from the secondary treatment tank at United Utilities wastewater treatment plant at Ellesmere Port, Cheshire, UK (lat. 53° 16′ 47.32″ N, long. 2° 53′ 50.65″ W) during four trips in February, June (2 trips) and September 2011. The Ellesmere Port plant receives mainly domestic wastewater from Ellesmere Port and the surrounding
suburban area and pre-treated effluent from a nearby oil refinery. The wastewater undergoes primary sedimentation which is followed by activated sludge treatment giving rise to secondary effluent that is normally discharged into the Mersey river basin. On each of the four sampling occasions, RMWSE samples were collected from three different locations of the secondary treatment tank by sweep sample in 2 L collection buckets. Samples were taken immediately to the University of Manchester and transferred to transparent 1 L glass bottles and kept in the laboratory overnight at 22 °C before microalgae isolation was begun. Twelve bottles were collected in total. 10 µL samples from each bottle were inoculated into 20 mL of autoclaved Tris-Acetate-Phosphate (TAP) medium, an artificial mixotrophic algal growth medium (Haris, 2009) in glass conical flasks, and cultured on an orbital shaker rotating at 2 Hz in conditions equivalent to those at the sampling site and thus optimal for the indigenous strains: 22 °C, 16 h light: 8 h dark and a photon flux of 150 µmol m⁻² s⁻¹. Samples were cultured until visible signs of algae growth; specifically, medium from any flask which looked green was examined by light microscopy to confirm the presence of algae. A 10-fold dilution series of the algae cultures was prepared and 20 mL volumes were inoculated onto TAP-agar plates and incubated for 14 d. Microalgae colonies corresponding to five distinct strains were selected and re-cultured in liquid TAP media and plated on TAP agar plates to ensure purity of the cells. The unknown strains observed by light microscopy were identified by morphological taxonomy. Microscopy images were acquired by using a Leica DMR microscope fitted with a Spot Explorer camera model 17.4. Taxonomy identification was performed according to the methods of John et al. (2002). Cell biovolumes were estimated as described by Hillebrand et al. (1999).
2.2.2. Ribosomal RNA sequencing and phylogenetic analysis

Microalgae genomic DNA was extracted using cetyl trimethyl ammonium bromide buffer (Doyle and Doyle, 1987). 18S-ITS1-5.8S-ITS2 rRNA sequence was amplified by PCR using 18SF 5’-TTAAGCCATGCATGTCTAAG-3’ and 18SR 5’-GACTACGACGGTATCTAATC-3’ primers and ITSF 5’-ACCTAGAGGAAGGAGAAGTGTAA-3’ and ITSR 5’-TTCCTCCGCTTTATGATATGC-3’ primers. PCR was performed as follows: each 25 µL of PCR reaction consisted of 2 µL of genomic DNA, 2.5 µL of 10 x PCR buffer (containing MgCl₂), 0.2 mM of dNTPs mixture, 5 µL of combinatorial enhancer solution (Ralser et al., 2006), 1 mM forward and reverse primers and 0.2 µL of Taq DNA polymerase. Final volume was made up to 25 µL with sterile distilled water. The PCR reaction was carried out in an AB-2720, 96 well Thermal cycler (Applied Biosystem, UK) and reaction condition were as follows: 35 times cycle of 95°C for 5min, 95°C for 30sec, 55°C for 1min and 72°C for 1min. Final elongation was at 72°C for 5min. Each primer pair produced a single band from each reaction as determined by visualisation on a 3% (w/v) agarose gel. PCR products were purified using a QIAquick PCR purification kit (Qiagen) using the manufacturer’s protocol. PCR products were sequenced by GATC-Biotech and aligned with 18S-ITS nucleotide sequences of selected unicellular Chlorophyceae microalgae obtained from the GenBank database (Table S1) by ClustalW alignment. Phylogenetic analysis was performed by the maximum likelihood method using RAxML v.7.1 (Stamatakis, 2006). Confidence in the tree was assessed using the fast bootstrap approach (Stamatakis, 2008) by performing 100 replications. Sequences showing the closest match by multiple alignment and phylogenetic analysis to the sequences from the isolated strains were compared by pairwise alignment using
EMBOSS Needle to determine sequence identity and confirm whether there was an exact match.

2.2.3. Strain cultivation

Batch cultures of the five isolated strains were grown in RMWSE or artificial TAP medium in triplicate on an orbital shaker at 120 Hz at 22°C, 16 h light:8 h dark and a photon flux of 150 μmol m\(^{-2}\) s\(^{-1}\), with identical starting cell densities in each flask as determined by optical density at 680 nm (OD\(_{680}\)). The total N and P concentrations of the RMWSE used for this growth were 75 mg/L and 1.7 mg/L respectively, and the pH was 7.5. The growth in wastewater of two controls, non-adapted strains from a culture collection were also compared: *Chlorella ellipsoidea* CCAP 211/33 and *Chlorella vulgaris* CCAP 211/79 were obtained from the UK Culture Collection of Algae and Protozoa (CCAP). The RMWSE was allowed to settle to remove large organic matter and decanted. Strains were cultivated in untreated RMWSE or RMWSE which was autoclaved at 121°C for 20 min. Microalgal strains were inoculated (1% v/v) into 200 mL flasks of RMWSE. To assess mixotrophic growth on various carbon sources, strains were grown in autoclaved TAP medium containing 0.1% (v/v) glacial acetic acid, or the same medium but with the acetic acid replaced with 0.1% (v/v) methanol or 0.1% (v/v) glycerol. Growth was compared in TAP medium lacking any external carbon source (TP medium). For growth under nutrient deficient conditions, the five isolates were grown in TAP medium under conditions of N or P deficiency or sufficiency as described previously (Webster et al., 2011). Phosphate (as potassium phosphate) was present at the sufficient concentration of 1 mM (as in standard TAP medium) or at the deficient concentration of 0.1 mM, 0.05 mM or 0.001 mM. Potassium concentration was
unchanged through the addition of KCl. Nitrogen (as ammonium chloride) was present at the sufficient concentration of 7 mM (as in standard TAP medium) or at the deficient concentration of 3.5 mM, 0.7 mM or 0.07 mM. All media was buffered to pH 7.0 with Tris. To start the cultures, 1 ml of early stationary phase cells at equal cell density that had been grown in nutrient sufficient conditions were centrifuged and washed three times in Milli-Q water to remove residual nutrients from the cell surface then inoculated into nutrient sufficient or deficient media. For each experiment cultures were grown in triplicate in a growth room on an orbital shaker at 120 Hz at 22 °C with a 16 h light:8 h dark light regime and a photon flux of approximately 150 μmol m⁻² s⁻¹

2.2.4. Oxidative stress analysis

For *in vivo* detection of oxidative stress, the production of ROS as a result of growing microalgae in RMWSE was detected by using the fluorescent stain 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma Aldrich). Cells cultured in TAP medium were centrifuged and resuspended in 50 mM potassium phosphate buffer (pH 7) then incubated in 50 μM DCFH-DA for 2 h at 25°C in the dark then washed twice with phosphate buffer to remove residual stain. Microalgae were grown in non-stressed TAP medium then incubated in untreated RMWSE for 12 h or in TAP medium with 25 mM H₂O₂ for 12 h in order to confirm the effectiveness of DCFH-DA to report intracellular ROS accumulation. The fluorescence of the cells was measured using a fluorescence spectrophotometer (Jasco FP750) at 485 nm excitation and 530 nm emission wavelengths. The percentage increase in ROS abundance relative to the non-stressed control treatment was calculated: 

\[
\text{[(DCFH-DA}} \text{]}
\]
fluorescence \(_{\text{treated}} - \text{DCFH-DA fluorescence}_{\text{control}}\) / DCFH-DA fluorescence \(_{\text{control}}\) × 100.

For growth comparison under artificial oxidative stress conditions, each strain was grown in TAP medium until late exponential phase (day 7). The cell density of each strain culture was equalised to an OD\(_{680}\) value of 2.0 by dilution with TAP medium then H\(_2\)O\(_2\) (5, 10, 15, 20 or 25 mM) or methyl viologen (0.01, 0.1, 0.5, 1 or 5 mM) was added. The oxidative stress response was determined 24 h later by measuring the relative percentage change (survival) in OD\(_{680}\), and by directly determining cell death by the accumulation of the cell death stain Evans Blue, as described (Zuppini et al., 2007), except that a final concentration of 0.1% (w/v) Evans Blue was used and evaluation of cell death was performed by light microscopy visualization.

APX activity was measured based on the rate of decrease in absorbance of ascorbate at 290 nm due to ascorbate oxidation. The isolated strains, plus \(C. vulgaris\) CCAP 211/79 were grown in TAP medium until late exponential phase then incubated for 12 h in untreated RMWSE, TAP medium with 25 mM H\(_2\)O\(_2\) or TAP medium alone (non-stressed control). 1 g of fresh cell pellets were recovered from each culture by centrifugation at 1500 \(g\) at 4\(\degree\)C for 5 min and each pellet was resuspended in 50 mM potassium phosphate buffer (pH 7), protease inhibitor cocktail (Sigma Aldrich) and 1 mM ascorbate then sonicated. The broken cells were centrifuged at 20,000 \(g\) for 30 min at 4\(\degree\)C and the cell extract was collected. Total protein in the cell extract was determined using a Bio-Rad Bradford assay kit, according to manufacturer’s instructions. A reaction mixture containing 50 mM potassium phosphate (pH 7) and 0.1 mM ascorbate was added to 100 \(\mu\)l of cell extract to a final volume of 1 ml in a quartz cuvette. The reaction was initiated by addition of 0.1 mM H\(_2\)O\(_2\) and APX
activity was determined by measuring the decline in absorbance at 290 nm for 3 min using a UV-visible light spectrophotometer (Aquamate Thermo Spectrometric). Specific APX activity (µmol ascorbate min⁻¹ mg⁻¹ protein) was calculated using the equation: $(\Delta A_{290\text{nm}} / (\varepsilon \times L)) \times (V_{\text{assay}} / V_{\text{sample}}) / P)$, where $\Delta A_{290\text{nm}}$ is the difference in absorbance per min, $\varepsilon$ is the extinction coefficient for reduced ascorbate (2.8 mM⁻¹ cm⁻¹), $L$ is the cuvette light path (cm), $V_{\text{assay}}$ is the total assay volume, $V_{\text{sample}}$ is the total volume of cell extract, $P$ is the cell extract protein concentration.

2.2.5. Microalgae growth and productivity analysis

Cell density was determined by OD₆₈₀ measurement using a Jenway spectrophotometer. For strains cultivated in RMWSE, RMWSE without added microalgae was used as a blank before taking measurements. OD₆₈₀ measurement was demonstrated to be a suitable method for quantifying cell density by initially comparing OD₆₈₀ values with cell number for each strain, determined by counting cells stained with Lugol's iodine on a Sedgewick-Rafter cell counting slide. OD₆₈₀ values and cell number showed a strong positive correlation ($R^2 = 0.974$, Fig. S1A). Growth rate ($\mu$) was determined at the exponential growth phase using the equation: $\mu = (\ln N_1 - \ln N_0) / (t_1 - t_0)$ where $N_0$ and $N_1$ are the OD₆₈₀ values at the early and late exponential phase, respectively, and $t_1$ and $t_0$ are the days corresponding to $N_0$ and $N_1$, respectively. Total chlorophyll was determined as described (Dean et al., 2010). Dry weight algal biomass was determined following centrifugation of a 50 mL sample at 1500 g for 20 min in a pre-weighed tube. The cell pellet was dried for 48 h at 60°C then weighed. For strains cultivated in RMWSE, the dry weight of any suspended solids/biota of RMWSE without added microalgae was subtracted to give the algal dry weight. OD₆₈₀ values and algal dry weight...
weight biomass showed a strong positive correlation ($R^2 = 0.977$, Fig. S1B) indicating the accuracy of the dry weight measurement as an algal biomass indicator.

Total lipid concentration was determined based on the Folch method (Folch et al., 1957). Dried biomass (100 mg) was homogenized with 2 mL of 2:1 (v/v) chloroform:methanol then sonicated for 1 h and filtered through Whatman glass microfiber filters. The filtrate was washed with 0.2% (v/v) sterile water and allowed to form a biphasic. The upper phase was rinsed with 3:48:47 (v/v) chloroform:methanol:saltwater. The lipid was dried and its weight determined.

Biomass and lipid productivity values were determined using the equations: biomass productivity (mg L$^{-1}$ d$^{-1}$) = WB/V × T, lipid content (%) = WL × 100/WB, and lipid productivity (mg L$^{-1}$ d$^{-1}$) = WL/V × T; where WB is dry weight biomass (mg), WL is lipid weight (mg), V is working volume and T is cultivation time. Neutral lipid content was determined using the fluorescent lipid stain Nile Red, as described previously (Dean et al., 2010). Each culture was diluted to an OD$_{680}$ value of 0.4. Relative fluorescence intensity of Nile Red was quantified using a fluorescence spectrophotometer (Jasco FP750) using 530 nm excitation and 575 nm emission wavelengths.

2.2.6. Fatty acid methyl ester (FAME) content

FAME content was determined at day 10 using a modified version of the method described by Indarti et al. (2005) and Wang et al. (2010). Cells were harvested by centrifugation at 1500 g for 10 min and then dried at 60°C for 48 h. Ten mL of methanol:concentrated sulfuric acid:chloroform (4.25:0.75:5) was added to each 100 mg of ground dried sample and heated in a sealed bottle at 90°C for 120 min, cooled,
then 2.5 mL of 2% (w/v) NaCl was added before centrifugation at 670 g for 10 min to form a bilayer. The bottom layer containing FAME was filtered through Whatman glass microfiber filters. The filtrate was run in positive ion mode on an Agilent Qtof 6510 mass spectrometer coupled to an Agilent 1200 series HPLC with a RP column (5 µm particle size). Filtrate (5 µL) was injected (0.2 mL min⁻¹) into 20% (v/v) acetonitrile (ACN)/0.1% (v/v) formic acid; after 1.5 min this was increased over 5 min to 80% (v/v) ACN as a system flush before returning to 20% (v/v) ACN. The source temperature was 350°C with 6 L min⁻¹ drying gas and a nebulizer of 30 psig. A pure FAME mix C₈-C₂₄ (Supelco) was used as a standard. The data was analyzed by Agilent Mass Hunter software and the NIST Mass Spectral Search Program. Fatty acid peak area was used for quantification by comparison to the standard. To assess FAME biodiesel quality, iodine value (IV), saponification value (SV) and cetane number (CN) was determined using the equations: SV = Σ (560 × Aᵢ)/MWᵢ; IV = Σ (254 × Aᵢ × D)/MWᵢ, where Aᵢ is percentage of fatty acid in FAME, MWᵢ is molecular mass of each of the constitute fatty acids and D is the number of double bonds; CN = (46.3 + 5458/SV) – (0.225 × IV) (Gopinath et al., 2009a).

2.2.7. Nutrient and biochemical oxygen demand (BOD) determination

N and P remediation of RMWSE by algal strains was determined from measurement of NH₄⁺-N and PO₄³⁻-P concentrations (mg/L) in the media before and 5 days after cultivation using a Skalar Sans Plus auto-analyser, using standard methodology (Skalar Analytical). Prior to measurement the culture aliquot was filter to remove algae cells and the auto-analyser was calibrated using standard solutions. BOD measurement was determined using a Jenway 9500 Dissolved Oxygen Meter following incubation at 20°C for 5 days in amber bottle.

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2.2.8. Statistical analysis

Unless otherwise stated, all data shown is representative or mean data of at least three replicate experiments. Differences between treatments were assessed using one-way or two-way ANOVA. When significant differences were detected at a 95% confidence level, the multi-range Tukey’s post-hoc test was applied. All statistical tests were performed using GenStat version 15.

3. Results

2.3.1. Isolation of microalgae strains from secondary effluent tanks

Five microalgae strains were isolated from RMSWE and were present during all sampling periods and therefore were dominant throughout the year. Light microscopy observation of the cell shape, size, flagella, colony formation and habitat (Fig. 1A) indicated that each strain was a distinct microalgae species of the division Chlorophyta.
Fig. 1. Morphological and phylogenetic characterisation of five microalgae strains isolated from secondary wastewater ponds. (A) Bright field images of the isolated strains. Scale bar = 2 µm. (B) A phylogenetic tree based on rRNA 18S and ITS nucleotide sequence obtained from unknown strains and sequence available for selected unicellular Chlorophyta microalgae. GenBank database accession numbers are given in Table S1. The species whose sequence was identical (100% identity) to the unknown strains are indicated by the shaded box. The tree was generated using maximum likelihood analysis of aligned sequences using 100 replications to determine tree confidence. Bootstrap percentage values are indicated at the tree nodes of branches and indicate confidence in tree node position. The branch length scale bar indicates evolutionary distance.
The cells average biovolume for each isolates enable further comparison index for identification (Table S2). Morphological characterisation allowed for preliminary identification of strains and compliments the phylogenetic analysis. Amplification of rRNA sequence from each strain allowed species identification by comparison with rRNA sequences of known microalgae (Fig. 1B). Each of the unknown strain's sequence shared 100% sequence identity with the identified matching sequence. The unknown strains were identified as A: *Chlamydomonas debaryana*, B: *Hindakia tetrachotoma*, C: *Chlorella luteoviridis*, D: *Parachlorella hussii*, and E: *Desmodesmus subspicatus* (formerly *Scenedesmus subspicatus*). *C. debaryana* and *D. subspicatus* belong to the class Chlorophyceae while *H. tetrachotoma*, *C. luteoviridis* and *P. hussii* belong to the class Trebouxiophyceae.

### 2.3.2. Growth of microalgae strains in secondary effluent

To test the stressful nature of the effluent, two selected non-adapted *Chlorella* strains from a culture collection were grown in the RMWSE and indeed shown to have poor growth with growth rates <0.08 d⁻¹ (Fig. 2A). In comparison, all five isolated strains grew very well in the untreated effluent in terms of growth rate (Fig. 2B), total chlorophyll concentration, as a physiological indicator of healthy cells (Fig. 3A), and biomass productivity (Fig. 3B). *D. subspicatus* performed the poorest for each parameter while *C. luteoviridis* and *P. hussii* had the highest cell density (*P*<0.01), specific growth rate (*P*<0.05), chlorophyll concentration (*P*<0.05), and biomass productivity (*P*<0.01), compared to the other three strains. Autoclaving as a means of treating wastewater potentially increases the growth of the microalgae in RMWSE.
Fig. 2. Specific growth rate determined during exponential growth phase of two non-adapted culture collection strains (*Chlorella ellipsoidea* CCAP 211/33 and *Chlorella vulgaris* CCAP 211/79) (A) and of the five isolated strains (*C. debaryana*, *H. tetrachotoma*, *C. luteoviridis*, *P. hussii*, *D. subspicatus*) (B) in untreated and autoclaved RMWSE. Growth curves of the five isolated strains in untreated RMWSE determined by optical density (OD) at 680 nm (C). Data values are means (±SE) of three replicate cultures per strain.
A 1.5- and 2.5-fold increase in growth of the *C. vulgaris* and *C. ellipsoidea* culture collection strains, respectively, was observed in autoclaved RMSWE (Fig. 2A). Growth of the indigenous strains was better than culture collection strains under autoclaved conditions but the isolated strains grew well in non-autoclaved, untreated wastewater and there was no significant decrease in growth compared to the autoclaved media (Fig. 2B). Furthermore, in the raw wastewater, *C. luteoviridis* and *P. hussii* growths remain linear in exponentially phase beyond 14 days of cultivation (Fig. 2C) suggesting that these strains are fully adapted to this media. All further RMSWE cultivation in the study was performed using untreated, non-autoclaved wastewater.
Fig. 3. Total chlorophyll concentration at day 10 (A) and biomass productivity determined during exponential growth phase (B) of the five isolated strains (C. debaryana, H. tetrachotoma, C. luteoviridis, P. hussii, D. subspicatus) in untreated and autoclaved RMWSE. Data are means (±SE) of three replicate cultures per strain.

2.3.3. Mixotrophic growth

To identify the mechanisms underlying the ability of C. luteoviridis and P. hussii to grow well in wastewater conditions, organic carbon utilisation was first examined. These two strains were assessed to examine whether they were more efficient than
the other indigenous strains at growing mixotrophically, and therefore able to utilise external carbon more efficiently. The strains were cultivated in artificial inorganic medium with only ambient CO$_2$ (air) present but not bubbled into the culture, and either without added external carbon source or with added acetate, methanol or glycerol. All five species could efficiently utilise acetate and methanol, as shown by significantly increased growth rate and biomass productivity compared to with no added carbon ($P<0.01$), although only $H$. tetrachotoma, $P$. hussii and $D$. subspicatus could efficiently utilize glycerol ($P<0.05$) (Fig. 4A and B). However, there was no significant increase in the ability of $C$. luteoviridis and $P$. hussii to utilise acetate, methanol and glycerol relative to the other strains.
Fig. 4. Heterotrophic growth capability of the five indigenous strains (*C. debaryana*, *H. tetrachotoma*, *C. luteoviridis*, *P. hussii*, *D. subspicatus*) in artificial inorganic media with different external carbon sources and no added carbon. (A) Biomass productivities were determined during exponential phase. (B) Specific growth rates per day were determined during exponential growth phase. Data values are means (±SE) of three replicate cultures per strain.

2.3.4. Oxidative stress tolerance

Oxidative stress tolerance was next evaluated in the isolated strains. The RMSWE provides a stressful environment that is likely to inhibit microalgal growth through the generation of oxidative stress. To confirm that wastewater induce oxidative stress...
within microalgae cells, a fluorescent reporter stain DCFH-DA for intracellular ROS production was used. DCFH-DA is oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS (He et al., 2002). The five isolated strains were grown in non-stressed conditions in TAP medium then transferred to RMSWE and incubated for 12 h after which ROS production was quantified. Similarly, a control experiment was carried out on the strain with the use of H$_2$O$_2$ as oxidative stress agent. In all strains including non-adapted culture collection strain C. vulgaris CCAP 211/79 and C. reinhardtii (data not shown) there was an increase in ROS generation following exposure to RMSWE relative to non-stressed conditions by approximately 130-170% (Fig. 5A). As expected, greater ROS generation by 328-857% was observed in those cultures treated with 25 mM H$_2$O$_2$ (Fig. 7A).
Fig. 5. Oxidative stress tolerance in *C. luteoviridis* and *P. hussii*. (A) ROS accumulation in the five isolated strains in response to untreated RMSWE exposure for 12 h as determined by DCFH-DA staining. (B) Increased survival of *C. luteoviridis* and *P. hussii* compared to other isolated strains determined by relative cell density (OD680) of late exponential phase cultures grown in TAP medium before and after incubation with 5 to 25 mM H$_2$O$_2$ for 24 h. (C) Percentage of live versus dead cells in populations of the five strains 24 h after treatment with 5 to 25 mM H2O2 determined by microscopic visualization of Evans Blue stained cells. (D) APX activity of the five isolated strains grown in direct oxidative stress conditions (12 h in TAP medium containing 25 mM H2O2) or untreated RMSWE conditions (12 h exposure). Data are means (±SE) of three replicate cultures per strain.

In order to examine whether the improved growth of *C. luteoviridis* and *P. hussii* in wastewater conditions was due to increased oxidative stress tolerance, all five strains were grown in TAP medium and artificially stressed by the addition of H$_2$O$_2$ (ranging from 5 to 25 mM) for 24 h and cell density was compared to prior to the oxidative stress induction to determine percentage survival. At 5 and 10 mM H$_2$O$_2$, the reduction in cell density for all strains was very small, but at 15 and 20 mM H$_2$O$_2$ there was a significant reduction (≥ 50%) in cell density of *C. debaryana*, *H. tetrachotoma* and *D. subspicatus* whilst there was almost complete survival (*P*<0.01).
of C. luteoviridis and P. hussii (Fig. 5B). At 25 mM H$_2$O$_2$ all strains showed reduced cell density, although C. luteoviridis and P. hussii still had 36% and 44% survival, respectively. To confirm that the increased survival of C. luteoviridis and P. hussii was due to reduced cell death, the strains were stained with the cell death indicator Evans Blue. In response to H$_2$O$_2$ treatment, a high proportion of C. debaryana, H. tetrachotoma and D. subspicatus cells showed strong Evans Blue staining, indicative of dead cells (Fig. 6). The percentage of live cells following 15, 20 and 25 mM H$_2$O$_2$ treatment is significantly higher (P<0.01) for C. luteoviridis and P. hussii (Fig. 5C). In addition, C. luteoviridis and P. hussii exhibited significant tolerance to methyl viologen-induced oxidative stress with robust growth (approximately 70% survival) following 1 mM methyl viologen treatment (Fig. 7B). In contrast the other three strains were very sensitive to this high methyl viologen concentration (Fig S2), although still significantly more tolerant compared to a sensitive strain such as C. reinhardtii (data not shown).
Fig. 6. Light microscopy visualisation of the five isolated strains stained with the cell death stain Evans Blue 24 h after treatment with 5 and 20 mM H\textsubscript{2}O\textsubscript{2}. Dead Evans Blue stained cells are indicated by arrows. Scale bar = 4 \(\mu\)m. Representative images are shown.
To test the hypothesis that the *C. luteoviridis* and *P. hussii* strains identified in this study are likely to have adapted to the secondary effluent conditions by increased oxidative stress tolerance through increased activity of anti-oxidant enzymes, APX activity was quantified in the five isolated strains under direct oxidative stress (H$_2$O$_2$ addition) and wastewater treatment conditions. Similar experiment was also carried with the control strains which were not adapted to RMWSE. In all strains, APX activity was equivalent between cells grown in 25 mM H$_2$O$_2$ TAP medium and RMSWE but was significantly higher ($P<0.01$) in *C. luteoviridis* and *P. hussii* compared to the three other isolated strains, and the non-adapted strains at 12h of incubations (Fig. 5D). When APX activity was compared between *C. luteoviridis* and *P. hussii*, and the non-adapted *C. vulgaris* strain, activity was substantially higher in these adapted strains compared to *C. vulgaris* in response to RMSWE exposure (Fig. 7C). In all strains APX activity was significantly lower when grown under non-stressed conditions, yet in the isolated strains activity was still significantly higher ($P<0.01$) compared to the control strain under non-stressed conditions.
Fig. 7. ROS accumulation in the five isolated strains in response to direct oxidative stress (12 h in AP medium containing 25 mM H₂O₂) as determined by DCFH-DA staining (A). Increased survival of C. luteoviridis and P. hussii determined by relative cell density (OD680) of late exponential phase cultures grown in TAP medium before and after incubation with 0.01 mM to 5 mM methyl viologen for 24 h (B). APX activity of C. luteoviridis and P. hussii, and a non-adapted strain (C. vulgaris CCAP 211/79), grown in non-stressed (TAP medium), direct oxidative stress (12 h in TAP medium containing 25 mM H₂O₂) or untreated RMSWE (12 h exposure) conditions (C). Data values are means (± SE) of three replicate cultures per strain.
3.5. **Oil yield characteristics**

One of the key criteria for selecting strains for biofuel feedstock is their lipid productivity, therefore it was critical to evaluate lipid characteristics of the strains. Among all the five isolated strains, *C. luteoviridis* and *P. hussii* had the highest mean percentage total lipid content of 27.7% and 35.7% of the dried weight biomass, respectively, in RMSWE conditions. Coupled with the high biomass productivity values for these strains (Fig. 3B), the total lipid productivity values of *C. luteoviridis* and *P. hussii* were significantly higher ($P<0.01$) in RMSWE conditions than the other three strains (Fig. 8A). To specifically quantify the relative accumulation of neutral storage lipid bodies within the cells, the fluorescent Nile Red stain was used (Dean et al., 2010). *C. luteoviridis* and *P. hussii* had markedly higher ($P<0.01$) storage lipid accumulation per cell than the other strains (Fig. 8B).
Fig. 8. Total lipid productivity determined during exponential phase (A) and cellular neutral lipid concentration determined by Nile Red staining at stationary phase (B) of the isolated strains (C. debaryana, H. tetrachotoma, C. luteoviridis, P. hussii, D. subspicatus) in untreated and autoclaved RMWSE. Data are means (±SE) of three replicate cultures per strain.

To compare this level of storage lipid accumulation in *C. luteoviridis* and *P. hussii* with the maximum potential lipid accumulation, the impact of nutrient starvation was assessed, as this is known to significantly induce storage lipid accumulation in many species of microalgae (Hu et al., 2008; Dean et al., 2010). As anticipated, starvation of both NH$_4^+$ and PO$_4^{3-}$ relative to replete concentrations of nutrients significantly inhibited ($P<0.01$) cell growth rate and biomass productivity of both
strains (Fig. 9). Interesting both strains were not markedly affected by intermediate concentrations (3.5 mM and 0.7 mM) of NH$_4^+$ that significantly inhibit growth in C. reinhardtii (Webster et al., 2011). Relative storage lipid accumulation in C. luteoviridis and P. hussii cells grown in 70 μM NH$_4^+$ and 1 μM PO$_4^{3-}$ concentrations was approximately to double to that observed in RMSWE conditions (Fig. 9 E and F), indicating that although the secondary wastewater cultivation provides a stressful environment it was not severe enough to induce maximal oil production in the algae.

**Fig. 9.** Specific growth rate determined during exponential phase of isolated C. luteoviridis and P. hussii strains grown in artificial TAP medium in response to N deficiency (A) and P deficiency (B). Biomass productivity of each strain cultivated in N deficiency (C) and P deficiency (D) TAP medium. Relative neutral lipid concentration in Nile Red stained C. luteoviridis and P. hussii cells at stationary phase in response to N deficiency (E) and P deficiency (F). Data values are means (±SE) of three replicate cultures per strain.
The fatty acid profile of the lipids accumulated by *C. luteoviridis* and *P. hussii* in RMSWE conditions was determined by quantification of FAME content. The majority of the lipids accumulated in both strains contained unsaturated 16 and 18 carbon fatty acids. Most fatty acids produced by *P. hussii* were C18:3, C18:2 and C18:1, with a smaller proportion of 16 carbon fatty acids, while *C. luteoviridis* also had a high proportion of C18:3 and C18:1 but also C16:0 fatty acids, and a smaller proportion of C18:2, C18:0 and C16:1 fatty acids (Fig. 10). Various chemical properties of the FAME biodiesel from *C. luteoviridis* and *P. hussii* were estimated from equations to determine the iodine value and saponification value (Gopinath et al., 2009a). These values can then be used to predict the cetane number of the FAME. Such predicted values have been shown to correspond closely to measured values (Gopinath et al., 2009a; 2009b). For *C. luteoviridis* a saponification value of 207.91, an iodine value of 71.63 and a cetane number of 56.43 were predicted, while for *P. hussii* a saponification value of 184.06, an iodine value of 57.03 and a cetane number of 63.12 were predicted.
Fig. 10. Abundance of specific fatty acid methyl esters (FAME) in C. luteoviridis and P. hussii grown in untreated RMWSE. Data are means (±SE) of three replicate cultures, and are representative of four independent experiments.

3.6. Remediation characteristics

The chemical characteristics of RMWSE from the treatment plant over a full year found that total N which is the summation of all the nitrogen sources detected was ranged between 33.3 - 75 mg L⁻¹ throughout the year of which 22.7 - 30.1 mg L⁻¹ was NH₄⁺, and PO₄³⁻ ranged between 1.4 - 1.7 mg L⁻¹ in April to September, and 0.1 - 0.9 mg L⁻¹ in October to March (Table S3). In addition the pH ranges from 7.5 to 7.7 and the BOD (7 - 16 mg/L) and COD (83 – 112 mg/L) were stable in most of the months except in January and Feburary when they were high. The remediation potential of the five strains in RMWSE was determined for NH₄⁺-N and PO₄³⁻-P removal and BOD reduction. BOD in RMWSE, relative to the activity in the absence of microalgae, showed a large decrease after 5 days of cultivation with each of the five strains but C. luteoviridis and P. hussii showed the highest reduction (>79%)
More than 80% NH$_4^+$-N was removed in RMWSE by *C. luteoviridis* and *P. hussii* after 5 days of cultivation, while remediation efficiency was slightly lower for the other three species (Fig. 11B). The removal of PO$_4^{3-}$-P followed a similar trend with highest removal by *C. luteoviridis* and *P. hussii* of approximately 80% after 5 days.

**Fig. 11.** Biochemical oxygen demand (BOD) reduction (A) and NH$_4^+$-N and PO$_4^{3-}$-P removal (B) by the five isolated strains (*C. debaryana*, *H. tetrachotoma*, *C. luteoviridis*, *P. hussii*, *D. subspicatus*) in untreated RMWSE after 5 days of cultivation, relative to RMWSE without added microalgae. Data are means (±SE) of three replicate cultures per strain.
2.4 Discussion

Microalgae strains from the genus of each of these five species have previously been shown to grow on wastewater conditions (Li et al., 2011, Zhou et al., 2011; Shimura et al., 2012). Chlorophyte algae such as *Chlorella* and *Chlamydomonas* are often dominant in wastewater stabilization ponds, with organic loading, and ammonia and sulphide tolerance indicated as potential factors in determining species dominance (Konig et al., 1987; Pearson et al., 1987); however, the exact mechanisms of wastewater tolerance are unclear and none of these specific species have previously been evaluated for biofuel or wastewater treatment characteristics. Non-adapted microalgae are very sensitive to wastewater. For example, growth of culture collection *Chlorella* strains was very poor on RMSWE with growth rates <0.08 day⁻¹ (Fig. 2A). The mean biomass productivity of 0.77 g L⁻¹ day⁻¹ by *P. hussii* is comparable to a *Chlamydomonas reinhardtii* strain grown in domestic wastewater centrate (Kong et al., 2010) and is better than values from previous secondary treated wastewater studies (Martinez et al., 2000; Orpez et al., 2009).

Microalgae growth in wastewater can be enhanced by autoclaving the water (Cho et al., 2011). We observed significant increase in growth rate of culture collection *C. vulgaris* and *C. ellipsoidea* when grown in autoclaved RMWSE compared to untreated RMWSE (Fig. 2A). Although algae and heterotrophic bacteria have a mutualistic relationship in facultative ponds with algae providing O₂ for the bacteria, which in turn provide CO₂ and inorganic N and P for the algae, wastewater microorganisms including some anaerobic bacteria and viruses can be toxic or outcompete microalgae species (Cho et al., 2011). Absence of microorganisms could be one explanation for improved growth in autoclaved media, although breakdown
of toxic organic compounds following autoclaving could be another reason. However, the isolated strains grew well in non-autoclaved, untreated wastewater and there was no significant increase in growth in autoclaved RMSWE (Fig. 2B and Fig. 3). Even under autoclaved conditions, growth of the indigenous strains was better than culture collection strains suggesting that other factors also influence strain toxicity to wastewater to which the indigenous strains have adapted.

To identify the mechanisms underlying the ability of *C. luteoviridis* and *P. hussii* to grow well in wastewater conditions, carbon utilisation was first examined. These two strains were assessed to examine whether they were more efficient than the other indigenous strains at growing mixotrophically, and therefore able to utilize external carbon more efficiently. Microalgae in general have the ability to change their metabolic processes in response to environmental conditions (Devi et al., 2012). Some algae can grow photoautotrophically by the use of light and CO₂ through photosynthetic processes and some heterotrophically by the use of organic carbon sources (Brennan and Owende 2010; Devi et al., 2012). In heterotrophic cultivation, cell growth and biomass and lipid productivity can be significantly influenced by the nutrients present in the cultivation medium (Devi et al., 2012; Borowitzka, 1999; Perez-Garcia et al., 2011; Brennan and Owende, 2010). For example, a study has found that lipid content of *Chlorella protothecoides* cultivated in heterotrophic conditions were three times higher than when cultivated in autotrophic condition (Miao and Wu, 2004). In another study, *Chlorella pyrenoidosa*, *C. vulgaris* and *C. sorokiniana* grown in glucose as a sole source of carbon showed an increased growth rate (Choix et al., 2012; Pleissner et al., 2013). Also, some studies have suggested
that waste glycerol from transesterification could be fed back to be used as a carbon source to enhance growth and biomass productivity of algae (Ethier et al., 2011).

The ability of microalgae to successfully utilise organic carbon compounds present in wastewater effluent and thus grow mixotrophically is critical to the success of algal wastewater strategies as it will allow substantial improvement in biomass productivity and higher lipid productivity (Xu et al., 2006; Zhou et al., 2012). The lack of significant increase in growth rate and biomass productivity of *C. luteoviridis* and *P. hussii* in acetate, methanol and glycerol relative to the others strains (Fig 4A and B), suggesting that differential carbon utilisation between the strains was not a critical factor.

The presence of abiotic chemical component such as heavy metals in wastewater can induce production of ROS (Pinto et al., 2003). These ROS can cause metabolic damage on the cell which can lead to cell death. This study established the presence of ROS generation in RMWSE as evidence in the percentage increase relative to the control (Fig 5A). This increase is synonymous to that induced with exogenous H$_2$O$_2$. This oxidative condition poses a challenge to growth of algae in RMWSE. The ability of algae to survive in high oxidative conditions will be important in determining strain suitability for RMWSE cultivation. In this study, *C. luteoviridis* and *P. hussii* showed high tolerance to oxidative stress. Therefore, it can be deduced that their high growth rates and increased biomass productivity in RMWSE is at least in part due to enhanced oxidative stress tolerance. Their oxidative stress tolerance was further confirmed by treating the strains with methyl viologen, which also induces internal ROS production (superoxide), is a more stable chemical than H$_2$O$_2$, and is highly toxic to most algae; for example, *C. reinhardtii* has an IC$_{50}$ for methyl
viologen of 0.03 µM (Tanaka et al., 2011). *C. luteoviridis* and *P. hussii* exhibited significant tolerance to methyl viologen-induced oxidative stress (Fig 7B). These results demonstrate that the resilience of these two strains to stress in wastewater conditions and their increased biomass productivity is at least in part due to enhanced oxidative stress tolerance. *Parachlorella* strains may be particularly tolerant to toxic environments. *Parachlorella* strains may be particularly tolerant to toxic environments. Recently, a strain of *Parachlorella* sp. *binos*, which is most closely related but not identical to *Parachlorella kessleri*, was isolated from activated sludge at a wastewater plant, could also grow in saline conditions, and showed significant radiation tolerance (Shimura et al., 2012).

Higher oxidative stress tolerance is an indicator of many extremophile species, including microalgae that have adapted to tolerate toxic environments. For example, *Chlamydomonas* sp. W80 and *Chlamydomonas* sp. HS5, which can grow in high saline environments, can tolerate oxidative damage caused by salinity by high expression of an APX enzyme that functions as an anti-oxidant (Tanaka et al., 2011). We observed that APX was high in the isolated strains even under non-stressed conditions (Fig. 7C; P<0.01) when compared to the control culture collection strains. This is different to the situation in *Chlamydomonas* sp. W80 and *Chlamydomonas* sp. HS5 where APX activity was identical regardless of whether the cells were stressed or unstressed but in both cases substantially higher than in the non-stress tolerant *C. reinhardtii* (Tanaka et al., 2011).

This study has therefore demonstrated that selected microalgae strains are able to provide high biomass productivity, however, if these strains are to be utilised as feedstock for biodiesel production, then it was important that lipid productivity
characteristics be determined. The lipid productivity values determined for the indigenous strains were substantially higher than those observed in many previous wastewater cultivation experiments (Pittman et al., 2011). Neutral storage lipid accumulation was slightly higher (P<0.05) under untreated wastewater conditions than autoclaved conditions (data not shown), probably due to the stress induction of lipids. Studies have shown that intracellular lipid content of algae increase when they are grown in either nutrient stress or environmental stress condition (Sharma et al., 2012; Adams et al., 2012; Bartley et al., 2013). For example *C. reinhardtii* and *S. subspicatus* grown in nutrient stress condition were observed to show significant increase in neutral lipid in comparison to when they were grown in non stress condition (Dean et al., 2010). In a similar study, TAG were found to significantly increase in *Nannochloropsis* sp following cultivation in a nutrient stressed medium (Pal et al., 2011). Lipid accumulation characteristics have not previously been examined for *C. luteoviridis* or *P. hussii* species when cultivated in either wastewater or nutrient starvation conditions; however, many studies have previously demonstrated that selected strains of *Chlorella* species are able to accumulate high concentrations of storage TAG lipids in response to stress (Xu et al., 2006; Hu et al., 2008; Wang et al., 2010; Pribyl et al., 2012), and this genus has been one of the most intensely studied for microalgal biofuel research. More recently, strains of *P. kessleri* were identified with high lipid content (~51% of dry weight) and lipid productivity (0.58 g L⁻¹ day⁻¹) when grown under nutrient limitation conditions (Pribyl et al., 2012; Li et al., 2013). *Parachlorella* strains may therefore be good candidates for further research into biofuel and wastewater applications (Li et al., 2013; Mizuno et al., 2013).
The FAME biodiesel quality is an important factor for chosen algae species for biofuel feedstock. Biodiesel with low cetane and saponification values are considered good quality fuel for an internal combustion diesel engine (Gopinath et al., 2009b). The cetane number usually decreases with increasing unsaturation of FAME. The quality standards for diesel fuel require a minimum cetane number of 40 and standards for biodiesel fuel suggest a minimum cetane number of 51 (Gopinath et al., 2009a). The values of 56.4 and 63.1 measured here for C. luteoviridis and P. hussii FAME, respectively, are therefore suitable for biodiesel usage and are equivalent to cetane number values that have been measured from palm oil and sunflower oil derived biodiesel (Gopinath et al., 2009a).

The dual use of algae in wastewater as a biofuel feedstock and a remediation agent is a unique attraction from an economic and sustainability point of view (Pittman et al., 2011; Chapter 1). In this study, remediation potential of the isolates was tested for N and P removal and BOD reduction (Fig. 11). Their N and P removal values were better or equivalent to those seen previously by algae from wastewaters (Kong et al., 2010; Wang et al., 2010; Johnson et al., 2010; Cho et al., 2011), however, it is important to note that the N and P concentrations vary markedly in these different wastewater types. The microalgae are also likely to be able to accumulate and assimilate other forms of N which will determine the strain’s total N remediation efficiency. Similarly, the BOD were significantly reduced in all the culture Future experiments will be able to evaluate the remediation potential of these strains on effluent from other steps in the treatment process such as the centrate, which has significantly higher N and P levels.
2.5 Conclusions

This study has shown that *C. luteoviridis* and *P. hussii* strains isolated from wastewater tanks can grow very efficiently in raw wastewater secondary effluent and display high biomass productivity and remediation efficiency. These strains are likely to have adapted to the secondary effluent conditions by increased oxidative stress tolerance which is due, at least in part to higher activity of the anti-oxidant enzyme APX. Oxidative stress tolerance is therefore an ideal trait to identify when screening strains for wastewater cultivation or may be a target for future engineering of strains. In addition, both strains display high lipid productivity values and fatty acid characteristics that indicate suitable biodiesel quality, demonstrating that they have potential as a future feedstock for biofuel applications which could be coupled to wastewater pollutant remediation.

Acknowledgments

The authors would like to thank the staff at United Utilities, particularly Dr. Son Le, Dave Leslie and Ruyi Hu for their advice, assistance and wastewater samples, provided under the terms of their grant agreement No. 265269 with the European Union Seventh Framework Programme (FP7-ENV.2010.3.1.1-2 ENV). We are grateful to Dr. David Knight for assistance with mass spectrometry, and to Rob Mansfield, Dr. Rachel Webster and Dr. Andrew Dean for technical advice and comments to the manuscript. This work was supported by the University of Manchester Sustainable Consumption Institute Doctoral Training Centre.
Fig. S1. Correlation plots of optical density (OD at 680 nm) against cell number (A) and OD680 against dried biomass (B) of *C. luteoviridis* grown in RMWSE. Data points are mean values of three replicate samples taken throughout the exponential growth period. Equivalent positive correlations between OD680 and cell number or dried biomass were observed for the other four isolated strains (*C. debaryana, H. tetrachotoma, P. hussii, D. subspicatus*), each with R² > 0.9.
**Fig S2.** Culture picture of C. debaryana, H. tetrachotoma, C. luteoviridis, P. hussii, D. subspicatus (from left to the right) after incubation with 0.01 mM to 5 mM methyl viologen for 24 h.
Table S1. Accession numbers of ribosomal RNA 18S and ITS nucleotide sequence of selected unicellular Chlorophyceae microalgae used for phylogenetic analysis and culture collection strain ID numbers.

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<th>Species</th>
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<sup>a</sup>Stock centres: ACOI, Coimbra Collection of Algae, Department of Botany, University of Coimbra, Portugal; CCAP, Culture Collection of Algae and Protozoa, Scottish Association for Marine Science, U.K.; SAG, Sammlung von Algenkulturen, Universität Göttingen, Albrecht-von-Haller-Institut für Pflanzenwissenschaften, Germany; UTEX, Culture Collection of Algae/MCDB, University of Texas, U.S.A.  
<sup>b</sup>Formly *Scenedesmus subspicatus*
Table S2. Mean biovolume of the five microalgae species when grown in RMSWE.

<table>
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<th>Strain</th>
<th>Species</th>
<th>Mean biovolume ±S.E.</th>
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<td>Strain A</td>
<td>Chlamydomonas debaryana</td>
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<td>Strain B</td>
<td>Hindakia tetrachotoma</td>
<td>28.74±5.02 µm³</td>
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<td>Strain C</td>
<td>Chlorella luteoviridis</td>
<td>31.70±1.21 µm³</td>
</tr>
<tr>
<td>Strain D</td>
<td>Parachlorella hussii</td>
<td>52.38±1.56 µm³</td>
</tr>
<tr>
<td>Strain E</td>
<td>Desmodesmus subspicatus</td>
<td>17.92±3.42 µm³</td>
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Table S3. Physiochemical characteristic of the wastewater secondary effluent United Utilities wastewater treatment plant at Ellesmere Port, Cheshire, U.K in 2010. Values are mean data derived from 8-18 samples per month

<table>
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<tr>
<th>Months</th>
<th>NH₄⁺ (mg/l)</th>
<th>NO₃⁻ (mg/l)</th>
<th>Total N (mg/l)</th>
<th>PO₄³⁻ (mg/l)</th>
<th>Suspended solids (mg/l)</th>
<th>BOD (mg/l)</th>
<th>COD (mg/l)</th>
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<td>February</td>
<td>28.679</td>
<td>19.240</td>
<td>68.708</td>
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<td>28.071</td>
<td>33.425</td>
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<td>April</td>
<td>26.953</td>
<td>23.277</td>
<td>74.936</td>
<td>1.692</td>
<td>23.286</td>
<td>16.060</td>
<td>88.286</td>
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<tr>
<td>May</td>
<td>30.121</td>
<td>26.221</td>
<td>74.967</td>
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<td>10.586</td>
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<td>June</td>
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<td>23.969</td>
<td>56.573</td>
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<td>November</td>
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<td>7.963</td>
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This chapter examines the role of acclimation for gaining tolerance to wastewater by microalgae. This is a follow-up study to Chapter 2 where I reported that indigenous algae grow well in wastewater potentially due to long term adaptation. The Chapter comprises of a paper manuscript to be submitted for publication.
Improvement of microalgae growth in wastewater through acclimation

Olumayowa Osundeko¹,², Helena Davies¹ and Jon K. Pittman¹

¹Faculty of Life Sciences, The University of Manchester, Michael Smith Building, Oxford Road, Manchester M13 9PT, UK
²Sustainable Consumption Institute, The University of Manchester, 188 Waterloo Place, Oxford Road, Manchester M13 9PL, UK

E-mail addresses: jon.pittman@manchester.ac.uk (J.K. Pittman), olumayowa.osundeko@postgrad.manchester.ac.uk (O. Osundeko).

Authors’ Contributions

Olumayowa Osundeko performed all the experimental work. Helena Davies helped in repeating growth experiments of the acclimated species in RMWSE. The manuscript was written by Olumayowa Osundeko and edited by Jon Pittman
Abstract

Unicellular green microalgae that had not been previously grown in wastewater and were closely related or identical species to the indigenous strains previously isolated from secondary effluent in Chapter 2, were obtained from a culture collection. The strains were acclimated in raw municipal wastewater secondary effluent (RMWSE) for 8 weeks. The acclimated strains had significantly higher growth rate, and total chlorophyll concentration than the non-acclimated strains except for *Chlamydomonas debaryana* and *Desmodesmus intermedius*. The result indicates that acclimation increased algae growth and tolerance to wastewater effluent and thereby increased their total biomass and lipid productivities. Although the percentage total lipid per dried biomass and neutral lipid per cell were higher in the non-acclimated algae than the acclimated ones, total lipid productivity values were significantly lower than the acclimated strains. Furthermore, no less than 70% of P and N were removed from the wastewater after 10 days of cultivation except for the *C. debaryana* culture in which less then 60% of N was removed. Also, the reductions of biochemical oxygen demand (BOD) by the algae were significantly improved by acclimation. Although acclimation improved the growth of all strains in wastewater, the result of this study also suggested that species specific factors were also important.

**Key words:** Biofuel; Microalgae; Acclimation; Wastewater bioremediation.
3.1 Introduction

Algae have been described as a sustainable source of fuel because they are capable of producing more lipid per biomass than most other plant feedstocks and require minimal agricultural land for cultivation (Chisti, 2007). However, recent life cycle analyses have questioned the sustainability of algae biofuel especially in terms of energy and water requirements (Sing and Olsen, 2011; Holma et al., 2013). Some of these concerns have been linked to high reliance on nutrients and fresh water for cultivation. The use of wastewater can potentially reduce the reliance on nutrient and freshwater (Pittman et al., 2011). Additional advantage of growing algae in wastewater includes their ability to remove nutrient as well as heavy metal from wastewater by cellular uptake (Xin et al., 2010; Chapter 1). The dual potential of algae for biofuel and remediation offers economic advantage to the strategy of cultivating algae in wastewater.

Despite the fact that wastewater provides a cheap and sustainable medium for growing algae for biofuel, the prospect still significantly relies on the capability of algae to grow in wastewater amidst competing biotic and abiotic factors (Hoffmann, 1998). In Chapter 2, we observed that wastewater can induce oxidative stress on algae and to be suitable for wastewater cultivation algae must possess a defence mechanism against oxidative stress. Wastewater chemical components usually vary with the type of influent in wastewater treatment plants and due to this heterogeneous nature, it may not be possible to assign a species for cultivation. Microalgae that are tolerance to oxidative stress are likely to be versatile for cultivation in wastewater effluent. The use of indigenous strains offers the advantage of adaptability to the prevailing biotic and abiotic competing factors in the
wastewater. Such strains are known to grow effectively in wastewater (McGinn et al., 2012; Wu et al., 2012; Chapter 2). Genetically modified (GM) algal strains are currently unlikely to be grown in wastewater for biofuel production or remediation due to several environmental legislative barriers and the potential loss of such strains to the environment (Xiong et al., 2010). However, indigenous strains can require extensive laboratory work to isolate therefore culture collection algae can be more accessible for cultivation. However, they often perform poorly in wastewater compared to when grown in synthetic medium (Kong et al., 2010; Li et al., 2011; Chapter 2). Culture collection strains are normally kept and preserved in optimised artificial medium that were well buffered and contain the right proportions of macro and micro nutrients. This is not the same as in wastewater effluent which is often heterogeneous in nutrient constituents and can contain toxins such as heavy metals, and biotic components such as other microbes (Pittman et al., 2011; Chapter 1). Thus, culture collection strains grow poorly in wastewater; for example, the growth of a culture collection *Chlorella vulgaris* in wastewater secondary effluent was significantly lower than that of the indigenous *Chlorella luteoviridis*. Therefore any process of improving algal growth in wastewater will be desirable.

Adaptation and acclimation are terms used to describe the changes a plant or animal undergoes to adjust to a new environment or condition. Whilst adaptation is about organism’s ability to change its physical and chemical make up in response to changes in its environment and usually caused by underlying hereditable genetic changes, acclimation is a temporary form of adaptation that may not be replicated in the progeny. Acclimation allows algae to adjust to a new environment prior to cultivation. For example, freshwater *Spirulina* was able to grow in seawater
following acclimation (Shao-chen, 2012). Also, Wang (1985) reported that algal tolerance to Zn toxicity increased following acclimation regardless of the origin of the algal source. In another study by Lau et al. (1996), physiological acclimation of microalgae to a wastewater environment prior to the cultivation in primary settled wastewater enhanced nitrogen (N) and phosphate (P) removal efficiencies. However, the impact of acclimation on biomass and lipid productivity on algae cultivated in wastewater remains unknown.

A recent study in our laboratory has isolated some new indigenous algae strains from wastewater treatment tanks (Chapter 2). Most of these algae have not been studied for wastewater cultivation and two of them; Parachlorella hussii and Chlorella leutuveridis, showed high propensity for biofuel production and remediation of N and P when cultivated in raw municipal wastewater secondary effluent (RMWSE). The success of these strains was linked to their oxidative stress tolerance. However, it was not known if these indigenous algae grew well in wastewater because of inherent species specific characteristics or because they have adapted to wastewater. Although culture collection strains have been used a lot in wastewater studies, when compared against indigenous strains they do not perform as well (as shown in Chapter 2). Therefore it is of interest to see if acclimation of these strains in the laboratory can improve their performance in wastewater.

Therefore, this study aimed to elucidate the impact of acclimation on growth, biofuel productivity and remediation capability of culture collection strain in wastewater secondary effluent. In doing this, microalgae species identical or similar to those reported in Chapter 2 were collected from a culture collection and two consecutive experiments were set up. The first experiment was to examine the growth profile,
biomass and lipid productivities and remediation capability of the algae before acclimation in wastewater effluent. In the second stage, the algae were subjected to an acclimation process and their growth, biofuel and remediation profiles were again measured. The comparison of algae before and after acclimation provided the basis for determining the acclimation impact on these algae for growth in wastewater effluent. In addition to these strains, some well studied culture collection strains for biofuel production that have been described to grow effectively in synthetic medium; *Chlorella vulgaris* (Cha et al., 2011), *Chlorella ellipsoidea* (Zhang and Hong 2013), *Neochloris pseudostigmatica* (Kawata et al., 1998) and *Botryococcus braunii* (Ranga Rao et al., 2012) were also included in this study. This will provide understanding in knowing whether acclimation in general could improve performance of algae (that normally grown in synthetic medium) in wastewater or whether there are any species-specific determinants of efficient wastewater growth. These experiments will serve as a basis for understanding the importance of acclimation in algae cultivation in wastewater for remediation and biofuel production.
3.2 Materials and Methods

3.2.1 Microalgae strains

Algae strains identical to those reported in Chapter 2 were obtained from the Culture Collection of Algae and Protozoa (CCAP): *Chlamydomonas debaryana* (CCAP 11/70), *Chlorella luteoviridis* (CCAP 211/3), and *Hindakia tetrachotoma* (CCAP 222/81). Culture collection strains of identical species to *Desmodesmus subspicatus* and *Parachlorella hussii* were not available, therefore the most closely related strains *Desmodesmus intermedius* (CCAP 258/38) and *Parachlorella kessleri* (CCAP 211/11G) were instead obtained. In addition, some well studied algae that have been described for their effective growth and oil productivity in synthetic media were also obtained from CCAP: *Chlorella ellipsoidea* (CCAP 211/33), *Chlorella vulgaris* (CCAP 211/79), *Neochloris pseudostigmatica* (CCAP 254/11) and *Botryococcus braunii* (CCAP 807/2). All strains were grown up to late exponential phase in Jaworski’s Medium (JM) (Thompson et al., 1988) synonymous in nutrient constituent to the RMWSE medium. Aliquot samples of each algae culture were subjected to the acclimation process in RMWSE (described below), following which these strains are referred to in this study as acclimated strains. The remaining samples were kept in JM medium and were not acclimated in RMWSE and are referred to as non-acclimated strains.

3.2.2 Acclimation in RMWSE

The physicochemical characteristic of the RMWSE used in this study are essentially identical to that described in Chapter 2. The RMWSE was collected from United Utilities Water PLC wastewater treatment plant at Ellesmere Port, Cheshire, UK.
Prior to using the RMWSE, the effluent was allowed to settle and settled particles were removed by decantation.

For the acclimation process, microalgae were cultured first in fresh medium containing 50% (v/v) JM and 50% (v/v) autoclaved RMWSE for 5 days. 1ml aliquot from these cultures was diluted with 10 ml of fresh autoclaved RMWSE. 20 µL of diluted algae culture was incubated on autoclaved RMWSE agar plates containing 15% (w/v) agar for 14 days. Distinct healthy algae colonies from each plate were selected and sub-cultured in either 10 ml of autoclaved or untreated RMWSE in a universal tube for 7 days. This was to select the most tolerant cells for each strain. Microalgae in autoclaved and untreated RMWSE from this selection were subjected to further acclimation for 8 weeks by continuous subculture in freshly autoclaved and untreated RMWSE, respectively, at an inoculate volume of 4% every 7 days. Algae growth during this process was on a orbital shaker rotating at 2 Hz, 22 °C, 16 h light:8 h dark and a photon flux of 150 µmol m$^{-2}$ s$^{-1}$. Optical density at 680nm was measured for each culture as an indicator for growth at every 7 days of the 8 week acclimation process. At each stage of the acclimation process, the presences of algae in the culture were routinely monitored by microscopic examination.

3.2.3 Strain cultivation

Batch cultures of the acclimated and non-acclimated strains in autoclaved and untreated RMWSE were set up in 200 ml glass flasks in triplicate on an orbital shaker at 2 Hz at 22°C, 16 h light : 8 h dark and a photon flux of 150 µmol m$^{-2}$ s$^{-1}$.

Algae that were grown in autoclaved or untreated RMWSE during the acclimation process were maintained in the respective autoclaved or untreated RMWSE media.
In the case of the autoclaved culture, RMWSE was autoclaved at 121°C for 20 min with the aim to reduce the biota content in the effluent before microalgae inoculation. The starting cell density of each inoculate was ~0.04 in each flask as determined by optical density at 680 nm (OD$_{680}$).

### 3.2.4 Microalgae growth analysis

Cell density of each culture was routinely determined at every 2 days of the cultivation by OD$_{680}$ measurement using a Jenway spectrophotometer. This has been demonstrated to be a suitable tool for monitoring algae growth and showed a positive correlation with cell number (Chapter 2). Prior to OD measurement, RMWSE without algae was used as a blank. Specific growth rates ($\mu$) were determined at the exponential growth phase as $\mu = (\ln N_1 - \ln N_0) / (t_1 - t_0)$ where $N_0$ and $N_1$ represent OD at the early and late exponential growth phases respectively. $t_1$ and $t_0$ are the days corresponding to the $N_0$ and $N_1$ respectively.

Total chlorophyll was determined at the late exponential growth phase, using same method described in Chapter 2. Ten ml of each of the culture was centrifuged at 1500 g for 20 min, and the pellet was re-suspended in 5 ml of 80% acetone. The extract OD was measured at 663.6 nm, 646.6 nm and 750 nm. Total chlorophyll was calculated by the formula described by Porra et al. (1989).

### 3.2.5 Biomass and lipid determination

Dried weight biomass was determined by centrifugation of 50 ml cultures in late exponential growth phase at 1500 g for 20 minutes in a dried pre-weighed tube. The resulting cell pellet was dried for 48 h at 60°C and then weighed. Weight contribution by RMWSE was determined by centrifugation of RMWSE without
algae in a pre-weighed tube and dried. The resulting weight was subtracted from the algae dried weight. Total lipid was determined by the Folch method (Folch et al., 1957) following same description as in Chapter 2. Biomass and lipid productivities and percentage lipid content were calculated using the equations below:

Biomass productivity (mg L$^{-1}$ d$^{-1}$) = $\frac{WB}{V \times T}$, lipid content (%) = $\frac{WL \times 100}{WB}$, and lipid productivity (mg L$^{-1}$ d$^{-1}$) = $\frac{WL}{V \times T}$; where WB is dry weight biomass (mg), WL is lipid weight (mg), V is working volume and T is cultivation time.

To determine the intracellular neutral lipid content in the algae, cells were stained with 0.25 μg ml$^{-1}$ Nile Red (9-diethylamino-5H-benzo (α) phenoxazine-5-one) (Invitrogen) dissolved in acetone. Relative fluorescence intensity of algae cells as a result of Nile Red staining was measured using a fluorescence spectrophotometer (Hitachi F-2000) at 530 nm excitation and 575 nm emission wavelengths.

3.2.6 Nutrient and biochemical oxygen demand (BOD) removal

Aliquots of each culture medium were collected to assess the extent of microalgae remediation of N and P and BOD reduction after 10 days of cultivation. NH$_4^+$-N and PO$_4^{3-}$-P concentrations in the culture before and after cultivation of acclimated and non-acclimated strains in untreated RMWSE were analysed using standard methods, as described in Chapter 2 (Skalar Analytical). BOD measurements were performed by measuring the dissolved oxygen before and after incubation of the culture aliquots at 20°C for 5 days with Jenway 9500 Dissolved Oxygen Meter.
3.2.7 Statistical analysis

All data shown is mean data of at least three replicate experiments. ANOVA analysis and Tukey’s post hoc analysis were used to determine the significance of difference wherever applicable. All statistic tests were done with GenStat version 15.

3.3 Results

3.3.1 Growth of acclimated and non-acclimated strains in secondary effluent

In an attempt to improve culture collection algae growth in RMWSE by acclimation, all algae strains including the control showed a steady increase in OD$_{680}$ measured at each week of the 8 weeks acclimation process (Fig. 1). However, the OD$_{680}$ of the strains remained almost the same at week 7 and week 8 of the acclimation process, which suggested that they had reached the peak of the acclimation process, except for $P$. kessleri and $C$. luteoviridis, for which OD$_{680}$ was still increasing by week 8. Further growth beyond 8 weeks was not measured in this study.
Fig 1: Optical densities of the culture as an indicator for algae growth taken at every 7 days before sub-culturing in fresh RMWSE during the 8 weeks acclimation of (A) *Chlamydomonas debaryana* (C.d), *Hindakia tetrachotoma* (H.t), *Chlorella luteoviridis* (C.l), *Parachlorella kessleri* (P.k) and *Desmodesmus intermedius* (D.i), and (B) control strains *Chlorella ellipsoidea* (C.e), *Chlorella vulgaris* (C.v), *Neochloris pseudostigmatica* (N.p) and *Botryococcus braunii* (B.b). Values are mean data derived from 3 replica of each culture.

In order to determine the effect of acclimation on algae growth in RMWSE, the OD_{680} of the culture were measured to determine their cell growth. The growth curves in Figure 2 show the variation of optical density for algae grown in untreated...
RMWSE with and without acclimation. It worth mentioning that in order to monitor
the presence of resident algae in untreated RMWSE, a control experiment with no
algae inoculate was set up simultaneously with other experiments in this study. No
significant algae presence was noticed in the control experiment during the first 15
days of cultivation; however, microscopic examination of the effluent after this
duration revealed that there were mixtures of algae species and diatoms. This
suggested that indigenous algae present in the wastewater during this experiment
were below detectable levels and any contributions by these algae were negligible.
Fig 2: Growth curves of *Chlamydomonas debaryana* (C.d), *Chlorella luteoviridis* (C.l), *Desmodesmus intermedius* (D.i), *Hindakia tetrachotoma* (H.t), *Parachlorella kessleri* (P.k), *Chlorella ellipsoidea* (C.e), *Chlorella vulgaris* (C.v), *Neochloris pseudostigmatica* (N.p) and *Botryococcus braunii* (B.b) determined by optical density measurement before acclimation (A) and after acclimation (B) in untreated RMWSE. All data presented are means ±SE of three replicates.

The non-acclimated strains showed high sensitivity to untreated RMWSE (Fig. 2A) as evidence by their low OD$_{680}$ as a cell density indicator during the growth cycle. However, following acclimation of the strain to untreated RMWSE, their culture cell...
densities were significantly increased (Fig. 2B). Furthermore, specific growth rates of the acclimated strains were significantly increased when grown in either autoclaved or untreated RMWSE (Fig 3A and 3B) (P<0.01, 1-Way ANOVA, post-hoc Tukey). The growth rate of the acclimated *H. tetrachotoma* and *C. luteoviridis* strains in both untreated and autoclaved RMWSE were double that of the non-acclimated strains. Similarly, the growth rate of acclimated *C. ellipsoidea*, *C. vulgaris*, *N. pseudostigmatica* and *B. braunii* were significantly higher than that of the non-acclimated strains (P<0.05). This is an indication that the acclimation process was able to improve algae growth in RMWSE, including those species that have not previously been identified by us in wastewater medium. Similarly, total chlorophyll concentrations of the strains as an indicator for healthy growth were significantly greater in all of the acclimated cultures than in the non-acclimated cultures of the same treatment (Fig. 3C and D) (P<0.01) except for *C. debaryana*. Acclimated *P. kessleri* was the significantly most healthy strain in RMWSE both in terms of growth rate and chlorophyll concentration reaching the highest values in both untreated and autoclaved cultures (P = 0.001, 2-Way ANOVA, post hoc Tukey). In addition, *C. debaryana* showed the poorest growth in both autoclaved and untreated RMWSE. Furthermore, the growth rate and total chlorophyll of all the strains were greater when RMWSE was autoclaved (Fig 3B and D).
3.3.2 Biomass and lipid productivity

High biomass productivity in RMSWE was dependent on the strain acclimation. The biomass productivity values of strains in autoclaved (Fig. 4A) and untreated cultures (Fig. 4B) were almost double after acclimation compared to non-acclimated strains (P<0.01) except for that of C. debayana, D. intermedius and B. braunii in untreated RMWSE (P<0.05). Correlating with the growth and chlorophyll analyses (Fig. 3), P.
*kessleri* and *C. luteoviridis* had the highest biomass productivity after acclimation. There was no statistical difference in the biomass productivity among acclimated *H. tetrachotoma, C. ellipsoidea, C. vulgaris* and *N. pseudostigmatica* in either autoclaved or untreated RMWSE. Although acclimation improved biomass productivity of *B. braunii* in autoclaved RMWSE (P<0.05), no significant increase in biomass was observed in the untreated RMWSE (Fig. 4B). Moreover, biomass productivity of acclimated *D. intermedius, B. braunii* and *C. debaryana* were the lowest in both autoclaved and untreated RMWSE in comparison to the rest of the acclimated strains in this study. The result clearly showed that acclimated *P. kessleri* and *C. luteoviridis* were the best biomass producers in RMWSE in either treatment.
Fig. 4. Total biomass productivity determined gravimetrically during exponential phase of the acclimated and non-acclimated strains of *Chlamydomonas debaryana* (C.d), *Chlorella luteoviridis* (C.l), *Desmodesmus intermedius* (D.i), *Hindakia tetrachotoma* (H.t), *Parachlorella kessleri* (P.k), *Chlorella ellipsoidea* (C.e), *Chlorella vulgaris* (C.v), *Neochloris pseudostigmatica* (N.p) and *Botryococcus braunii* (B.b) cultured in autoclaved (A) and untreated (B) RMWSE. Data are means (±SE) of three replicate cultures per strain.
In some instances when algae undergo stress such as nutrient limitation stress, intracellular lipid increases but biomass productivity reduces thereby resulting in lower lipid productivity (Dean et al., 2010). In this study, the percentage total lipid content of algae in autoclaved (Fig. 5A) and untreated RMWSE (Fig. 5B) were higher in the non-acclimated strains than the acclimated strains, however, this difference was only statistically significant in *H. tetrachotoma* and *C. vulgaris* (P<0.05). Furthermore, the spectroscopy measurement of the fluorescence intensity of the cells following Nile Red staining as an indicator for the presence of intracellular neutral lipid also showed that most of the non-acclimated strains had significantly higher intracellular lipid than the acclimated strains (P<0.05) except for *P. kessleri* and *C. luteoviridis* in autoclaved RMWSE (Fig. 5CA) and except for *C. luteoviridis* and *B. braunii* in untreated RMWSE (Fig. 5D).

*B. braunii* has the highest intracellular lipid content especially in autoclaved RMWSE than the rest of the algae in this study. Apart from *B. braunii*, the amounts of neutral lipid content of the non-acclimated strains in autoclaved RMWSE were considerably similar. In addition, neutral lipid content of the non-acclimated *P. kessleri*, *D. intermedius*, *N. pseudostigmatica* and *B. braunii* were the highest when grown in untreated RMWSE which probably suggested that these strains might be naturally high lipid producers.
Fig. 5. Percentage total lipid content per dried biomass of the acclimated and non-acclimated strains Chlamydomonas debaryana (C.d), Hindakia tetrachotoma (H.t), Chlorella luteoviridis (C.l), Parachlorella kessleri (P.k) and Desmodesmus intermedius (D.i), and control strains Chlorella ellipsoidea (C.e), Chlorella vulgaris (C.v), Neochloris pseudostigmatica (N.p) and Botryococcus braunii (B.b) cultured in autoclaved (A) and untreated (B) RMWSE. Intracellular neutral lipid determined by fluorescence intensity following Nile Red staining of the acclimated and non-acclimated strains cultured in autoclaved (C) and untreated (D) RMWSE. Data are means (±SE) of three replicate cultures per strain.

Despite the high neutral lipid concentration in most of the non-acclimated strains grown in RMWSE, their lipid productivity values were significantly lower than for the acclimated strains in the same condition except in the case of C. debaryana and D. intermedius in autoclaved RMWSE (Fig. 6A) and C. debaryana and H. tetrachotoma in untreated RMWSE (Fig. 6B). Although the neutral lipid content of acclimated B. braunii was highest in autoclaved RMWSE, its lipid productivity was significantly lower than those of P. kessleri and C. luteoviridis (P<0.01).
Biomass appeared to be the driving force for the lipid productivity. Acclimated strains that had highest biomass productivity as a result of increased growth rate therefore had enhanced overall lipid productivity. Therefore acclimated *P. kessleri* and *C. luteoviridis* had the highest lipid productivity in both cultivation conditions than other strains in this study (P<0.05).
Fig. 6. Total lipid productivity during exponential phase of the acclimated and non-acclimated strains *Chlamydomonas debaryana* (C.d), *Hindakia tetrachotoma* (H.t), *Chlorella luteoviridis* (C.l), *Parachlorella Kessleri* (P.k) and *Desmodesmus intermedius* (D.i), *Chlorella ellipsoidea* (C.e), *Chlorella vulgaris* (C.v), *Neochloris pseudostigmatica* (N.p) and *Botryococcus braunii* (B.b) cultured in autoclaved (A) and untreated (B) RMWSE. Data are means (±SE) of three replicate cultures per strain.
3.3.3 Impact of acclimation on remediation capability

The remediation capability of the algae strains were tested by measuring the percentage NH$_4^+$ and PO$_4^{3-}$ removal and BOD reduction as a result of algae growth for a period of 10 days before and after acclimation in untreated RMWSE (Fig. 7). Microalgae that were similar or identical to the indigenous species reported in Chapter 2 were used for this study. Physico-chemical characteristics of the RMWSE from the United Utilities treatment plant was quantified and found to contain total nitrogen ranging from 33.3 mg L$^{-1}$ to 75 mg L$^{-1}$ and PO$_4^{3-}$ ranging from 0.1 mg L$^{-1}$ to 1.7 mg L$^{-1}$ (Chapter 2). The BOD was found to be stable throughout the year and was in the range of 7.8 mg L$^{-1}$ to 33.4 mg L$^{-1}$. In this study, the initial PO$_4^{3-}$, NH$_4^+$ and BOD of the RMWSE were 1.03 ± 0.14 mg L$^{-1}$, 25.06 ± 3.1 mg L$^{-1}$ and 13.42 ± 0.59 mg L$^{-1}$, respectively. The PO$_4^{3-}$-P and NH$_4^+$-N removal rates from RMWSE by the strains (Fig. 7A and B) were significantly higher in the acclimated cultures than the non-acclimated cultures (P<0.01). The non-acclimated strains removed less than the half of the PO$_4^{3-}$-P and NH$_4^+$-N in RMWSE. After acclimation, more than 75% of PO$_4^{3-}$-P and 60% of NH$_4^+$-N were removed from the RMWSE medium by the strains. *P. kessleri* showed the most improved remediation capability following acclimation and more than 80% of PO$_4^{3-}$-P and NH$_4^+$-N were removed.
Fig. 7. Reduction in PO$_4^{3-}$-P (A), NH$_4^+$-N (B) and biochemical oxygen demand (BOD) (C) as a result of growth of non-acclimated and acclimated *Chlamydomonas debaryana* (C.d), *Hindakia tetrachotoma* (H.t), *Chlorella luteoviridis* (C.l), *Parachlorella kessleri* (P.k) and *Desmodesmus intermedius* (D.i) in untreated RMWSE. Data are means (±SE) of three replicate cultures per strain.

Although, acclimation did not significantly improve *C. debaryana* and *D. intermedius* growth rate in untreated RMWSE (Fig. 2A), interestingly, their capability to remove PO$_4^{3-}$-P and NH$_4^+$-N were significantly improved (P<0.01). However, it worth noting that the background microbial contribution to the removal of N and P and reduction of BOD were not quantified in this study.

Furthermore, BOD reduction was significantly enhanced by acclimation (P<0.01) except for *C. debaryana* (Fig. 7C). The percentage BOD reduction in the non-acclimated *C. debaryana* was approximately 50% and higher than those other non-
acclimated strains despite their poor growth in untreated RMWSE. Moreover, more than 70% of BOD was reduced as a result of growth of acclimated *H. tetrachotoma, C. luteoviridis, P. kessleri* and *D. intermedius* in RMWSE unlike for the non-acclimated strains where less than 40% reduction of BOD was recorded.

### 3.4 Discussion

Wastewaters often contain biotic and abiotic contaminants which can negatively impact algae growth (Pittman et al., 2011; Olguín, 2012). Algae that can tolerate these conditions are likely to be ideal for wastewater cultivation either for biofuel and/or remediation purposes. In Chapter 2 we identified indigenous strains that grew well in wastewater and their strong growth was attributed partly to tolerance to oxidative stress. However, it was unclear whether this growth was also due to these specific species or whether this tolerance was just due to general, non-species specific adaptation (or acclimation) to the wastewater conditions.

The initial growth of non-acclimated strains in untreated RMWSE was poor with low doubling time. *C. debaryana* for example barely showed any significant increase in cell density in untreated RMWSE, as determined by optical density (OD$_{680}$) measurement over 10 days (Fig. 2A). This trend was also observed with other non-acclimated strains in this study. This suggested that the non-acclimated algae were unable to grow effectively in wastewater. This result is in line with the result in Chapter 2 which showed that non-acclimated culture collection *C. vulgaris* grown in RMWSE showed poor growth rate compare to indigenous *C. luteoviridis*. In another study, non-acclimated *C. vulgaris* were observed to have lower growth rate and poor
capability to remove inorganic N and P from primary settled wastewater than when it was acclimated (Lau et al., 1996).

In this study, we attempted to mimic the wastewater exposure conditions of the indigenous strains by acclimating culture collection strains of the same or similar species to 8 weeks of wastewater exposure with the aim of understanding the impact of acclimation on algae growth, productivity and remediation capability in RMWSE.

The steady increment in the culture OD$_{680}$ during the acclimation period showed that continuous cultivation of algae strains in wastewater effluent can influence their response to the wastewater condition (Fig. 1). Surprisingly, after just 8 weeks acclimation, growth rate of algae in wastewater conditions were generally improved, with $P. kessleri$ reaching the highest growth rate values in both the untreated (Fig. 3A) and autoclaved RMWSE (Fig. 3B). The algae growth in untreated RMWSE was rapid with barely any lag phase which suggested that cell divisions were rapid unlike for the non-acclimated strains (Fig. 2). In other words, the acclimation process shortened the time lag for strain adaptation in RMWSE and enhanced their growth rate. Previous studies have showed that algae can be adapted to grow in extreme toxic conditions. For example, Scenedesmus sp. acclimated to petroleum contamination was reported to grow effectively in petroleum contaminated medium unlike the non-acclimated strain (Carrera-Martinez et al., 2011).

Acclimation improved the growth rate of all the algae strains in both autoclaved and untreated RMWSE including $C. ellipsoidea$, $C. vulgaris$, $N. pseudostigmatica$ and $B. braunii$ that were not previously identified in the United Utilities secondary effluent. This suggested that acclimation can be very useful in the wastewater cultivation of algae that are not indigenous to wastewater. Furthermore, the growth rates of $C.
*debaryana* and *D. intermedius* were the lowest in comparison to other strains either in untreated or autoclaved RMWSE (Fig. 3A and B). Previously, I showed that indigenous *C. debaryana* and *D. subspicatus* were not tolerant to oxidative stress unlike *P. hussi* and *C. luteoviridis* and had low anti-oxidant enzyme response following induced oxidative stress (Chapter 2). With the assumption that *D. intermedius* and *P. kessleri* may be similar to *D. subspicatus* and *P. hussii*, we can infer that the relatively poor growth of acclimated *C. debaryana* and *D. subspicatus* in RMWSE was least partly due to their species-specific sensitivity to oxidative stress. Furthermore, *P. kessleri* showed the best growth profile compared to the other strains both in acclimated and non-acclimated forms in RMWSE. By comparing the growth rates of the algae strains in RMWSE before and after acclimation, it is possible to conclude that acclimation improves algae growth in wastewater, however, there is a clear indication that species-specific characteristics are also a key factor.

A further growth analysis by total chlorophyll concentration measurement of the acclimated and non-acclimated algae in untreated (Fig. 3C) and autoclaved RMWSE (Fig. 3D) also suggested that acclimation had made algae more physiologically active and more adaptive to the RMWSE environment. It is interesting therefore that after 8 weeks acclimation the algae appear to have undergone a process which increases their tolerance to RMWSE condition as evident in their increased growth rate and chlorophyll concentrations. In all the cultures, acclimated *P. kessleri* had the highest growth rate, total chlorophyll concentration than the rest of the strain suggesting that it is the most tolerant to RMWSE. Other studies have found strains of this genus to be tolerant to extreme conditions. For example, *P. kessleri* isolated
from wastewater effluent were reported to have high propensity for growth in saline, high temperature, oxidative, acidic and alkaline conditions and efficient in accumulating radionuclide particles (Shimura et al., 2012).

Moreover, the growth rates of the acclimated strains appeared to be greater in autoclaved RMWSE than in untreated RMWSE, which suggested that the presence of biota in the untreated RMWSE may be inhibiting algae growth. In addition autoclaving might also change the nutrient composition of RMWSE, however, this was not verified in this study. Algae grown on autoclaved centrate wastewater were shown to have much higher growth rate than in untreated raw centrate wastewater (Cho et al., 2011, Chapter 2). This suggested that autoclaving may have reduced biota competition in RMWSE and thereby enhanced algae growth. However, autoclaving wastewater for algae cultivation in commercial terms will be impractical. The relationship between microbial biota and algae cell growth in RMWSE remains unclear. A report has shown that bacteria for example can be beneficial to algae grown in wastewater in terms of nutrient removal and chemical oxygen demand reduction (Su et al., 2011). However, more studies are still needed to fully understand the algae-bacteria relationship in wastewater. In our previous study, we found that treatment of RMWSE did not have any significant impact on the growth of indigenous algae species (Chapter 2). However, it worth noting that those indigenous strains have been well adapted to the wastewater condition presumably for over a long period of time unlike the relatively short 8 weeks acclimation in this study. It is possible that acclimation over a longer period of time might further improve algae in their ability to overcome biota competition in the RMWSE. These
results also substantiate the hypothesis that acclimation can be an important process in cultivation of a non-indigenous species in wastewater effluent.

The biomass productivities of the strains were significantly increased in both the autoclaved and untreated RMWSE as a result of acclimation including for *C. ellipsoidea*, *C. vulgaris*, *N. pseudostigmatica* and *B. braunii* except for *C. debaryana* and *D. intermedius*. Although biomass productivity of the acclimated *B. braunii* was significantly increased in autoclaved RMWSE (Fig. 4A), it was lower in untreated RMWSE just like those of *C. debaryana*, and *D. intermedius* (Fig. 4B). This showed that these strains might be sensitive to biotic contaminants. In addition, biomass productivity of strains in this study correlated with their growth profile in RMWSE. For example, the growth rate of acclimated *C. debaryana* and *D. intermedius* (Fig. 2) were the lowest amongst the acclimated strains and therefore their biomass productivity values were also the lowest in both autoclaved and untreated RMWSE.

The result showed that biomass productivity was strongly dependent on a strain’s growth profile. The biomass of *P. kessleri* and *C. luteoviridis* were the highest which is a reflection of their higher growth rate (Fig. 4). Acclimation in most cases increased biomass productivity of the strains but this improvement was still less than the values of the similar indigenous strains in Chapter 2. Although the biomass productivity of the indigenous strains were higher than these acclimated strains, it was clear in Fig. 4 that acclimation improved biomass productivity by almost 2-fold in both autoclaved and untreated RMWSE except for *C. debaryana* and *D. intermedius*. The lack of improvement in the biomass productivity of these two strains may be partly due to species specific characteristics. For example, indigenous *C. debaryana* and *D. subspicatus* were generally the worst performers on RMSWE.
out of the five algae studied in Chapter 2 and were the poorest in terms of stress
tolerance. In contrast, the biomass productivity of acclimated *C. luteoviridis* and *P.
keesleri* were the highest in both autoclaved and untreated RMWSE, which is similar
trend to our previous study for *C. luteoviridis* and *P. hussii*. It is therefore likely that
the species of these genera are tolerant to wastewater conditions.

The biomass productivities of acclimated *C. ellipsoidea, C. vulgaris* and *N.
pseudostigmatica* in untreated RMWSE (Fig. 4B) were greater than in previous
studies where these species from culture collections have been cultivated in
wastewater. For example, biomass productivity in secondary wastewater effluent for
*C. vulgaris* was about 0.013 g L\(^{-1}\) d\(^{-1}\) (Kim et al., 2010), for *C. ellipsoidea* was about
0.03 g L\(^{-1}\) d\(^{-1}\) (Yang et al., 2011) and for *Neochloris* sp. was 0.04 g L\(^{-1}\) d\(^{-1}\) (Levine et
al., 2011). This study therefore showed that acclimation can improve the
performance of these culture collection strains in wastewater cultivation.

Despite improved biomass productivity as a result of acclimation for *C. ellipsoidea,
C. vulgaris, N. pseudostigmatica* and *B. braunii*, these values were still lower than
for *H. tetrachotoma, C. luteoviridis* and *P. kessleri*. This suggested that some species
are naturally more tolerance to RMWSE than the other and this should be considered
as an important criterion in selection of algae for wastewater cultivation. For
example, in a study by Chen et al. (2012), *Chlorella* sp. and *Scenedesmus* sp.
performed better and dominated the pond culture than other algae strains cultivated
in anaerobic digest effluent. This was partly due to their natural tolerance to
wastewater condition.

In this study, the percentage lipid content of all non-acclimated strains were higher
than the acclimated types in both autoclaved (Fig. 5A) and untreated RMWSE (Fig.
Similarly, intracellular neutral lipid content of the algae grown in both autoclaved and untreated RMWSE (Fig. 5C and D) measured by Nile Red staining showed that the non-acclimated strains appeared to have more TAG content than the acclimated strains. I hypothesise that response to stress induced by the prevailing condition of RMWSE caused increased cellular lipid content of the non-acclimated strains. In contrast, the lipid productivity of the non-acclimated strains was considerably lower than the acclimated strains. It is well known that cellular lipid content of algae increases when subjected to stress conditions, however, this usually bring about a decrease cell population and lower biomass and therefore low lipid productivity. I established in Chapter 2 that RMWSE induced oxidative stress on algae. Oxidative stress has been reported to increase cellular lipid content in _C. sorokiniana_ (Zhang et al., 2013). Other stressors such as nitrogen and phosphorus starvation have also been known to induce high lipid synthesis in algae. For example, Liu et al. (2013) reported that _Dunaliella_ sp. grown in nitrogen-depleted medium had a final lipid content that was 78% more than the unstressed cells. A similar result was also reported by Dean et al. (2010) for _C. reinhardtii_ which has increased cellular lipid content following nitrogen stress. However, nitrogen starvation significantly inhibits cell growth and reduces total biomass and thereby lower lipid productivity is achieved. In this study, it was clear that the lipid productivity (Fig. 6) of the algae relies greatly on their biomass productivities. Furthermore, neutral lipid content (Fig. 5) was relatively higher in all the strains when they were cultivated in untreated RMWSE than in the autoclaved medium. I suggest that this might be partly related to the contribution of biota presence in the untreated effluent.
Algae that are naturally rich in lipid and tolerant to wastewater condition will be most desirable for cultivation in RMWSE for biofuel applications (Griffiths and Harrison, 2009). *P. kessleri* and *C. luteoviridis* had highest productivity of lipid than all the other strains including *B. braunii* which is known to be a high oil yielding species and can contain more than 50% lipid content per cell when grown in synthetic medium (Cheng et al., 2013). A recent study showed that *P. kessleri* contain high lipid content of about 51% of dried weight and lipid productivity of 0.58 g L\(^{-1}\) d\(^{-1}\) when grown in nutrient depleted conditions (Pribyl et al., 2012). This study therefore supports our previous finding that *Parachlorella* sp. and *C. luteoviridis* are ideal candidate species as a biofuel feedstock in wastewater cultivation.

Remediation of N and P in RMWSE by algae was examined. Acclimated strains are physiological more active than the non-acclimated strains and thereby rapidly assimilate N and P. More than 60% of P and N were removed from RMWSE by the acclimated strains after 10 days cultivation (Fig. 7). Surprisingly, despite the lower growth rate of some of these strains, like *C. debaryana* in untreated RMWSE, they were able to remove a considerable amount of N and P. The possible presence of nitrifiers such as *Alcaligenes faecalis* (Liu et al., 2012) and *Klebsiella pneumonia* (Padhi et al., 2013) in the RMWSE might also contribute to the increased removal efficiency of N by algae. For example, studies have shown that nitrifiers are capable of adhering to an algae cell and can account for 12.5% removal of ammonium from wastewater (Lau et al., 1996).

In the previous study, more than 80% of N and P were removed in just 5 days of cultivation by the indigenous *P. hussii* and *C. luteoviridis* (Chapter 2) but it is worth
noting that those strains are more productive in RMWSE based on their higher growth, biomass productivities than strains in this study. The algae in this study could have achieved a similar extent of nutrient removal after a longer time of acclimation. In addition, acclimation also significantly enhanced the reduction of BOD in RMWSE by these strains at the end of 10 days cultivation. This result highlighted the importance of acclimation for the improvement of algae remediation capability.

Finally, the cellular changes that occurred during the acclimation process remain unknown and will be of interest for further study. One of the speculative changes is likely to include their stress tolerance, including oxidative stress tolerance. Also, it will be interesting to know if the acclimation process caused genetic mutation in addition to the physiological acclimation. For example, Ritter et al. (2010) suggested that based on the physiological and proteomic observations of the brown algae *Ectocarpus siliculosus* acclimated for 6-10 days to copper stress, a genetically adapted strain copper contaminated sites may have been developed. Therefore, the impacts of acclimation on the algae metabolic machineries including their oxidative tolerance response will be ideal future work of this study.

### 3.5. Conclusion

This study provides evidence that an acclimation process is an important approach for enhancing algae tolerance to wastewater cultivation for biofuel production and remediation purposes. This is particularly advantageous for the use of non-indigenous strains that have high potential for oil yield but are likely to grow poorly
in wastewater effluent. The acclimation process can serve a way to improve strain performance in wastewater. This study therefore opens up a new method of improving the spectrum of algae capability in wastewater cultivation. Further tests to evaluate the changes in species at the molecular level as a result of acclimation will be of future interest.

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Chapter 4

Chapter 4 covers algae growth improvement in wastewater secondary effluent by nutrient amendment and elucidated the continuous cultivation potential of the best two algae strains identified and characterised in Chapter 2; *Chlorella luteoviridis* and *Parachlorella hussii*. Moreover, the growth, remediation and biomass production potential of these algae in wastewater were examined under scale-up conditions by cultivation in an open pond system over a full season cycle in the United Kingdom. This is the first experimental assessment of open pond cultivation of microalgae in the UK for dual purposes of biofuel production and wastewater remediation. The chapter comprises of a version of a manuscript which has been submitted to Bioresource Technology journal for peer review. The Chapter has results included in the main manuscript and as supplementary information.
Implications of sludge liquor addition for wastewater-based open pond cultivation of microalgae for biofuel generation and pollutant remediation

Olumayowa Osundeko\textsuperscript{1,2} and Jon K. Pittman\textsuperscript{1}\* 
\textsuperscript{1}Faculty of Life Sciences, The University of Manchester, Michael Smith Building, Oxford Road, Manchester M13 9PT, UK 
\textsuperscript{2}Sustainable Consumption Institute, The University of Manchester, 188 Waterloo Place, Oxford Road, Manchester M13 9PL, UK 

*Corresponding author. Tel: +44 161 275 5235; fax: +44 161 275 5082.  
E-mail addresses: jon.pittman@manchester.ac.uk (J.K. Pittman),  
olumayowa.osundeko@postgrad.manchester.ac.uk (O. Osundeko).

Key words: Biomass; Lipid; Microalgae; Wastewater remediation, Pond cultivation

Authors’ Contributions

Olumayowa Osundeko performed all the experimental work and wrote this manuscript. Manuscript was edited by Jon Pittman.
Abstract

Five algae strains were previously isolated from a wastewater secondary treatment tank; *Chlamydomonas debaryana*, *Desmodesmus subspicatus*, *Hindakia tetrachotoma*, *Chlorella luteoviridis* and *Parachlorella hussii* were examined for growth improvement in raw municipal wastewater secondary effluent (RMWSE) by addition of nutrient substances. In this study, 25% of liquor, derived from dewatering of activated sludge, optimised the growth and biomass productivities of *C. luteoviridis* and *P. hussii*. However, *C. debaryana*, *D. subspicatus*, and *H. tetrachotoma* were sensitive to the RMWSE enriched with liquor. Further studies showed that *C. luteoviridis* and *P. hussii* can be cultivated in wastewater in a continuous system for steady production of biomass and nutrient removal. The capability of *C. luteoviridis* and *P. hussii* for cultivation in an open pond system was studied during four seasons in the United Kingdom. The result showed that these algae are capable of all year cultivation and their best outputs were observed in the summer and spring and growth was maintained in autumn and winter. More than 78% of NH$_4^+$-N and PO$_4^{3-}$-P were removed from wastewater by these algae after 10 days during the summer and spring cultivations. Biomass productivity by *C. luteoviridis* in the summer, spring, autumn and winter peaked at 0.33, 0.29, 0.09 and 0.03 g L$^{-1}$ d$^{-1}$, respectively and that of *Parachlorella hussii* peaked at 0.42, 0.40, 0.11 and 0.06 g L$^{-1}$ d$^{-1}$, respectively. This result showed the robustness and capability of these two strains for wastewater works and outlined their dual purpose use for remediation and biofuel production on a commercial scale in UK.
4.1 Introduction

The advantages of algae as a biofuel feedstock over other plant sources has been well documented (Chisti, 2007 and 2008; Amin 2009; Khan et al., 2009; Brennan and Owende 2010; Demirbas 2010; Mata et al., 2010; Bajhaiya et al., 2010; Amaro et al., 2011; Wu et al., 2012; Ahmad et al., 2011). One of these advantages is faster growth rate, reduced agricultural land requirement and ability to grow in sub-optimum media such as wastewater effluent. However, due to high water, nutrient and energy requirements for algae cultivation, a number of life cycle analyses showed that cost and environmental balances are negative (Clarens et al., 2010; Sturm and Lamer, 2011 and Campbell et al., 2011). Algal biodiesel production costs still need to reduce significantly to be competitive with fossil fuel which currently stands at US$112/barrel (OPEC). A niche of opportunity may exist whereby algae are grown in wastewater for remediation and biomass as a by-product. The use of wastewater for algal cultivation offers a solution to the problems of high nutrient and water requirements.

Municipal wastewater for example, typically contains high concentrations of nutrients including nitrogen (N) and phosphorus (P), high level of BOD and heavy toxic metals. Treating this effluent to a safe limit before discharging to the receiving water bodies is part of legislation in many countries. Effluent from the secondary treatment often contains high concentrations of nutrients especially phosphate which in most cases are above statutory limits for discharge. Therefore, further removal of these nutrients remains an integral part of the wastewater treatment work. Tertiary treatments can help to further polish the effluent to a higher standard before discharge or recycling for domestic or agricultural uses (Abdel-Raouf et al., 2012).
Chemical and physical-based technologies are known for removal of nutrients from secondary wastewater effluent; however, they rely on significant amounts of energy and chemical usage which can be economically and environmentally less attractive (Hoffmann, 1998). For example, for most commercial wastewater processing, phosphate is precipitated from wastewater using chemicals to form a solid insoluble fraction. This can possibly introduce a chemical pollutant to the effluent if not managed properly.

Algae are efficient in removing N and P and reducing BOD and therefore can offer a cheap and sustainable tertiary treatment of wastewater effluent (see Chapter 1). The use of algae for wastewater treatment and biofuel production was first proposed over 5 decades ago (Oswald and Golueke, 1960). Recently, algae have been extensively studied especially as a biofuel feedstock, however, the dual use of algae for wastewater remediation and biofuel production has received relatively little attention. Integrating microalgae cultivation in an existing wastewater treatment plant offers an inexpensive and economically feasible way of commercial treatment of wastewater (Lundquist et al., 2010; Menger-Krug et al., 2012). Biomass produced from this process can be an added value to the wastewater treatment process. Despite the vast potential of algae as a remediation agent and biofuel feedstock in wastewater works (Chapter 1; Pittman et al., 2011; Spolaore et al., 2006; Ruiz-Marin et al., 2010), commercial cultivation of algae still encounters fundamental difficulties such as poor growth, culture instability, need for efficient harvesting, environmental impact and land availability (Guieysse et al., 2013; Singh and Olsen, 2011). Therefore, overcoming these challenges remains an important step toward large scale cultivation of algae.
Poor growth of algae in wastewater secondary effluent can be partly due to the low nutrient concentration in the effluent. In some cases, essential nutrient concentrations in the wastewater secondary effluent can be low (see Chapter 2), in particular trace mineral nutrients and can result in poor growth, low biomass and low lipid productivity (Christenson and Sims, 2011). Therefore, there is need to supplement these nutrient in wastewater to achieve high algae growth and biomass productivity. Additions of commercial fertilizers can negatively increase the carbon footprint of algae cultivation and also the price of algae-derived biofuel. The use of wastewater centrate liquor offers a sustainable and economical approach for increasing nutrient constituents in wastewater. Centrate liquor is the liquid product of the thickening and dewatering process of activated sludge (referred to as “liquor” in this manuscript) and has the characteristics of rich nutrients including phosphorus, ammonium and COD (Li et al., 2011). Liquor has been known to support algae grow for higher biomass productivity than wastewater secondary effluent (Wang et al., 2010; Kong et al., 2010; Li et al., 2011). However, liquor has high COD, suspended solid concentration, high turbidity and high concentration of toxic metal which can be detrimental to algae cultivation (Li et al., 2011). Dilution of liquor can reduce its toxic effect and turbidity (González et al., 1997) and thereby promote higher algae growth. Kong et al. (2010) studied the growth of *C. reinhardtii* in liquor diluted with freshwater and fortified with trace element ingredients. However, the uses of freshwater and synthetic nutrients are likely to negatively impact on the sustainability of such cultivation approach. Therefore, the mixture of wastewater secondary effluent and liquor offers a more sustainable alternative. Up till now, no study has examined the use of secondary effluent and centrate mixture for algae
growth either for remediation and or biofuel purpose. Furthermore, it is important to evaluate which strains can grow well in liquor.

Culture stability and growth are essential characteristics for large scale cultivation of algae in wastewater. Previous studies have shown that algae that are tolerant to oxidative stress are efficient for cultivation in wastewater either for biomass production and remediation purposes (Chapter 2). For instance, *P. hussii* and *C. luteoviridis* were observed to grow effectively under extreme oxidative conditions and in raw wastewater secondary effluent in the laboratory batch cultivation. In another study, indigenous *P. kessleri* isolated from cyclone overflow water collected from bitumen extraction facilities was reported to be effective in removal of toxic metal from oil sands tailings pond water (Mahdavi et al., 2012). The stability of algae species for long term cultivation in wastewater can be studied in a continuous cultivation process. This can provide useful information for its commercial evaluation and enhance higher volumetric productivity of biomass over a long period. For example, Ruiz-Marin et al. (2010) compared growth of *S. obliquus* under semi-continuous culture conditions with continuous addition of fresh wastewater and in batch culture conditions. They found that initial growth over four cultivation cycles was much higher than the batch culture, possibly due to eventual nutrient depletion in the batch culture.

Algae can be cultivated in open ponds or in closed systems such as photobioreactors. However, the choice of cultivation system relies on the factors such as cost and reliability (Odlare et al., 2011). Outdoor open pond cultivation can be in the form of a large tank pond or raceway style High Rate Algal Ponds (HRAPs) with long, shallow, looped channels and powered by paddlewheels to circulate water through
the system. HRAPs are shallow oxidation ponds that encourage the effective mixing and growth of suspended microalgae (Hoffmann, 1998). Open pond cultivation systems are usually simpler and cheaper to run than closed systems. However, evaporation and culture control remains a challenge.

Cultivating algae in wastewater in open cultivation systems has a much smaller environmental footprint compared to other non-wastewater cultivations which rely on freshwater and fertilizer (Park et al., 2011). Cultivation of algae in an open pond will rely on climatic condition such as temperature and sunlight. For example, in the Northern hemisphere like the UK where average temperature and sunlight intensity are highly variable over the seasons, growing algae in an open pond will face challenges of temperature and light intensity especially during autumn and winter. Recent life cycle analysis of biodiesel production from C. vulgaris in the UK suggests that cultivation in typical open pond raceways would be significantly more environmentally viable than in closed air-lift tubular (Stephenson et al., 2010).

However, no assessment has been made in the UK of an algae dual use system for biofuel production and removal of nutrient from wastewater effluent at pilot scale in an open pond cultivation system. It has been estimated that $8 \times 10^4$ ha of derelict or contaminated land exists in the UK as of 2005 (Bardos et al., 2009). Stephenson et al. (2010) estimated that if all of this land were to be used to grow algae in an open pond system, 40 tons ha$^{-1}$ year$^{-1}$ of TAG can be achieved, and 3.2 million tons of biodiesel could be produced per year which represents 12% of the diesel required for road transport in the UK during 2008. Therefore, this study aimed to evaluate the potential of using algae for dual purposes; for biofuel production and wastewater
treatment using an open pond cultivation system in the UK. This study will provide a base for further techno-economic evaluation of this process in wastewater works.

This study was therefore carried out with following aims:

1. To carry out a laboratory study on growth improvement of five previously isolated algae strains by addition of nutrients in the form of liquor, inorganic fertilizer, phosphate and ammonium compounds. This main focus was to determine a sustainable method of improving algae growth and productivity in RMWSE whilst providing insight for possible scale-up in pond cultivation.

2. To evaluate the efficacy and potential of *P. hussii* and *C. luteoviridis* for a semi-continuous cultivation system in laboratory conditions with a focus on understanding their resilience and stability for nutrient removal from wastewater and for biofuel feedstock.

3. To study nutrient removal efficiency and biofuel productivity of *P. hussii* and *C. luteoviridis* in open pond cultivation in wastewater effluent and to repeat the pond cultivation process in a seasonal cycle (autumn, winter, spring and summer) in order to evaluate the commercial prospect of algae cultivation in wastewater for bioremediation and biofuel production in the UK.
4.2 Materials and Methods

4.2.1 Wastewater characteristics and microalgae strains

Wastewaters used in this study were obtained from United Utilities Water Plc treatment plant at Ellesmere Port, Cheshire, UK (lat. 53° 16′ 47.32″ N, long. 2° 53′ 50.65″ W) during 2011 to 2013. The Ellesmere Port plant receives mainly domestic wastewater from Ellesmere Port and the surrounding suburban area and pre-treated effluent from a nearby oil refinery. The raw municipal wastewater secondary-treated effluent (RMWSE) and the sludge liquor from dewatering of the activated sludge were sampled from the same treatment tanks throughout the study. All samples were taken to the University of Manchester, allowed to settle and decanted before it was used for culturing. Physico-chemical analyses were carried out on the wastewaters. Analysis of the sludge liquor is shown in Table S1. The indigenous wastewater microalgae strains *Chlamydomonas debaryana*, *Chlorella luteoviridis*, *Desmodesmus subspicatus*, *Hindakia tetrachotoma* and *Parachlorella hussii* were isolated from a wastewater secondary treatment tank as described (Chapter 2). Prior to cultivation, the algae were cultured in 2 L bottles of raw wastewater secondary effluent for up to 8 days equivalent to their exponential growth stage (as shown in Chapter 2). The cultures were subjected to continuous orbital shaker rotating at 2 Hz and at 22 °C, with 16 h light:8 h dark and a photon flux of 150 μmol m⁻² s⁻¹.
4.2.2 Growth improvement in RMWSE

The growth improvement of the five algae in RMWSE as a result of the addition of nutrients was investigated in 4 scenarios in 250 ml batch culture systems. In the first scenario, liquor was added to RMWSE at relative concentrations of 10%, 20%, 25%, 30% and 40% (v/v).

In the second scenario, the RMWSE was enriched by addition of 243.13 ml of inorganic liquid fertilizer (Wilko Liquid Tomato Feed) which increased the total concentration of ammonium and phosphate in the media to 97.5 mg L\(^{-1}\) and 13.9 mg L\(^{-1}\), respectively, equivalent to that of 25% (v/v) liquor (Table S2). In the third scenario, ammonium chloride was added to the RMWSE which increased the total ammonium concentration in the media to 97.5 mg L\(^{-1}\). In the fourth scenario, 43.45 mg L\(^{-1}\) potassium phosphate was added to RMWSE to increase the final phosphorus concentration in the RMWSE to 13.8 mg L\(^{-1}\). A control experiment was set up with no additional nutrients added. The pH of each medium was set to 7.5 with MES. Each of the five algae strains were inoculated into three flasks of each media and placed on an orbital shaker rotating at 2 Hz at 22 °C, 16 h light: 8 h dark and a photon flux of 150 μmol m\(^{-2}\) s\(^{-1}\). No additional CO\(_2\) was added. Starting cell densities in each culture were identical as determined by optical density measurement at 680 nm (OD\(_{680}\)).

4.2.3 Semi-continuous cultivation

*P. hussii* and *C. luteoviridis* were cultivated in 250 ml glass flasks in a controlled laboratory condition to investigate their ability to be grown in a continuous system. The continuous cultivation was initiated from a batch culture of RMWSE and 25%
(v/v) liquor mixture. The cultivation was carried out in triplicate on an orbital shaker rotating at 2 Hz at 22 °C, 16 h light: 8 h dark and a photon flux of 150 μmol m\(^{-2}\) s\(^{-1}\), with identical starting cell densities in each flask. After 8 days of cultivation which corresponded to the mid-exponential phase of their cell growth, 50% (v/v) of the algae culture suspension were removed and replaced with fresh base medium every 2 days at the volume rate of 0.25 d\(^{-1}\). The cultivation conditions were kept constant through the experiment and the integrity of the culture was monitored by microscopic visualisation to examine the presence of inoculated algae in the culture. Samples were taken for optical density, biomass and lipid productivity, phosphorus and ammonium quantifications before the addition of fresh medium every 2 days of the initial 8 days batch cultivation and thereafter every 24 hours during the continuous cultivation mode. Percentage phosphorus and ammonium removals were calculated by \((N_0 - N_t) \times 100/N_0\). Where \(N_0\) is the initial N or P concentration and \(N_t\) is the N or P concentration at time t.

4.2.4 Open pond cultivation of algae

Identical ponds fitted with three airlift pumps (Fig. 1) were installed at the University of Manchester Experimental Botanical Grounds (lat. 53° 26′ 41.59″ N, long. 2° 12′ 59.21″ W) in an unheated glasshouse with open windows and no artificial lighting. The flow rate of the pumps in each pond was 2000 L h\(^{-1}\) for optimal mixing. Pond medium pH and temperature were measured by remote probes embedded in the pond. Light intensity was measured at the pond surface. The cultivation medium was RMWSE +25% (v/v) liquor to a pond depth of 12 cm giving a total volume of \(~150\) L. The concentrations of PO\(_4^{3-}\)-P and NH\(_4^+\)-N in the ponds at the start of cultivation for each season are shown in Table S3. 2 L of pre-cultured exponential phase P.
*P. hussii* or *C. luteoviridis* in RMWSE +25% (v/v) liquor to a cell density of OD$_{680}$ 3.9 (Fig. S1) were inoculated into each pond and cultivated for 25 d during each season: summer (July to August 2012), autumn (October to November 2012), winter (January to February 2013) and spring (April to May 2013). Pond integrity was routinely monitored during the cultivation by visual light microscopy observation to determine loss of initial inoculants and invasion of other microorganisms by measuring the percentage of inoculated algae of the total microorganisms in each volume of sample at each time point. Evaporation rates were measured using the equation: $V_0 - V_t/(V_0T)$. $V_0$ is the initial pond volume at the start of cultivation; $V_t$ is the volume of the culture at duration $T$.

**Fig. 1.** (a) Schematic diagram of the open pond design fitted with three airlift pumps and a probe (P) for measuring the pond temperature and a pH meter (M). The culture depth (CH) was maintained at 10 cm. (b) Representative images of the *P. hussii* and *C. luteoviridis* ponds during the summer season.
4.2.5 General microalgae growth and productivity analysis

Culture growth was estimated by measuring optical density at 680 nm (OD$_{680}$) of the culture at daily intervals using a Jenway spectrophotometer. Before taking the optical density measurement, the culture media without added algae for each experiment was used as a blank for the spectrophotometer measurement. Growth rate ($\mu$) was determined at the exponential growth phase using the equation $\mu = (\ln N_1 - \ln N_0)/(t_1 - t_0)$ where $N_0$ and $N_1$ are the OD$_{680}$ values at the early and late exponential phase, respectively, and $t_1$ and $t_0$ are the growth duration corresponding to $N_0$ and $N_1$, respectively.

Total chlorophyll and Nile red fluorescence as a measure of neutral lipid in algae cells were determined as described in Chapter 2. Biomass was determined following centrifugation of a 50 ml culture suspension at 1500 g for 20 min in a pre-weighed tube. The cells were washed three times with distilled water and dried at 60 °C for 48 hours, then weighed to obtain the biomass concentration. The dry weight of any suspended solids/biota of cultivation media without added microalgae was subtracted from algae dried biomass of the cultures. The measurement of the lipid content was determined gravimetrically using a modified Folch method (Folch et al., 1957). Biomass and lipid productivity of the algae in the culture were determined using the equations: biomass productivity (mg L$^{-1}$ d$^{-1}$) = WB/V x T, lipid content (%) = WL x 100/WB, and lipid productivity (mg L$^{-1}$ d$^{-1}$) = WL/V x T; where WB is dry weight biomass (mg), WL is lipid weight (mg), V is working volume and T is cultivation time.
4.2.6 Nutrient and biochemical oxygen demand (BOD) determination

Ammonium and phosphate removals was measured at the late exponential growth stage in batch culture flask experiments, at the end of the batch mode and thereafter every day and before addition of fresh base medium in the continuous cultivation experiments, and at day 5 and day 10 of the pond cultivation experiments. 10 ml of algae suspension was centrifuged and the supernatant was filtered using a 0.2 µm Millipore filter to ensure all supernatants and algae were removed. N and P removal from RMWSE by algal strains was determined by the measurement of ammonium and phosphate concentration in the media before inoculation of algae and in the filtered culture suspension as described in Chapter 2. BOD measurement was performed by measuring the dissolved oxygen before and after incubation at 20°C for 5 days with Jenway 9500 Dissolved Oxygen Meter. The percentage removal was calculated as \((C_0 - C_1) \times 100/C_0\) where \(C_0\) and \(C_1\) are the mean nutrient concentration or BOD of the media before and after algae cultivation, respectively.

4.2.7 Replication and Statistical analysis

All data shown is representative or mean data of at least three replicate experiments or replicate samples, in the case of the pond experiments, unless stated otherwise. One-way ANOVA (p<0.05) was used wherever applicable and Tukey’s post hoc analyses were used to determine the significance of difference wherever applicable. All statistical tests were performed using GenStat version 15.
4.3 Results

4.3.1 Algae growth and productivity in RMWSE enriched with nutrient substances

Although algae are capable of growth in wastewater secondary effluent either for remediation or biofuel production, nutrient concentration in secondary effluent is low compared to other effluents like primary effluent and to other artificial growth media like TAP medium. This can potentially be limiting to gaining maximal productivity in terms of commercial productivity of biomass required for biofuel production. In this study, all five algae strains reported in Chapter 2 were examined for growth improvement, first by addition of increasing concentrations of liquor. The liquor is the product of dewatering of activated sludge generated during secondary treatment and is often rich in nutrients especially nitrogen and phosphorus (Table S1). The mean concentrations of ammonium and phosphate in the liquor used were 180.05 mg L\(^{-1}\) and 99.03 mg L\(^{-1}\) respectively.

As seen previously (Chapter 2) *C. luteoviridis* and *P. hussii* grew better than the other three strains in RMWSE with no added liquor (Fig. 2a). When the growth of other three algae declined at day 12 of cultivation for example, the growth of *C. luteoviridis* and *P. hussii* continued in the exponential stage. There were no obvious differences in the growth pattern of *C. luteoviridis* and *P. hussii*.

In this study, liquor was added to RMWSE at concentrations of 10%, 20%, 25%, 30% and 40%. As result of this addition of liquor, the final ammonium and phosphate concentration of the media increased (Table S2). Addition of liquor at 20% and above caused a significant reduction in growth of *C. debaryana*, *D. subspicatus* and *H. tetrachotoma*, with almost no growth in the +40% liquor medium.
In contrast, addition of 10% to 30% liquor led to significant improvements in the growth of *C. luteoviridis* and *P. hussii*, with maximal growth of both strains in the +25% liquor medium. By day 12 there was an approximately 3.5-fold increase in cell density relative to the control culture (*p*<0.01). Cell density of *C. luteoviridis* and *P. hussii* declined rapidly by day 15 in the +20% and +25% liquor media (Fig. 2c and 2d) due to nutrient starvation after the rapid cell growth. Higher proportions of liquor up to 40% had a detrimental effect on *C. luteoviridis* and *P. hussii* growth relative to the media with lower liquor proportions, but this was still not significantly reduced compared to the control RMWSE medium without liquor (Fig. 2f). In addition, there was slight increase in the OD of *C. debaryana*, *D. subspicatus* and *H. tetrachotoma* at day 15 which may be partly due to contamination.
Fig. 2. Growth curves of isolated microalgae strains as determined by OD$_{680\text{nm}}$ measurement in RMWSE enriched with (a) 0% liquor, (b) 10% liquor, (c) 20% liquor, (d) 25% liquor, (e) 30% liquor and (f) 40% liquor. All data presented are means ±SE of three independent cultures.

Further increments in the percentage of liquor content in RMWSE above 25% to 40% were observed to have a detrimental effect on both *C. luteoviridis* and *P. hussii* growth (Fig. 2f). Thus the growth of *C. luteoviridis* and *P. hussii* were optimum at 25% of liquor (Fig. 2d). In addition, the specific growth rates of *C. luteoviridis* and
*P. hussii* in RMWSE enriched with 25% liquor (Fig. 3a) were significantly higher than those of *C. debaryana, D. subspicatus* and *H. tetrachotoma* in the same growth condition (*p*<0.001).

To assess whether the increased growth of *C. luteoviridis* and *P. hussii* by 25% liquor addition was solely due to higher N and/or P availability, growth was compared in RMWSE supplemented with liquid fertiliser, NH₄Cl alone or K₂HPO₄ alone, with the final concentrations of NH₄⁺-N and PO₄³⁻-P being identical to the +25% liquor medium (Table S2). When cultivation was assessed on the basis of growth rate, chlorophyll concentration or biomass productivity, *C. luteoviridis* and *P. hussii* always performed significantly better (*p*<0.05) in the liquor medium compared to the other media (Fig. 2). The only exception was for growth rate of both strains in the PO₄³⁻-P medium which showed no significant difference with the liquor medium (Fig. 2a).

For all other strains, growth was significantly increased as a result of the fertiliser, NH₄⁺-N and PO₄³⁻-P enrichments relative to the liquor treatment (Fig. 2), but compared to the basal RMWSE medium, significant growth improvement was only observed for these strains following fertiliser or PO₄³⁻-P addition but not by NH₄⁺-N addition (Fig. 2a), indicating that PO₄³⁻-P concentration in the secondary wastewater was limiting, as inferred from the high N/P ratio (Table S2).
Fig. 3. Growth rate (a), total chlorophyll concentration (b), biomass productivity (c) and lipid productivity (d) of isolated microalgae strains at day 10 of cultivation in RMWSE alone (control) and with added nutrients in the form of 25% liquor, inorganic fertilizer mix, ammonium chloride, or potassium phosphate. All nutrient additions increased the total NH$_4^+$-N concentration by 22.5 mg L$^{-1}$ and/or total PO$_4^{3-}$-P concentration by 12.4 mg L$^{-1}$. All data are means ±SE of three independent cultures.

Enrichments of RMWSE with ammonium, inorganic fertilizer and phosphate did not significantly increase the biomass productivity of all the algae tested in this study when compared to the control except for *C. debaryana* which showed improved biomass productivity in RMWSE enriched with phosphate ($p<0.05$). Just like the growth rate, biomass productivity of *C. debaryana*, *D. subspicatus* and *H. tetrachotoma* was significantly lower in RMWSE with liquor enrichment.

Lipid productivity of all the algae recorded in this experiment however, did not increase as a result of any of the RMWSE enrichments (Fig. 3d). Surprisingly, the
lipid productivity of all the algae were significantly lower in RMWSE enriched with ammonium compare to the control ($p<0.05$). In addition the lipid productivity of *P. hussii* was lower in the culture enriched with fertilizer and phosphate which suggested that lipid productivity was not triggered by addition of these nutrients.

**Fig. 4.** Cellular neutral lipid content in response to liquor addition determined by Nile Red staining of the isolated microalgae strains grown in RMWSE alone or enriched with liquor. Nile Red staining was performed with $1 \times 10^5$ cells of each strain at day 10 of cultivation and relative fluorescence was quantified. a.u. = arbitrary units. Data are means ±SE of three replicate cultures.

The previous study has shown that RMWSE can induce oxidative stress on algae due to induced production of reactive oxygen species (Chapter 2). We observed significant increase in the intracellular lipid content of *P. hussii* and *C. luteoviridis* (Fig. 4) when 10% of liquor was added to RMWSE ($P<0.05$). In addition, 20% of liquor also increased the cellular neutral lipid content of *C. luteoviridis*. However, further increase in the liquor amount to 40% caused a gradual decrease to cellular neutral lipid content. In contrast, there were no changes in neutral lipid content of *C.*
debaryana and H. tetrachotoma relative to control particularly when 10%, 30% and 40% liquor were added to RMWSE. However, a reduction was observed in C. debaryana and D. subspicatus when 20% and 25% liquor was added to RMWSE, respectively.

4.3.2 Remediation characteristics of algae in RMWSE enriched with liquor

The remediation capabilities of algae in RMWSE enriched with liquor were examined in terms of their ability to remove NH$_4^+$-N and PO$_4^{3-}$-P. After 5 days of cultivation, more than 60% of the initial N and P concentrations in the control culture were removed by C. debaryana, D. subspicatus and H. tetrachotoma and more than 80% of these nutrient concentrations were removed by C. luteoviridis and P. hussii (Fig. 5a and b). Further increments in liquor concentration in RMWSE up to 25% did not impair the ability of C. luteoviridis and P. hussii to remove N and P from the media. However, the ability of C. debaryana, D. subspicatus and H. tetrachotoma to remove phosphate reduced as the concentration of liquor increased, except for C. debaryana at 10% liquor. Interestingly, the ability of C. debaryana to remove ammonium decreased as the amount of liquor in RMWSE increased.
Fig. 5. Remediation of NH$_4^+$-N (a) and PO$_4^{3-}$-P (b) after 5 days of cultivation in response to liquor addition by the isolated microalgae strains grown in RMWSE alone or enriched with liquor. Data are means ±SE of three replicate cultures.

The efficiency of *C. luteoviridis* and *P. hussii* for nutrient removal was reduced slightly in the 30% liquor medium containing >100 mg L$^{-1}$ NH$_4^+$-N and >15 mg L$^{-1}$ PO$_4^{3-}$-P. The remediation efficiencies of the other strains were likewise reduced as nutrient concentrations increased (Fig. 5).
4.3.3 Semi-continuous cultivation

To evaluate cultivation of the best performing strains further, a continuous cultivation experiment was conducted in 250 ml glass flask in a laboratory condition. The initial growth of this cultivation experiment was carried out in a batch mode up to day 8 of cultivation (Fig. 6a) which represented the mid-exponential growth stage of both algae strains in RMWSE enriched with 25% (Fig. 2d). Fresh growth medium containing 48.73 mg L\(^{-1}\) ammonium and 6.90 mg L\(^{-1}\) phosphate were continuously fed into the culture every two days at a rate of 0.25 d\(^{-1}\). During the continuous mode (day 8-18), the OD\(_{680\text{ nm}}\) value varied from 1.531 to 2.919 in the *P. hussii* culture and 1.207 to 2.641 in the *C. luteoviridis* culture. In addition, the growth peaks of the algae were steady during the continuous cultivation mode. The growth pattern of *C. luteoviridis* and *P. hussii* were similar, which indicated that the two species have similar growth characteristic in RMWSE (Chapter 2).
Fig. 6. Cell growth (a), biomass yield (b) and lipid yield (c) of *P. hussii* and *C. luteoviridis* during semi-continuous cultivation in RMWSE enriched with 25% liquor. All values are mean data ±SE of 3 independent cultures.
The biomass yield of *C. luteoviridis* and *P. hussii* remained steady and peaked at 6.01 to 7.99 g L\(^{-1}\) and 5.32 to 8.44 g L\(^{-1}\), respectively, over the 10 d (Fig. 6b). This gave a mean biomass removal every 2 d of 3.57 and 3.65 g L\(^{-1}\) for *C. luteoviridis* and *P. hussii*, respectively; equivalent to a mean biomass productivity of 1.78 and 1.83 g L\(^{-1}\) d\(^{-1}\). Lipid productivity (Fig. 6c), NH\(_4\)\(^+\)-N removal (Fig. 7a) and PO\(_4\)\(^{3-}\)-P removal (Fig. 7b) values of both strains were also steady during the 10-day continuous cultivation period, with a mean lipid productivity of approximately 0.39 and 0.42 g L\(^{-1}\) d\(^{-1}\) for *C. luteoviridis* and *P. hussii*, and more than 70% of NH\(_4\)\(^+\)-N and PO\(_4\)\(^{3-}\)-P continuously removed from the media every 2 d. Although the nutrient remediation by the strains was equivalent to that observed in batch mode, the biomass and lipid productivity was greater. The result from this experiment showed the capability of these algae to remove NH\(_4\)\(^+\)-N and PO\(_4\)\(^{3-}\)-P from the growth media during continuous cultivation.
Fig. 7. Remediation of NH$_4^+$-N (a) and PO$_4^{3-}$-P (b) by *Chlorella* *luteoviridis* and *Parachlorella hussii* during semi-continuous cultivation in RMWSE enriched with 25% liquor. Each data represent mean value of three experimental replicates.
4.3.4 **Algae cultivation in an open pond system**

Following demonstration of efficient cultivation of *C. luteoviridis* and *P. hussii* in secondary effluent amended with 25% liquor in flask experiments, open pond experiments were performed to evaluate strain performance following scale-up to 150 L and in a temperate environment in the UK during all four seasons. To avoid the additional variable of pond dilution and contamination through rainfall, which can be high in north-west England, the ponds were situated in an unheated, semi-enclosed glasshouse. Pond water daytime temperature was significantly higher during spring and summer than in winter and autumn, ranging between 15 to 25°C in summer and -6 to 3°C in winter (Table 1A). Likewise, daytime light intensity was significantly higher during spring and summer than in autumn and winter (Table 1B). Mean pond evaporation rate was higher in the summer (1.47 L d⁻¹) and the spring (1.19 L d⁻¹) than in autumn (0.45 L d⁻¹) and winter (0.12 L d⁻¹).
Table 1A. Daytime water temperature of the algal ponds during the *P. hussii* (*P.h.*) and *C. luteoviridis* (*C.l.*) seasonal cultivation experiments. Values are mean data from 2 measurements.

<table>
<thead>
<tr>
<th>Day</th>
<th>Autumn</th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
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<td></td>
<td><em>P.h.</em></td>
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<td><em>P.h.</em></td>
<td><em>C.l.</em></td>
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<td></td>
<td>(°C)</td>
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<tr>
<td>3</td>
<td>5</td>
<td>5</td>
<td>-1</td>
<td>-2</td>
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<tr>
<td>6</td>
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<td>3</td>
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<tr>
<td>29</td>
<td>8</td>
<td>8</td>
<td>-3</td>
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Table 1B. Daytime light intensity measured at the surface of the algal ponds during the *P. hussii* and *C. luteoviridis* seasonal cultivation experiments. Values are mean data ±SE from 6 measurements.

<table>
<thead>
<tr>
<th>Day</th>
<th>Autumn</th>
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<th>Spring</th>
<th>Summer</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(μmol m⁻² s⁻¹)</td>
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<tr>
<td>6</td>
<td>96.37 ± 13.37</td>
<td>110.62 ± 8.57</td>
<td>768.64 ± 48.94</td>
<td>909.31 ± 44.35</td>
</tr>
<tr>
<td>13</td>
<td>235.63 ± 18.37</td>
<td>144.22 ± 4.61</td>
<td>515.56 ± 18.19</td>
<td>884.63 ± 64.34</td>
</tr>
<tr>
<td>20</td>
<td>98.76 ± 10.35</td>
<td>122.47 ± 5.47</td>
<td>638.64 ± 48.44</td>
<td>1005.43 ± 73.27</td>
</tr>
</tbody>
</table>

Each pond contained RMWSE +25% liquor although there was variation in initial nutrient and BOD concentration (Table S3) and starting pH (Table 2) between each batch of liquor, which was collected fresh at the start of each seasonal experiment.
Table 2. The pH for *P. hussii* and *C. luteoviridis* pond routinely measured during the pond cultivation in summer (17th of July to 11th of August 2012), autumn (19th of October to 13th of November 2012), winter (22nd of January to 16th of February 2013) and spring (24th of April to 19th of May 2013) in the UK.

<table>
<thead>
<tr>
<th>Day</th>
<th>Autumn</th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
</tr>
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<tr>
<td></td>
<td><em>P. h.</em></td>
<td><em>C. l.</em></td>
<td><em>P. h.</em></td>
<td><em>C. l.</em></td>
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<td></td>
<td>(pH unit)</td>
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<tr>
<td>0</td>
<td>8.27</td>
<td>8.22</td>
<td>8.29</td>
<td>8.27</td>
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<tr>
<td>8</td>
<td>7.93</td>
<td>8.02</td>
<td>8.34</td>
<td>8.29</td>
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<tr>
<td>11</td>
<td>8.24</td>
<td>8.29</td>
<td>8.55</td>
<td>8.57</td>
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</tbody>
</table>

4.3.4.1 Growth of *P. hussii* and *C. luteoviridis* in open pond cultivation

It worth noting that these pond scale experiments were performed in uncontrolled, unsterile conditions, therefore the algae were expected to grow in the pond amongst populations of bacteria and zooplankton. However, microscopic examination showed that the initial algal strain inoculates were the dominant organism in the pond throughout the cultivation period. Quantification of pond integrity found that for both strains, the initial inoculate remained the dominant organism throughout the cultivation period. 92-98% of the microalgae present in the pond by the end of cultivation were the original strain (Fig. 8A and B). Algae growth rate values were significantly higher in the summer and spring than in the autumn and winter (Fig. 8C). This result is also supported by the total chlorophyll measurement (Fig 8D) which indicated that *P. hussii* and *C. luteoviridis* are more photosynthetically active during the summer and spring. The growth rate values of *P. hussii* are higher than *C.*
luteoviridis in autumn and summer cultivations (P<0.05) but no significant
difference was observed during spring and winter cultivations.

3.3.2 Biomass and lipid productivities

The biomass productivity of both P. hussii and C. luteoviridis in the pond increased
progressively as cultivation duration increased, especially during summer and spring
(Fig. 9). Biomass productivity values were higher in the summer and spring than in
autumn and winter (Fig. 9B and C). Biomass productivity of *P. hussii* for example at day 16 during summer and spring cultivations were $0.415 \pm 0.031$ g L$^{-1}$ d$^{-1}$ and $0.340 \pm 0.048$ g L$^{-1}$ d$^{-1}$ respectively, equivalent to 4.71 and 3.86 g m$^{-2}$d$^{-1}$ respectively. However, in winter and spring, their biomass productivity values declined to $0.068 \pm 0.005$ g L$^{-1}$ d$^{-1}$ and $0.106 \pm 0.006$ g L$^{-1}$ d$^{-1}$. Similar trends were observed for the biomass productivity of *C. luteoviridis* suggesting that the environmental condition is a major factor for algae biomass production. The results showed a similar trend to the algae growth rate during these seasons (Fig. 8C).

![Diagram](image-url)

**Fig. 9.** Biomass productivity of *P. hussii* and *C. luteoviridis* in RMWSE enriched with 25% liquor in an open pond cultivation system in Summer 2012 (A), Autumn 2012 (B), Winter 2013 (C) and Spring 2013 (D). Data are means (±SE) of three replicate samples of the pond cultures per strain.
Culture pH decreased as the cultivation duration increased in summer and spring and increased during autumn and winter cultivations (Table 2). The initial pH and nutrient constituents of the culture medium varied and this was because the liquor samples were collected freshly at the start of each experiments. The initial pH and nutrient constituent of each culture media used at every season is shown in Table S3.

Intracellular neutral lipid determined by fluorescence intensity measurement following Nile Red staining suggested that *P. hussii* and *C. luteoviridis* cell contain more neutral lipid during spring cultivation (Fig. 10A). Although neutral lipid might be higher in the algae cell during the spring cultivation than in summer, the total lipid concentration per biomass measured at day 16 of the cultivation (Fig 10 B) was significantly higher for both strains during the summer than in the spring and other cultivations seasons (P< 0.05).
Fig. 10. Intracellular lipid (A) determined by measuring fluorescence intensity of the cell sample following Nile red staining of *P. hussii* and *C. luteoviridis* at day 10 of cultivation in the open pond system and lipid productivity g L⁻¹ d⁻¹ (B) measured at day 16 of the pond cultivation and in Summer 2012 (A), Spring 2013 (B), Autumn 2012 (C) and Winter 2013 (D). Data are means (±SE) of three replicate samples of the pond cultures per strain.

Lipid productivity appeared to be directly dependent on the biomass productivity of algae. Lipid productivity of *P. hussii* and *C. luteoviridis* were significantly lower in autumn and winter cultivations (P<0.01).
3.3.3 Remediation potential

The initial nutrient concentrations and BOD values of the culture medium shown in Table S3 allowed the calculation of total removal efficiency of *P. hussii* and *C. luteoviridis*. Nutrient removal by the algae (Fig. 11A and B) was higher during the spring and summer cultivations than in the autumn and winter cultivation (*p*<0.05). More than 75% of the initial N and P concentration in the pond were removed by the algae at 10 days of the cultivation in the spring and summer. In most of the cultivation experiments, more nutrients were removed by the algae at day 10 than day 5 (*P*<0.05) except in autumn and spring where there were no differences. There were no significant difference in the nutrient removal efficiency of *P. hussii* and *C. luteoviridis* in any of the pond cultivation studies except in the autumn period (*P* = 0.08). BOD of the culture showed a significant decrease after 10 days cultivation of *P. hussii* and *C. luteoviridis* in the summer and spring unlike in the autumn and winter periods (Fig. 11C). The BOD reduction followed a similar trend as the growth rate of the algae suggesting importance of algae growth in reduction of wastewater BOD. Although a control pond experiment without an algal culture was not performed in this study, the microscopic examination of the culture suggested that the inoculated algae strains were the dominant species in the ponds and therefore the main remediation agent in the ponds.
Fig. 11. Remediation of $\text{PO}_4^{3-}$-P (a) and $\text{NH}_4^+$-N (b) at day 5 and 10 and BOD reduction (c) at day 10 during cultivation of *P. hussii* and *C. luteoviridis* during pond cultivation in each season. All values are mean data ±SE of 3 replicate samples per strain.
4.4 Discussion

4.4.1 Nutrient enrichment

The ability of algae to grow in wastewater secondary effluent for either biofuel or remediation purposes has been well documented (Wang and Lan, 2011; Pittman et al., 2011; Chapter 1). However, secondary effluents are generally lower in inorganic nitrogen and phosphorus than synthetic growth medium like TAP which can limit optimum growth of algae. Liquor for example, a product from activated sludge dewatering is rich in nutrients and therefore may be used as a cheap source of nutrient to boost algae growth in secondary effluent. Therefore in this study, improving nutrient concentrations of RMWSE by addition of different nutrient sources (liquor, inorganic fertilizer, phosphate and ammonium) was examined for optimum growth of algae for biomass production and removal of nutrients.

In the case of liquor, an experiment to determine the optimum proportion of liquor for addition to RMWSE was conducted. *C. luteoviridis* and *P. hussii* culture optical densities increased as the concentration of liquor in RMWSE increased and attained highest optical density at 25% liquor. Therefore it can be inferred that 25% liquor in RMWSE was optimal for cell growth (Fig. 2A to D), despite the liquor addition providing a lower N/P ratio (Table S2), which is optimal for many green algae (Tilman et al., 1986), addition of liquor caused a reduction in growth of *C. debaryana*, *D. subspicatus* and *H. tetrachotoma*.

Liquor is very rich in nutrients including NH$_4^+$-N, PO$_4^{3-}$-P and K, and can therefore alleviate any macronutrient deficiencies that may be caused by relatively nutrient-poor secondary effluent. However, liquor also includes other organic and inorganic...
compounds which can inhibit algae growth (Table S1). For example, high concentration of transition metals such as Fe$^{2+}$ and Zn$^{2+}$ can induce oxidative stress (Suzuki et al., 2012). Furthermore, the metals can inhibit photosynthesis and cause morphological changes to the algal cell (Clijsters and Vanasse, 1985; Pena-Castro et al., 2004). The liquor used here also has a relatively high concentration of Na (0.4 g L$^{-1}$) which can be toxic to freshwater algae (Prieto et al., 1996). Thus, when the concentration of liquor in the medium increases, it can be assumed that the accumulation of intracellular reactive oxygen species also increases. We have previously shown that C. luteoviridis and P. hussii are highly tolerant to oxidative stress unlike C. debaryana, D. subspicatus and H. tetrachotoma (Chapter 2). It is therefore not surprising that these three species were extremely sensitive to liquor addition while C. luteoviridis and P. hussii displayed no sensitivity up to 40% liquor. However, neither of these strains could be grown to high density on 100% liquor (data not shown), which has been observed with some microalgae (Li et al., 2011). It should be noted that the liquor used in this study is not directly comparable to liquor from the other studies such as that of Li et al. (2011) due to the much higher concentrations of metals and Na in this effluent (Table S1), making it potentially more toxic. Furthermore, these strains were isolated from and maintained in RMWSE and had therefore not adapted to growth in raw liquor. It may be possible that following acclimation of these strains in higher concentrations of liquor eventual growth in 100% liquor medium is possible. Nevertheless, the growth rate and biomass productivity values observed in the liquor medium by C. luteoviridis and P. hussii (Fig. 2) are either equivalent or better than those reported previously for microalgae grown in activated sludge liquor conditions (Dominguez Cabanelas et al., 2013; Li et al., 2011). For example, C. vulgaris grown in concentrated liquor had an
equivalent growth rate of 0.23 - 0.38 d\(^{-1}\) but a relatively lower biomass productivity of 0.1 - 0.2 g L\(^{-1}\) d\(^{-1}\) (Dominguez Cabanelas et al., 2013) compared to the values of 0.84 and 0.97 g L\(^{-1}\) d\(^{-1}\) for \textit{C. luteoviridis} and \textit{P. hussii}, respectively.

In a comparison to the control culture with no added nutrient substances, addition of 12.4 mg L\(^{-1}\) PO\(_4^{3-}\)-P, equivalent to P concentration in 25\% liquor in RMWSE showed that the growth rate of all the algae increased (Fig. 3A). However, no significant growth improvement was observed in any of the algae in terms of growth rate and total chlorophyll when RMWSE was enriched with either inorganic fertilizer or ammonium alone. This suggests that phosphates may be the limiting nutrient in RMWSE. PO\(_4^{3-}\)-P is an essential nutrient and inadequate availability in the culture medium can limit algal growth (Dean et al., 2008).

Although enrichment of RMWSE with phosphate increased the algae growth rate, biomass productivity of these strains remained relatively the same as the control (Fig. 3C). However, apart from for \textit{C. debaryana}, biomass productivity of all the strains remained unchanged following PO\(_4^{3-}\)-P addition relative the RMWSE control medium (Fig. 3c), indicating that PO\(_4^{3-}\)-P at this concentration had a relatively minor consequence and higher concentrations may be needed to improve microalgal biomass. Of all the treatments in this study, it appears that 25\% liquor in RMWSE is optimum for \textit{C. luteoviridis} and \textit{P. hussii} growth and biomass productivity (Fig. 3). This result suggests that other constituents in the liquor in addition to NH\(_4^+\)-N and PO\(_4^{3-}\)-P may be enhancing growth of \textit{C. luteoviridis} and \textit{P. hussii}. This could include essential metal nutrients such as Fe but also could include organic carbon, which is high in liquor, as indicated by the high BOD, COD and suspended solids concentration (Table S1). We previously found that \textit{C. luteoviridis} and \textit{P. hussii} can
efficiently utilise external carbon and grow mixotrophically (Chapter 2), which would potentially increase growth relative to the auxotrophic conditions in the RMWSE + inorganic fertiliser medium.

The addition of liquor did not have a marked effect on cellular lipid production. As we have seen previously (Chapter 2), *C. luteoviridis* and *P. hussii* accumulate more triacylglycerol (TAG) as determined by Nile Red fluorescence than the other strains in RMWSE, and this was also seen following 10% to 30% liquor addition (Fig. 4). However, the was no significant increase in cellular lipid content following liquor addition; indeed high (40%) concentrations of liquor inhibited TAG biosynthesis. We hypothesise that further addition of liquor might induce cell death in the algae. It was therefore not surprising that further increase in liquor concentration to 40% caused a significant drop in intracellular lipid of *C. luteoviridis* and *P. hussii* in comparison to 10% liquor in RMWSE (*P*<0.001).

Environmental stresses including nutrient limitation and salinity (Hu et al., 2008; Dean et al., 2010; Siaut et al., 2011) are known to induce TAG biosynthesis, but liquor-induced stress and perhaps oxidative stress induced by wastewater exposure does not trigger lipid biosynthesis, suggesting that only specific abiotic stresses induce lipid accumulation in microalgae. Consequently there was no increase in lipid productivity in *C. luteoviridis* and *P. hussii* in response to liquor addition (Fig. 3d). Furthermore, lipid productivity in these strains was significantly lower following enrichment with NH$_4^+$-N or PO$_4^{3-}$-P compared to control media (*p*<0.05). This may be related to a surplus of nutrient in the cell and thereby high lipid production was not triggered. As shown previously, the fatty acid methyl esters that are produced in these two strains have equivalent characteristics to palm oil and sunflower oil-
derived biodiesel (Chapter 2) and the lipid productivity values are better or equivalent to those observed in other wastewater cultivation studies (Pittman et al., 2011). Further study is needed to determine the impact of liquor-derived stress, which may induce oxidative stress, on algae lipid biosynthesis. However, due to these relatively low oil yields, energy generation from this algal biomass may be more viable by anaerobic digestion rather than biodiesel generation (Menger-Krug et al., 2012).

In the previous study, we found that *C. luteoviridis* and *P. hussii* were able to remove more than 80% of N and P when grown in RMWSE (Chapter 2). Interestingly, in this study, they also remove almost 80% of N and P as the nutrient concentration in the media increases. This showed the versatility of these algae in wastewater remediation. In contrast, removal efficiency of *C. debaryana*, *D. subspicatus* and *H. tetrachotomai* reduced as the concentration of liquor in the media increases. This can be attributed to the poor growth rate of these algae at these elevated concentrations. A previous study has shown that the efficiency of algae in removing nutrient from wastewater can be directly or indirectly dependent on their growth rate (Olgun, 2003). It is therefore not surprising that the nutrient removal by these algae including *C. luteoviridis* and *P. hussii* decreased at 30% of liquor. However, it was observed that despite the poor growth of *H. tetrachotoma* in increasing liquor concentration, their phosphate removal capabilities were not significantly reduced up to 25% liquor concentration in comparison to the control. One of the possible reasons could be link to algae-bacteria relationship in the culture. For example, the presence of *Azospirillum brasiliense* has been reported to increase phosphate removal by *Chlorella vulgaris* (De-Bashan et al., 2002). However, the
mechanism of nutrient removal by algae still requires further study. Similar to the outcome of Chapter 2, *C. luteoviridis* and *P. hussii* remained the best performing strain of all the five algae in this study, and their growth, biomass production as well as remediation capability were optimum in RMWSE enriched with 25% liquor. This is evidence that these algae strains are highly efficient for remediation and biofuel production when grown in wastewater. However, their resilience for continuous cultivation needs to be established for commercial application in wastewater work.

### 4.4.2 Continuous cultivation

The laboratory and pond cultivation experiments evaluated microalgae growth, productivity and remediation in a batch mode. While this provided a useful validation of the microalgal strain performance, any potential commercial cultivation of microalgae would likely be performed in a continuous or semi-continuous process. Therefore, an experiment was performed to evaluate semi-continuous cultivation of *P. hussii* and *C. luteoviridis* in RMWSE +25% liquor in the laboratory. The microalgal growth was initiated and cultivated until day 8, which represented the mid-exponential growth stage of both strains. A 50% volume of the culture was removed and fresh RMWSE containing 25% liquor at mid exponential growth phase. The growth results of this experiment showed that the two algae were able to grow, produce biomass and lipid in a steady state. The result suggested the resilience of these algae...
for a continuous cultivation system. For algae to be relevant in wastewater work, it must be able to remove nutrient at a steady rate from wastewater effluent. This study showed the suitability of *C. luteoviridis* and *P. hussii* for continuous removal of nutrient from wastewater effluent. No less than 70% of N and P in the media were removed every 2 days during the continuous cultivation (Fig. 7A and B). The potential of the use of algae for sustainable low cost wastewater treatment has been well reported (Oswald et al., 1959; Oswald 1988; Ruiz-Marín et al., 2010; Rawat et al., 2011; Park et al., 2011; Pittman et al., 2011; Lohrey and Kochergin, 2012). Algae can assimilate a significant amount of nitrogen and phosphorus for synthesis of proteins, nucleic acids and phospholipids synthesis (Munoz and Guieysse, 2006) and therefore offer a sustainable means for remediation of wastewater.

**4.4.3 Cultivation of algae in an open pond system**

Mass cultivation of microalgae for biofuel production or wastewater treatment depends heavily on the performance of the microalgae strains used. Microalgae can be sensitive to wastewater as a result of a combined effect of ammonia and pH by uncoupling the electron transport in photosystem II (Munoz and Guieysse, 2006). Therefore a careful selection of algae that are well adapted to the wastewater condition is crucial for successful cultivation of algae in wastewater.

Fundamental factors that influence pond cultivation especially in an open system include climatic conditions (especially temperature and solar light intensity), suitability of algae strain and culture management (Rodofi et al., 2009). It is important that the algae strain for cultivation in wastewater is robust to withstand uncontrollable physio-chemical factors including shear-stress generated by culture mixing and environmental stress such as temperature.
The initial laboratory experiment in this study has established the efficacy and suitability of *C. luteoviridis* and *P. hussii* for wastewater cultivation. Therefore, to evaluate the commercial possibility of cultivating these algae in wastewater for remediation and biomass production, a pilot pond experiment was set up in an open pond system over four consecutive seasons. The aim was to assess the ability of these strains to remove nutrient and for biofuel production and the suitability of such a process in a temperate climate as in the North West of the UK.

The algae pond used in this study was fitted with three air lift pump for continuous circulation of algae culture in the pond (Fig. 1). The pondwater depth was not greater than 10 cm and allowed light penetration and circulation of the culture. Also, in situ temperature and pH probes were fitted to allow monitoring of the state of the culture. In situ culture temperatures and light intensities measured at surface of the pond during experiment showed that temperature and light intensity in summer and spring are likely to favour algae growth than those observed in the winter and autumn (Table 1A and B). Operating conditions of the pond including temperature, light intensity and pH can influence algae growth. Light intensity is among several parameters influencing the growth of photosynthetic organisms such as microalgae. For example, efficiency of *Scenedesmus obliquus* for biomass production and remediation in a brewery wastewater were greatly dependent on light intensity and aeration (Mata et al., 2012). In another example, Ho et al. (2012) reported that the biomass productivity of *S. obliquus* increased significantly as the light intensity increased from almost 100 to 400 μmol·m⁻²·s⁻¹. Temperature is also another factor that influences cellular metabolic rates and photosynthesis. At low temperature various cellular temperature dependent components can be affected or slow down as in the
case of *S. obliquus* grown in diluted olive mill wastewater at various temperature ranges (Hodaifa et al., 2010).

The microscopic observation of the algae population in the culture pond (Fig. 8A and B) showed that the alga could maintain competitive advantage and dominate the culture pond over 20 days cultivation. At least 92-98% of the original strains were present at the end of cultivation. This was not because locating the ponds in a glasshouse prevented competition; during the summer cultivation period, a pond in an adjacent glasshouse growing *Chlamydomonas reinhardtii* in fertiliser medium exhibited a culture crash and contamination then replacement by a different algal species (data not shown). Rodolfi et al. (2009), showed that strain robustness and capacity to dominate the culture pond is an essential characteristic of an alga for outdoor cultivation. High growth rate is also necessary for algae to overcome the competing effect of grazer zooplankton like rotifers and cladocerans which can proliferate in algae ponds and thereby reduce biomass production (Cragg et al., 2012; Smith et al., 2010). Microscopic examination of the cells showed some of them formed cell clusters during all the cultivation seasons (Fig S2). This might be related to increased extracellular polysaccharides in the algae cell (not measured) which is known to be favoured by the high concentration of carbon and nitrogen in the culture media (Miqueleto et al., 2010; Liu et al 2013). However, this can be advantageous in harvesting of the algae biomass by flocculation.

The specific growth rates of *C. luteoviridis* and *P. hussii* in the summer and spring (Fig. 98C) were lower than those obtained in the laboratory conditions (Fig. 3A). The environmental conditions of the pond cultivation are not controlled and are dictated by the prevailing weather condition unlike in the laboratory experiment.
The impact of the weather condition on algae growth was more obvious in the winter and spring when the temperature and light intensity were lower. Consequently, the total chlorophyll concentration (Fig. 8D) was also lower during winter and autumn, suggesting that algae were physiologically less active during these seasons.

Thus, a possible influence of weather condition on algae growth biomass is more evident in the winter and autumn cultivations (Fig. 9 A-D). In a recent report by Slegers et al. (2013), a model based framework was used to study the effect of light intensity and temperature on microalgae production in large scale open ponds under different climatologic conditions and they found that climate condition was an important factor for biomass productivity.

The culture pH decreased during the summer and spring and increased in the winter and autumn cultivations (Table 2). This can be related partly due to the evaporation rate which was higher in the summer and spring than in autumn and winter. It worth noting that fresh media was not continuously fed into the pond during the cultivation process. Initial pH of the medium was approximately 8.3 to 8.6 but pond pH decreased by the end of cultivation in summer and spring to approximately 7.3, and increased during autumn and winter to approximately 8.8. Strong algal growth generally promotes pond water alkalization (Park et al., 2011). However, previous study of *C. vulgaris* growth in a pH range of 7.5 – 10.6 observed that growth was favoured when the pH was nearer to 7.5 than towards 10.6, due to the adverse effect of alkaline pH (Goldman et al., 1982). Kong et al. (2010) also observed that growth of *C. reinhardtii* in liquor medium was optimal at pH 7.5. Pond pH above 8.5 can also inhibit the growth of aerobic heterotrophic bacteria that mediate organic matter breakdown in the wastewater to release carbon for algae growth (Park et al., 2011).
Thus, in addition to the adverse light and temperature conditions, increased pondwater pH may also explain why algal biomass productivities were poorer in the winter and autumn.

In a similar trend, cellular neutral lipid content and lipid productivity by the algae were best during the summer and spring (Fig. 10 A and B). In the summer for example, more than 0.4 and 0.3 g L\(^{-1}\) d\(^{-1}\) of lipid which is equivalent to 454 and 341 mg lipids m\(^{-2}\) of pond per day were produced by \textit{P. hussii} and \textit{C. Luteoviridis}, respectively. However, it worth noting that despite the lower growth, biomass and lipid production in autumn and winter, are still higher than those reported in a similar experiment carried out in Sweden where extra illumination was applied to augment the low light intensity in a pond culture (Larsdotter et al., 2010).

Nutrient removal by both strains was equivalent and was higher during the spring and summer than in autumn and winter (\(p<0.05\)). Previous study in Sweden also showed that P removal by algae was lower in autumn than in spring and winter cultivation in a pond system (Larsdotter et al., 2010). During the summer, approximately 60\% of the NH\(_4^+\)-N and PO\(_4^{3-}\)-P in the pond were removed by the strains after 5 d of cultivation and approximately 80-90\% after 10 d (Fig. 11A and B). There was no statistical difference in the nutrient removal efficiency of \textit{P. hussii} and \textit{C. luteoviridis} except in autumn (\(p=0.08\)). Both strains reduced significantly more BOD in the summer and spring than in autumn and winter (Fig. 11c). Previous study in Chapter 2 showed that \textit{P. hussii} and \textit{C. luteoviridis} can grow heterotrophically. Not surprising that despite the low sunlight in the autumn and winter they were able to remove considerable amount of NH\(_4^+\)-N and PO\(_4^{3-}\)-P from wastewater, although their nutrient removal was significantly lower in autumn and
winter than in the summer and spring. It worth noting that a control pond without an algal culture was not operated in this study, the microscopy examination of the cultures suggested that the microalgae were the dominant species in the ponds and therefore the main remediation agent. However, it is worth recognising that nutrient removal might also involve other chemical and biological processes. For example, some NH$_4^+$-N may have precipitated from the pond, a phenomenon known to occur at elevated pH greater than 8.5 (Craggs et al., 1997). Despite the poor growth of the microalgae during winter cultivation when the culture pH was $>8.5$, more than 50% reduction of the initial NH$_4^+$-N and PO$_4^{3-}$-P concentration were observed after 10 d. In addition, more than 40% of the initial BOD was reduced by both strains in the winter, indicating that *P. hussii* and *C. luteoviridis* can be cultivated all year round in the UK for efficient wastewater remediation.

### 4.5 Conclusion

This study has demonstrated that sludge liquor is a sustainable nutrient amendment to enhance productivity of secondary wastewater-cultivated microalgae. However, due to the toxic nature of liquor, robust strains are required that are well adapted to tolerate such condition. The continuous cultivation of *C. luteoviridis* and *P. hussii* showed that they are capable of long term steady growth for nutrient removal and biomass production. Pilot scale cultivation of *C. luteoviridis* and *P. hussii* in an open pond system highlighted their suitability for commercial cultivation in wastewater. Environmental factors such as temperature and light intensity still remain a determining factor for the performance of these strains in such cultivation system. The results from this pond study show that the algae cultivation in an open pond
system for biomass and nutrient removal is suitable in the UK particularly during summer and spring when nutrient removal and biomass production are optimum. However, their performances are weaker in the winter and autumn.

Acknowledgments

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Supplementary figure and tables

Table S1: Chemical characteristic of wastewater liquor

<table>
<thead>
<tr>
<th>NH4⁺ mgL⁻¹</th>
<th>P mgL⁻¹</th>
<th>BO D mgL⁻¹</th>
<th>COD mgL⁻¹</th>
<th>SS mgL⁻¹</th>
<th>Cd mgL⁻¹</th>
<th>B mgL⁻¹</th>
<th>Na mgL⁻¹</th>
<th>Mg mgL⁻¹</th>
<th>K mgL⁻¹</th>
<th>Ca mgL⁻¹</th>
<th>Mn mgL⁻¹</th>
<th>Fe mgL⁻¹</th>
<th>Cu mgL⁻¹</th>
<th>Zn mgL⁻¹</th>
<th>Ag mgL⁻¹</th>
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<tbody>
<tr>
<td>&gt;180</td>
<td>99.03</td>
<td>458</td>
<td>3234</td>
<td>2296</td>
<td>0.08</td>
<td>4.65</td>
<td>4155</td>
<td>480</td>
<td>793.</td>
<td>70.0</td>
<td>0.92</td>
<td>6.99</td>
<td>0.17</td>
<td>1.23</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table S2. The concentration of PO₄³⁻-P and NH₄⁺-N in the culture media following enrichments with liquor v/v.

<table>
<thead>
<tr>
<th>RMWSE +</th>
<th>NH₄⁺ mg L⁻¹</th>
<th>PO₄³⁻ mg L⁻¹</th>
<th>N/P ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% liquor</td>
<td>74.96</td>
<td>1.43</td>
<td>52.42</td>
</tr>
<tr>
<td>10% liquor</td>
<td>83.96</td>
<td>6.38</td>
<td>13.16</td>
</tr>
<tr>
<td>20% liquor</td>
<td>92.96</td>
<td>11.33</td>
<td>8.20</td>
</tr>
<tr>
<td>25% liquor</td>
<td>97.47</td>
<td>13.91</td>
<td>7.01</td>
</tr>
<tr>
<td>30% liquor</td>
<td>101.97</td>
<td>16.29</td>
<td>6.26</td>
</tr>
<tr>
<td>40% liquor</td>
<td>110.97</td>
<td>21.24</td>
<td>5.22</td>
</tr>
</tbody>
</table>

Table S3. PO₄³⁻-P, NH₄⁺-N and BOD characteristics of the pond media (RMWSE +25% liquor) used for P. hussii and C. luteoviridis cultivation during each season. Values are mean data ±SE from 3 samples.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Autumn</th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg L⁻¹)</td>
<td>(mg L⁻¹)</td>
<td>(mg L⁻¹)</td>
<td>(mg L⁻¹)</td>
</tr>
<tr>
<td>PO₄³⁻</td>
<td>13.81 ± 3.67</td>
<td>21.68 ± 5.83</td>
<td>24.72 ± 4.68</td>
<td>15.35 ± 1.69</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>97.47 ± 5.2</td>
<td>113.47 ± 13.62</td>
<td>101.81 ± 3.05</td>
<td>120.11 ± 6.83</td>
</tr>
<tr>
<td>N/P ratio</td>
<td>15.60</td>
<td>11.57</td>
<td>9.10</td>
<td>17.30</td>
</tr>
<tr>
<td>BOD</td>
<td>24.86 ± 1.94</td>
<td>37.51 ± 6.53</td>
<td>29.33 ± 8.59</td>
<td>33.47 ± 8.66</td>
</tr>
</tbody>
</table>
Fig. S1. Representative images of the *P. hussii* cultivation in open pond system during the summer season, 2012.
Fig. S2. Representative light microscopy images of samples from the *P. hussii* and *C. luteoviridis* ponds during the seasonal cultivation experiments used to determine culture integrity. Samples are from day 13 of cultivation. Bar = 5 µm.
Chapter 5

This final chapter comprises of a brief discussion on the sustainability of biofuel from microalgae particularly by cultivation in wastewater, and a general discussion of this thesis and future work recommendations.
5.1 Sustainability of Microalgae Grown in Wastewater for Biofuel

As the world population is set to increase by a record high in 2050 (UN World Population Prospects, 2012) and oil prices are near an all time high (Jammazi and Aloui, 2012), finding a sustainable energy source is a major focus in this 21st century. The need for alternative fuel sources has grown more urgently; scientists around the globe continue to seek various methods for generating energy. Some of these methods include a variety of renewable or low atmospheric pollution technologies such as solar, wind, hydroelectric, geothermal, nuclear and biofuel based. With the potential for an energy crisis looming and the continual concern over the negative effects that fossil fuels have on the environment, it is clear that fossil fuels are not a sustainable energy source. However, renewable bioenergy that can directly replace liquid fossil fuel are still in their early developmental stages.

Many of the bioenergy options currently being explored, such as crop-based biofuel for energy production, come with concerns of competition with food production, water, fertiliser and farmland. However, finding a sustainable balance in energy production, food and water supply remains a critical challenge that needs to be resolved. In recent years, a crop-based biofuel approach has been widely debated (Nonhebel, 2012; Bahel et al., 2013) due to its impact on the global food supply and agriculture. For example, the use of corn as a biofuel feedstock in the US has been correlated with a rise in the price of corn by over 60% in 2007 (WDR, 2008). Microalgae are likely to be better feedstock toward actualization of sustainable biofuel capable of meeting the global demand for fuel than most land-based plant sources (Chisti, 2007). They are capable of higher lipid productivity than land-based plants and in certain conditions algae can accumulate up to 60% of its biomass as
lipid (Xin et al., 2010). According to NASA research, microalgae have the potential for producing 2,000 - 5,000 gallons of oil/acre/year which is more than triple that of oil palm crops for example (Trent, 2011). Algae grow faster than most plant-based crops and are capable of producing high biomass within a short period. Algae can potentially serve as a sustainable source for renewable fuel. In addition it could be used to capture carbon dioxide from power plants; for the production of human and animal food, pharmaceuticals, cosmetics, and organic fertilizers; aquaculture; and soil nutrient recovery (Menetrez, 2012; Chapter 1).

Even though algae cultivation has been tipped to be a better option for biofuel feedstock generation, it comes with challenges of high nutrient demands and competing with water resources, in particular for freshwater algae. In fact, recent life cycle analysis suggested that algae might be performing poorly from a sustainability point of view (Slade and Bauen, 2013; Sing and Olsen, 2011; Holma et al., 2013). The suggestion from these life cycle studies was that growing algae in wastewater can improve on its environmental performance and economic output. However, unlike plant biofuels, large scale-up studies of algae cultivation in wastewater for biofuel are quite rare. Whilst growing algae on wastewater resolve some of these challenges, the question still remains, how sustainable will algae grown in wastewater for biofuel and remediation purposes be in terms of overall economy, environmental and ethical considerations? In this concluding Chapter, I will briefly review the economic, environmental and societal potential of microalgae biofuel produced on wastewater in a context of sustainability, and discuss the results of this PhD study.
5.1.1 Economic considerations

Algae grow rapidly and can produce 50 times more oil per acre compared to other plant sources, and can fix greenhouse gas (Chisti, 2007). However, the economic feasibility of algal biofuel is still one of its impediments, though there are some positive points which can help to tip the balance. The current state of algae biofuel from an economic point of view is still unattractive. For example, the estimated cost of a barrel of algae based fuel using current technology is in the range of US$300-2600 (Hannon et al., 2010) compared to the current petroleum price of US$106/barrel (OPEC). However, with the use of wastewater for cultivation and the possible remediation credit, and combining algae biomass with other applications such as animal feed, bio-fertiliser, it is expected that the cost of algae-based biofuel will reduce. Depending on the species and growth conditions, algae can yield a wide array of byproducts which include lipids, carbohydrates, and proteins (Menetrez, 2012).

Analysis of the microalgae biofuel production strategy have shown that the use of wastewater as a cultivation medium could decrease water requirement by 90% and eliminate nutrient requirements (Yang et al. 2011; Clarens et al. 2010) and thereby reduce the overall cost. Acién et al. (2012) performed a cost evaluation of biomass production from *Scenedesmus almeriensis* cultivated in a 3 m$^3$ tubular photobioreactor and found that cultivation cost accounted for 47.41% of the total cost of production. They recommended that it is important to reduce the cost of running a photobioreactor, and use wastewater and flue gases in order to make microalgae biofuel production economically attractive. Growing algae in wastewater can offset concerns such as water resources and nutrients.
Harvesting of algae from wastewater is also a major concern. In general, the cost of algae biomass recovery from wastewater can be considerable high. Harvesting of algae can be by sedimentation or flocculation, filtration or centrifugation (Georgianna and Mayfield, 2012; Chapter 1). Research to improve harvesting of algae from wastewater has focussed on biofloculation, immobilization and centrifugation (Zhang et al., 2008; Brennan and Owende, 2010). Drying of algae before lipid extraction also contributes significantly to the cost of algae biofuel and poses a challenge for commercial development. Efficient wet extraction could reduce the need for dewatering and drying (Levine et al., 2010).

Although algae cultivation on land has been suggested to take up less space than land based plants, in places like Europe where land availability is limited, algae cultivation may still impact on land availability for agriculture. Also acquiring such land for algae cultivation might require additional cost in terms of planning and permission. Therefore by integrating algae cultivation into an existing wastewater treatment process can alleviate this challenge and also reduce the cost of cultivation and heating supply to algae culture (Lundquist et al., 2010).

Algae grown in wastewater provide more economic benefits than just the production of biofuel. Combining algae cultivation on wastewater for biofuel production also serves the dual purpose of removing pollutants from the wastewater thereby providing a saving in water treatment costs and this credit can help in improving the overall economy output. The world is estimated to produce up to 2440 billion m$^3$ wastewater by year 2025 (Shiklomanov, 1999). Additionally, it is expected that 80 percent of this might be untreated (Gabel, 2012). This means that not only is there
already ample medium for growing microalgae, but as algae assimilate nitrogen and phosphorous, they are serving to treat the wastewater and reclaim nutrients.

The concept of creating biofuels from algae was evaluated in detail for the first time back in the 1970s. In response to the realization of upcoming shortages of fossil fuels, the U.S. government funded a large research program of thousands of strands of algae. Since then, commercial interest in algae has shifted from a food related product to fuel with its potential for sustainable biofuel feedstock. For example, Solazyme became the first company to produce jet fuel approved by American Society for Testing and Materials (ASTM) and in 2010 sold 150,000 gallons at a cost of US$10 million for 150,000 gallons or US$67/gallon to the US navy (Menetrez, 2012). However, further reduction in cost of algae based fuel to US$4 per gallon will improve its economical competitiveness with petroleum base fuel. By improving the growth techniques, strain selection and the use of wastewater for cultivation, efficient harvesting method and processing will drive the cost of biodiesel produced from algae oil closer to being competitive with other sources that are produced from feedstocks such as palm, soybean, corn and petroleum. In addition, while crude oil sources will eventually deplete, algal biofuel sources will be renewable, and research in this aspect is growing fast. There is also a growing market for the use of algae’s bi-product for animal and fish feed, food and food additives, health and beauty products, and pharmaceuticals (Trent, 2011). Expanding and using the market for these bi-products would serve to both increase profit and drive down the net cost for producing biofuels.
5.1.2 Environmental considerations

Microalgae biofuel has been criticised for performing poorer than other plant-based biofuel in terms of energy requirement, green house gas emission and water use (Clarens et al., 2010). Cultivation of microalgae in either a photobioreactor or in an open system such as raceway pond requires considerable amounts of energy for mixing. The net energy requirement of a system has been defined as the ratio of the total energy output from the system over energy input for the entire production system (Razon and Tan, 2011). Net energy analysis of microalgae biofuel production is important as a diagnostic measure of identifying performance and improvement options that may be necessary. One of the arguments for producing biofuel from microalgae is that microalgae can potentially produce a sustainable net surplus of energy. However, a recent report has raised concerns that microalgae biodiesel may not deliver more energy than is required to produce it (Lohrey and Kochergin, 2012). The majority of the energy demand has been attributed to the harvesting process and oil extraction (Lam and Lee, 2012). However, using wastewater as an alternative to fertilizer and wet extraction instead of dry extraction can improve its total environmental performance (Razon and Tan, 2011; Lohrey and Kochergin, 2012).

Over 10 billion liters of sewage are produced every day in England and Wales which is treated and managed by sewerage companies, and strictly regulated by the Environment Agency (Ofwat, 2006). The European Water Framework Directive; WFD 2000/60/EC was established on the basis of protecting all European waters, and requires all Member States to assess the ecological status of all water bodies, based especially on the status of the biological elements as well as hydromorphological and physico-chemical quality (Borja et al., 2013). Pollutant
controls into freshwater bodies has helped reduce loading of nitrogen, phosphorus and toxic inorganic and organic pollutants, which otherwise cause eutrophication and toxicity to aquatic ecosystems and can have direct or indirect consequences to human and animal health. The ability of algae to remove contaminants from wastewater as a form of remediation thus has clear environmental benefits. Recent study has shown that integrating microalgae systems can substantially improve energy balance of municipal wastewater treatment plants without any external source input (Menger-Krug et al., 2012). However, the effective energy balance of this process in wastewater treatment plants still relies on efficient harvesting process of the algae.

Algae farms located close to large industrial factories can also help to sequester carbon dioxide released from industrial processes using it to grow while helping to reducing GHG in the air. Although, the environmental benefit of biofuel from algae grown in wastewater is promising, there are still some hurdles to cross. A life cycle analysis of the environmental performance of wastewater-grown algae based on pilot scale experimental results still needs to be examined. Some recent life cycle results indicated that microalgae is not yet fairing well compared to plant-based biofuel (Sturm and Lamer 2011; Campbell et al 2011; Lardon et al 2009; Clarens et al 2010). For example, a stochastic life cycle assessment of algae-based biofuel in comparison with switchgrass, canola, and corn, concluded that algae might not be performing better than other sources from an environmental point of view (Clarens et al., 2010). Reportedly one of the main culprits for microalgae’s high rate of emissions is the fertilizer used for cultivation, in addition to harvesting and drying. In growing corn, for example, the farmer is able to rotate his crops with soybeans for natural fertilizer by N fixation. However, growing algae in PBR or HRAP means that nearly all
fertilizer must be provided from an outside source. Although microalgae are capable of producing more oil than rapeseed for example, algae will require about 55–111 times more N fertilizer to produce equivalent 8–16 tons/ha/year of rapeseed oil (Demirbas, 2011). Such quantities of N and P could severely impact its environmental performance. Unfortunately these fertilizers are petroleum-based and typically non-environmentally friendly (Howell, 2010). This is where turning to wastewater for growing algae has another benefit.

Wastewater-cultivated algae have N and P available in the wastewater to feed on. In fact, many reports including this thesis have shown the potential of this approach. Using wastewater also eliminates one of the other main environmental concerns of growing freshwater algae: use of water. Once again, growing the algae in farms requires more water use than a crop plant. Though this is partly set off by its large productivity and smaller need for land, of course using valuable water is as much of a concern. If wastewater-grown algae (a) uses second-hand water versus valuable freshwater, (b) does not need any petroleum-based fertilizers coming from outside sources, and (c) can absorb some of the carbon dioxide generated by the bacteria in the wastewater, it already overcomes some of the environmental concerns.

Furthermore, algae cultivation in mixing tanks or bioreactors is an energy-consuming process and contributes to its total carbon footprint consideration (Lam and Lee, 2012). In OMEGA modules for example, algae were grown off shore in a semi-permeable membrane, with the wave energy naturally mixing the algae, and temperature control is kept by the temperature of the surrounding water (Wiley et al., 2013). The semi-permeable membranes in the modules take advantage of the saline content in the ocean, allowing water and wastewater to pass through without the
algae or pollutants, again using natural versus energy-driven processes. The water which passes through is clean enough to be released back into the water bodies without adverse impact on its surroundings. The idea at some point would be to also recover the wastewater in an energy-friendly form, further boosting the positive environmental impact of this structure.

The harvesting of algae biomass from wastewater still requires high amounts of energy. Lundquist et al. (2010) suggested that digesting algae biomass in an already existing anaerobic digestion system present in the wastewater treatment plant for biogas production can help offset this challenge. However, more research is still needed to make biofuel from algae grown in wastewater more energy-efficient.

5.1.3 Social considerations

Both social and cultural aspects of algae use for biofuel and remediation are hard to quantify and are therefore often not addressed in many discussions. However, these aspects will play an important role in the implementation of technology and impact policy making. Biofuel has been accepted in many countries because of the fact that it contributes to energy security and helps in mitigating GHG emission. In addition it provides employment and can increase rural incomes (WDR, 2008). Brazil is currently at the forefront of biofuel generation probably due to sustained government support through direct subsidies and land availability.

As lined out by the Nuffield Council on Bioethics (2011) in accordance with worldwide human rights policies, using wastewater for the production of algal biofuel and by-products shows the potential to pass the tests of (a) not endangering food security or displacing people from their right of land, (b) providing positive
effects on environmental security and sustainability, and (c) contributing to the reduction of greenhouse gas emissions. The greater advantages of algae-derived biofuel over fossil fuel can make a difference in perspective of biofuel and cost to society (Kovacevic and Wesseler, 2010). The driving force in society attitude toward algae based biofuel is still the cost of the fuel. However, there is an increasing momentum with government and society driven by the instability in major OPEC countries and ever increasing price in fossil fuel. In addition, policies on biofuel should be improved to support the most efficient fuel options for the society. In order to ensure net societal benefits, a comprehensive multi-agency assessment involving governments, Universities, companies and members of the public is needed to map out the standards required for safe operation of algae cultivation in wastewater.

5.1.4 Future and Overview of Algae Biofuel Sustainability

The current consensus by many studies is that algal biofuels are currently unsustainable except when generated from algae grown in wastewater due to their requirements of growth. More promisingly there is continued growth in funding from several private organisations, and an increase in companies which are developing the algal biofuels. While alternative energy sources for ground transport also focusing on other processes such as hydrogen fuel cells, aviation and shipping fuel is expected to continue to rely solely upon liquid fuels, which also means that the aviation industry is strongly interested in invested efforts to replace fossil fuels (Trent, 2011). Efforts to make the process more economically stable are in turn helping to make the process more environmentally stable. The remediation benefit by algae in wastewater module systems can create additional markets helps to boost its economic sustainability versus solely growing microalgae for fuels. In practice,
using microalgae biomass collected from growth in wastewater has many benefits over alternative land options, but currently still does not come close to competing with fossil fuels. In the UK Parliamentary Office of Science and Technology’s biofuel report it is estimated that commercial production of algal biofuel will not be feasible before 2020 (Allen, 2011). However, as fossil fuel supplies continue to dwindle and research for algal biofuel production continues to grow, there is potential for improved sustainability and economic competitiveness.

5.2 General discussion

In this thesis I examined the potential of growing algae in wastewater for sustainable biofuel production. Growing algae in wastewater is still hampered with lots of difficulties including non-optimal strains, poor growth, non-efficient harvesting, environmental stresses, among others. Although the use of algae for wastewater treatment has been proposed for over 5 decades (Oswald et al., 1957), algae are still rarely used for wastewater treatment around the world. One of the concerns of using algae for treatment of wastewater includes poor growth, strain sensitivity, and inefficient recovery of the algal cells (Park et al., 2011; Pittman et al., 2011). Finding a suitable strain that can grow well in wastewater effluent and remove considerable amounts of nutrient pollutants and produce biomass for biofuel is an essential step of this promising technology. Up until now, no study has elucidated the mechanism by which algae tolerate wastewater cultivation. In Chapter 2 of this thesis, I described the isolation of algae species from a wastewater secondary treatment tank that were characterized as \textit{Chlamydomonas debaryana}, \textit{Hindakia tetrachotoma}, \textit{Chlorella luteoviridis}, \textit{Parachlorella hussii} and \textit{Desmodesmus subspicatus} (Fig. 1 in Chapter 2).
The ability of these strains to grow in wastewater was tested in autoclaved and untreated raw municipal wastewater secondary effluent (RMWSE). Although, it will be unsustainable to grow algae in wastewater effluent in autoclaved condition, for the purpose of this study, it allowed an understanding of the negative impacts exerted on the algae by other biota in the effluent. Two of the strains, *C. luteoviridis* and *P. hussii*, could grow very effectively in raw wastewater comparable to other strains in this study. In the initial study, I observed a substantial increase in the growth rate of non-indigenous algae obtained from the Culture Collection of Algae and Protozoa (CCAP) in autoclaved RMWSE than in untreated effluent (Fig. 2A in Chapter 2). This may be partly due to decreased microbial load or the breakdown of toxic compounds in the effluent when autoclaved. Previous research has shown that effective utilization of *Chlorella* sp. for biofuel and remediation were optimum when bacteria and other microorganisms were greatly reduced or eliminated from the effluent (Cho et al., 2011).

In contrast, I observed that the growth rates of the indigenous species were not significantly affected by RMWSE treatment. This suggested that other underlying factors might be involved in allowing the growth of these algae in RMWSE which therefore prompted further investigations. Firstly, we investigated the capability of these indigenous strains to break down organic carbon for mixotrophic growth. Previous studies have highlighted the importance of algae to utilize organic carbon for successful cultivation in wastewater. For example *Auxenochlorella protothecoides* lipid and biomass productivities and nutrient removal in wastewater were greatly improved when cultivated hetero-photoautotrophically (Zhou et al., 2012b). The result of this study showed that all the indigenous algae strains were
able to utilize organic carbon effectively which indicated their ability to grow mixotrophically (Fig. 4 in Chapter 2). This therefore raised a further question of what other factors might possibly enhance the growth of these strain in RMWSE.

A next experiment showed that wastewater induces oxidative stress within the algae cell, as shown with the use of a fluorescent reporter stain DCFH-DA that detected the presence of intracellular ROS. The presence of ROS can induce oxidative damage to the cell thereby altering its cellular metabolic pathway and other essential cellular functions (Tanaka et al., 2011). Algae like other microrganisms possess a capacity to adjust cellular processes to favour their survival when subject to stress conditions (Grossman, 2000). This survival mechanism is important for an alga to survive in harsh environmental conditions like wastewater. In the study, I observed a buildup of ROS in algae cell when cultivated in RMWSE in relative to the control strain (Fig. 5 in Chapter 2). This ROS triggered the expression of antioxidants such as ascorbate peroxidase (APX) in algae cell to protect the cell against oxidative damage and maintains the redox state of the cell.

Increased activities of APX were found to be highest in *C. luteoviridis* and *P. hussii* compared to other three strains (Fig. 5D in Chapter 2). On the other hand, *C. debaryana, H. tetrachotoma* and *D. subspicatus* showed lower APX expression and increased cell death in an oxidative induced medium. Not surprising that the growth, biomass and lipid productivity of *C. debaryana, H. tetrachotoma* and *D. subspicatus* were significantly lower than those of *C. luteoviridis* and *P. hussii*.

*C. luteoviridis* and *P. hussii* were not just good for biomass and lipid production but they also removed significant amounts of N and P from RMWSE and reduce its BOD. Therefore, it was concluded that *C. luteoviridis* and *P. hussii* in this study are
efficient for wastewater cultivation for dual purpose of biofuel production and remediation partly due to their oxidative stress tolerance. Oxidative tolerance is therefore an important characteristic alga should possess for its successful cultivation in wastewater.

The tolerance of algae to wastewater effluent was hypothesized to be also partly due to their long acclimation in the wastewater conditions. Therefore a further test was conducted to examine the role of acclimation in the growth of algae in wastewater effluent. In Chapter 3, we obtained CCAP strains; *Chlamydomonas debaryana, Hindakia tetrachotoma, Chlorella luteoviridis, Parachlorella kessleri, Desmodesmus intermedius*, that have not been previously grown in wastewater and were closely related or identical to those indigenous strains isolated from secondary effluent in Chapter 2. In addition, four other CCAP strains (*Chlorella ellipsoidea, Chlorella vulgaris, Neochloris pseudostigmatica* and *Botryococcus braunii*) that have been described for their effective growth and oil productivity in synthetic media were tested for acclimation. The algae were acclimated in RMWSE for 8 weeks which involved a series of sub-culturing in RMWSE. The growth rate, total chlorophyll, biomass and lipid productivity and remediation potential of the strains were studied in autoclaved and untreated RMWSE. The result of the study showed that the growth parameters of all the strains tested increased after acclimation (Fig. 2 and Fig. 3 in Chapter 3). Increased growth parameters of these strains after acclimation suggested an increased overall fitness of the algae cultured in wastewater effluent. In addition, biomass productivity of these strains in autoclaved and untreated RMWSE were greatly increased after acclimation in comparison to the non-acclimated strains (Fig. 4 in Chapter 3), except that of *C. debayana, D. intermedius* and *B. braunii* in
untreated RMWSE. Furthermore, just like in Chapter 2, I found that treatment of the effluent by autoclaving improved the growth and productivity of the algae. This result therefore showed that acclimation/adaptation is an important factor for algae success in wastewater cultivation. In addition, the growth of the other four algae (C. ellipsoidea, C. vulgaris, N. pseudostigmatic and B. braunii) that are normally grown in synthetic medium also improved significantly in RMWSE after acclimation. Furthermore, this demonstrated that many strains can potentially be engineered for wastewater growth. It is obvious that acclimation increases algae tolerance to wastewater condition. However, it is not known if the oxidative stress tolerance of the strains was also increased as a result of the acclimation process. Our results are in line with a previous study on acclimation of C. vulgaris which showed significant growth improvement and increased capability to remove nutrient from wastewater secondary effluent (Lau et al., 1996). In another study, Raphidocelis subcapitata acclimated to 65 µg L⁻¹ of Zn for 100 days was reported to have 3-fold increased zinc tolerance (Muyssen and Janssen, 2001). However, despite the improved growth rate and productivity of almost all the algae tested in this study, C. luteoviridis and P. kessleri appeared to be the best performing strains in all comparisons. This suggested that in addition to increased tolerance of the acclimated algae to wastewater, an inherent species specific characteristic is also an important factor. Bischof et al. (1998) acclimatized some brown algae (Alaria esculenta, Laminaria saccharina, and Saccorhiza dermatodea) to UV radiation in arctic coastal water. In their study they found that the tolerance of the macroalgae after acclimation increased but the degree of tolerance varies significantly among the species. Furthermore, the ability of all strains to remove N and P and reduce BOD was also enhanced after acclimation (Fig. 7 in Chapter 3). Probably acclimation
improved the algae’s physiological activity and thereby ability to rapidly assimilate N and P.

However, the growth capabilities of the acclimated CCAP algae in RMWSE were still lower to those of indigenous species reported in Chapter 2. It worth noting that those indigenous strains have been well adapted over many years to the wastewater condition unlike the 8 weeks acclimation of the CCAP algae in this study. I project that prolonged acclimation process may further improve the performance of these algae potentially to the level of the indigenous strains. This study therefore showed evidence that acclimation can improve algae capabilities for wastewater cultivation.

In order to make alga cultivation in wastewater more sustainable and economically viable, there is a need to improve productivity and growth performance. Previous studies have shown that algae growth in wastewater was improved by bubbling CO$_2$ into the culture medium (Chinnasamy et al., 2009; Woertz et al., 2009) or by increasing culture temperature (Chinnasamy et al., 2009; Sakai et al., 1995). However, in this thesis, I studied the improvement of algae capacity for growth, biomass and remediation in RMWSE by addition of nutrients in different forms such as ammonium, phosphate, inorganic fertilizer and centrate (liquor). Liquor is the effluent from dewatering of activated sludge and is very rich in nutrient especially N and P. However, it also contains high concentration of toxic substances which can negatively impact algae growth (Wang et al., 2010; Li et al., 2011).

The initial study of Chapter 4 examined the impact of liquor addition to RMWSE on growth and productivities of the indigenous algae strains isolated in Chapter 2. In this experiment, varying concentrations of liquor were added to RMWSE and the growth of alga at each concentration was measured. The result of this experiment
showed that *C. debaryana*, *D. subspicatus* and *H. tetrachotoma* growth in RMWSE were severely affected by the addition of liquor (Fig. 2 in Chapter 4). In contrast, *C. luteoviridis* and *P. hussii* growth were favoured by increasing liquor concentration in RMWSE. The growth of *C. luteoviridis* and *P. hussii* were optimum when 25% liquor was added to RMWSE. The optical densities of *C. luteoviridis* and *P. hussii* RMWSE culture containing 25% v/v liquor were doubled that of control media with no liquor. However, further increments of liquor concentration in RMWSE up to 40% were observed to impact *C. luteoviridis* and *P. hussii* growth negatively.

The detrimental effect of addition of liquor in RMWSE on *C. debaryana*, *D. subspicatus* and *H. tetrachotoma* can be related to their oxidative stress tolerance. In Chapter 2 we observed that *C. luteoviridis* and *P. hussii* has higher expression of an antioxidant in response to ROS induced by RMWSE unlike in the other three strains. It is therefore not surprising that they were able to tolerate increasing stress, potentially oxidative stress, induced by the increasing liquor concentration in RMWSE. The remediation capability of *C. luteoviridis* and *P. hussii* did not significantly change as a result of addition of liquor (Fig. 5A and B in Chapter 4).

Interestingly, despite the poor growth of *C. debaryana*, *D. subspicatus* and *H. tetrachotoma* in RMWSE containing liquor, their remediation potential remained relatively the same except when 30% liquor was added.

In a similar experiment conducted with the use of inorganic fertilizer, ammonium chloride and potassium phosphate with equivalent concentration of N and P to that of the 25% liquor medium, we observed that the algae growth rate increased with addition of phosphate in comparison to the control (Fig. 3A in Chapter 4). Despite this growth improvement, it was still significantly lower than the growth observed
with 25% liquor. In addition, no significant increase in biomass and lipid productivity was observed by addition of phosphate to RMWSE. This suggested that although phosphorus might be limiting in RMWSE, other nutrient constituents such as metal nutrients and carbon might as well be limiting. Not surprisingly, addition of ammonium and inorganic fertilizer did not significantly increased algae growth or productivities.

Interestingly, increased stress as a result of addition of liquor did not trigger lipid synthesis in *C. debaryana, H. tetrachotoma* and *D. subspicatus* (Fig. 3D in Chapter 4). This can be related to a surplus of nutrient in the culture and thereby high lipid production was not triggered in the algae cell. Furthermore, I observed that oxidative stress can induce cell death (Chapter 2). Therefore it can be inferred that at elevated liquor concentration, algae might be dying when they are unable to protect their cell from oxidative damage as a result of intracellular ROS. It was obvious that *C. luteoviridis* and *P. hussii* are the best performing strains in RMWSE and their growth and productivity were optimized in RMWSE when 25% liquor is added. This study highlighted the versatility of these strain for commercial cultivation in wastewater for remediation and biofuel production.

Further experiments were carried out in Chapter 4 to examine the capability of *C. luteoviridis* and *P. hussii* for continuous cultivation in RMWSE containing 25% liquor. We observed that a mean biomass removal every 2 d of 3.57 and 3.65 g L\(^{-1}\) for *C. luteoviridis* and *P. hussii*, respectively; equivalent to a mean biomass productivity of 1.78 and 1.83 g L\(^{-1}\) d\(^{-1}\), can be achieved on a continual basis (Fig. 6A in Chapter 4). In addition, more than 70% of N and P can be removed every 2 days during continuous cultivation (Fig. 7 in Chapter 4) similar to previous studies (Park
et al., 2011; Lohrey and Kochergin, 2012). Furthermore, lipid yield of *C. luteoviridis* and *P. hussii* on the other hand was steady during the continuous cultivation (Fig. 6B and C in Chapter 4).

To understand the commercial capability of *C. luteoviridis* and *P. hussii* in wastewater for remediation and biofuel production in the UK, pilot scale monoculture pond experiments were conducted during all seasons from 2012 to 2013. *C. luteoviridis* and *P. hussii* grew effectively in the pond scale cultivation especially in the summer and spring. In addition, their biomass and lipid productivities were favoured in the summer and spring than in winter and autumn when temperature and light intensity were lower.

Specific growth rates of *C. luteoviridis* and *P. hussii* in the summer and spring for example (Fig. 8C in Chapter 4) were lower than those we observed in the laboratory batch experiment (Fig. 3A in Chapter 4). The environmental conditions in the pond cultivation were not controlled and were dictated by the prevailing weather condition unlike in the controlled laboratory experiment. Remediation capability of the strain showed that the vast majority of N and P and significant BOD reduction can be achieved all year round by cultivation of these algae in open pond system in the UK. Although, remediation were more favored in the summer and spring than in the winter and autumn (Fig. 11 in Chapter 4).

Interestingly, *C. luteoviridis* and *P. hussii* dominated the culture pond in all the cultivation durations and were not outcompeted by other biota which is normally a challenge in high rate algae ponds (Rodolfi et al., 2009; Cragg et al., 2012; Smith et al., 2010). In addition to their ability to grow fast and dominate the culture in the pond, we ensured that the starting inoculums were higher to reduce the lengthy lag
phase and prevent hijacking of the culture by grazer zooplankton like rotifers and cladocerans which can proliferate in an algal pond and impair algal growth. Fundamental factors which influenced algae growth and productivities in the pond cultivation were temperature and solar light intensity. Not surprisingly, growth characteristics and biomass productivity were highest in the summer and spring than in the winter and autumn.

Evidence from these experiments showed the potential C. luteoviridis and P. hussii for commercial cultivation in wastewater in the UK and also the resilience and capability of these algae for sustainable treatment solution and biofuel production.

5.3 Future work

Although this thesis has made good progress in answering some of the questions with regard to algae cultivation in wastewater with specific interest in remediation and biofuel production, there are still much to learn. Some of the scientific questions raise by this thesis will still require further work. Therefore, continued research is important in order to gain a more in-depth understanding of this area of research.

In this study I elucidated that oxidative stress tolerance is an important mechanism that enable algae to effectively grow in wastewater effluent, however, more study is still needed to be done to understand the regulation mechanisms of ROS scavenging and the transcriptional factors that switch on expression of antioxidants in algae. A number of experiments were carried out at the initial stage of this study which was not included in this thesis. Some of these studies include understanding phosphorus stress response regulation in Chlamydomonas reharrdii. Such principle can be
applied in the case of oxidative stress response. However, it will first require identifying possible transcriptional factor(s) that are involved in increasing expression of antioxidant enzymes as a result of oxidative stress. This can provide understanding on possible genetic target for improving algae growth in wastewater. It is also desirable to understand the relationship between lipid biosynthesis and oxidative stress induction in algae.

Although algae growth and productivity increased after acclimation in wastewater, however, it was unclear if this acclimation was as a result of increasing stress tolerance or adaptation whereby a genomic change might have occurred. A further study needs to be carried out to investigate changes that have taken place as a result of the continuous growth of the algae in RMWSE. This can be done by a complete gene sequence of the algae before and after acclimation. This will also be informative for future genetic target to improve algae growth in wastewater.

The pond experiment in this study showed that *C. luteoviridis* and *P. hussii* are capable of all year cultivation in an open pond system for bioremediation and biofuel production; however, this study did not consider the impact of other biota in the effluent because no control pond was set up. Therefore, it will be desirable to understand the interaction between algae and other biota in the pond and the contributing factor of biota to algae remediation output. One of the important aspects of algae cultivation in wastewater that still require more research and development is harvesting and processing of microalgae following cultivation. Therefore, further study needs to be carried out on recovery of algae biomass from wastewater which was not covered in scope of this thesis. Due to the duration set for this research, some studies were not covered. These include an experiment to dissect
out the effects of light level and temperature in the seasonal productivity variation in an open pond system. Also, continuous cultivation of *C. luteoviridis* and *P. hussii* all year round will be desirable.

A long term future work of this thesis will include an investigation of the potential of *C. luteoviridis* and *P. hussii* for larger pilot scale cultivation. This can be done by integrating algae cultivation in wastewater treatment plants. This will further help in bespoke evaluation of the technical feasibility of this process in wastewater industries. An evaluation study of the biomass produce from algae grown in wastewater effluent for downstream processing will be desirable.
Appendix

Award deriving from this PhD

*Nominate for UK Scopus Young Researcher Award 2013.

The award is an initiative of Elsevier and run in conjunction with the US-UK Fulbright Commission, with the aim of developing UK research talent. They recognise the achievements of young researchers and the institutes that foster them.

Peer-reviewed published and accepted papers deriving from this PhD


My activities at Sustainable Consumption Institute, Manchester

This PhD was part of the Sustainable Consumption Institute (SCI) Doctoral Training Centre, University of Manchester. Therefore, in addition to the research work, I was required to fulfill some duties and activities that are part of the formal doctoral training program. SCI is a multidisciplinary centre and carries out research on major national and international issues with regards to sustainability, climate change and encouraging consumers to adopt more sustainable lifestyles. The institute brings together global expertise, with specific research programmes at the University of Manchester as well as other leading world research centres. It is also provides a platform for the extensive doctoral training programme for postgraduate students of the next generation of researchers in their choosing area of studies in one of the research focuses of the institute. Therefore, as part of Doctoral Training Programme, in addition to the research work performed in the Faculty of Life Sciences, I devoted 20% of my time during the first year, 10% during the second year and 5% for the remaining two years to SCI activities. We met regularly every Monday at the Institute for various activities and tasks in addition to group meetings.

Certificate presentation after two weeks intensive course on sustainability and climate change
Below are just some of activities I have been involved in at the SCI doctoral training centre:

- I undertook a short course on sustainability led by 2007 Noble Prize winner and IPCC Vice-Chair Professor Mohan Munasinghe. This also included a competitive group presentation to a panel on policy stakeholders on issue related to key aspect of sustainability (certificate was issued at the end of the course).


- I also participated in an inter-disciplinary group-authored of a paper with the aim to become familiar with academic journal submission processes and standards: R. Hiteva, **O. Osundeko**, M. Sharmina, N. Truelove. Mapping Elements of University Research Lifecycle (Yet to be published).

- I assisted in delivering a lecture for undergraduate students on Environmental Sciences BSc programme.

- I also undertook a policy task which involved a team-authored policy report on a relevant sustainability topic. This task aimed at understanding the complexities of policy-making process.

- I also participated in research mapping task. In this task, I mapped my PhD in the context of global research community on algae biofuel and connected this to a reflection and assessment of the SCI research strategy and themes. I also took part in the horizon scanning task and post-doc. application. This helped
in indentifying international frontiers and to predict potential direction of algae biofuel research. In addition I also learned about writing post-doctoral proposal.

- I made oral and poster presentation at TESCO headquarter. TESCO is one of the major funders of SCI and they co-funded my PhD study with Biotechnology and Biological Sciences Research Council (BBSRC)

- I organised (with 8 others) the first Manchester Sustainable Food Festival held at Manchester Museum that attracted over 700 people including Charity organisations, NGOs, School, interest groups. This was part of public engagement task.

- Finally, I took part in the employability task which included a reflection on career and employment options. Also included simulation activities on job application and interview processes.
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OPEC, Organization of the Petroleum Exporting Countries


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