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Sustainable Biodiesel Production from Microalgae Cultivated with Piggery Wastewater

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Kieli
Englanti

Julkaisun nimike
Kestävä biodieseltuotanto sikalan jätevedellä kasvatetusta mikrolevästä

Tiivistelmä

Vastauksena energiakriisiin, globaalisen lämpenemiseen ja ilmaston muutoksen haasteisiin, mikrolevien käyttöön biodieselin tuotannossa on kiinnitetty paljon huomiota pyrittäessä kohti kestävää kehitystä. Mikrolevien käyttö biodieselin raaka-aineena sisältää monia etuja liittyen ympäröivään luontoon, ruokaturvallisuuteen ja maankäyttöön. Yhdistämällä makean veden mikrolevän *Chlorella zofingiensis* viljely ja sikaloiden jätevesien käsittely saadaan lupaava sovellutus sekä ravinteiden erottamiseen että biodieselin tuotantoon. 1900 mgL^{-1} COD:llä laimennettu sikaloiden jätevesi sisältää optimaalisen ravinnepitoisuuden levän *C. zofingiensis* viljelyyn: ravinteiden poisto jätevedestä sekä biomassan, lipidien ja biodieselin tuotto ovat edullisimmillaan. Viljelyvettä kierrätettäessä havaittiin, että ravinteiden puute saattoi lisätä lipidien kertymistä. Tällöin N- ja P-rajallisessa ympäristössä havaittiin korkein FAME tuotto 10,95 %:n kuiva-ainepitoisuudella samalla, kun viljely tuotti korkeimmat biodieseltuotot noin 20 mg L^{-1} $päivä^{-1}$. Myös havaittiin, että viljelyvesi voitiin kierrättää kaksi kertaa levän kasvattamiselle optimaalisena ravinneliuoksena.

Mahdollisuuksia levän tuotantoon eri suuruusluokissa kokeiltiin käyttäen laimennettuja sikalajätevesiä optimaalisella pitoisuudella. Kokeissa havaittiin, että NaClO:n käyttö on tehokas ja helppo tapa esikäsitellä sikalan jätevettä ilman havaittavia vaikutuksia ravinteiden poistoon ja biomassan, lipidien sekä biodieselin tuotantoon. Kontrolloimattomissa olosuhteissa ulkona *C. zofingiensis* voi kasvaa ja kerätä runsaasti biomassaa ja lipidejä. Tulosten mukaan stabiili biomassan tuotto 1,314 $gL^{-1}päivä^{-1}$ saavutetaan, kun 50 % mikrolevän viljelystä korvataan uudella jätevedellä aina 1,5 päivän jälkeen. Tämä yhdistettynä levän viljelyveden kierrätykseen johtaa johtopäätökseen, että levän *C. zofingiensis* viljely sikalan jätevedessä ravinteiden keräämiseksi ja biodieselin tuottamiseksi voidaan toteuttaa eri suuruusluokan yksiköissä.

Levän *C. zofingiensis* viljely sikalan jätevedessä biodieselin tuottamiseksi toteuttaa kestävän kehityksen periaatteita ympäristön ja erityisesti veden käytön kannalta. Kustannustehokkuutta pitäisi tulevaisuudessa parantaa esimerkiksi levän biologisia ominaisuuksia kehittämällä. Mikrolevän käyttö biodieselin tuotantoon käyttäen jätevettä on kestävää kehitystä ajatellen ilmeisen lupaavaa, mutta vain jos sen taloudellista kannattavuutta voidaan parantaa riittävästi suuren mittakaavan tuotannossa.

Asiasanat
Mikrolevä, *Chlorella zofingiensis*, sikalan jätevesi, ravinteiden erottaminen, levän viljelyveden uudelleenkierto, lipidi, biodieseltuotanto, kestävyys

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Abstract

In response to the world energy crisis, global warming and climate change, microalgal biodiesel production has received much interest in an effort to search for sustainable development. Using microalgae as a biofuel feedstock holds many advantages in relation to the environment, food security and land use. The integrated approach, which combines freshwater microalgae *C. zofingiensis* cultivation with piggery wastewater treatment, is a promising solution for nutrient removal and biodiesel production. Diluted piggery wastewater with 1900 mg L^{-1} COD provided an optimal nutrient concentration for *C. zofingiensis* cultivation, where advantageous nutrient removal and the highest productivities of biomass, lipid and biodiesel were presented. When recycling harvest water to re-cultivate *C. zofingiensis*, it was found that nutrient limitation could favor lipid accumulation. The N- and P-limited medium showed the highest FAME yield at 10.95% of dry weight, while the N-limited culture and P-limited culture shared the highest biodiesel productivity at around 20 mg L^{-1} day^{-1}. It was also shown that harvest water could be 100% recycled twice to prepare a full nutrient medium to re-grow *C. zofingiensis*.

The potential to scale up production was tested, using the diluted piggery wastewater with the optimal concentration to grow *C. zofingiensis*. It was found that using NaClO is an effective and easy way to pretreat piggery wastewater without any obvious impacts on the nutrient removal and the productivity of biomass, lipid and biodiesel. In an uncontrolled outdoor environment *C. zofingiensis* could grow well to robustly accumulate biomass and lipids. The results also indicated that the semi-continuous feeding operation, replacing 50% of microalgae culture with fresh wastewater every 1.5 days, could provide a stable net biomass productivity of 1.314 g L^{-1} day^{-1}. These findings plus the potential of harvest water recycling can lead to the conclusion that *C. zofingiensis* cultivation in piggery wastewater for nutrient removal and biodiesel production is potentially scalable.

Therefore, *C. zofingiensis* cultivation in piggery wastewater for biodiesel production can realize environmental sustainability, especially water sustainability. However, its cost-effectiveness should be further enhanced in future via methods such as algal biological property improvement. Undoubtedly, microalgae biodiesel production using wastewater is an apparently promising solution offering all-round sustainability. However, this can only be realized if the economic viability of large-scale production is improved.

Keywords

Microalgae, *Chlorella zofingiensis*, Piggery wastewater, Nutrient removal, Harvest water recycling, Lipid, Biodiesel production, Sustainability

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"*I hear and I forget. I see and I remember. I do and I understand.*"

Vaasa, November 2013

Liandong Zhu

Contents

ACKNOWLEDGEMENTS VII

1 INTRODUCTION 1
1.1 Background 1
1.1.1 Energy crisis 1
1.1.2 Global warming and climate change 3
1.1.3 Water pollution 5
1.1.4 Sustainable development 6
1.2 Towards bioenergy 8
1.3 Advantages of microalgae as the biofuel feedstock 10
1.3.1 Some agro-biofuels are unsustainable 10
1.3.2 Strengths of microalgae as a biofuel feedstock 11
1.4 Microalgae cultivation technology 12
1.4.1 Microalgae biology 12
1.4.2 Culture parameters 13
1.4.2.1 Light 14
1.4.2.2 Temperature 14
1.4.2.3 Nutrients 14
1.4.2.4 CO_2 addition and O_2 removal 15
1.4.2.5 Mixing 16
1.4.2.6 pH and salinity 16
1.4.3 Culture vessels 16
1.4.4 Biomass harvest and drying 20
1.5 Downstream processing 20
1.5.1 Microalgal lipid extraction 20
1.5.2 Microalgal biodiesel conversion 21

2 RESEARCH QUESTION AND STRUCTURE 22
2.1 Topic selection and research objective 22
2.2 Research question, sub-questions and papers 23
2.3 Interconnections of the four papers 25
2.4 Research approach and methods 27
2.5 Research process 28
2.6 Significance of this work 31

3 SUMMARY OF THE ARTICLES 33
3.1 Summary in brief and contribution 33
3.1.1 Papers' information 33
3.1.2 Contributions of this work 34
3.2 Sustainability concerns 35
3.2.1 Sustainability impact factors selection 35
3.2.2 Sustainability concerns 35
3.3 Integration of microalgae cultivation with piggery wastewater treatment 38
3.3.1 Microalgal growth 38

3.3.2 Nutrient removal....39
3.3.3 Chemical contents, and lipid and biodiesel productivity....40
3.3.4 FAME profile....41
3.4 Recycling harvest water to re-grow algae....42
3.4.1 Recycling harvest water to re-grow algae under nutrient limitation....42
3.4.2 Times for harvest water recycling....45
3.5 Scale-up potential of cultivating *C. zofingiensis*....45
3.5.1 Nutrient removal in autoclaved culture VS NaClO-pretreated culture....46
3.5.2 Indoor VS outdoor cultivation....46
3.5.3 Semi-continuous operation for algae production....49

4 DISCUSSION AND FUTURE RESEARCH....50
4.1 Technology feasibility....50
4.2 Net energy ratio (NER) analysis....52
4.3 Economics and economic improvement methods....55
4.4 Research limitation....58
4.5 Future research....59

5 CONCLUSIONS....61

REFERENCE....62

Figures

Figure 1. World total primary energy supply from 1971 to 2010 by region and fuel type (Mtoe)....1
Figure 2. Proportion of diesel vehicles among new cars in Western Europe. 2
Figure 3. World energy consumption by energy sources (a) and proved reserves of energy resources for consumption (b). 3
Figure 4. Status of CO_2 emissions in 2004 and the outlook in 2030, by country....4
Figure 5. Water quality changes during water uses in a time sequence. 6
Figure 6. Sustainability concepts and their inter-relationships....7
Figure 7. CO_2 cycle for fossil fuel and biofuels.. 8
Figure 8. Flexible biofuels production from microalgae. 10
Figure 9. Algae growth phases....13
Figure 10. Examples of open pond systems: a. Small pond for Spirulina culture, Asia; b. *Dunaliella salina* ponds of Cognis, Australia; c. Center-Pivot ponds for the culture of Chlorella in Taiwan; d. Open raceway-type culture ponds at Earthrise in California; e. Paddle wheel of a raceway pond; f-g: Raceway ponds in Foshan, China....18

Figure 11. Examples of closed cultivation systems: a. 'Big Bag' culture of microalgae; b. Bubble column reactor; c. Tubular bubble column photobioreactors; d-f. Tubular reactor system; g-h. Experimental photobioreactor; i. Lab-scale PBRs; j. Pilot-scale PBRs. 19
Figure 12. Transesterification of oil to biodiesel ($R_{1–3}$ are hydrocarbon groups). 21
Figure 13. A concept of the integration of microalgae production with wastewater treatment. 26
Figure 14. Logical relations of the four articles. 27
Figure 15. Research approach applied in this dissertation. 28
Figure 16. Life cycle process of microalgae production for biodiesel conversion. 29
Figure 17. Research process flow of this thesis. 31
Figure 18. A framework for sustainability impact analysis. 35
Figure 19. *C. zofingiensis* cultivated in piggery wastewater under six nutrient concentration levels within ten days. 39
Figure 20. FAME profile of *C. zofingiensis* grown in piggery wastewater under six nutrient concentration levels within ten days. 42
Figure 21. The growth of *C. zofingiensis* when recycling harvest water as the media with different nutrient conditions. 43
Figure 22. Growth performance changes of *C. zofingiensis* with harvest water recycling times in a full medium. 45
Figure 23. Nutrient removals by *C. zofingiensis* grown in piggery wastewater pretreated by autoclave and NaClO under indoor conditions. 46
Figure 24. Growth curves for *C. zofingiensis* grown under indoor and outdoor conditions with different pretreatments. 47
Figure 25. Lipid content and productivity of *C. zofingiensis* grown under indoor and outdoor conditions with different pretreatments. 48
Figure 26. Fatty acids methyl ester profile of *C. zofingiensis* grown under indoor and outdoor conditions with different pretreatments. 48
Figure 27. Growth curve of *C. zofingiensis* during semi-continuous operation process under indoor conditions. 49
Figure 28. Proposed scale-up scheme for *C. zofingiensis*-based biofuel production using piggery wastewater. 52
Figure 29. Cultivation of *C. zofingiensis* in pilot-scale tbcPBRs using artificial wastewater in winter in southern China. 54
Figure 30. Cultivation of *C. zofingiensis* in pilot-scale tbcPBRs using artificial wastewater in summer in southern China. 54
Figure 31. Value pyramid for algae product markets. 56
Figure 32. Biorefinery option concepts for algal biofuels and high-value products. 56
Figure 33. The cost gap between microalgal biodiesel and crude oil. 58
Figure 34. Pollution phenomena during algae production. 60
Figure 35. A potential integrated biorefinery approach 60

Tables

Table 1. Yields of bio-oils produced from a variety of crops and algae.....11
Table 2. Characteristics comparison of open ponds and photo-bioreactors. 17
Table 3. Information contained in the four papers including authors and their responsibilities. 33
Table 4. Research aims, methods, findings and contributions to the research area. 34
Table 5. Potential sustainability concerns of microalgae production for biofuel usage. 36
Table 6. Growth parameters of *C. zofingiensis* in tbcPBRs under six nutrient concentration levels within ten days. 39
Table 7. Wastewaters treatment by various microalgae reported in the literature and this work. 40
Table 8. Chemical contents of biomass, and lipid and biodiesel productivity of *C. zofingiensis* in tbcPBRs under six nutrient concentration levels within ten days.....41
Table 9. Growth parameters and lipid production of *C. zofingiensis* when recycling harvest water at a degree of 100% or 50% under different nutrient conditions. 43
Table 10. Summary of FAME profile for *C. zofingiensis* when recycling harvest water at a degree of 100% or 50% under different nutrient conditions. 44
Table 11. Growth parameters of *C. zofingiensis* in tbcPBRs under indoor and outdoor conditions with different pretreatments.....47
Table 12. NER analysis for the *C. zofingiensis*-based oil production in southern China.....53

Abbreviations

B	Boron
Ca	Calcium
CH_4	Methane
$C_6H_{12}O_6$	Glucose
CO	Carbon Monoxide
Co	Cobalt
CO_2	Carbon Dioxide
COD	Chemical Oxygen Demand
C16:0	Palmitic Acid Methyl Ester
C16:1	Palmitoleic Acid Methyl Ester
C18:0	Stearic Acid Methyl Ester
C18:2	Octadecadienoic Acid Methyl Ester
C18:3	Octadecatrienoic Acid Methyl Ester

C20:1	Eicosenoic Acid Methyl Ester
C20:2	Eicosadienoic acid Methyl Ester
C22:1	Docosenoic Acid Methyl Ester
C24:0	Tetracosanoic Acid Methyl Ester
C24:1	Tetracosenoic Acid Methyl Ester
FAME	Fatty Acids Methyl Ester
Fe	Iron
HHV	Higher Heating Value
K	Potassium
Mg	Magnesium
MJ	Megajoule
Mn	Manganese
Mo	Molybdenum
NaClO	Sodium Hypochlorite
NER	Net Energy Ratio
N_2	Nitrogen
NH_3	Ammonia
NO_3^-	Nitrate
NO_x	Nitrogen Oxides
O_2	Oxygen
P	Phosphorus
PO_4^{3-}	Phosphate
SO_x	Sulfur Oxides
SS	Suspended Solid
TAG	Triacylglycerol
tbcPBR	Tubular Bubble Column Photobioreactor
TN	Total Nitrogen
TP	Total Phosphate
Zn	Zinc

This thesis consists of five chapters and four following articles:

I Zhu, L. & Ketola, T. (2012). Microalgae production as a biofuel feedstock: risks and challenges. *International Journal of Sustainable Development & World Ecology* 19, 268–274. 73

II Zhu, L., Wang, Z., Shu, Q., Takala, J., Hiltunen, E., Feng, P. & Yuan, Z. (2013). Nutrient removal and biodiesel production by integration of freshwater algae cultivation with piggery wastewater treatment. *Water Research* 47, 4294–4302..... 81

III Zhu, L., Takala, J., Hiltunen, E. & Wang, Z. (2013). Recycling harvest water to cultivate *Chlorella zofingiensis* under nutrient limitation for biodiesel production. *Bioresource Technology* 144, 14–20. 91

IV Zhu, L., Wang, Z., Takala, J., Hiltunen, E., Qin, L., Xu, Z., Qin, X. & Yuan, Z. (2013). Scale-up potential of cultivating *Chlorella zofingiensis* in piggery wastewater for biodiesel production. *Bioresource Technology* 137, 318–325. 99

1 INTRODUCTION

1.1 Background

1.1.1 Energy crisis

The world population has grown from 2 billion during the Second World War to 7 billion in the 21st century (Avni and Blazquez 2011). There is no denying the fact that energy is one of the most important basic elements for the development and even the survival of human society. Energy is of significant importance to both economic and social development (Zhang et al. 2011). Currently, the world is witnessing increasing energy supply along with the development of the world's economy through industrialization, urbanization and modernization (Figure 1). All of the net growth has occurred in developing countries with emerging economies, like the BRICS countries (Brazil, Russia, India, China and South Africa). For instance, China alone has accounted for 71% of global energy consumption growth, while energy consumption by OECD (Organization for Economic Co-operation and Development) has declined, led by a sharp decline in Japan (BP 2012).

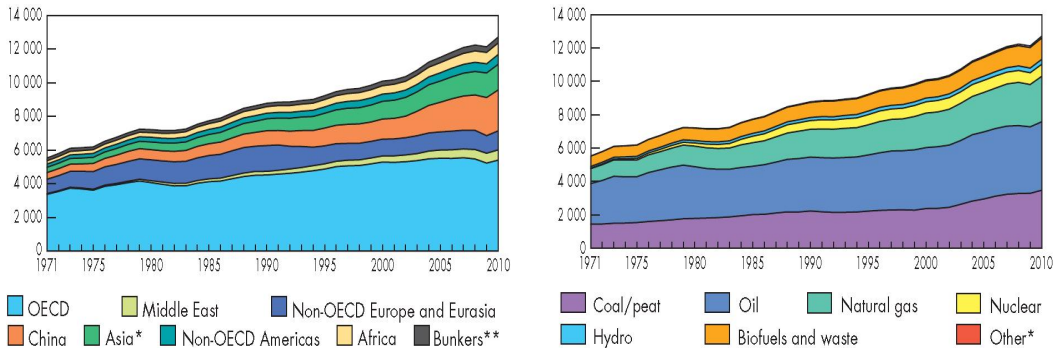

Figure 1. World total primary energy supply from 1971 to 2010 by region and fuel type (Mtoe) (IEA 2012a). Mtoe means million tons of oil equivalents. Asia excludes China. Bunkers include international aviation and marine bunkers. Other includes geothermal, solar, wind, heat, etc.

In the current traditional primary energy consumption structure, fossil fuels account for 88.1% with a dominant role, where crude oil consists of 34.8%, coal 29.2% and natural gas 24.1%, while the shares of hydroelectricity and nuclear energy are very small, only 6.4% and 5.5%, respectively (Ong et al. 2011). Glob-

ally there has also been growing demand for diesel, which is derived from crude oil during refining. Gasoline powered cars have the dominant market share in the United States, where the share of diesel cars in new car sales was a mere 2.68% from 2010 onwards (Fosten 2012). However, in European countries, especially Austria, Spain, France and Italy (Eichlseder and Wimmer 2003), as shown in Figure 2, the market share of diesel-based cars has exceeded 50% since 2006. The increase in the number of diesel vehicles will lead to an increase of demand for fossil diesel, if alternative energy cannot be developed and put into practice. By the end of 2009 diesel had captured over 55% of the new vehicle market in Europe (Schipper and Fulton 2013).

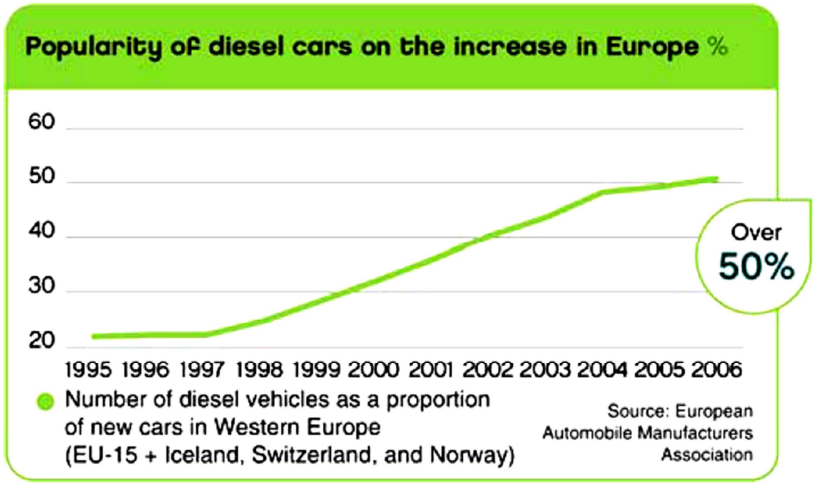

Figure 2. Proportion of diesel vehicles among new cars in Western Europe (Neste Oil 2006).

Total energy consumption will increase year by year from now on, as a result of significant population and economic growth in the developing countries, especially in China and India. According to the estimation of the International Energy Agency (IEA), global energy consumption will witness a 53% increase by 2030 (Ong et al. 2011). Figure 3(a) shows past and future consumption of various energy sources from 1970 to 2030, as evaluated by the IEA (IEA 2006; Saito 2010). Comparing the forecasted energy demand and available resources of crude oil, it is undeniable that future energy demand cannot solely be met by fossil fuels. In 2004, the quantity of accessible crude oil resources was estimated to be about 171.1 billion tons. Based on the current consumption of about 11.6 million tons of crude oil per day, it is expected that the entire available resources will suffice only for a fairly short time period (Shafiee and Topal 2009; Vasudevan and Briggs 2008). Analyzing global oil depletion, the UK Energy Research Centre even concluded that a peak of conventional oil production will be reached between 2020 and 2030, when readily-available resources will be used up (Sorrell et al. 2009).

According to Ong et al. (2011), the global proven reserves for crude oil and natural gas are estimated to last for 41.8 and 60.3 years, respectively, based on the current production rates. Saito (2010) estimates the duration of fossil resources consumption on the basis of the reserves available at the end of 2006, as shown in Figure 3(b). He suggests that the total energy consumption of developing countries will exceed that of the developed countries in 2030, and will continue to increase dramatically. It is therefore questionable whether there will be enough fossil fuels for human beings to consume in the future.

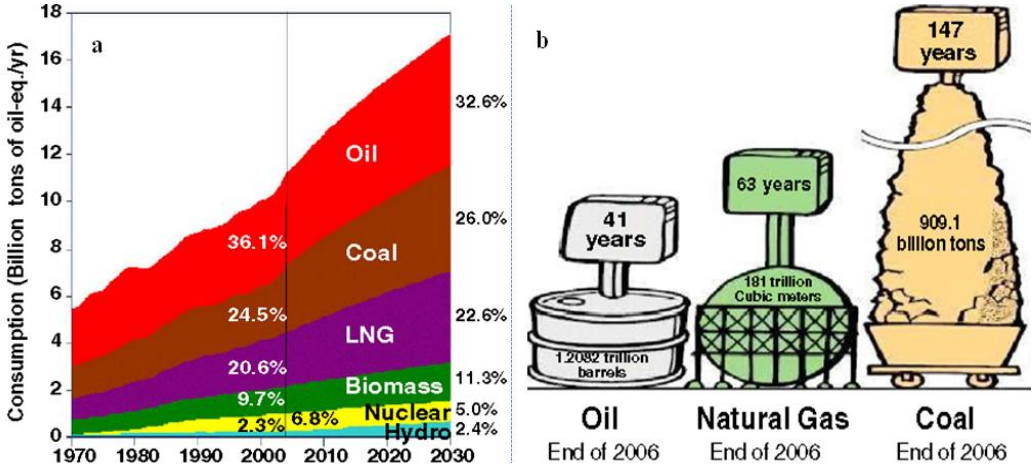

Figure 3. World energy consumption by energy sources (a) and proved reserves of energy resources for consumption (b) (Saito 2010).

1.1.2 Global warming and climate change

Although the world is going through an energy crisis in terms of depletion of resources, it is true that new oil and gas reserves have constantly been found. Most exciting have been the new geological surveys that show that as much as a fifth of the world's exploitable gas and oil reserves lie under the Arctic ice (McCarthy 2008). Potential oil and gas refining will therefore increase fossil fuel reserves, thus risking an exponential increase in the greenhouse effect, which could result in all kinds of catastrophes for the Earth and its inhabitants. Natural disasters linked to global warming can cause tremendous damage to local areas in terms of the economy, people's health and safety, and transportation (Cai et al. 2012).

CO_2 emissions have clearly increased in the last 35–40 years, and the total amount of CO_2 emissions related to the burning of fossil fuels has reached about 26 billion tons (Saito 2010). The statistical data show that at present CO_2 concentration in the atmosphere is about 380 ppm, compared to 280 ppm before the Industrial Revolution. Figure 4 shows CO_2 emissions in 2004 and the estimated

emissions in 2030 for different countries. The total global CO_2 emissions in 2030 will be 1.6 times higher than in 2004. One evident problem is the high number of on-road diesel vehicles, since emissions from these engines significantly contribute to the atmospheric levels of the most important greenhouse gas, CO_2, as well as other urban pollutants such as CO, NO_x, unburned hydrocarbons, particulate matters, and aromatics (Kalam et al. 2003). The use of conventional fossil fuels can cause fast-rising CO_2 emissions (Krumdieck et al. 2008; Roman-Leshkov et al. 2007), and with the ever-increasing pace of modern industrialized development this trend will continue if a feasible alternative energy source cannot be found in time.

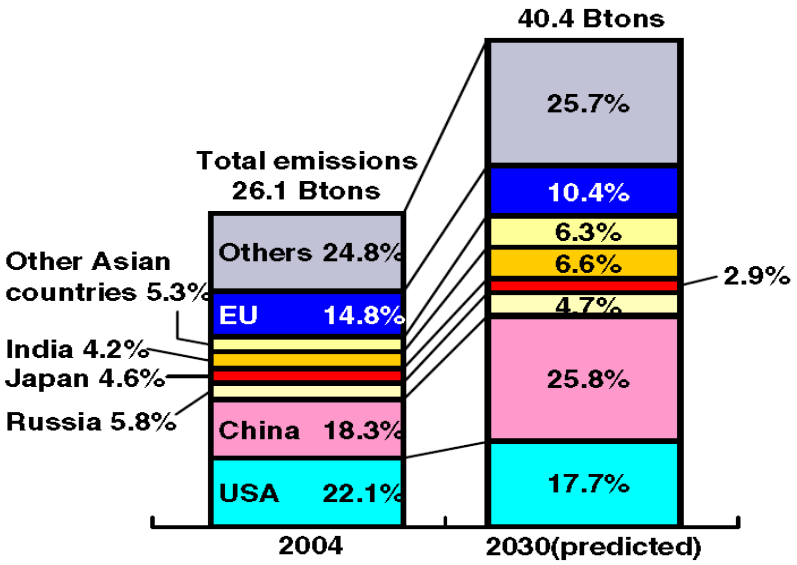

Figure 4. Status of CO_2 emissions in 2004 and the outlook in 2030, by country (Saito 2010).

The Inter-governmental Panel on Climate Change (IPCC) has demonstrated that a temperature increase of 2°C above preindustrial levels will dramatically increase the risk of severe climate change impacts (EPA 2006). In the EU-15 member countries transport-related greenhouse gases (GHG) emissions accounted for 21% of the total EU-15 GHG emissions in 2008, an increase of 20% from 1990 (EEA 2011). Several studies have shown that the two-degree limit for temperature rise will be broken during the next couple of decades if GHG emissions continue to intensify (EPA 2006; MTC 2008). If international efforts can achieve effective international agreements and GHG emissions can be decreased at least by half by 2050 in an attempt to mitigate climate change, the temperature rise can be kept at 2°C (MTC 2008). These tasks are challenging and require mutual action internationally to mitigate GHG emissions.

1.1.3 Water pollution

Water pollution is a major global problem. There are three main pollution sources: agriculture, industry, and municipalities. Agricultural wastewater is the biggest polluter, since agriculture accounts for more than 70% of global water use (GEO 2007). A large amount of fertilizers and pesticides is used during agricultural production, and these can cause the contamination of groundwater and surface waters through run-off. Animal wastes are another contributor of pollution in some areas. Industrial wastewater contains a lot of inorganic and organic matters, as well as heavy metals such as lead, mercury, and cadmium. Municipal wastewater is a representative organic wastewater, which contains a lot of organic matters and organisms like bacteria.

Once the wastewater flows into a waterbody without treatment, it can cause disasters for the respective ecosystems. Nutrient imbalance in water can give rise to eutrophication, threatening the development and stability of biodiversity. In developing countries, up to 90 % of wastewater flows into rivers, lakes and seas without any treatment, threatening people's health and food security and affecting access to safe and clean water for drinking and bathing (WWAP 2012). It is estimated that 700 million Indians have no access to a proper toilet (The Economist 2008). Around 90% of cities in China suffer from some degree of pollution by wastewater, and nearly 500 million Chinese in total cannot access clean drinking water (The New York Times 2007). Fully industrialized countries continue to struggle with water pollution problems as well. For example, in the USA 45% of assessed streams and 47% of assessed lakes are classified as polluted waterbodies, according to a national report on water quality in the United States (EPA 2007). Another example is that 97% of groundwater samples in France do not meet standards for nitrates (UN WWAP 2009).

It has been suggested that water pollution is the leading worldwide contributor to deaths and diseases and that it directly or indirectly deprives the lives of more than 14,000 people daily (West 2012). For instance, 1,000 Indian children die of sickness every day due to dirty water (The Economist 2008). Wastewater must be treated before discharge, according to the quality requirement of the usage (Figure 5).

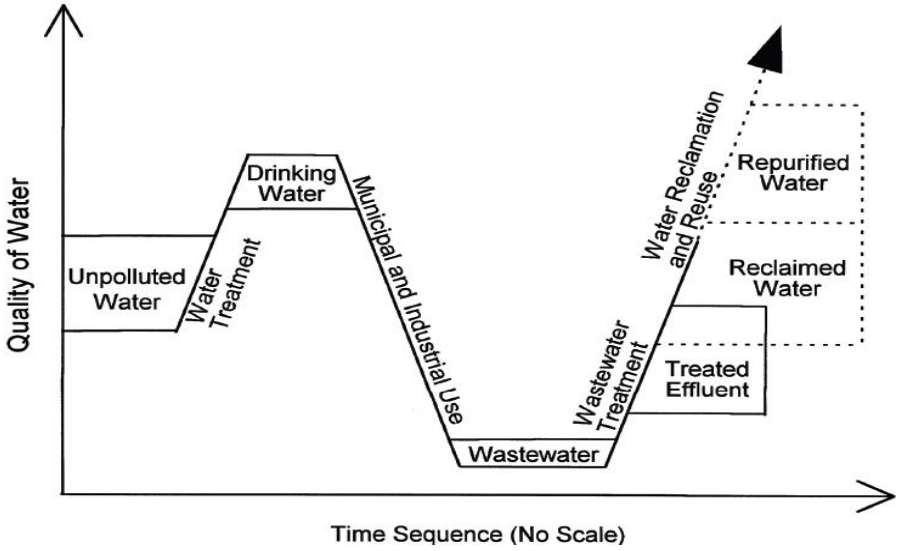

Figure 5. Water quality changes during water uses in a time sequence (Asano and Bahri 2009).

There are many methods that have been developed to treat wastewater. These include activated sludge treatment methods, constructed wetlands, artificial floating beds (Zhu et al. 2011a), and others. Currently, there is a lot of on-going research on the treatment of industrial, municipal and agricultural wastewaters by microalgae culture systems (Yang et al. 2008; Zhang et al. 2012; Samori et al. 2013). When cultivating *Arthrospira platensis* in olive-oil mill wastewater it has been found that the maximum removal of chemical oxygen demand (COD) was 73.18%, while phenols, phosphorus and nitrates in some runs were completely removed (Markou et al. 2012). Ruiz-Marin et al. (2010) compared two species of microalgae growing as immobilized and free-cells to test their abilities to remove total nitrogen (TN) and total phosphate (TP) in batch cultures with urban wastewater. Kothari et al. (2012) found that *Chlorella pyrenoidosa* could remove about 80–85% TP and 60–80% of TN from dairy wastewater.

1.1.4 Sustainable development

Recently, more and more concerns have been expressed regarding sustainable development. Sustainable development refers to a mode of human development where an activity can meet the needs of the present generation in an environmentally friendly manner while maintaining options for future generations (Bruntland 1987). The concept of sustainable development can be divided into four parts: environmental sustainability, economic sustainability, social sustainability, and cultural sustainability (Figure 6).

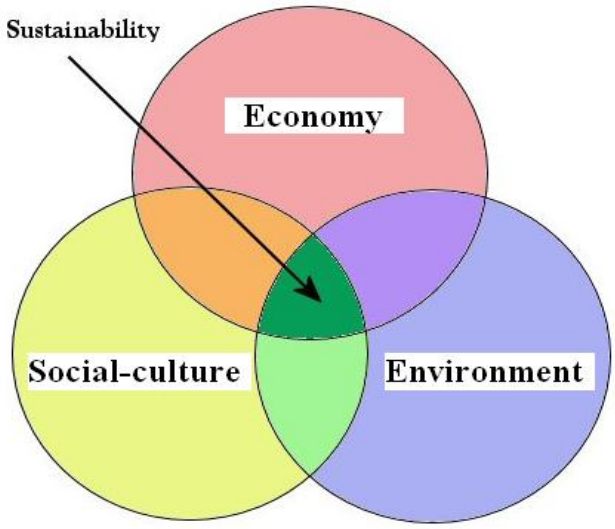

Figure 6. Sustainability concepts and their inter-relationships.

Today more than ever before, unpredictable environmental issues strongly bound with economic, social and cultural impacts are dominating the international agenda, and much importance has been attached in particular to the sustainability of industry. Identifying the core environmental, economic, social and cultural impacts is the first step in supporting the development of a sustainable industry. Unsustainable aspects can be identified using the techniques of risk assessments (Gupta et al. 2002) and environmental impact assessments (Salvador et al. 2000). Potential risks can thus be forecast and then either mitigated or eliminated to some degree.

It is a long-term goal to achieve sustainable economic development along with sustainability of energy. Many significant problems lie in energy production and consumption, such as shortage of resources, low energy efficiency, high emissions, damage to environment, and lack of effective management systems (Zhang et al. 2011). As an example of the scale of the challenge, from 1990 to 2006 China observed an increase of nearly 6% annually in CO_2 emissions, ending up with 5.65 billion tons CO_2 in 2006, accounting for 20.3% of the global amount (Jiang et al. 2010). Therefore, it is a long journey for developing countries to optimize energy structures, improve energy efficiency, enhance environmental protection, and carry out efficient energy management in pursuit of sustainable development.

Biofuels have become a hot research topic due to their advantages over fossil fuels (Figure 7). The desire to reduce reliance on foreign oil imports, to improve energy security and to reduce the effects of global warming and climate change has sparked a lot of interest in terms of research and development (R&D) of alter-

native fuels (Coplin 2012). Policymakers, academics, business representatives, and members of relevant associations are pushing development of biofuels for various reasons. Some think of biofuels as a substitute for high-priced petroleum, while others emphasize their potential to extend available energy resources to confront the increasing world demand for fuels in the transportation sector. Others see biofuels as a substitute for carbon-neutral energy or as an economic opportunity for business. Nonetheless, there are still some skeptical voices arguing that not all biofuel types are sustainable. Many of the biofuels which are currently being supplied have been criticized on the basis of potential adverse effects on the natural environment, food security and land use.

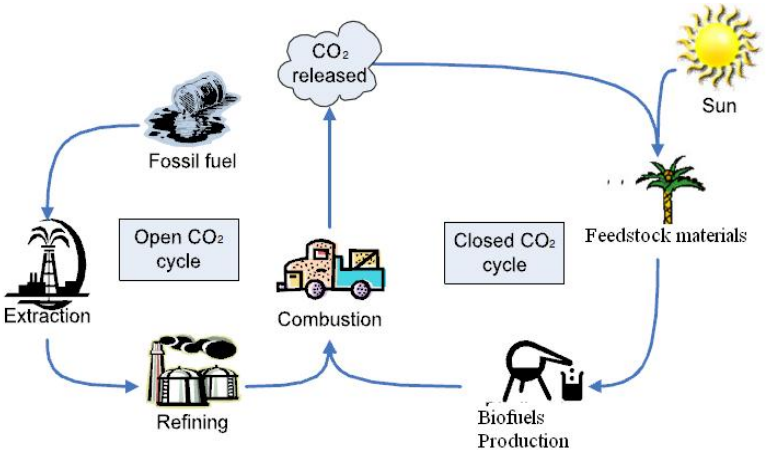

Figure 7. CO_2 cycle for fossil fuel and biofuels. Modified from Ng et al. (2009).

1.2 Towards bioenergy

In response to the challenges outlined above, renewable energies have received a lot of attention and will hopefully become one of the main energy sources for the world. According to calculations, renewable energy in 2010 covered only 13% of the global primary energy demand (IEA 2012b).

Bioenergy is thought of as the renewable energy with the highest potential to satisfy the energy needs of modern society for both developed and developing countries (Ong et al. 2011). At present, bioenergy contributes around 10–15% of the world energy use (Demirbas et al. 2009). Biofuels, mainly in the form of biodiesel, bioethanol, biogas and biohydrogen, have therefore received increasing attention (Antoni et al. 2007; Johnson and Wen 2010).

Biodiesel, which is usually produced from either animal fat or oil crops, such as soybean, corn, rapeseed, palm, and castor bean, is a non-toxic, renewable and biodegradable fuel, and thus one of the potential alternatives to fossil fuel. Nevertheless, this feedstock has low oil yield and entails high demand for land, water and fertilizer.

Bioethanol is considered to have the potential to replace the fossil-derived petrol (Prasad et al. 2007). Bioethanol is produced via fermentation using a variety of sugars, which are derived through hydrolyzing starch from, for instance, corn, sugarcane and sorghum. Bioethanol from lignocellulosic feedstock is also being developed (Dwivedi et al. 2009). Lignocellulosic feedstock includes woody sources such as aspen, energy crops such as switchgrass, agricultural wastes such as corn stover (Huang et al. 2009), as well as dairy and cattle manures involved in a few studies (Chen et al. 2004).

Biogas generation is widely used for the treatment of all kinds of wastes (Pham et al. 2006). Biomass used for anaerobic digestion can be obtained from (1) terrestrial sources including mechanically sorted and hand-sorted municipal solid wastes, various types of fruit and vegetable solid wastes, leaves, grass, wood and weed, and (2) aquatic sources including both marine and freshwater biomass, such as seaweed and sea-grass (Zamalloa et al. 2012).

Biohydrogen is a clean biofuel type, since it can be used in a fuel cell with water as the only exhaust product and without any pollutant emissions. Large-scale electrolysis of water is possible, but costs more energy than can be generated by hydrogen. However, several bacteria, such as purple non-sulfur bacteria (Lee et al. 2002; Bianchi et al. 2010), can use a wider range of organic substrates (such as food wastes, agricultural residues and wastewaters) and light to produce hydrogen.

Microalgal biofuels have received a great deal of attention. Algae, which can absorb CO_2 photo-autotrophically, are ideal candidates for CO_2 sequestration and greenhouse gas mitigation during algae-based biofuels production. Microalgae have been found to have several constituents, mainly including lipids (7–23%), carbohydrates (5–23%), proteins (6–52%), and some fat (Brown et al. 1997). Previous studies have shown that microalgae are consequently a versatile feedstock for the production of biofuels including biodiesel, bioethanol, biogas, biohydrogen, and many other fuel types like biobutanol, bio-oil, syngas, and jet fuel (Li et al. 2008; Koller et al. 2012) via thermochemical and biochemical methods (Figure 8).

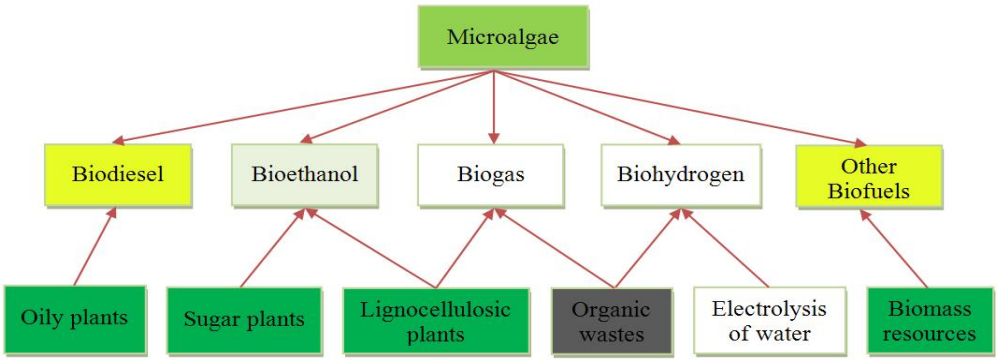

Figure 8. Flexible biofuels production from microalgae (Zhu et al. 2012).

1.3 Advantages of microalgae as the biofuel feedstock

1.3.1 Some agro-biofuels are unsustainable

The feedstock used for biofuel production mainly includes the following materials: straw, sugarcane, wood materials, wood wastes, manure, energy plants, and many other co-products or byproducts from a wide range of agricultural processes (Zhu et al. 2012). According to the feedstock differences, biofuels can be classified into three types: the first generation, the second generation and the third generation. Biodiesel and bioethanol are the most popular types of first- or second-generation biofuels. Biodiesel is made from, for example, canola and palm, and bio-ethanol from crops such as sugarcane and corn starch. It is believed that biofuels production can bring job opportunities and increase farmers' incomes, especially in developing countries. Meanwhile, it can also reduce a country's reliance on crude oil imports (Zhu et al. 2012). As such, biofuels production is of strategic importance to the future development of our society.

Nevertheless, biofuels, which are derived from food or non-food crops, are not thought of as renewable and sustainable energy types. The growth of these non-food crops targeted for biofuels production will lead to competition for arable farmland with food crops. Farms are limited and should be used to grow food crops. If the food crops grown in farmlands are used to produce biofuels, it will affect food security, and food prices will increase rapidly, subsequently impacting the access of poor populations to food (von Braun et al. 2008). Microalgal biofuels can deal with most of the concerns connected to first- and second-generation biofuels, and are thus referred to as third-generation biofuels. Microalgal biofuels are currently attracting a lot of research attention (Lam and Lee 2012).

1.3.2 Strengths of microalgae as a biofuel feedstock

There is no denying the fact that there exist some disadvantages to employing microalgae as the biofuel feedstock, for instance, the expensive nature of start-up and harvest and drying (more detailed information is exhibited in Table 5 in Chapter 3). However, several obvious advantages have been identified.

First, microalgae can grow very fast and have a high photosynthesis rate. Compared to all the terrestrial crops investigated until now, one unit of growing area of microalgae can produce much more biomass and oil, as shown in Table 1. It is expected that 50 times more biomass can be produced from microalgae than that from switchgrass, which is the fastest growing terrestrial crop (Demirbas 2006). The doubling time for microalgal biomass during the exponential growth phase can be as short as 3.5 h (Chisti 2007), and up to 20–22 g dry weight·m^{-2}·day^{-1} of average productivity has been achieved in raceway ponds.

Table 1. Yields of bio-oils produced from a variety of crops and algae (Avagyan 2008).

Substance	Gallons of oil per acre per year
Corn	15
Cotton	35
Soybeans	48
Mustard seed	61
Sunflower	102
Rapeseed (canola)	127
Jatropha	202
Oil palm	635
Microalgae	
Based on actual biomass yields;	1,850
Theoretical laboratory yields	5,000–15,000

Second, less freshwater is required to grow microalgae than for land crops. In addition, water used in the process can be largely recycled for the algal biomass production system.

Third, the algal biofuel industry has low land occupancy. Unproductive land, such as arid or semiarid areas, infertile farms, saline soils, polluted land, and other land with low economic value (e.g. deserts) can be used to establish microalgae growth and biofuel refineries. The advantage here is that the biofuels do not compete with food crops for farmland.

Fourth, all kinds of wastewaters, such as municipal, agricultural and industrial wastewaters, can be utilized to culture microalgae. The wastewater provides nutrients to form algal biomass; thus, wastewater can be purified mainly by algal cell uptake, physical processes and microbial activity (Zhu et al. 2011a; Zhu et al. 2013). This provides a new measure for wastewater treatment, since the inorganic and organic matters in wastewaters can also be degraded.

Fifth, microalgae systems can be filled with flue gas (rich in CO_2) as a carbon source, since some species can tolerate CO_2, NO_x, SO_x, dust, and other elements in flue gas (Imhoff et al. 2011).

Sixth, the methods to harvest and pretreat microalgae are easy, although the costs are high, thus accelerating the biofuel production process in practice.

Seventh, many value-added chemicals, like protein and glycerol, can be co-produced during the biofuel production process (Nilles 2005). For example, more than 400,000 tons of glycerol could be simultaneously co-produced when 1 billion gallons of algal biodiesel are produced (Oswald 1988).

Eighth, engineering tools can be applied to microalgae. The genes of the algal cells can be modified and mutated via a certain technological method or by changing growth conditions. This can significantly increase the biomass quantity and algal oil content.

Ninth, several biofuel types, mainly in biodiesel, bioethanol and biogas, can be produced from microalgae. Thus, microalgae are versatile.

Finally, the physical and fuel properties (e.g. density, viscosity, acid value, heating value, etc.) of biodiesel from microalgal oil are generally comparable to those of fossil fuel diesel.

1.4 Microalgae cultivation technology

1.4.1 Microalgae biology

Microalgae, which grow in aquatic environments, are simple microscopic heterotrophic and/or autotrophic photosynthetic organisms, ranging from unicellular to multi-cellular in form. In contrast to aquatic plants, microalgae do not have real embryos, roots, stems or leaves. They are able to use water, sunlight, and CO_2 to synthesize biomass through photosynthesis (Ozkurt 2009). The synthetic biomass can then further be converted into biodiesel, fertilizer and other useful products. More than 40,000 different species of microalgae have been identified (Fuentes-Grunewald et al. 2009), and most of them have a high content of lipids, account-

ing for between 20 and 50% of their total biomass (Chisti 2007). The overall reaction process can be summarized as follows:

$$6CO_2 + 12H_2O + \text{photons} = C_6H_{12}O_6 + 6O_2 + 6H_2O$$

Apart from sunlight and CO_2, water, nitrogen and phosphorus are the three major inputs for algae growth. Major nutrients such as N and P alone contribute to about 10–20% of algae biomass (Benemann 1996). As well as macro-ingredients including N, P, Mg, Na, Ca, and K, micro-ingredients like Mo, Mn, B, Co, Fe and Zn are also required. In general, the growth of microalgae goes through four phases (Figure 9): lag phase, exponential phase, stationary phase and lysis phase.

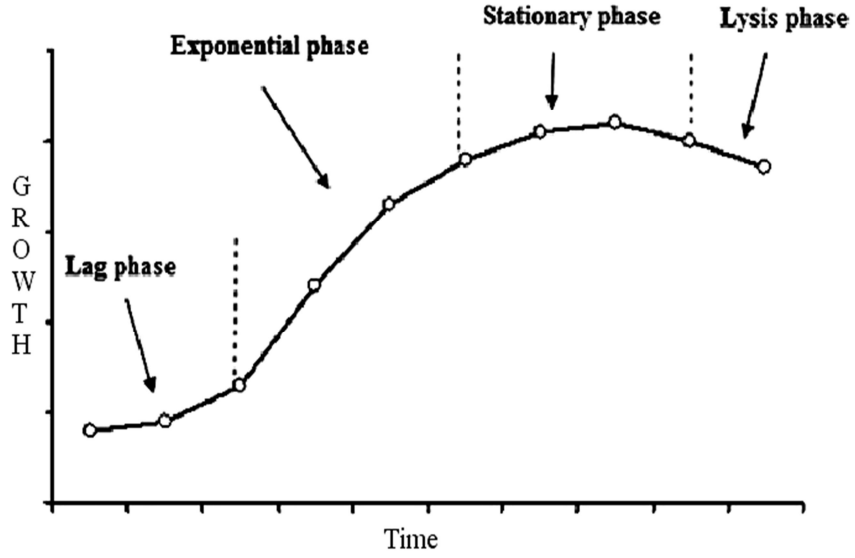

Figure 9. Algae growth phases (Moazami et al. 2012).

1.4.2 Culture parameters

Specific environmental conditions, which vary between microalgae species, are required in order to successfully cultivate microalgae. Factors that influence microalgae growth include (Mata et al. 2010): abiotic factors such as light intensity, temperature, O_2, CO_2, pH, salinity, nutrients (N, P, K, etc.) and toxins; biotic factors such as bacteria, fungi, viruses, and competition for abiotic matters with other microalgae species; operational factors such as mixing and stirring degree, width and depth, dilution rate, harvest frequency, and addition of bicarbonate. The generally most important factors are described in the following sections.

1.4.2.1 Light

Light is the energy input source for the photosynthesis of microalgae. Light accessibility and intensity is one of the key parameters impacting the growth performance of microalgae culture. When the light intensity is at a fairly low level, for instance, below the compensation point, there is no net growth (Long et al. 1994; Alabi et al. 2009; Ye et al. 2012). After the compensation point, as the light intensity increases, the growth can increase until the light saturation point where the photosynthesis rate is the maximum. After this point, no increase in growth rate will appear when increasing the light intensity, since it will cause photoinhibition (Henley 1993; Ye et al. 2012).

When the microalgae culture concentration is low, every microalgal cell can capture the light. The microalgal cells might lack self-shading, which could cause photoinhibition (Alabi et al. 2009). To avoid this, the light intensity should not be too high. When the microalgae culture concentration is high, it is not possible for the light to penetrate deeply into the culture; also, only the top layer can absorb the available light, leaving the rest in the dark – this is called over-shading. The top layer might face light saturation and inhibition, since most microalgae reach light saturation at around 20% of solar light intensity (Pulz 2001; Torzillo 2003). Proper mixing is one solution to these issues, allowing the cells to move around, thus efficiently increasing photosynthesis.

1.4.2.2 Temperature

Temperature is another key limiting factor, especially for outdoor cultivation systems. Generally, microalgal growth increases exponentially as temperature increase to an optimal level, after which the growth rate declines. Temperatures below the optimal range and above freezing will not kill microalgae, and many microalgae can easily tolerate temperatures up to 15°C lower than their optimal (Mata et al. 2010). Keeping cultures at temperatures above the optimal will result in total culture loss (Alabi et al. 2009). Generally, temperature must remain within 20 to 30°C to achieve ideal growth (Chisti 2007). In outdoor systems, overheating issues might occur, and thus water-cooling systems should be considered to make sure the temperature will not exceed the optimal range.

1.4.2.3 Nutrients

Generally, the composition of microalgae is $CH_{1.7}O_{0.4}N_{0.15}P_{0.0094}$ (Oswald 1988). Thus, the macronutrients should contain nitrogen and phosphorus (silicon is also required for saltwater algae). In addition, trace metals, such as, Fe, Mg, Mn, B, Mo, K, Co and Zn, are also needed. The nutrients used can be supplied in the

form of simple, easily available agricultural fertilizers. However, significant costs will be incurred here.

Several studies have reported that N or P deficiency or limitation during microalgae cultivation can improve the lipid accumulation and transformation for most species (Khozin-Goldberg and Cohen 2006; Hu et al. 2008; Devi and Mohan 2012; Feng et al. 2012). In practice, microalgae are cultured in full media with enough nutrients in the early stages, while in later stages nutrient deficiency or limitation needs to be designed to improve the lipid content. Ito et al. (2012) found that nitrogen deficiency conditions could cause a decrease in amino acids in algal cells to 1/20 the amount or less, while the quantities of neutral lipids increased greatly. Devi and Mohan (2012) suggested that the stored carbohydrates from the growth phase might channel towards the formation of triacylglycerides (TAGs), leading to efficient composition for biodiesel production.

Recently most kinds of wastewater have been tested for microalgae cultivation. The N and P removal mainly results from the uptake of microalgal cells during growth (Su et al. 2011). Moreover, microorganisms (if existing in microalgae culture) can also contribute to the nutrient degradability. Ammonia (NH_4^+), nitrate and nitrite can be degraded via nitrification (Eq. (1)) and denitrification (Eq. (2)) by some special bacteria (Zhu et al. 2011a), as shown in the following equations (Eq. (1) plus Eq. (2) is equal to Eq. (3)). Inorganic nitrogen in the form of nitrate after nitrification can be absorbed by algal cells or continues to be degraded into gas nitrogen. For phosphorus reduction, physical and chemical reactions such as absorption, ion exchange and sedimentation or precipitation play a very important role (Ruiz-Marin et al. 2010). Phosphate can also be degraded to some degree through microbial activities (Kim et al. 2005; Oehmen et al. 2007). In addition, if the pH of microalgae culture increases, it will also contribute to the P removal via P precipitation (Ruiz-Marin et al. 2010). Metal ions such as calcium, aluminum and iron can react with phosphate and settle down. For example, Eq. (4) shows that phosphate reacts with ionic calcium and is removed as a solid.

$$NH_4^+ + 2O_2 + 2HCO_3^- \rightarrow NO_3^- + 3H_2O + 2CO_2 \quad (1)$$

$$5C + 4NO_3^- + 2H_2O \rightarrow CO_2 + 4HCO_3^- + 2N_2 \quad (2)$$

$$4NH_4^+ + 8O_2 + 5C + 4HCO_3 \rightarrow 2N_2 + 10H_2O + 9CO_2 \quad (3)$$

$$10\ Ca^{2+} + 6\ PO_4^{3-} + 2\ OH^- \leftrightarrow Ca_{10}(PO_4)^*6(OH)_2 \downarrow \quad (4)$$

1.4.2.4 CO_2 addition and O_2 removal

The microalgal biomass contains a high proportion of carbon, around 45–50% (Alabi et al. 2009). CO_2, plus acetic acid, sugar, etc., is the carbon source for pho-

tosynthesis. Algal growth limitation might occur if algal culture is supplied only from air, which only contains 0.033% CO_2. Extra CO_2 can be blended with air and injected into algae cultures via gas addition facilities (Mata et al. 2010). CO_2 is expensive, so the use of it can increase the costs. In practice, air can be introduced into a deep level underwater via air stones to improve the efficiency of CO_2. Another method is to introduce CO_2-rich industrial flue gas into the cultures.

During photosynthesis, CO_2 is used and O_2 is generated. If O_2 cannot be emitted into the air and its concentration exceeds saturation, it will cause photo-oxidative damage to chlorophyll reaction centers, thus inhibiting the process of photosynthesis and reducing biomass productivity (Alabi et al. 2009). In open algae systems, this phenomenon will not happen, since there is an interface between atmosphere and medium and O_2 can be emitted easily and freely. Nonetheless, as to the closed systems such as closed PBRs, additional facilities such as gas exchangers are required (Mata et al. 2010).

1.4.2.5 Mixing

As already discussed in Section 3.2.1, when the culture concentration is high, the light cannot penetrate, thus reducing biomass productivity. Therefore, mixing is necessary to make sure all algal cells are suspended with identical access to light. Mixing is also useful to mix nutrients and help the cells' uptake of these nutrients. Additionally, mixing can also make gas exchange more efficient.

1.4.2.6 pH and salinity

Usually, suitable pH value for algae culture is 6–8 (Zeng et al. 2011). However, different sources of media have different pH values. Also, influenced by CO_2, the pH values are changeable during cultivation. However, algae species seem to be more tolerant of the broad range of pH values. Lam and Lee (2012) cultivated *Chlorella vulgaris* in media with pH values of 3, 4, 5, 6, 7, 8 and 9, and came to the conclusion that there was no great difference in the growth characteristics of the algae. Of course, the tolerance ability of is species-dependent.

Due to evaporation, salinity might increase during algae production. Too high a degree of salinity is harmful for algae cells since it might change their shape and structure due to the water pressure between media and cells (Mata et al. 2010).

1.4.3 Culture vessels

Microalgae can be manually cultivated. In total, it has been found that more than 50,000 microalgae species exist; only about 30,000 species, however, have been

studied and analyzed until now (Richmond 2004). From a technological point of view the most practical and mature way to cultivate microalgae is to use ponds and photobioreactors (Chisti 2007). The main differences between open ponds and photobioreactors are summarized in Table 2. Figure 10 and 11 exhibit some typical prototypes of open ponds and photobioreactors.

Table 2. Characteristics comparison of open ponds and photobioreactors (Zhu et al. 2011b).

Parameter	Open pond	Photobioreactor
Land requirement	High	Variable
Water loss	Very high, may also cause salt precipitation	Low, and may be high if water spray is used for cooling
Hydrodynamic stress on algae	Very low	Low-High
Gas transfer control	Low	High
CO_2 loss	High, depending on pond depth	Low
O_2 inhibition	Usually low enough because of continuous spontaneous outgassing	High (O_2 must be removed to prevent photosynthesis inhibition)
Temperature	Highly variable	Cooling often required
Startup	6–8 weeks	2–4 weeks
Construction costs	High – US $ 100,000 per hectare	Very high – US $ 1,000,000 per hectare: PBR plus supporting systems
Operating costs	Low – paddle wheel, CO_2 addition	Very high – CO_2 addition, Ph-control, oxygen removal, cooling, cleaning, maintenance
Limiting factor for growth	Light	Light
Control over parameters	Low	High
Technology base	Readily available	Under development
Risk of pollution	High	Low
Pollution control	Difficult	Easy
Species control	Difficult	Easy
Weather dependence	High – light intensity, temperature, rainfall	Medium – light intensity, cooling required
Maintenance	Easy	Hard
Ease of cleaning	Easy	Hard
Susceptibility to overheating	Low	High
Susceptibility to excessive O_2 levels	Low	High
Cell density in culture	Low – between 0.1 and 0.5 g l^{-1}	High – between 2 and 8 g l^{-1}
Surface area-to-volume ratio	High	Very high
Applicability to variable species	Low	High
Ease of scale-up	High	Variable (bubble column and tubular PBRs are easy)

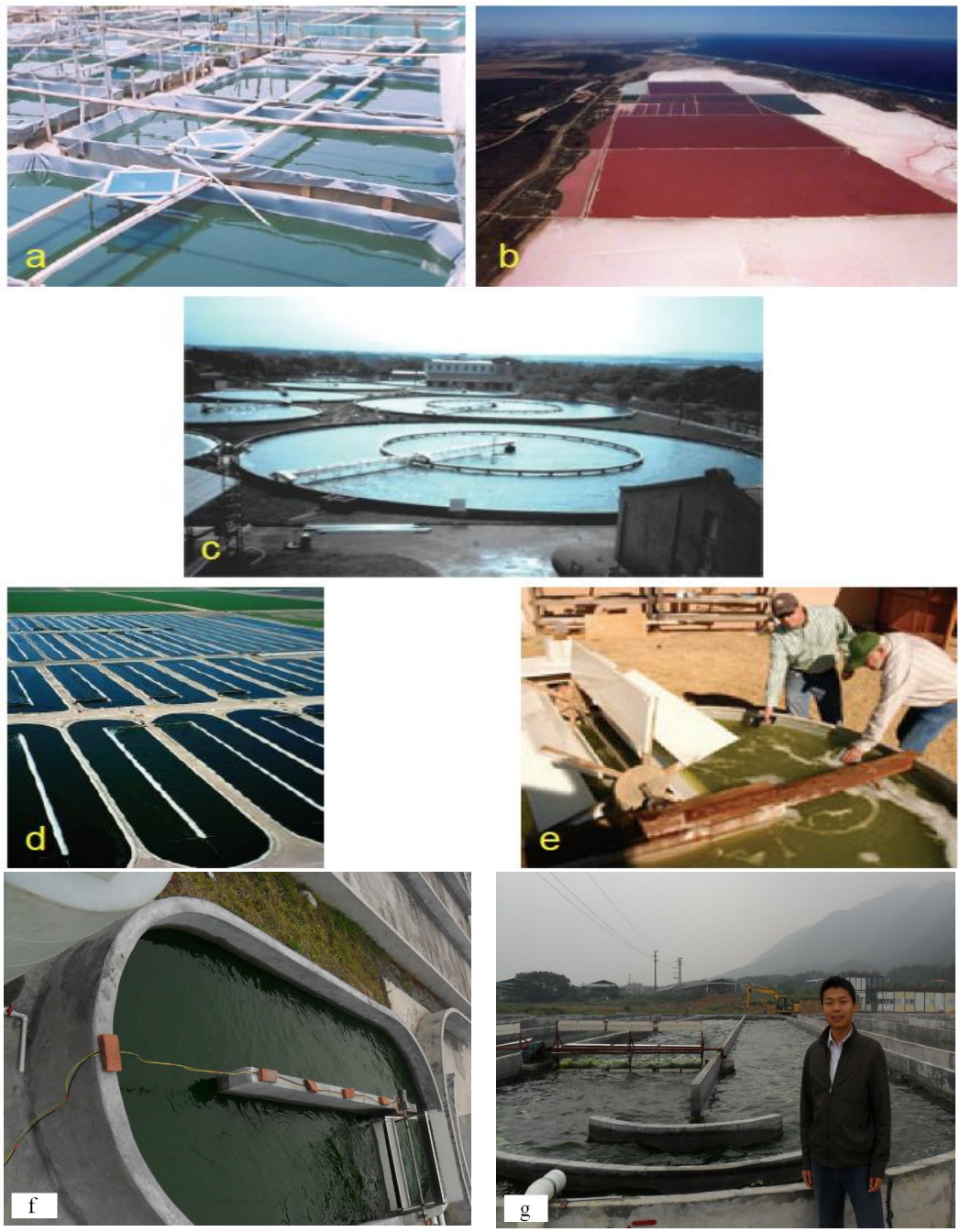

Figure 10. Examples of open pond systems (Source: a–e by FAO 2009; f by Liandong Zhu; g by Shuhao Huo). a. Small pond for Spirulina culture, Asia; b. *Dunaliella salina* ponds of Cognis, Australia; c. Center-Pivot ponds for the culture of Chlorella in Taiwan; d. Open raceway-type culture ponds at Earthrise in California; e. Paddle wheel of a raceway pond; f–g: Raceway ponds in Foshan, China.

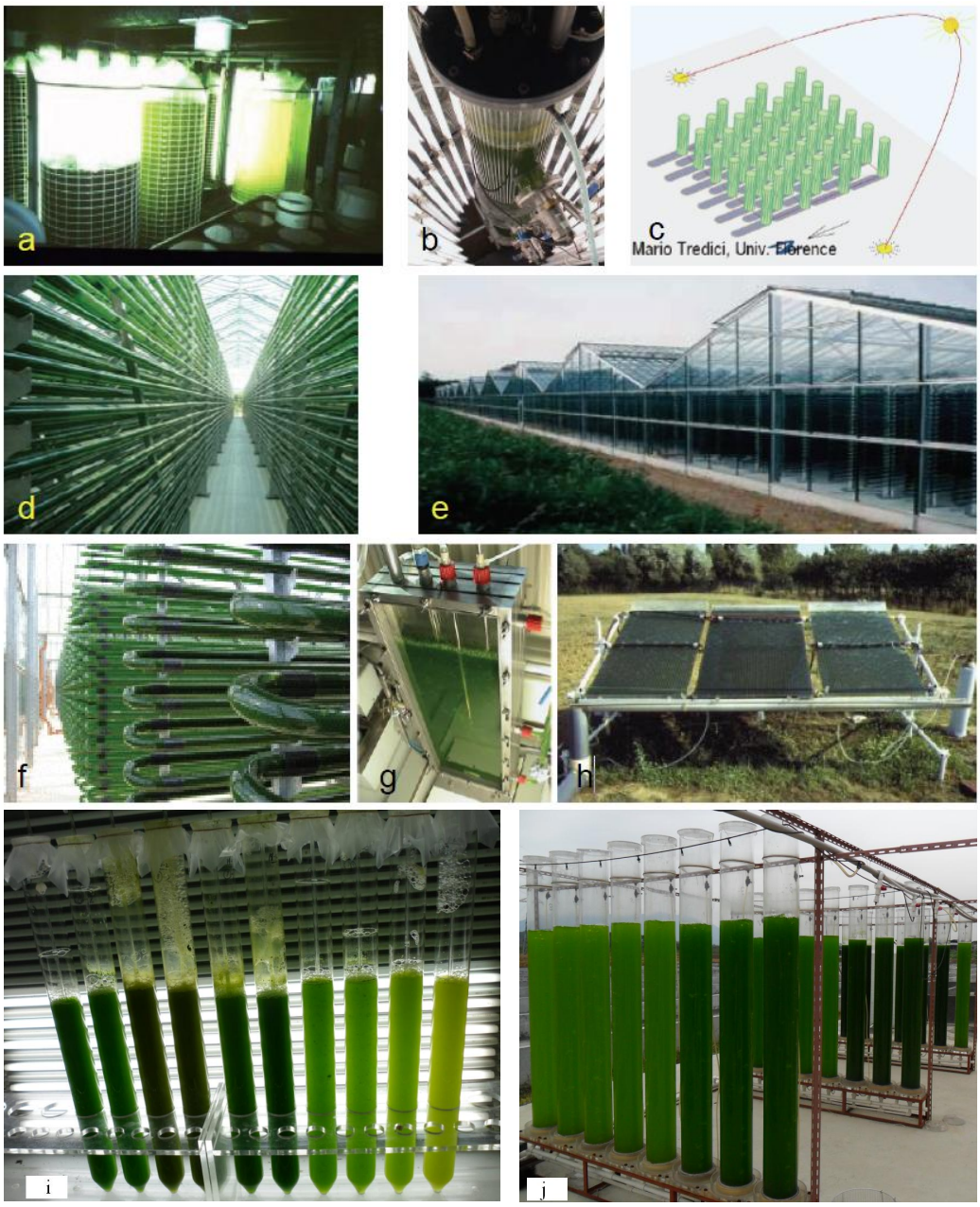

Figure 11. Examples of closed cultivation systems (a–h by FAO 2009; i–j by Liandong Zhu). a. "Big Bag" culture of microalgae; b. Bubble column reactor; c. Tubular bubble column photobioreactors; d–f. Tubular reactor system; g–h. Experimental photobioreactor; i. Lab-scale PBRs; j. Pilot-scale PBRs.

1.4.4 Biomass harvest and drying

Microalgae concentrations are always low, and their size is only a few micrometers (1 to 30 μm), which makes the harvesting and further concentration of algae difficult and therefore expensive (FAO 2009). It has been suggested that harvesting including drying contributes 20–30% of the total biomass production costs (Mata et al. 2010). The harvesting cost could be significantly reduced by optimizing various processes; however, current studies are not conclusive enough to propose such optimal harvesting processes. Thus, further R&D efforts are still required.

Basically, most common harvesting methods include sedimentation, filtration, flotation and centrifugation, sometimes with an additional flocculation step or a combination of flocculation–flotation (Mata et al. 2010). The aim of harvesting is to obtain slurry with at least 2–7% of total solid matters (SEI 2009); the microalgae can achieve up to 20% of total solid matters when using centrifugation (US Department of Energy 2010).

After harvesting the next step is dewatering and drying. Drying needs lots of energy and thus is the economic bottleneck of the entire process. The most common methods include spray-drying, drum-drying, freeze-drying and sun-drying (Mata et al. 2010). Sun-drying is cheap, but it is geography-dependent and will require extra space and considerable time.

1.5 Downstream processing

1.5.1 Microalgal lipid extraction

Lipid can be extracted by both chemical methods and mechanical methods. Lipids can be released by solvent extraction from the dried biomass. Several organic solvents can be used, for example, hexane, ethanol (96%), or a hexane–ethanol (96%) mixture, and up to 98% of lipids can be extracted (Mata et al. 2010). Super-critical extraction is also employed in practice, such as Subcritical Water Extraction and Supercritical Methanol Extraction (US Department of Energy 2010).

Mechanical disruption is a method that is initially employed to disrupt the cell membrane by grinding, pressing, beating, or crushing prior to the application of the extraction solvents (US Department of Energy 2010). In addition, extraction methods such as ultrasound and microwave have also been studied for oil extraction (Mata et al. 2010).

1.5.2 Microalgal biodiesel conversion

Biodiesel can be produced by three common routes: acid-catalyzed transesterification, base-catalyzed transesterification and chemical-catalyzed transesterification of fatty acids to alkyl esters. During production, alkaline or acidic, homogenous or heterogenous chemical catalysts can be used in the process. Base-catalyzed transesterification is the established means of processing biodiesel and is the overwhelming option used in industry for economic and technical reasons. The overall biodiesel production reaction is as follows:

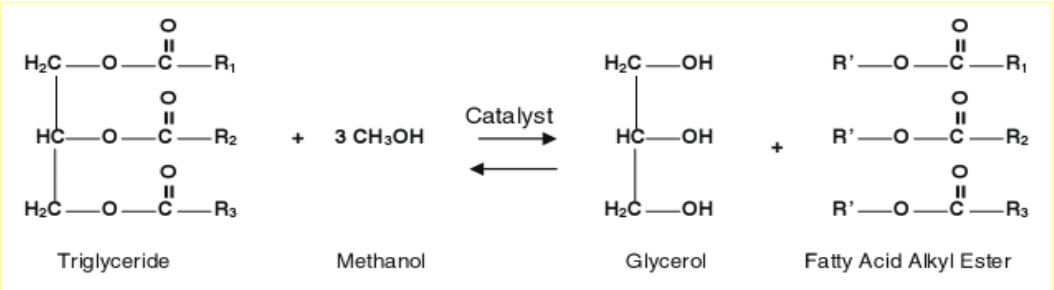

Figure 12. Transesterification of oil to biodiesel ($R_{1–3}$ are hydrocarbon groups).

As indicated in Figure 12, one molecule of each triglyceride in the algal oil reacts with three molecules of methanol to produce three molecules of methyl esters, the biodiesel product, and one molecule of glycerol (Aikins et al. 2009; Mata et al. 2010).

2 RESEARCH QUESTION AND STRUCTURE

2.1 Topic selection and research objective

As already explained in the Introduction, the world is currently facing serious environmental and energy problems. Global warming and climate change, water pollution, and the energy crisis are dominating the global scientific agenda. Thus, in an attempt to work towards sustainable development, the work presented here investigates a sustained solution to deal with issues related to both environment and energy.

Biodiesel produced from microalgae is one of the options to relieve the urgent demand mentioned above. Biodiesel production from microalgae holds a lot of advantages in terms of the impact on the natural environment, food security and land use, and microalgae have been proposed by many researchers as a promising feedstock (Chisti 2007; Avagyan 2008; Feng et al. 2011). Microalgae, which are rich in lipids, starch, and protein, can be utilized as a non-food-based feedstock for biofuels (mainly in the form of biodiesel, bioethanol and biogas) and chemical production. Some microalgae species can also be grown in wastewater by uptake of nitrogen and phosphorus (Chen et al. 2012; Kothari et al. 2012; Markou et al. 2012; Samori et al. 2013). In contrast to agriculture-based biofuel plants, microalgae grown in wastewaters can consume significantly less freshwater and improve water quality by removal of nutrients. Thus, no or limited chemical fertilizers, which are easily dissolved in rainwater or run-off, need to be added into the algae production system.

Based on environmental and energy considerations, this thesis *combines biodiesel production from microalgae with wastewater treatment in a sustainable manner*. In this research, the sustainability of microalgae production will be disclosed, piggery wastewater with different nutrient levels will be used to cultivate freshwater algae *Chlorella zofingiensis* to reveal the nutrient removal ability and the productivity of biomass, lipids and biodiesel, the harvest water will be recycled to re-cultivate *C. zofingiensis* to examine the effect on the growth of algae, and potential for scale-up will be investigated as well.

To summarize, the objectives of this dissertation are: (1) to investigate and minimize the sustainability impact factors before the establishment of microalgae facilities, (2) to evaluate the efficiency of the integration of *C. zofingiensis* cultivation with piggery wastewater treatment, (3) to examine the feasibility of recycling harvest water to re-produce microalgae, and (4) to reveal the possibility of scaling

up *C. zofingiensis* production for biodiesel conversion. The conclusions will follow to show how sustainable microalgae biodiesel production can become.

2.2 Research question, sub-questions and papers

Today, microalgae-based biodiesel is receiving more attention than ever before in efforts to search for renewable and alternative energy. However, there are widely voiced concerns about the sustainability of this energy source. To support the algal biodiesel industry, sustainably producing microalgal biomass for biodiesel conversion is becoming more and more important. To generate biodiesel from microalgae cultivated in wastewater is a promising concept to make microalgal biodiesel production more sustainable. So *what kind of sustainability can be achieved during biodiesel production from microalgae cultivated in wastewater*? There are several sub-questions that follow from this.

The following sections map out these sub-questions, examine the background to them, and outline the papers that will attempt to offer solutions. There are four sub-questions, four backgrounds, and four papers to answer each sub-question.

Problem setting I: Nowadays, the unpredictable environmental issues strongly bound with the economic, social and cultural impacts of the energy sector are dominating the international agenda. In order to be sustained, an industry should be environmentally sound, economically viable, socially just and culturally acceptable. In this regard, identifying and exploring the potential benefits and impacts related to microalgae-derived biodiesel production, systematically, can be seen as a first step towards supporting a biofuel industry. Consequently, all these aspects can be indentified and predicted in advance in order to make the best of benefits and minimize the potential impacts associated with microalgae production activities.

Sub-question I: Form a broad perspective, what kind of sustainability concerns (including the environmental, economic, social and cultural dimensions) the microalgae production has?

Title of Paper I: Microalgae production as a biofuel feedstock: risks and challenges

Problem setting II: In order to reduce the use of chemical fertilizers, ongoing research has been carried out, including on the use of manure and wastewaters rich in nitrogen and phosphorus as nutrient sources to cultivate microalgae. In this situation, compared to agriculture-based biofuel plants, microalgae can consume

significantly less freshwater and improve water quality, since only limited amounts of chemical fertilizer, which is easily dissolved in rainwater or run-off, need to be applied to the microalgae system.

Sub-question II: Is it effective to use piggery wastewater to cultivate algae *C. zofingiensis*? How efficient is this method for biomass accumulation, biodiesel production, and N and P removal?

Title of Paper II: Nutrient removal and biodiesel production by integration of freshwater algae cultivation with piggery wastewater treatment

Problem setting III: Microalgae biomass harvest can improve the quality of the treated wastewater and provide biomass to produce biodiesel as well, while leaving a lot of harvest water. Many previous studies have suggested that the harvest water should be recycled into the microalgae system with the purpose of water and nutrient reuse. However, there are no experimental data on the feasibility of recycling harvest water to re-cultivate microalgae. For instance, the influences of harvest water recycling on the growth of microalgae still need to be surveyed. During microalgae growth some metabolites including toxins can be released, which might cause inhibition of microalgae production when harvest water is circulated into the cultivation system. This might impact the biomass and lipid productivities and fatty acid methyl ester composition of biodiesel as well. In addition, after recycling several times, the harvest water is susceptible to contamination by fungus and bacteria, another inhibition factor for microalgae growth, total algal lipid content and lipid production rate. It is therefore unknown how many times or to what extent the harvest water can be recycled at a degree of 100%.

Sub-question III: What is the situation when the harvest water is recycled into the microalgae production system? What are the effects?

Title of Paper III: Recycling harvest water to cultivate *Chlorella zofingiensis* under nutrient limitation for biodiesel production.

Problem setting IV: Most of the studies on microalgal biodiesel production have been carried out only on lab or pilot scale, and microalgae are therefore not yet being commercially produced on a large scale. In addition, in real operations it is energy-intensive and complex to sterilize wastewaters by autoclave before the introduction of microalgae species. Moreover, previous studies have demonstrated that most feeding modes are batch operations, which are usually time-consuming, causing limited capacity and ability to treat wastewaters effectively. All of these factors are bottlenecks for the scale-up of microalgae cultivation us-

ing wastewaters in practice, showing that this apparently promising approach is still in its infancy.

Sub-question IV: Is it feasible or viable to amplify the production of microalgae for biodiesel production?

Title of Paper IV: Scale-up potential of cultivating *Chlorella zofingiensis* in piggery wastewater for biodiesel production

2.3 Interconnections of the four papers

Bioenergy will have a more and more important role to play in our daily lives as we seek to achieve sustainable development. Much importance has been attached to the sustainability of bioenergy. Identifying the core environmental, economic, social and cultural impacts associated with microalgae production is the first step to support the development of a sustainable algae-based biodiesel industry. Sustainability of microalgal biodiesel production is a huge topic, since many issues related to environment, economy, society and culture are involved. Paper I explores the environmental, economic, social and cultural impacts of microalgae production for biofuel usage throughout the life cycle from a *broad* perspective, using risk analysis and environmental impact analysis, so that some risks can be forecast and then either mitigated or eliminated to some degree.

When narrowing the topic of sustainability to the environmental dimension and then to the water aspect, issues over on water sustainability are of great concern since water is one of the most important factors in human survival. In terms of microalgae cultivation for biodiesel production, wastewater is a good source of water and nutrients for the culture (Figure 13). In an attempt to achieve sustainability in water usage, three key challenges should be taken into consideration: the feasibility of the integration of microalgae production with wastewater treatment should be evaluated (Paper II), recycling harvest water for microalgae reproduction should be trialed and the experiment described (Paper III), and the potential for scale-up of this technology should be explored (Paper Ⅳ).

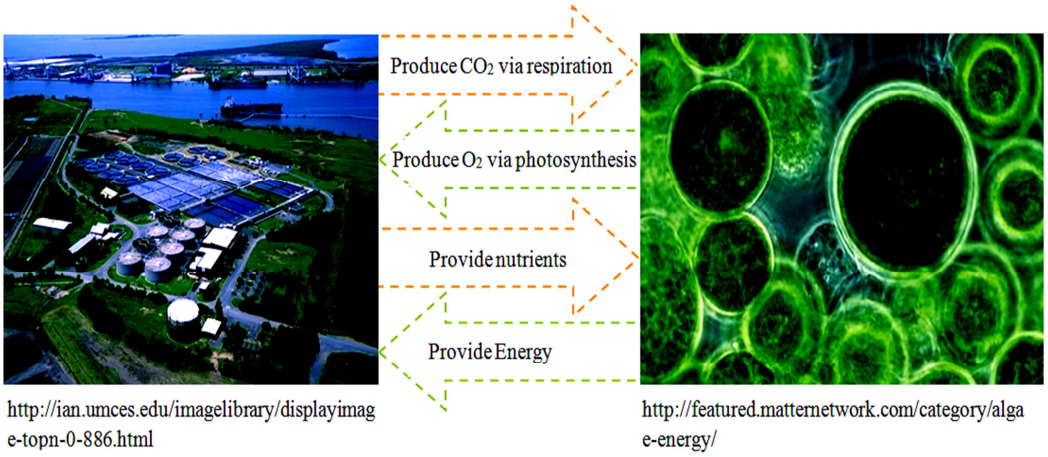

Figure 13. A concept of the integration of microalgae production with wastewater treatment.

Paper I explores the sustainable concerns related to microalgae production in a broad manner. Paper II reveals relevant nutrient removal abilities and specifies the productivities of biomass, lipids and biodiesel when cultivating *C. zofingiensis* cultures with different nutrient concentrations. Consequently, an optimal dilution of piggery wastewater for algal cultivation is determined. Paper III finds out the algal performance when recycling harvest water for *C. zofingiensis* re-cultivation under nutrient limitation conditions. This paper also reveals the times for harvest water recycling at a degree of 100%. Paper IV testifies that NaClO can be an easy method for wastewater pretreatment before the introduction of microalgae, and that *C. zofingiensis* can grow well outdoors. Importantly, this paper also clarifies that semi-continuous feeding is an efficient way to maintain stable and high biomass productivity.

The direct relations of the four papers are as follows. Water concern is one highlighted aspect in Paper I, which is the "roof" of Paper II, III and IV. The results of Paper II provide the optimal dilution ratio of piggery wastewater to carry on the scale-up potential experiments in Paper IV. The harvest of biomass described in Paper II can provide harvest water for the culture as described in Paper III. Paper II and Paper III can serve as guidance and a foundation for further scale-up trials to cultivate *C. zofingiensis* using piggery wastewater (Paper IV). All in all, the relationships between the four articles can be portrayed as a "funnel" shape, as displayed in Figure 14.

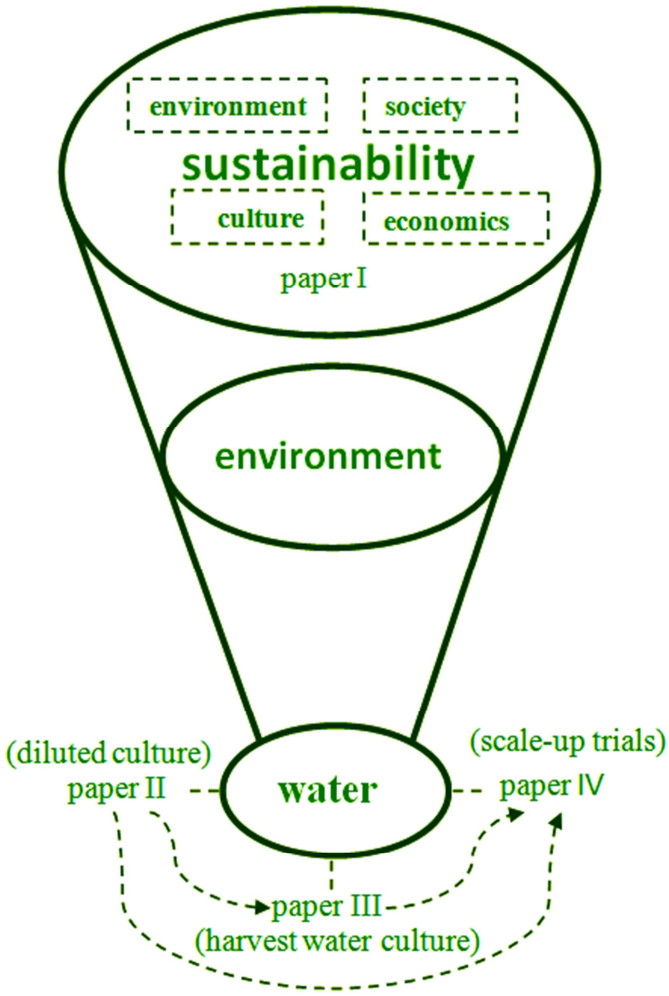

Figure 14. Logical relations of the four articles.

2.4 Research approach and methods

Towards sustainable microalgal biodiesel production, in this study piggery wastewater is introduced to cultivate *C. zofingiensis*. Firstly, Paper I points out the sustainability concerns related to microalgae production for biodiesel conversion from a board point of view. Then, the process for integration of *C. zofingiensis* production with piggery wastewater treatment for biodiesel conversion and nutrient removal is determined. The harvest water from biomass harvest is collected for reproduction of *C. zofingiensis*, and some performances are investigated. Finally, the amplification potential of cultivating microalgae species in wastewaters is verified, showing that this approach is apparently promising for pilot-scale or large-scale application.

From a theoretical viewpoint, Arbnor & Bjerke (2009) have put forward three levels of knowledge creation (analytical view, systems view and action view), which can be used for the basic approach in the present work (Figure 15). Paper I to IV move from basic knowledge to more advanced knowledge. Each step represents a clear example of progress or a new discovery; however, to make microalgal biodiesel sustainable on an industrial scale there is still a long way to go, especially on the commercial side.

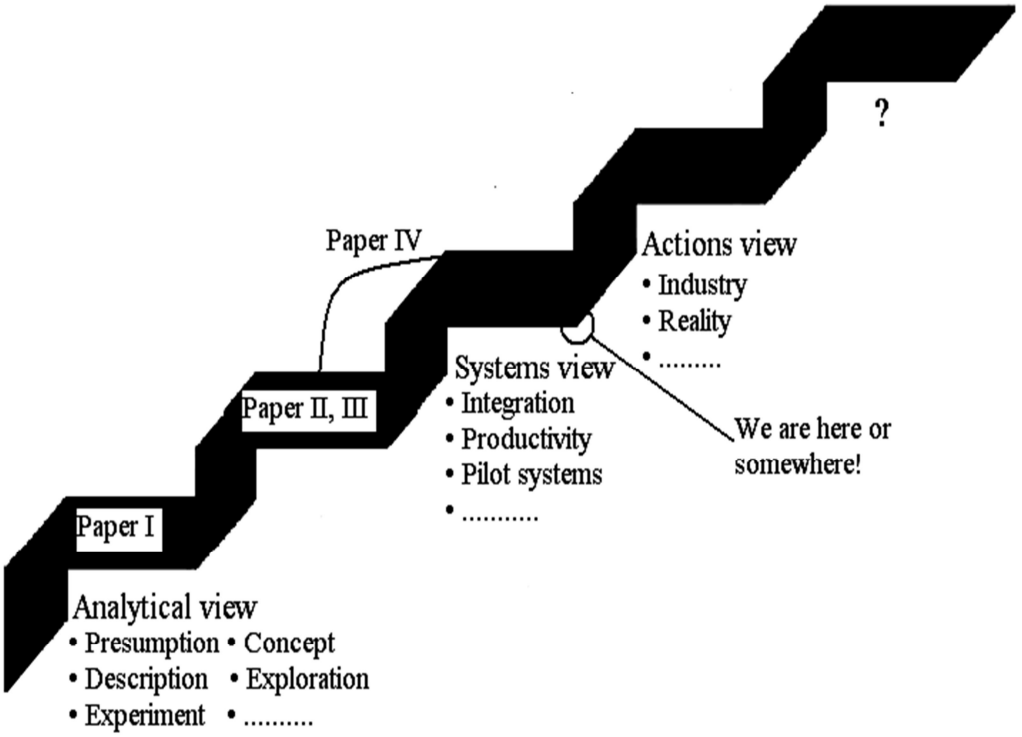

Figure 15. Research approach applied in this dissertation.

The methods applied in this work are mainly based on qualitative and quantitative investigation. Paper I is an exploratory study, and thus uses qualitative methods. Paper II, III and IV are based on experimental results, that is, a series of data, and therefore lie in the scope of quantitative investigation.

2.5 Research process

Cultivation of microalgae in wastewater is one solution to realize sustainable biodiesel production in practice, since it not only recovers energy but also has environmental merits in terms of purifying wastewater. Generally, microalgae cultivation for biodiesel production will go through the process shown in Figure 16. To begin with, suitable species should be selected. Then, microalgae are grown in

closed systems or open ponds, followed by harvesting and drying. Afterwards, a downstream process called biodiesel refinery is carried out with lipid extraction and lipid transesterification to obtain the fatty acid methyl esters (FAME).

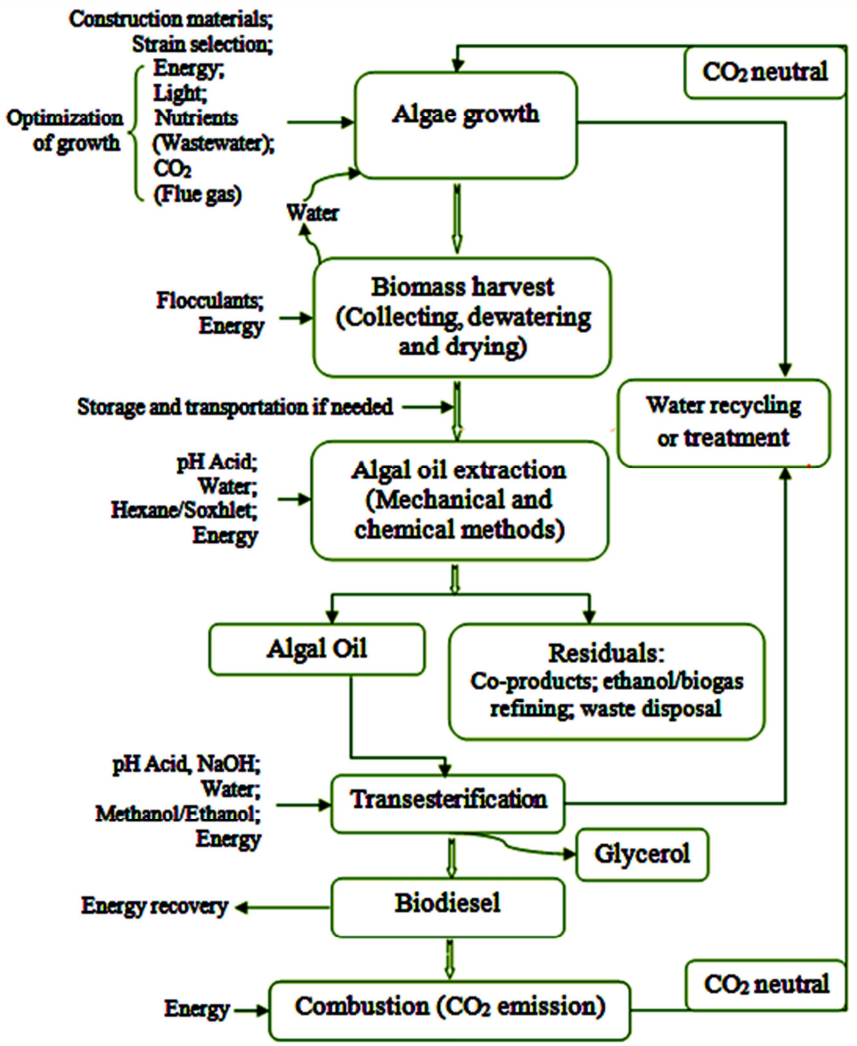

Figure 16. Life cycle process of microalgae production for biodiesel conversion.

When selecting strains, there are several factors to be considered, such as the desired end product, growth rate, optimal temperature range, lipid content, adaption to wastewater culture, and responses to stress conditions like nutrient deprivation. The last factor is the most important indicator, since some species will not accumulate lipids under stress conditions. For example, under nutrient deficiency *Dunaliella salina* increases carbohydrates from 16 to 56% and decreases its lipid content from 25 to 9% (Alabi et al. 2009). In this study *C. zofingiensis* was selected as the culture species, which belongs to the Chlorella family, a genus of single-

cell green algae with a spherical shape and about 2 to 10 μm in diameter. Previous studies show that *C. zofingiensis* contains 17.9 to 65.1% lipid of dried cell weight, has a specific growth rate of 0.30 to 2.15 day^{-1}, and can accumulate neutral lipids under nutrient starvation or limitation (Feng et al. 2012; Huo et al. 2012).

In this study, piggery wastewater from a pig farm was selected as the culture medium and a tubular bubble column photobioreactor (tbcPBR) as the culture carrier. Growth characteristics and nutrient removal were investigated during the experiment. After several days' cultivation, microalgae biomass was collected. In this study centrifugation was the harvest method and an oven served as the dryer. Afterwards, a modified method developed by Bigogno et al. (2002) was applied to quantify the total lipid content by using methanol with 10% DMSO, ether and hexane. Finally, the content of FAME was analyzed following a one-step extraction-transesterification method developed by Indarti et al. (2005), after suitable modifications, whereby neutral lipid was transferred to FAME using methanol with 2 wt.% of concentrated sulfuric acid (H_2SO_4) as the catalyst.

From the overall design point of view, this thesis follows a strict process, as shown in Figure 17. The success of each step will influence the next procedure's implementation, in an effort to augment the cultivation of *C. zofingiensis* for biodiesel production. For example, when designing a microalgae culture system, many factors need to be considered, such as CO_2, light intensity, nutrient concentrations, and so on. Other factors like location will also affect the results. Once the design is successful, the integration of *C. zofingiensis* cultivation with piggery wastewater can be achieved successfully. If the design fails, reasons should be found and the cultivation systems should be re-designed accordingly.

According to the process, the economics of the scale-up systems should be surveyed to discover the commercial situation for this technology. Methods to improve the cost-effectiveness should be put forward to work towards the large-scale commercial implementation of microalgae production for biodiesel conversion.

Finally, the research question will be answered in the Conclusion, where it will be shown how sustainable microalgae biodiesel production could become.

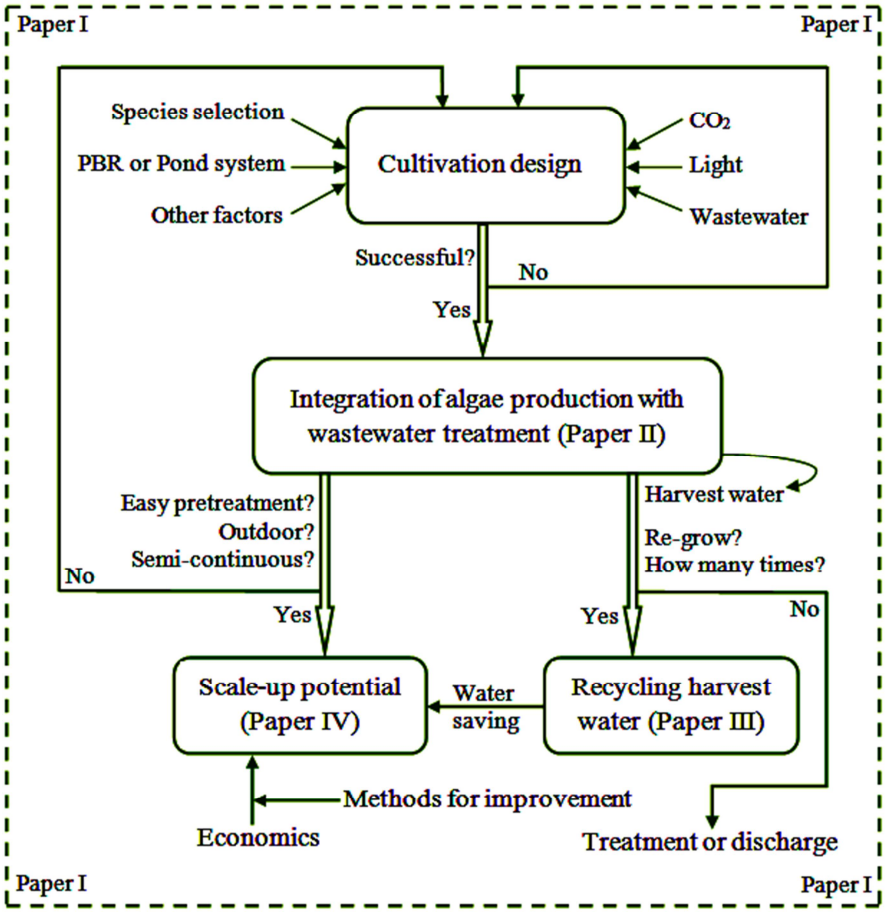

Figure 17. Research process flow of this thesis.

2.6 Significance of this work

Microalgal biofuel is not yet produced on a commercial scale due to the high costs resulting from system start-up, operation, maintenance and management. In addition, an economically viable algae-to-biodiesel project might initially depend on government subsidies and the future price of crude oil. Moreover, over-pursuit of commercial profits from microalgae production at this stage might cause some indirect problems, such as land use change or land use overexpansion.

However, economic and technological approaches have been suggested to overcome oil dependence on foreign sources and strong scientific commitment has been devoted to this paramount challenge (Demain 2009). Today more than ever before, unpredictable global environmental issues strongly bound with the social and economic impacts of the energy sector are dominating the international agenda. That the petroleum-based economy is getting closer and closer to the end of its lifecycle has become more and more evident. Consequently, it is very im-

portant to be able to forecast and prevent any shortfall in future supply, and to provide access to new bioenergy alternatives for the marketplace. To meet these goals, potential sources of alternatives need to be easily accessible and widespread, with attractive extraction efficiency. Microalgae biomass, capturing solar energy by photosynthesis, can be developed into an alternative source of feedstock. The implementation of the present research will be significant in several ways:

To start with, the developed approach has environmental benefits. Microalgae can assimilate organic pollutants (mainly nitrogen and phosphorus) from wastewater into cellular constituents such as lipid, protein and carbohydrate, thus achieving pollutant reduction in an environmentally friendly way. Nutrients absorbed can be removed from wastewater via biomass harvest. Moreover, since microalgae can fix CO_2 and release O_2, their use leads to a decrease in the harmful emissions of CO_2. This is advantageous in view of the fact that currently fossil fuels contribute to the increase of CO_2 emissions.

In the second place, the research can provide some evidence as to whether or not it is efficient and sustainable at present to produce biodiesel from microalgae cultivated in wastewater. If the answer is yes, then it will be practical and reasonable to make full use of this kind of feedstock. Researchers' horizons and aspirations in the utilization of microalgae biofuels will be broadened.

Finally, with respect to biofuels, various replaceable options are useful to help protect the food security of the poor, in particular in the developing world, where already more than 800 million people (excluding China) suffer from hunger and malnutrition (FAO 2007). Governments could decrease the support given to first-generation agro-fuels (Koning & Mol 2009), and encourage the development of microalgae biodiesel sources in every possible aspect.

Overall, from a sustainability perspective, this research is of vital importance. Energy companies like the Finnish Fortum Corporation can potentially benefit too. In the face of the energy crisis, global warming and global climate change, the energy sectors must shoulder some special responsibility to widen the use of renewable energy in an environmentally friendly manner. The findings of this research can offer technical guidance for the energy industry as regards the requisite construction investments for facility establishment, instrument installation, and so forth. A certain amount of research and innovation through well-funded R&D programs is still required in order to decrease the costs and increase the yields.

3 SUMMARY OF THE ARTICLES

3.1 Summary in brief and contribution

3.1.1 Papers' information

This thesis is the compilation of four articles, all published in peer-reviewed international journals. The author designed the experiments independently, carried out the empirical work with some assistance from co-authors and colleagues, and wrote and revised the articles independently. Table 3 summarizes the information contained in the four papers.

Table 3. Information contained in the four papers including authors and their responsibilities.

Paper	Paper I	Paper II	Paper III	Paper IV
Title	Microalgae production as a biofuel feedstock: risks and challenges	Nutrient removal and biodiesel production by integration of freshwater algae cultivation with piggery wastewater treatment	Recycling harvest water to cultivate *Chlorella zofingiensis* under nutrient limitation for biodiesel production	Scale-up potential of cultivating *Chlorella zofingiensis* in piggery wastewater for biodiesel production
Journal	Int. J. Sustain. Dev. World Ecol.	Water Res.	Bioresour. Technol.	Bioresour. Technol.
Publisher	Taylor and Francis	Elsevier	Elsevier	Elsevier
Authors	Zhu L. & Ketola T.	Zhu L., Wang Z., Shu Q., Takala J., Hiltunen E., Feng P. & Yuan Z.	Zhu L., Takala J., Hiltunen E. & Wang Z.	Zhu L., Wang Z., Takala J., Hiltunen E., Qin L., Xu Z., Qin X. & Yuan, Z.
Responsibilities	The first author wrote and revised the paper; the second author proposed the four dimensions and provided some comments during revision	The first author designed and implemented the tests, and wrote and revised the paper; Shu Q. helped in the tests; the other authors provided experimental facilities, as well as comments or suggestions	The first author designed and implemented the tests, and wrote and revised the paper; the other authors provided experimental facilities, as well as comments or suggestions	The first author designed and implemented the tests, and wrote and revised the paper; Qin L. & Qin X. assisted in the tests; the other authors provided experimental facilities, as well as comments or suggestions

3.1.2 Contributions of this work

This thesis contains several novelties and innovations. Table 4 shows the contributions of this thesis to the research area.

Table 4. Research aims, methods, findings and contributions to the research area.

Paper	Paper I	Paper II	Paper III	Paper IV
Title	Microalgae production as a biofuel feedstock: risks and challenges	Nutrient removal and biodiesel production by integration of freshwater algae cultivation with piggery wastewater treatment	Recycling harvest water to cultivate *Chlorella zofingiensis* under nutrient limitation for biodiesel production	Scale-up potential of cultivating *Chlorella zofingiensis* in piggery wastewater for biodiesel production
Aims	To explore the potential risks associated with microalgae production from the sustainability perspective, including environmental, economic, social and cultural impacts	To determine an optimal dilution of piggery wastewater for algal cultivation; to reveal relevant nutrient removal abilities; and to specify the productivities of biomass, lipids and biodiesel	To find out the algal performance when recycling harvest water for *C. zofingiensis* recultivation under nutrient limitation conditions; also to reveal the times for harvest water recycling	To compare algae cultivation (1) in piggery wastewater pretreated by autoclave and NaClO, and (2) under both indoor and outdoor conditions; also to determine biomass productivity in a semi-continuous feeding operation
Methods	Qualitative and exploratory study	Quantitative and empirical study	Quantitative and empirical study	Quantitative and empirical study
Main Findings	Sustainability concerns related to microalgae production including environmental, economic, social and cultural dimensions were explored in detail	The diluted piggery wastewater with 1900 mg L^{-1} COD provided an optimal nutrient concentration for *C. zofingiensis* cultivation, presenting the advantageous nutrient removal and the highest productivities of biomass, lipid and biodiesel	The N- and P-limited medium observed the highest FAME yield, while the N-limited culture and P-limited culture shared the highest biodiesel productivity; 100% harvest water could be recycled twice with the addition of sufficient nutrients	It is greatly possible to amplify the cultivation of *C. zofingiensis* in piggery wastewater, since wastewater can be easily pretreated by NaClO; algae can grow well outdoors; the semi-continuous feeding run was efficient in biomass productivity
Contributions	The paper explores the potential risks related to algae production from the sustainability perspective, systematically and explicitly	The paper evaluates an integrated approach combining *C. zofingiensis* cultivation with piggery wastewater treatment; the biodiesel yields are quantified as well	The paper investigates the harvest water recycling for the re-production of algae and discovers the times for harvest water recycling	The paper explores the technical scale-up potential of algae production for biodiesel conversion in a systematic way

3.2 Sustainability concerns

3.2.1 Sustainability impact factors selection

Research on microalgae production in open or closed systems for biofuel usage has recently boomed, and many discoveries and technological breakthroughs have been achieved theoretically and/or empirically (Zhu et al. 2011b). Currently, there is no large-scale commercial operation in the production of biodiesel from microalgae feedstock (Sander and Murthy 2010), although commercial microalgae farming corporations on a relevant scale, such as Earthrise, Cyanotech, and Oilgae, are emerging. In this situation, identifying the core environmental, economic, social and cultural impacts associated with microalgae production is the first step in supporting the development of a sustainable biofuel industry. Thus, research into the sustainability of such production systems is of great importance before a complete industrial-scale process for biofuel production from microalgae is built.

From a sustainability point of view, this exploratory study maps out the environmental (water, land, biodiversity and greenhouse gases), economic, social and cultural risks of microalgae production for biofuel usage (Figure 18), systematically and explicitly, using risk analysis and environmental impact analysis. Through the relative analyses we can point out the unsustainable aspects of microalgae production, so that relevant problems can be forecast and then either mitigated or eliminated to some degree.

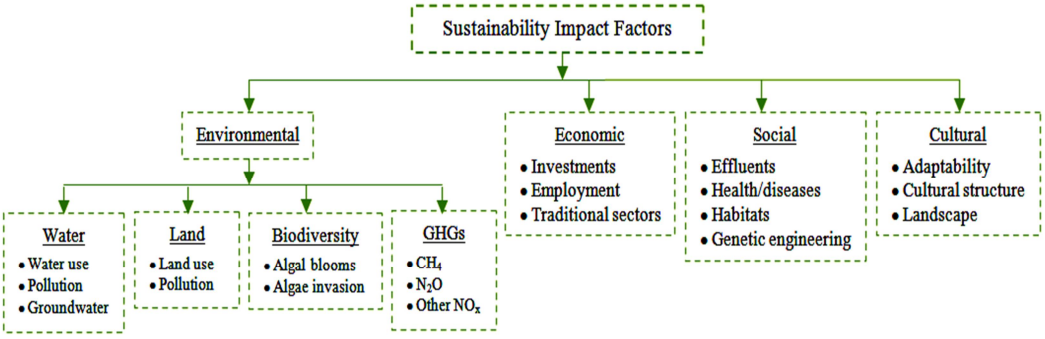

Figure 18. A framework for sustainability impact analysis (Fig. 1, Paper I).

3.2.2 Sustainability concerns

The sustainability concerns associated with the production of microalgae for biofuel usage are summarized in Table 5.

Table 5. Potential sustainability concerns of microalgae production for biofuel usage.

Environmental dimension	Economic dimension	Social dimension	Cultural dimension
+Less water is required; +Wastewater can be used; +Water used in the systems can be recycled; +Unproductive lands can be used for plant construction; +Microalgae-based biofuels are carbon neutral; +Combustion gas like flue gas can provide CO_2 as a carbon resource for microalgae cultivation	+Increases employment and incomes; +Additional economic values from byproducts or co-products; +Increases income taxes	+Job creation; +Energy security	+ Algal biofuels concept has been added into cultural structures as a new cultural element and ingredient
–Water resource abuse; –Damage to waterways; –Groundwater may not be recharged effectively; –Soil pollution, land use overexpansion, land service expectancy decrease, and soil erosion; –Detrimental effect on local ecosystem, causing eutrophication, algal blooms, fish kills, and biological invasion; –Greenhouse gases (e.g., NO_x, CH_4) and NH_3 emissions	–Start-up phase is expensive; –Requires overwhelming investment; –Might cause depression or shrinkage in the traditional algal industry	–Local poultry, wildlife and people might suffer from water pollution; –Safety effects of genetically modified algae may not be immediately apparent or known; –Diseases (e.g., malaria) spread; –Animal habitat interruption	–Requires time for people to adapt to and accept unconventional microalgae utilization; –Personnel changes in work force might harm the existing cultural structures; –Landscape aesthetics might be affected

Environmentally, there are several benefits related to microalgae production (Zhu et al. 2011b). From a water use point of view, microalgae production can decline the consumption of fresh water since many microalgae species can grow in saline and brackish water, seawater, and other salt water like saline groundwater. Manure and wastewaters rich in N and P can provide a nutrient source to cultivate microalgae, so that no or limited chemical fertilizers need to be added into the culture systems. Furthermore, water can be reused effectively through recycling. In terms of land use, microalgae systems can be established on non-farmlands, such as arid or semiarid earth, saline soils, infertile farmland, polluted land, and other types of land with low economic value (e.g., deserts). In addition, large areas offshore can be utilized to grow microalgae, so that less land needs to be used. As to greenhouse gases, CO_2 released during combustion can be captured and mitigated again via photosynthesis by microalgae; thus, microalgae-based biofuel is carbon neutral. Moreover, combustion gas (e.g., flue gas) enriching CO_2 can be

transferred into cultivation systems to provide a carbon resource for microalgae cultivation.

Economically, microalgae production can increase employment and incomes. Additional economic value can be obtained from by-products or co-products from biofuel conversion. Moreover, microalgae production can increase income taxes for all kinds of government. From a social standpoint, microalgae production can create jobs, especially in developing countries. Furthermore, the microalgae-based biofuel industry could play a significant role in improving nations' energy security and reducing countries' reliance on crude oil imports (Flamos et al. 2010). Culturally, the microalgal biofuels concept has been added into cultural structures as a new cultural element and will influence the development of algal culture.

However, there are some risks and challenges as well, primarily connected to water, land, biodiversity, and greenhouse gases. Firstly, there exist potential water safety risks, such as water resource abuse, water pollution, and groundwater recharge deficiency. Secondly, unreasonable construction will lead to land use overexpansion, land pollution and service expectancy reduction. Thirdly, microalgae production may exert a detrimental effect on the local ecosystem, causing algal blooms and biological invasion. Finally, it may emit unexpected greenhouse gases (nitrogen oxides [NO_x], methane [CH_4]) and ammonia (NH_3).

Economically, the start-up phase and operation are expensive, and will require overwhelming investment from bioenergy companies, the car industry and governments. In addition, more people may be laid off because of the increasing automation. Furthermore, microalgae production for biofuel extraction might cause depression or shrinkage in the traditional algal industry. From the perspective of society, the water contamination caused by microalgae production will jeopardize the health of local poultry, wildlife and people. Moreover, the safety of genetically modified algae may not be immediately apparent or known. Another public health risk is that the microalgae cultivation system might become a medium for mosquito eggs and larvae, leading to wider transmission of diseases such as malaria. From a cultural point of view, microalgae production for biofuel extraction is a new concept especially for developing countries, so time is required for their citizens to adapt to this biomass fuel as an alternative to conventional fossil fuel. Furthermore, personnel changes in the work force might harm the existing cultural structures, and landscape aesthetics might be affected by mechanical activities as well.

Undoubtedly, the environmental, economic, social and cultural benefits involved in microalgae production can significantly contribute to a sustainable industry,

sooner or later. However, in order to sustain a microalgal biofuel industry in the long term, the associated risks must be forecast and addressed through relevant measures. Efficient government policies, proactive company behaviors and positive public participation will have an important role to play in mitigating or even eliminating all kinds of potential risks. Only in this way might a microalgal biofuel industry enjoy prosperity in a sustainable manner.

3.3 Integration of microalgae cultivation with piggery wastewater treatment

As shown in the microalgal biomass molecular formula – $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$ (Grobbelaar 2004) – nitrogen (N) and phosphorus (P) are important nutrients for algal growth. Thus, microalgae production involves enormous amounts of N and P fertilizers. According to Markou and Georgakakis (2011), the quantity of N required as fertilizer is estimated to be 8–16 tons N/ha. In order to reduce the use of chemical fertilizers, the present study used piggery wastewater rich in nitrogen and phosphorus as a nutrient source to cultivate *C. zofingiensis*. An optimal dilution of piggery wastewater for algal cultivation was determined, relevant nutrient removal abilities were revealed, and the productivities of biomass, lipids and biodiesel were specified.

3.3.1 Microalgal growth

The growth performance of *C. zofingiensis* cultivated in the piggery wastewater with the COD concentration of 3500, 2500, 1900, 1300, 800 and 400 mg L^{-1} is illustrated in Figure 19. During 10-day cultivation, all cultures went through all the growth phases except the lysis phase. The growth parameters of *C. zofingiensis* in tbcPBRs under six nutrient concentration levels are shown in Table 6.

The specific growth rate μ of cultures in 3500, 2500, 1900 and 1300 mg L^{-1} COD piggery wastewater reached 0.287, 0.322, 0.340 and 0.431 day^{-1}, respectively. Rapid growth with μ of about 0.5 day^{-1} and doubling time of about 1.4 days happened in the media with 800 and 400 mg L^{-1} COD. The piggery wastewater treatment with the initial COD concentration at 1900 mg L^{-1} witnessed the highest final biomass increase (2.962±0.192 g L^{-1}) and productivity (296.16±19.16 mg L^{-1} day^{-1}), while the cultures in 800 and 400 mg L^{-1} COD piggery wastewater shared the obviously lowest biomass increases and biomass productivities.

Table 6. Growth parameters of *C. zofingiensis* in tbcPBRs under six nutrient concentration levels within 10 days (Table 2, Paper II).

Culture (mg L^{-1} COD) *	Specific growth rate μ (day^{-1})	Doubling time (days)	Biomass increase (g L^{-1})	Biomass productivity (mg L^{-1} day^{-1})
3500	0.287	2.42	2.646	267.81
2500	0.322	2.15	2.733	273.33
1900	0.340	2.04	2.962	296.16
1300	0.431	1.61	2.166	216.63
800	0.487	1.42	1.603	160.34
400	0.492	1.41	1.063	106.28

* COD concentration was used to present the initial nutrient concentration in all cases in Subsection 3.3.

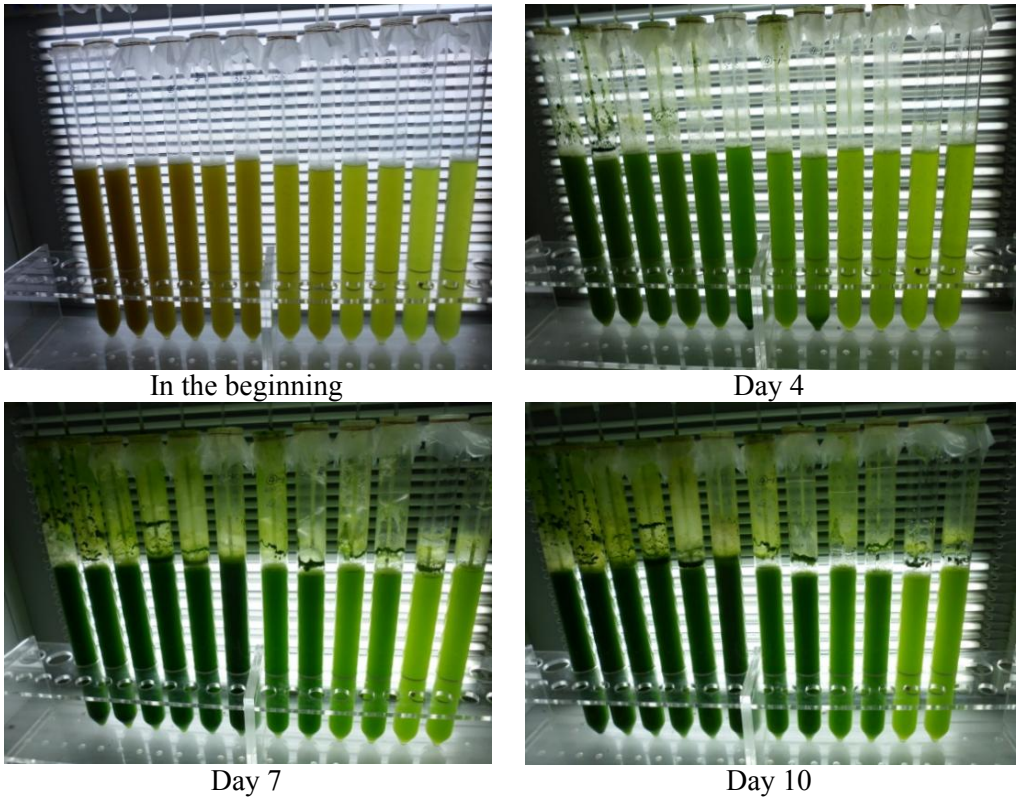

Figure 19. *C. zofingiensis* cultivated in piggery wastewater under six nutrient concentration levels within 10 days.

3.3.2 Nutrient removal

The removal of COD, TN and TP in wastewater treatment by various microalgae reported in the literature and the present study is exhibited in Table 7. High-percentage removal of COD, TN and TP was achieved during all the treatments in

this study. The main contributors to high nutrient removal are (1) nutrient uptake by microalgal cells to accumulate biomass, and (2) microalgal–bacterial interaction, which can also degrade nutrients. Moreover, the increase of pH in the cultures in this study could help contribute to the precipitation of phosphorus.

Table 7. Wastewater treatment by various microalgae reported in the literature and the present study.

Wastewater source	Microalgae	Reactor	Culture time, d	Removal, %			Reference
				COD	TN	TP	
Soybean processing	*Chlorella pyrenoidosa*	PBR	5	77.8	88.8	70.3	Su et al. (2011)
Municipality	*Chlorella minutissima*	Oxidation pond	15	>75.0	>41.0	30.0	Bhatnagar et al. (2010)
Piggery effluent	*Chlorella vulgaris*	Plastic bag	10	–	>78.0	100	Kumar et al. (2010)
Urine	*Spirulina platensis*	PBR	8	–	99	99	Yang et al. (2008)
Dairy wastewater	*Chlorella pyrenoidosa*	Flask	15	–	60–80%	80–85%	Kothari et al. (2012)
Pig farm (3500 mg L^{-1} COD)	*Chlorella zofingiensis*	tbcPBR	10	74.3	78.7	85.0	In this study
Pig farm (2500 mg L^{-1} COD)	*Chlorella zofingiensis*	tbcPBR	10	78.2	81.0	89.2	In this study
Pig farm (1900 mg L^{-1} COD)	*Chlorella zofingiensis*	tbcPBR	10	79.8	82.7	98.2	In this study
Pig farm (1300 mg L^{-1} COD)	*Chlorella zofingiensis*	tbcPBR	10	76.5	77.8	98.6	In this study
Pig farm (800 mg L^{-1} COD)	*Chlorella zofingiensis*	tbcPBR	10	65.8	70.9	99.4	In this study
Pig farm (400 mg L^{-1} COD)	*Chlorella zofingiensis*	tbcPBR	10	67.3	69.0	100.0	In this study

3.3.3 Chemical contents, and lipid and biodiesel productivity

The sugar, protein and lipid contents of the microalgal biomass were determined and the lipid and biodiesel productivity calculated as shown in Table 8.

As shown in the table, as the initial nutrient concentration increased, the lipid content decreased from 45.81% to 33.91%, but the protein content increased from 11.29% to 19.82%. This tendency for change in the protein content in algal cells was in line with the research conducted by Chen et al. (2012), who cultivated *Chlorella* sp. with diluted animal waste. However, there was no evident difference in sugar percentage among the algae cultured in different nutrient concentrations.

Table 8. Chemical contents of biomass, and lipid and biodiesel productivity of *C. zofingiensis* in tbcPBRs under six nutrient concentration levels within 10 days.

Culture (mg L^{-1} COD)	Lipid content (%)	Sugar (%)	Protein (%)	Lipid productivity (mg L^{-1} day^{-1})	FAME content (%)	Biodiesel productivity (mg L^{-1} day^{-1})
3500	33.91	24.93	19.82	90.81	8.80	23.56
2500	36.48	26.24	18.79	99.71	9.97	27.26
1900	37.33	24.81	15.85	110.56	10.18	30.14
1300	37.38	25.11	14.21	80.98	10.01	21.67
800	42.16	27.00	13.03	67.59	10.84	17.38
400	45.81	27.26	11.29	48.69	11.15	11.85

The highest FAME yield appeared in the 400 mg L^{-1} COD culture where 11.15 g-biodiesel/100g-dry weight was derived from *C. zofingiensis*, while the lowest yield occurred in the 3500 mg L^{-1} COD medium (8.80% of dry weight). The differences in lipid and biodiesel productivity of *C. zofingiensis* among all the treatments mainly resulted from the differences in biomass productivity. The 1900 mg L^{-1} COD culture showed the highest productivities of biomass, lipid and biodiesel.

3.3.4 FAME profile

C. zofingiensis in all cultures had shorter carbon chains for fatty acids, as shown in Figure 20. The C16–C18 of *C. zofingiensis* in the 3500, 2500, 1900, 1300, 800 and 400 mg L^{-1} COD cultures represented a major portion of fatty acid methyl esters compositions, accounting for a total of 89.17%, 87.75%, 84.51%, 92.31%, 97.28% and 80.31%, respectively. The high percentage of C16–C18 can improve the quality of biodiesel (Huang et al. 2010).

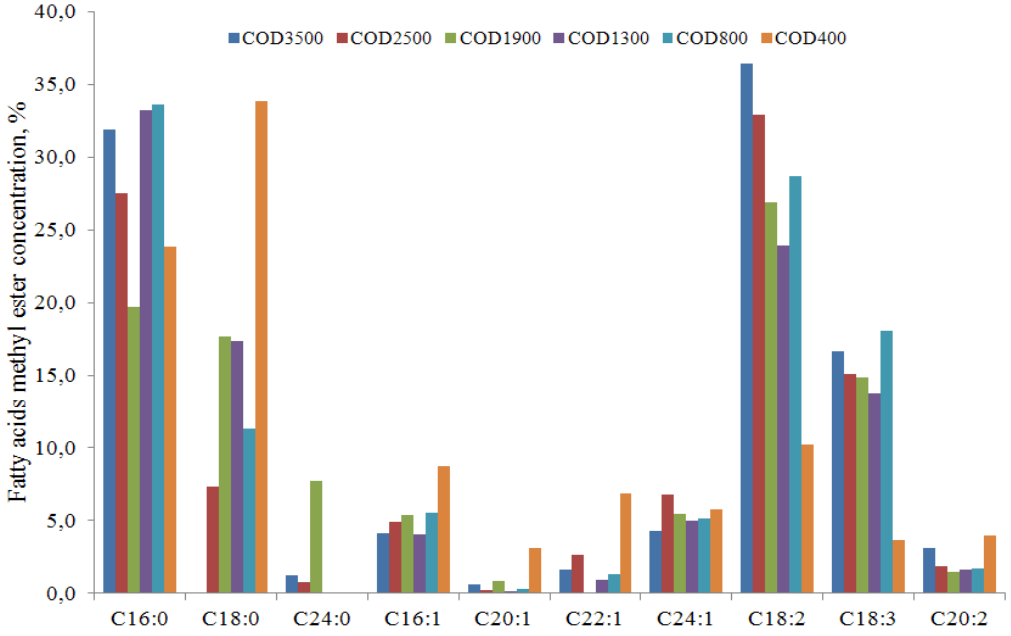

Figure 20. FAME profile of *C. zofingiensis* grown in piggery wastewater under six nutrient concentration levels within 10 days.

3.4 Recycling harvest water to re-grow algae

Many previous studies have recommended that harvest water should be recycled into the microalgae system not only for water reuse but also for nutrient recovery (Lam and Lee 2012b). Nonetheless, researchers still know little about the feasibility of recycling harvest water to re-cultivate microalgae, on the basis of the available experimental data. Previous studies have also reported that N or P deficiency or limitation can promote lipid accumulation (Hu et al. 2008; Devi and Mohan 2012; Feng et al. 2012). In this part of the investigation, harvest water from the production of *C. zofingiensis* in piggery wastewater was used to prepare the media and freshwater microalgae *C. zofingiensis* was re-cultivated under nutrient limitation. The objectives were to find out the productivity of biomass and lipids, and the composition of fatty acids under the above conditions, and to discover how many times the harvest water can be circulated effectively at a degree of 100%.

3.4.1 Recycling harvest water to re-grow algae under nutrient limitation

The growth characteristics of *C. zofingiensis* cultivated in the harvest water media for eight days are shown in Figure 21. From Figure 21 it can be seen that *C. zofingiensis* in all treatments with 100% or 50% harvest water could grow well.

The lag phase of algal growth was short in all treatments. This was possibly because the harvest water still contained some un-harvested algal cells, which could accelerate the growth of algae (Lam and Lee 2012b). Still, the un-harvested algal cells had already adapted to the wastewater environment, so once the nutrients were accessible, the microalgae could use the nutrients immediately.

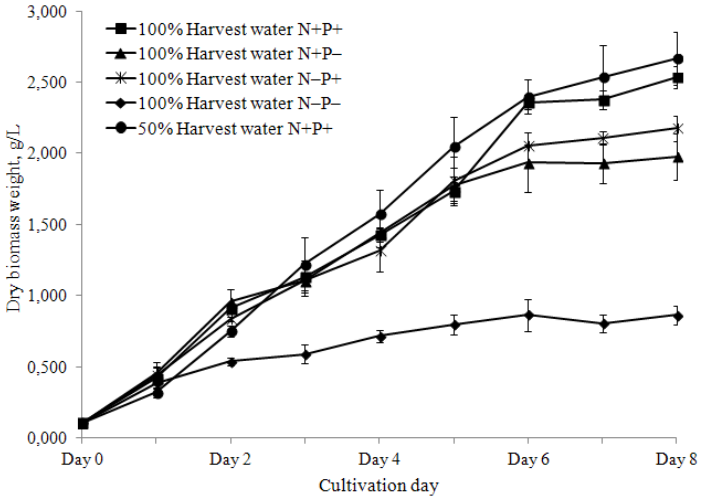

Figure 21. The growth of *C. zofingiensis* when recycling harvest water as the media with different nutrient conditions (Fig. 1, Paper III).

C. zofingiensis in the full media with 50% harvest water had the highest specific growth rate μ of 0.403 day^{-1} with the shortest doubling time of 1.72 days and the highest biomass productivity (Table 9). Nutrient-limited cultures showed much higher lipid content (41.21% – 46.21% of dry weight) than nutrient-full cultures (around 26% or 27% of dry weight). Influenced by the biomass productivities, the sequence of lipid productivities in cultures was not identical to that of the lipid contents.

Table 9. Growth parameters and lipid production of *C. zofingiensis* when recycling harvest water at a degree of 100% or 50% under different nutrient conditions (Table 3, Paper III).

Recycling classification*	Specific growth rate μ (day^{-1})	Doubling time (days)	Biomass productivity (mg L^{-1} day^{-1})	Lipid content (%)	Lipid productivity (mg L^{-1} day^{-1})
$HW_{100\%}$ N+P+	0.345	2.01	253.48	26.17	66.34
$HW_{100\%}$ N+P–	0.289	2.39	197.73	42.71	84.44
$HW_{100\%}$ N–P+	0.307	2.26	217.58	41.21	89.65
$HW_{100\%}$ N–P–	0.334	2.08	86.30	46.21	39.88
$HW_{50\%}$ N+P+	0.403	1.72	266.66	26.90	71.73

* $HW_{100\%}$ and $HW_{50\%}$ show that the ratios for harvest water are 100% and 50% in all cases in this sub-section.

Ten types of fatty acids resulted from the algae cultivated in the harvest water media (Table 10). The main FAME ingredients were C16:0, C18:2 and C18:3. Long-chain fatty acids (C22:0, C22:1 and C24:0) were detected in small quantities in all cultures. The FAMEs containing 16 or 18 carbons (C16:0, C18:0, C16:1, C18:2 and C18:3) accounted for from 81.95% (100% harvest water N–P– culture) to 94.76% (50% harvest water N+P+ culture) of the total FAMEs, which could help improve biodiesel quality (Huang et al. 2010).

The FAME yields ranged from 6.16% to 10.95% of the dry cell weight with the following relationship: nutrient-limited cultures > nutrient-full cultures. Still, affected by the biomass productivities, the 100% harvest water N–P– culture, which observed the highest FAME yields, shared the lowest biodiesel productivity at 9.45 mg L^{-1} day^{-1}. However, no additional nutrient needs to be added into the culture system with N and P limitation, which will save costs. It is worthy of note that the 50% harvest water N+P+ culture is a preferable solution to grow algae since substantial biodiesel productivities (16.42 mg L^{-1} day^{-1}) can be achieved, the costs of nutrient supply can be reduced, and environmental advantages can be obtained via wastewater treatment.

Table 10. Summary of FAME profile for *C. zofingiensis* when recycling harvest water at a degree of 100% or 50% under different nutrient conditions (Table 4, Paper III).

FAME composition		$HW_{100\%}$ N+P+	$HW_{100\%}$ N+P–	$HW_{100\%}$ N–P+	$HW_{100\%}$ N–P–	$HW_{50\%}$ N+P+
Saturated fatty acids (% of total FAME)	C16:0	31.88	41.35	42.34	38.93	32.92
	C18:0	3.94	6.73	7.14	7.15	5.06
	C24:0	3.80	1.15	4.42	0.61	2.98
	Subtotal	39.63	49.23	53.90	46.69	40.97
Monoenoic fatty acids (% of total FAME)	C16:1	4.42	1.45	0.45	5.58	4.40
	C20:1	2.90	3.93	5.20	0.31	4.40
	C22:1	4.33	1.33	1.47	1.08	1.69
	C24:1	2.89	2.83	3.91	2.52	4.48
	Subtotal	14.55	9.54	11.03	9.49	14.96
Polyenoic fatty acids (% of total FAME)	C18:2	30.67	27.25	21.68	29.21	24.85
	C18:3	11.33	13.29	12.84	13.89	14.72
	C20:2	3.82	0.70	0.54	0.72	4.50
	Subtotal	45.82	41.24	35.07	43.82	44.07
C16–C18 (% of total FAME)		82.25	90.06	84.46	94.76	81.95
Total (% of dw)		6.72	10.07	9.49	10.95	6.16
Biodiesel productivity (mg L^{-1} day^{-1})		17.03	19.91	20.66	9.45	16.42

3.4.2 Times for harvest water recycling

Figure 22 addresses the question of whether the harvest water can be fully recycled multiple times.

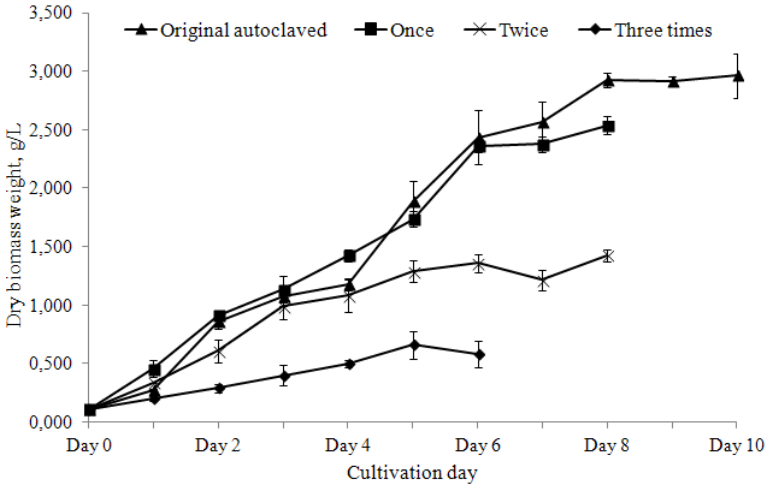

Figure 22. Growth performance changes of *C. zofingiensis* with harvest water recycling times in a full medium (Fig. 3, Paper III).

As Figure 22 shows, after recycling the harvest water twice under full nutrient conditions, there was an evident inhibition phenomenon in the culture. The inhibitive factors could be some harmful metabolites with continuous accumulation, salinity stress due to evaporation, and/or inhibitory bacteria. Thus, harvest water could be 100% recycled twice to prepare a full nutrient medium to re-cultivate *C. zofingiensis*.

3.5 Scale-up potential of cultivating *C. zofingiensis*

It has been shown above that an integrated approach which combines *C. zofingiensis* cultivation with piggery wastewater treatment can be successfully achieved; it has subsequently been verified that harvest water can be recycled to re-produce *C. zofingiensis*. Nonetheless, microalgae cultivation for biodiesel production is still in its infancy, although it is an apparently promising approach. The bottlenecks mainly lie in three scenarios. First, most relevant findings have been drawn from lab studies, and thus microalgae are not yet being commercially produced on a large scale for bulk application. Second, wastewater pretreatment (usually by sterilization via autoclave) before the introduction of microalgae is energy-intensive and complex in real operation. Last but not least, batch operation, which is the prevailing feeding mode, is time-consuming, limiting capacity

and ability to treat wastewaters. In this part of the investigation, in order to reveal the potential of amplifying microalgae production for biodiesel conversion, (1) sodium hypochlorite (NaClO) was used to pretreat the piggery wastewater, (2) outdoor cultivation was determined, and (3) a semi-continuous feeding operation was employed.

3.5.1 Nutrient removal in autoclaved culture VS NaClO-pretreated culture

Figure 23 illustrates nutrient removal in the cultivation of *C. zofingiensis* with autoclaved medium and NaClO-pretreated medium under indoor conditions. From the figure, it can be seen that there was no obvious difference in nutrient removals when cultivating *C. zofingiensis* with autoclaved medium and NaClO-pretreated medium. After 10-day cultivation, *C. zofingiensis* grown in autoclaved piggery wastewater was able to remove 79.8% COD, 82.7% TN and 98.2% TP; using NaClO-pretreated piggery wastewater to culture *C. zofingiensis*, 78.3% COD, 84.5% TN and 95.3% TP removal was observed. Thus, from a nutrient removal point of view NaClO pretreatment can replace the autoclave method and be used to pretreat wastewaters before the introduction of microalgae in practice.

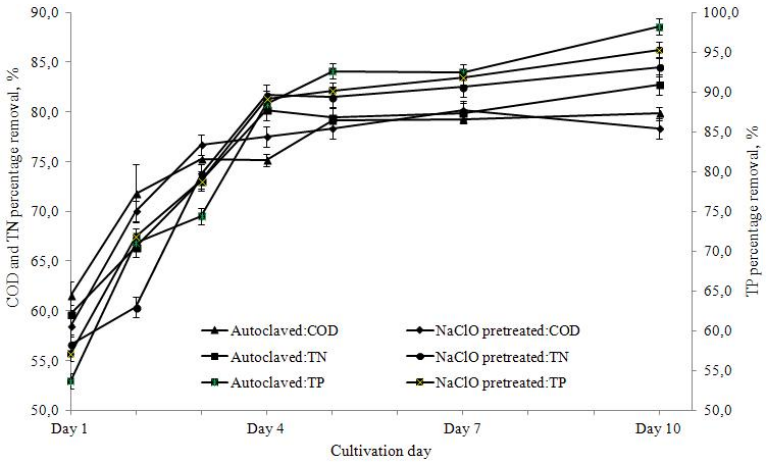

Figure 23. Nutrient removals by *C. zofingiensis* grown in piggery wastewater pretreated by autoclave and NaClO under indoor conditions (Fig. 1, Paper IV).

3.5.2 Indoor VS outdoor cultivation

The growth characteristics of *C. zofingiensis* grown under indoor and outdoor conditions with piggery wastewater are exhibited in Figure 24. It can be seen that

there was no evident difference in the growth of microalgae cultivated in autoclaved piggery wastewater and NaClO-pretreated piggery wastewater.

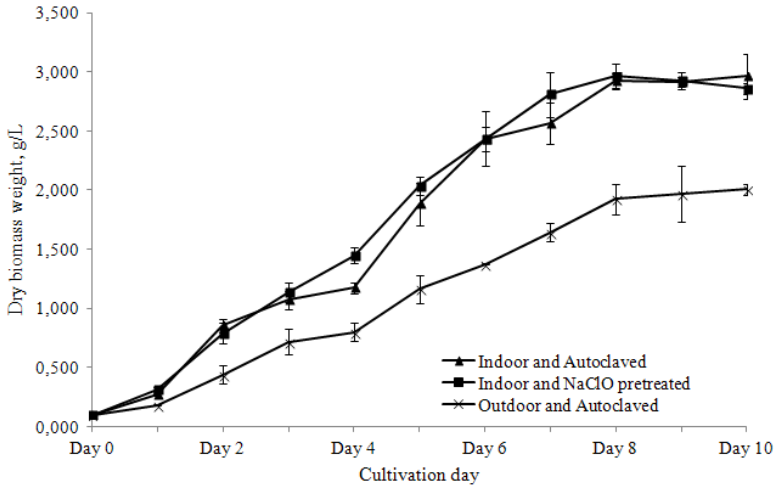

Figure 24. Growth curves for *C. zofingiensis* grown under indoor and outdoor conditions with different pretreatments (Fig. 2, Paper IV).

Under outdoor conditions, weather, temperature and light intensity are inconsistent. Amazingly, *C. zofingiensis* was able to survive and adapt well outdoors, with a comparable specific growth rate of 0.337 day^{-1}, although less biomass productivity was observed (Table 11). Nonetheless, outdoor cultivation with easy access to sunlight is the most viable solution, since it can reduce the energy input and thus improve cost effectiveness (Lam and Lee 2012b).

Table 11. Growth parameters of *C. zofingiensis* in tbcPBRs under indoor and outdoor conditions with different pretreatments (Table 2, Paper IV).

Culturing condition	Specific growth rate μ (day^{-1})	Doubling time (days)	Biomass productivity (mg L^{-1} day^{-1})
Indoor and Autoclaved	0.340	2.04	296.16
Indoor and NaClO-pretreated	0.320	2.17	285.96
Outdoor and Autoclaved	0.337	2.06	200.58

The microalgae cultivated outdoors had a little higher lipid content (around 35%) than indoor algae (around 33%), as shown in Figure 25. However, the lipid productivity of indoor microalgae was higher than that of outdoor microalgae since outdoor microalgae had lower biomass productivity.

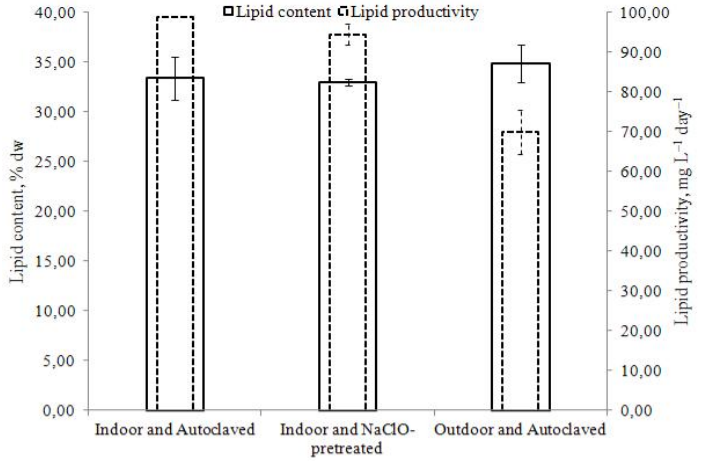

Figure 25. Lipid content and productivity of *C. zofingiensis* grown under indoor and outdoor conditions with different pretreatments (Fig. 4, Paper IV).

The FAME composition of *C. zofingiensis* cultivated under indoor and outdoor conditions with different pretreatments for 10 days is exhibited in Figure 26. In all cases, the main FAME ingredients were C16:0, C18:0, C18:2 and C18:3. The FAMEs containing 16 or 18 carbons (C16:0, C18:0, C16:1, C18:2 and C18:3) occupied 71.79 % to 84.97% of total FAME. For each pretreatment method (either autoclave or NaClO) there was no obvious difference in the FAME profile of *C. zofingiensis* under indoor operation. The proportion of unsaturated fatty acid methyl esters from outdoor *C. zofingiensis* was higher than that from indoor algae, which could lead to improved engine performance in cold climates since the pour point can be decreased.

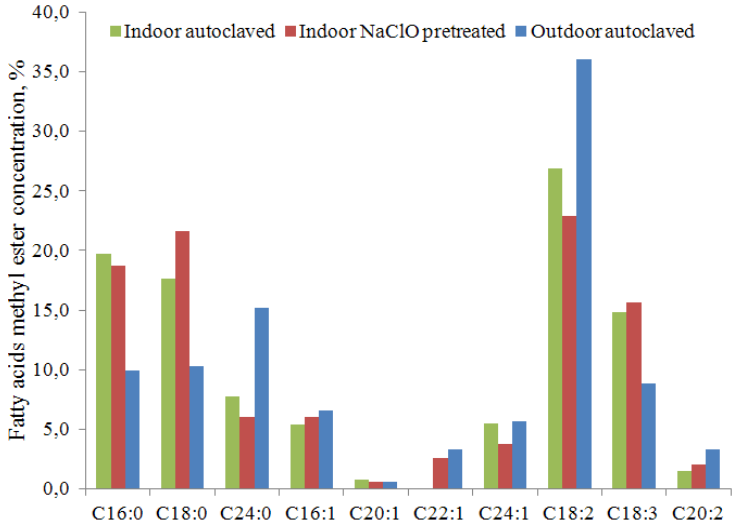

Figure 26. Fatty acids methyl ester profile of *C. zofingiensis* grown under indoor and outdoor conditions with different pretreatments.

The results also show that the FAME yields of *C. zofingiensis* grown in the autoclaved and NaClO-pretreated media were close, with 11.15 and 10.18 g-biodiesel/100g-dry weight, respectively. The FAME yield of algae cultivated outdoors was a little lower than that of indoor algae, reaching up to 9.19 g-biodiesel/100g-dry weight.

3.5.3 Semi-continuous operation for algae production

During the extensive experiment, the semi-continuous operation was first carried out in batch mode for six days and then in semi-continuous mode with 50% of algae culture solution harvested and the same amount of fresh wastewater replenished every 36 h for a period of nine days. The wastewater used in batch and semi-continuous operation was 1900 mg L^{-1} COD culture. The results showed that the daily biomass productivity during semi-continuous operation verified the robustness of the culture strategy, since the average biomass concentration was achieved at 1.971 g L^{-1}, with a net productivity of 1.314 g L^{-1} day^{-1}, which was higher than the results in the batch culture, as shown in Table 11. According to Figure 23, it can be extrapolated that most of the pollutants can be removed during semi-continuous operation.

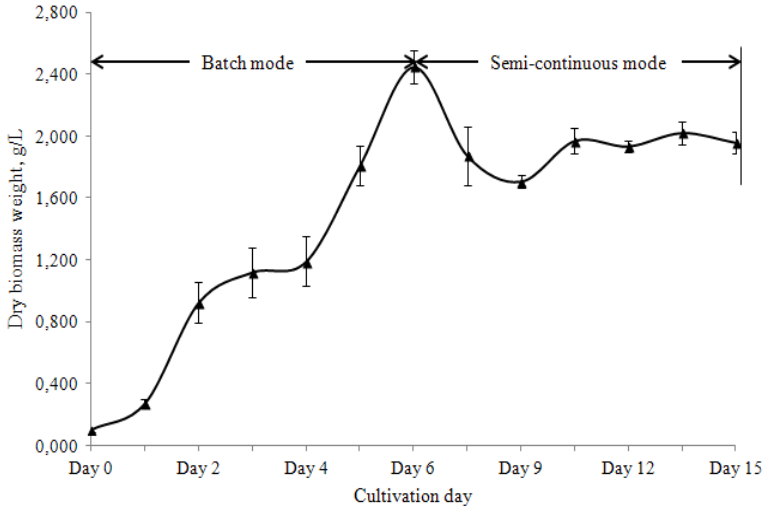

Figure 27. Growth curve of *C. zofingiensis* during semi-continuous operation process under indoor conditions (Fig. 5, Paper IV).

The findings in the present sub-section 3.5 can serve as a foundation for further scale-up trials using piggery wastewater for *C. zofingiensis* biomass and biodiesel production.

4 DISCUSSION AND FUTURE RESEARCH

4.1 Technology feasibility

In the quest for sustainable development, microalgal biodiesel, as a renewable and sustainable energy type, has enjoyed a surge in popularity (Chisti 2007; Chen et al. 2012; Feng et al. 2012; Ito et al. 2012). The potential risks of microalgae production have been predicted in Paper I. However, microalgal biodiesel applications do appear to be highly economically convenient – but only in conjunction with wastewater treatment (Samori et al. 2013). Meanwhile, with the development of the poultry industry, pollution by animal wastewaters is becoming an increasingly severe problem where disposal is inappropriate. Thus, microalgae production can be productively integrated with poultry wastewater for biodiesel conversion for future usage. From a technical point of view, *C. zofingiensis* cultivation in piggery wastewater for scaled-up production is feasible.

First, Paper II provided the basic data for the technology for this process. Paper II introduced and determined an integrated approach to combine *C. zofingiensis* production with piggery wastewater treatment. Contaminants were removed efficiently, with COD, TN and TP removal ranging from 65.81% to 79.84%, from 68.96% to 82.70% and from 85.00% to 100%, respectively (Table 7). The lipid and biodiesel productivity ranged from 48.69 to 110.56 mg L^{-1} day^{-1} and from 11.85 to 30.14 mg L^{-1} day^{-1}, respectively (Table 8). The diluted piggery wastewater with 1900 mg L^{-1} COD provided an optimal nutrient concentration for *C. zofingiensis* cultivation, showing advantageous nutrient removal and the highest productivities of biomass, lipid and biodiesel. This optimal nutrient concentration was subsequently used in Paper IV.

Second, harvest water recycling can efficiently save freshwater, which can speed up the scale-up process, especially in water-restrained areas. Paper III investigated harvest water recycling for *C. zofingiensis* re-cultivation. The 50% harvest water N+P+ culture was a preferable solution to grow *C. zofingiensis*, since substantially high biodiesel productivities of 16.42 mg L^{-1} day^{-1} can be achieved (Table 10) and wastewater could be treated, and at the same time the costs of nutrient supply could be reduced. Still, the experiment also demonstrated that 100% of the harvest water could be recycled twice with the addition of sufficient nutrients. These findings can help provide guidance for operation in practice.

Third, the performances in algal growth (Figure 24 and Table 11) and nutrient removal (Figure 23) were not obviously affected by the wastewater pretreatment

methods with either NaClO or autoclave. Thus, NaClO pretreatment can replace autoclave, since NaClO sterilization provides an easier and cheaper way for wastewater pretreatment before the introduction of microalgae. During the experiments, it was found that without sterilizing the wastewater, the system was easily polluted by a local algae species called *Scenedesmus* sp. and protozoa such as rotifer. Therefore, wastewater pretreatment is a necessary step before the introduction of *C. zofingiensis*.

Fourth, *C. zofingiensis* was able to adapt and grow well under uncontrolled conditions, where weather, temperature and light intensity were inconsistent. This makes sense since outdoor cultures have easy access to sunlight, and improves cost-effectiveness.

Finally, the semi-continuous feeding operation replacing 50% of algae culture with fresh wastewater every 1.5 days was able to provide a stable net biomass productivity of 1.314 g L^{-1} day^{-1}. The retention time for wastewater under the semi-continuous operation is much shorter than under batch mode, which can improve the daily processing capacity of wastewater treatment plants. From Figure 23, it can be seen that nutrient reduction was evident even in the beginning phase. Thus, it can be extrapolated that most of the wastewater nutrients can be removed during the semi-continuous feeding operation.

On the basis of the above discussion, it can therefore be concluded that is eminently possible to amplify the cultivation of *C. zofingiensis* in piggery wastewater in practice. The proposed scale-up scheme for *C. zofingiensis*-based biodiesel production using piggery wastewater is exhibited in Figure 28. The physical layout requirement for the integrated approach is that the algal ponds must be located close to the pig farm to reduce transportation. Due to less capital cost in open pond systems than in PBR, the proposed scale-up system will use open pond as the production facility. In order to make use of post-extracts and sustain microalgal biodiesel production, the lipid leftovers, together with other residuals rich in proteins and carbohydrates (Huo et al. 2012), can be used for biogas production via anaerobic digestion. Sialve et al. (2009) calculate the theoretical methane yields for lipids, proteins and carbohydrates as 1.014, 0.851 and 0.415 L g^{-1}, respectively. The residues after anaerobic digestion can be supplied as the nutrient sources for the re-cultivation of algae, or can be sold as fertilizers. Apart from biodiesel, biogas and fertilizer as shown in Figure 28, value-added products including bio-ethanol, high-value protein, cosmetics, etc., can also be produced in algae biomass production.

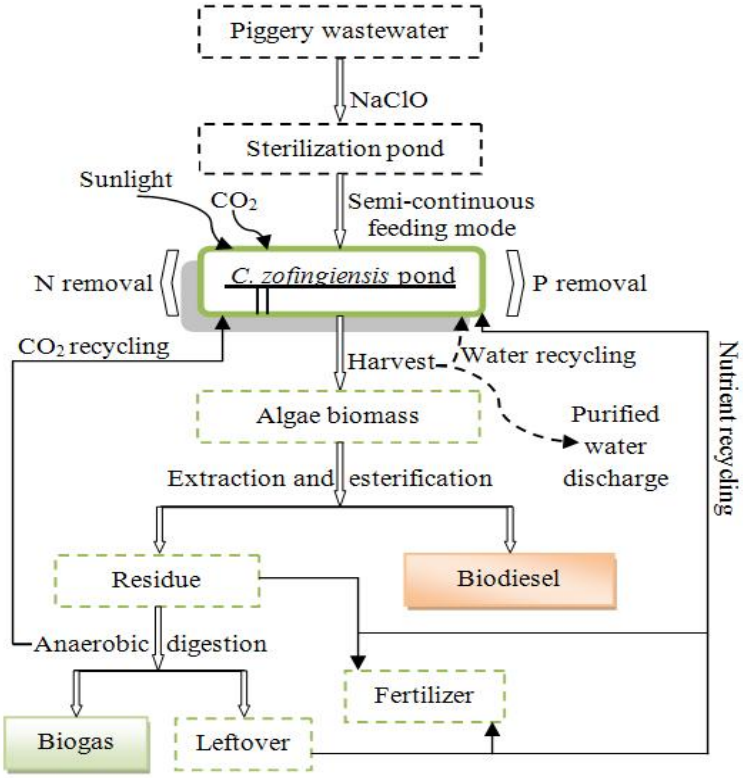

Figure 28. Proposed scale-up scheme for *C. zofingiensis*-based biofuel production using piggery wastewater (Fig. 5, Paper II).

4.2 Net energy ratio (NER) analysis

The potential balance of microalgal biodiesel in energy varies widely depending on the cultivation system, energy savings realized by the co-products, and so on. Energy inputs may include electricity, heat, pressure and other forms of energy needed to operate technologies. Lardon et al. (2009) found that the energy used in microalgal oil extraction was 0.37 MJ/MJ biodiesel for wet extraction and cultivation under low-nutrient conditions, while energy used for transesterification was 0.0024 MJ/MJ biodiesel, assuming HHV of 38 MJ/kg microalgal biodiesel. The net energy ratio (NER) assessment is therefore of great importance in the present study. This part of the investigation examined the NER related to microalgal biodiesel production in order to testify the energy balance of the process.

The NER in this case has been defined as the ratio of the total biodiesel produced over the energy required during operations (Singh and Gu 2010):

$$NER = \sum \text{Energy produced} / \sum \text{Energy required.}$$

The higher the NER is, the more positive the chain will be. In this work, the NER is calculated in four scenarios: ① semi-continuous operation at small scale (indoors); pilot-scale operation in ② winter and ③ summer (outdoors, Figure 29–30); ④ one estimated operation (semi-continuous operation at pilot scale in summer). The NER calculation is based on the estimation that the dry biomass productivity over a three-month period is 1 ton. The results are shown in Table 12.

Table 12. NER analysis for *C. zofingiensis*-based oil production in southern China.

Parameter	①	②	③	④ [a]
Cell density (g L^{-1})	1.971	1.004	1.221	1.971
Biomass productivity (mg L^{-1} day^{-1})	1314.00	66.94	305.25	1314.00
Cultivation time per run (d)	1.5	15.0	4.0	1.5
tbcPBR volume required for biomass production of 1 ton (m^3)	8.5	166.0	36.4	8.5
tbcPBR unit treatable volume (L)	0.8	33.0	33.0	33.0
tbcPBR unit amount	176	838	49	11
Area (m^2)	9	204	12	3
Wastewater needed (m^3)	507	996	819	507
Energy consumption by wastewater filling (MJ)	170	338	275	170
Energy consumption by water pumping for cooling (MJ)	–	–	194	–
Energy consumption by air pumping (MJ)	12442	93312	5832	1166
Energy consumption by air conditioning (MJ)	1458	–	–	–
Energy consumption by illumination (MJ)	1166	–	–	–
Energy consumption by harvest (MJ)	1010	1980	1624	1010
Energy consumption by drying (MJ)	864	864	864	864
Total consumption for biomass production (MJ)	17110	96494	8789	3210
Calorific values of Chlorella biomass (MJ kg^{-1}) [b]	24	24	24	24
Energy produced as biomass (MJ)	23500	23500	23500	23500
NER for biomass production	***1.37***	***0.24***	***2.67***	***7.32***
Energy consumption by extraction and conversion (MJ)	1008	1008	1008	1008
Total consumption (MJ)	18118	97502	9797	4218
Average oil percentage by estimation (% of dw)	30	30	30	30
Oil yield (kg)	300	300	300	300
Calorific values of oil (MJ kg^{-1}) [c]	37	37	37	37
Energy produced as oil (MJ)	11100	11100	11100	11100
NER for oil production	***0.61***	***0.11***	***1.13***	***2.63***

[a] Scenario ④ is based on the data obtained in Scenario ①, and thus its data are estimated values.
[b] 24 MJ kg^{-1} is used according to Illman et al. (2000).
[c] The calorific value of rapeseed crude oil at 37 MJ/kg is used (Sialve et al. 2009).

As it can be seen from Table 12, the pilot-scale operation in winter is not economically feasible since the NER is far less than 1. The NERs for biomass production in Scenario ①, ③ and ④ are more than 1. Nonetheless, when oil is produced, the NER is decreased by more than 50%. The NER for pilot-scale oil production in summer is just a little over 1. The estimated scenario ④ has much higher NERs for biomass and oil production, reaching 7.32 and 2.63, respectively. This scenario should be further verified in practice. Rodolfi et al. (2009) report that the NER for algae production in flat-panel photobioreactors is just barely above 1 due to the costs for mixing and harvesting. Another study has shown that the NERs for oil production in flat-plate photobioreactors, tubular photobioreactors and the raceway ponds are 0.07, 1.65 and 3.05, respectively (Jorquera et al. 2010). These values will be a little high since the authors do not consider the biomass harvest costs. In contrast, Huesemann and Benemann (2009) point out that the only process with an NER > 1 is to use raceway ponds to produce microalgae.

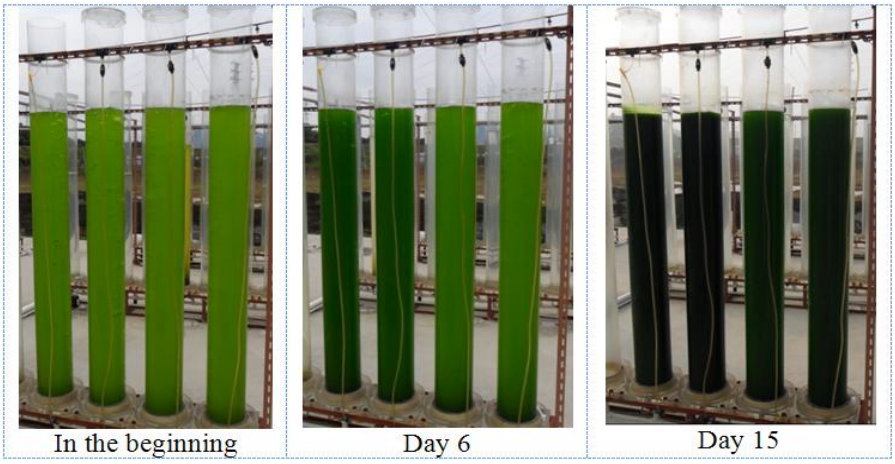

Figure 29. Cultivation of *C. zofingiensis* in pilot-scale tbcPBRs using artificial wastewater in winter in southern China (photos by Liandong).

Figure 30. Cultivation of *C. zofingiensis* in pilot-scale tbcPBRs using artificial wastewater in summer in southern China (photos by Qing Shu).

4.3 Economics and economic improvement methods

This work has verified that *C. zofingiensis* cultivation with piggery wastewater for biodiesel production and pollutant removal is environmentally sustainable. However, from the sustainability point of view, profitability is also a very important dimension, which can determine the direction and future of microalgal biodiesel. Currently, there is no commercial microalgal biodiesel production at a large scale (Chisti 2007; Sialve et al. 2009; Mata et al. 2010; Feng et al. 2011). The main issues lie in the expensive capital costs and operation costs. Assuming that CO_2 is available at no cost, Chisti (2007) suggests that the estimated cost of producing microalgal biomass is US$ 2.95 and US$ 3.80 per kg for photobioreactors and raceways, respectively. However, if the annual biomass production capacity is raised to 10,000 t, the cost will decrease to roughly US$ 0.47 and US$ 0.60 per kg for photobioreactors and raceways, respectively, because of economy of scale (Chisti 2007). Assuming that oil content is 30% of dried weight and the oil recovery process contributes 30% of the total costs, the cost of algal oil would be something like US$ 2.00 and US$ 2.59 per liter for photobioreactors and raceways, respectively. The current commodity prices in mid-2013 for gasoline and heating oil in the US are US$ 0.76 and US$ 0.79 per liter, respectively (http://oilprice.com/). Thus, we are still some way from microalgal oil commercialization due to the cost gap. Assuming that the cost of crude oil accounts for 52% of the final selling price (Chisti 2007), and that the final selling price of the microalgal oil is between US$ 2.00 and US$ 2.59 per liter, as mentioned above, for microalgal oil to be competitive to crude oil the crude oil price would have to be between US$ 165 and US$ 214 per barrel. As of mid-2013, however, the average crude oil price is about US$ 110 per barrel; in 2008 the price peaked at a mere US$ 130 per barrel.

However, environmental merits achieved via wastewater pollutant reduction and CO_2 mitigation should also be included as a beneficial value in practice. This work demonstrates that *C. zofingiensis* can effectively reduce nutrients, which can improve environmental credits. As shown in Table 12, large amounts of wastewater can be treated. The scenario ③ ④ can reduce the price of 1 ton of biomass by US$ 327.6 and US$ 202.8, respectively, if the credit for wastewater treatment at US$ 0.40 per m^3 is counted (Feng et al. 2011). In addition, limited or no fertilizers, which are easily dissolved in rainwater or run-off, need to be applied to a microalgae system when employing wastewater as the nutrient source. This can also be taken as an added environmental advantage for the algae-based wastewater treatment method. Finally, if the CO_2 is supplied by combustion gas like flue gas from power plants, carbon can be sequestered simultaneously. All of these factors add value to microalgal biodiesel production in practice.

However, in order to make algal biodiesel production more economically feasible, improvements still need to be put forward. There are several principal possible methods to improve the economics of microalgal biodiesel production in practice, described below.

(1) Versatile production of biofuels and byproducts or co-products. As shown in Table 12, the NER for biomass production is twice the NER for oil production, and thus the residues should be used in order to enhance the economics of micro-algal oil production. As illustrated in Figure 28, apart from biodiesel and biogas, valued-added products including bio-ethanol, high-value proteins, and cosmetics can also be produced. Biofuels are one application, but nutritious feed, food and other materials more valuable than fuels can be produced as well (Figure 31 and 32).

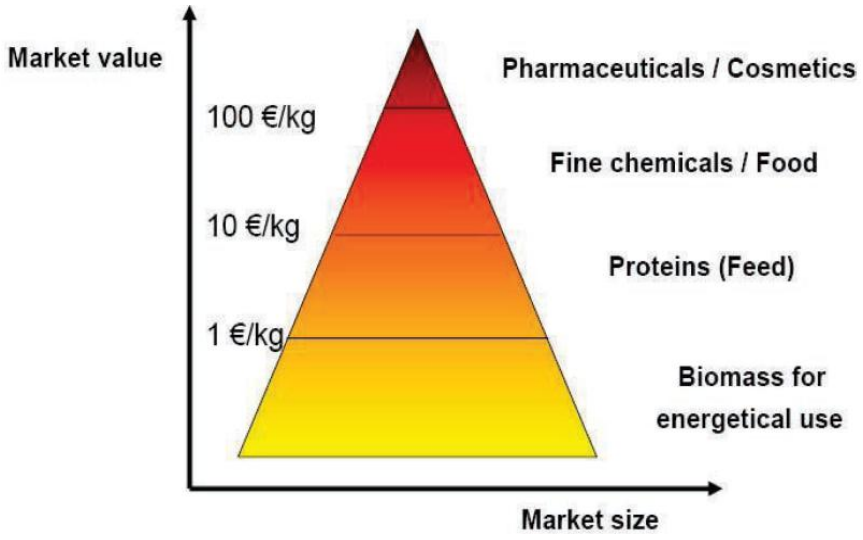

Figure 31. Value pyramid for algae product markets (SEI 2009).

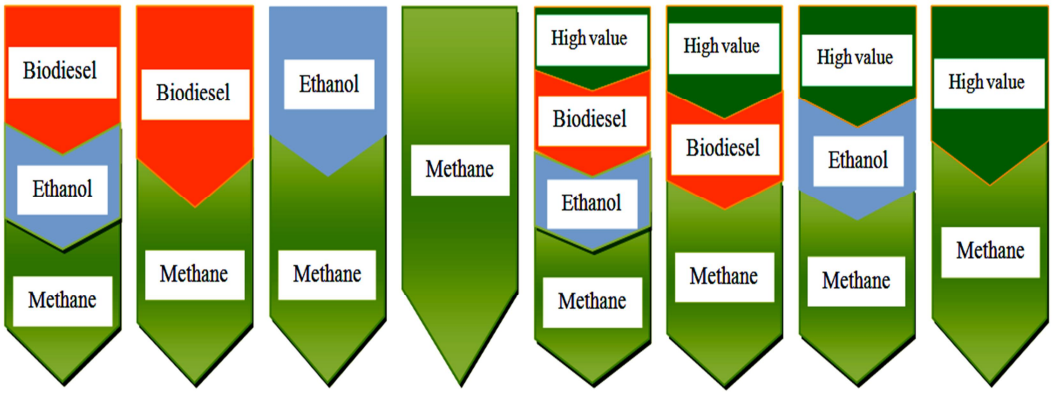

Figure 32. Biorefinery option concepts for algal biofuels and high-value products.

By-products or co-products from the biodiesel production process can also improve its cost-effectiveness. As far as biodiesel esterification is concerned, the main by-product is glycerol. Glycerol is an expensive and versatile chemical with over 1500 known commercial applications (SEI 2009). More than 400,000 tons of glycerol can be co-produced when extracting 1 billion gallons of biodiesel (Nilles 2005). In addition, poly-unsaturated fatty acids (PUFAs) are a potential co-product of microalgal biodiesel production. Microalgal PUFAs, which are rich in omega-3 fatty acids, are a vegetable alternative to, e.g., fish oils. The PUFAs would be extracted prior to oil esterification, as these fatty acids are not the most efficient ingredients for esterification (SEI 2009).

(2) Improving algal biological properties. There are two measures to achieve the goal. One is to develop cultivation techniques, focusing for example on stress levels (e.g., nutrient starvation) and light control to achieve the desired levels of lipids, protein and other commercially significant materials. In the present study, if the average oil content could be improved to 50%, the NERs for oil production in Scenario ①, ②, ③ and ④ would increase from 0.61, 0.11, 1.13 and 2.63 to 1.02, 0.19, 1.89 and 4.39, respectively. Another method is to use genetic and metabolic engineering to, for example, increase photosynthetic efficiency, enhance biomass growth rate, trigger the accumulation of oil, improve temperature tolerance to reduce the expense of cooling, reduce photoinhibition, etc.

(3) Reducing energy input. The best stages at which to reduce energy input are harvest and drying. Costs related to harvest contribute 20–30% to the total cost of the biomass production (Mata et al. 2010), but these costs can be significantly reduced by optimizing various processes. Further R&D efforts are still required here. Drying needs lots of energy and thus is a principal economic bottleneck of the process as a whole. Sun-drying is geography-dependent, but is cheap and can be used in sunlight-rich areas.

(4) Cultivation system engineering. For both PBRs and open pond systems, light penetration into the deeper levels and mixing are two key factors which can affect the system efficiency. The concentration of cells, optical properties of the culture, intensity of turbulence, diameter of the tube, and the external irradiance level will influence the light penetration and thus the photosynthetic efficiency of microalgal cells (Molina et al. 2001). Some microalgae prefer to grow attached to the internal wall of cultivation systems, thus preventing light penetration into the culture and reducing system productivity (Chisti 2007). Thus, robust methods for wall growth control are necessary. By mixing, light–dark cycling in the cultures can be achieved. In cultures with high biomass density, mixing can reduce photoinhibition of the upper layer of algal cells and allow every algal cell equal ac-

cess to light. However, mixing can affect algal growth: if the culture is mixed too fast (light–dark cycling is too frequent) the microalgae will be damaged. This is because some algae are more sensitive to shear damage (shear sensitivity) under high-speed flow (Mazzuca et al. 2006). Thus, high-efficiency cultivation systems with advanced mixing should be designed to generate optimal light–dark cycling.

(5) Policy support. Apart from technological research, the likeliest future influence on microalgal biodiesel practice will be government policies on carbon dioxide emissions and tax. Substantial investment and subsidies will also be required to increase cost-effectiveness, limit risks, and make this practice a reality.

Through the development of technology and with the help of government policy, the cost gap can be shortened or closed. The average crude oil price in 2002 was US$ 26 per barrel (Gallagher 2011) and increased at an annual rate of 14% to US$ 110 per barrel by the middle of 2013, despite the global economic downturn. Especially since the crude oil price might go up in future due to the limited reserves, there is hope that the gap can be closed (Figure 33).

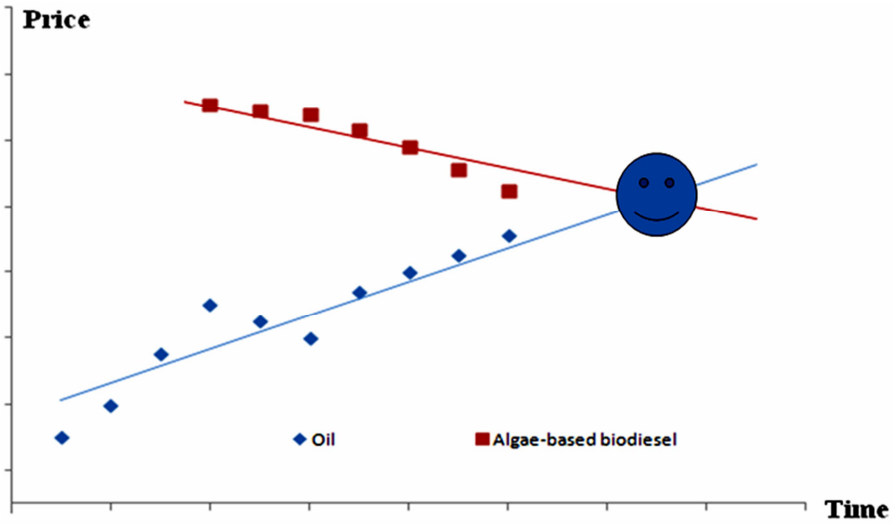

Figure 33. The cost gap between microalgal biodiesel and crude oil.

4.4 Research limitation

All the experiments presented in the present study were carried out using tbcPBR. However, the capital cost for tbcPBR is higher than for open pond systems, although its productivity is high. Thus, it is necessary to assess the respective situation when using ponds to produce *C. zofingiensis*. If the pond productivity is high, then the economics can be improved due to low costs in start-up phase. Methods

to improve the algae growth performance in pond systems should also be researched.

Although this dissertation verifies that *C. zofingiensis* can be grown in piggery wastewater for biodiesel conversion, there is no guarantee that all microalgae species can adapt to piggery wastewater. For different species, nutrient removal, microalgal growth performance, lipid accumulation and fatty acid composition are very variable.

The lipid and biodiesel productivities were not determined under semi-continuous operation. Under this mode, high and stable biomass productivity of 1.314 g L^{-1} day^{-1} was achieved. However, the lipid content and FAME yields are still unknown and should be investigated to support this culture strategy.

4.5 Future research

This work has demonstrated that the cultivation of freshwater microalgae *C. zofingiensis* in piggery wastewater for biodiesel production is promising and can be considered for large-scale production. To support a microalgal biodiesel industry, future research is still needed, including but not limited to the following points.

(1) Resistance to organic shock loads. When using algal technology to treat wastewater, the ability to resist the introduced organic shock loads should be further verified since the discharge of wastewater might fluctuate considerably. Additionally, the biomass productivity might be affected and thus should be further investigated as well.

(2) Species pollution control. Without sterilization of wastewater, *C. zofingiensis* was able to grow, but the culture was easily polluted by a local species *Scenedesmus* sp., rotifer, etc. (Figure 34). Although wastewater can be sterilized beforehand using NaClO, there is no guarantee that, during long-term operation, the system (especially the open system) will not be contaminated by other algae species, protozoa, or bacteria. Pollution control will be demanding and thus costly, and this should therefore be a further focus for future research. If pollution control is successful, NaClO for wastewater pretreatment will no longer be required.

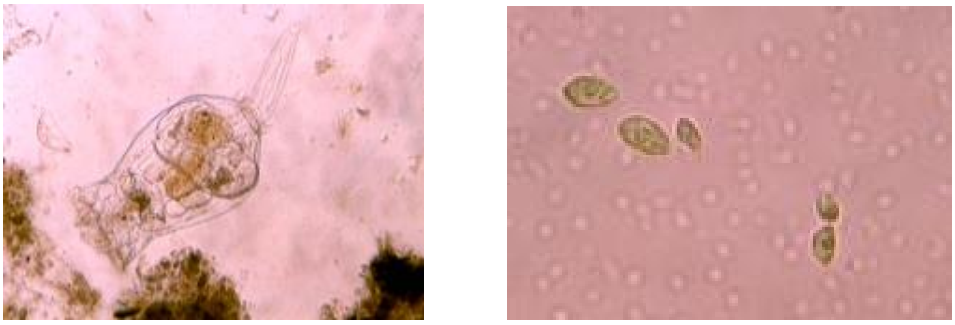

Figure 34. Pollution phenomena during algae production (photo by Qing Shu, Left: Rotifer; Right: *Scenedesmus* sp.).

(3) Metabolites. After recycling harvest water twice at a degree of 100%, the inhibition phenomenon occurred. One interesting future study would seek to discover which inhibitive factors (e.g., harmful metabolites and bacteria) are important here.

(4) Combined biofuels production to use residues. Algae are rich in lipids, sugar and protein. Lipids in algae can be refined into biodiesel, while the carbohydrates can be converted into ethanol. Ethanol can be obtained from microalgae by converting the starch (the storage component) and cellulose (the cell wall component). Carbohydrates, proteins and fats in microalgae tissues can be made into methane. The potential integrated biofuel chain approach for the continuous production of three biofuels, as shown in Figure 35, should be further researched.

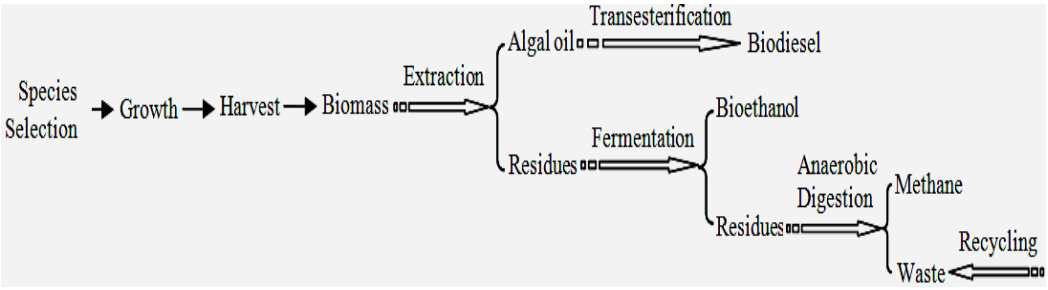

Figure 35. A potential integrated biorefinery approach (Zhu et al. 2012).

(5) Winter performance. As shown in Table 12, the winter performance of *C. zofingiensis* is poor, where the air temperature and light intensity varied from 5.7°C to 20.0°C and from 6 to 1810 μmol m^{-2} s^{-1}, respectively. Thus, how to improve the productivity in winter in subtropical monsoon zones is also an urgent issue.

5 CONCLUSIONS

Working towards a sustainable microalgal biofuel industry, this study has investigated the potential risks associated with microalgae production from a broad perspective, including the environmental, economic, social and cultural dimensions. It was shown that the relative benefits can be fully exploited and that the potential impacts can be foreseen and minimized or even eliminated to some degree.

Using piggery wastewater to cultivate freshwater microalgae *C. zofingiensis*, integrating biodiesel production with wastewater treatment, is a promising solution to help ease the world energy crisis and climate change. Pollutants including COD, TN and TP in piggery wastewater were efficiently removed and biomass and lipids were accumulated simultaneously. The diluted piggery wastewater with 1900 mg L^{-1} COD provided an optimal nutrient concentration for *C. zofingiensis* cultivation, where advantageous nutrient removal and the highest productivities of biomass, lipid and biodiesel were presented.

Harvest water from *C. zofingiensis* production can be used to prepare media with full nutrients and nutrient limitation. These media were reused to produce *C. zofingiensis*. Nutrient-limited cultures showed much higher lipid content (41.21% – 46.21% of dry weight) than nutrient-full cultures (26% of dry weight). The N- and P-limited medium observed the highest FAME yield, at 10.95% of dry weight, while the N-limited culture and P-limited culture shared the highest biodiesel productivity, at around 20 mg L^{-1} day^{-1}. Harvest water from algae production could be 100% recycled twice with the addition of sufficient nutrients.

The optimally diluted piggery wastewater with 1900 mg L^{-1} COD was used to carry out the scale-up potential experiments. It was found that NaClO can be effectively used to pretreat piggery wastewater without any obvious impacts on the nutrient removal and the productivity of biomass, lipids and biodiesel. *C. zofingiensis* could grow well outdoors to robustly accumulate biomass and lipids. The semi-continuous feeding operation by replacing 50% of algae culture with fresh wastewater every 1.5 days was able to provide a stable net biomass productivity of 1.314 g L^{-1} day^{-1}. These findings plus the success of harvest water recycling can serve as a foundation for further scale-up trials using piggery wastewater for *C. zofingiensis* biomass and biodiesel production.

Thus, *C. zofingiensis* cultivation in piggery wastewater for biodiesel production is environmentally sustainable, especially at the level of water sustainability, and thus is a promising solution to solve both environmental and energy issues. However, its economics should be further improved during the scale-up process via methods such as algal residue reuse and biological property improvement.

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Microalgae production as a biofuel feedstock: risks and challenges

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From a sustainability perspective, the potential risks associated with microalgae production for biofuel extraction will be investigated in this paper, including the environmental, economic, social and cultural dimensions. Environmentally, four main concerns are mapped out: first, there are potential water safety risks, such as water resource abuse, water pollution and groundwater recharge deficiency; second, unreasonable construction will lead to land-use overexpansion, land pollution and service expectancy reduction; third, microalgae production may exert a detrimental effect on the local ecosystem, causing algal blooms and biological invasion; finally, microalgae might emit unexpected greenhouse gases [nitrogen oxides (NO_x), methane (CH_4)] and ammonia (NH_3). From an economic risk standpoint, microalgae production requires an overwhelming investment due to the expensive start-up and operation. Socially, contaminant discharge will threaten the health of local animals and people. Moreover, the safety effects of genetically modified algae may not be immediately apparent or known. In addition, over time, microalgae may become a medium for mosquitoes to spread disease. From a cultural point of view, it requires time for people in developing countries to adapt algal oil to their daily life as an alternative to conventional fossil fuels. Furthermore, personnel changes in the workforce may harm existing cultural structures, and landscape aesthetics may be affected by system construction. Taking the above challenges into consideration, efficient government policies, proactive company behaviours and positive public participation will play an important role in minimising or even eliminating these potential risks.

Keywords: microalgae production; biofuel; impact; risk; challenge

Introduction

There has been ever-increasing demand for diesel supply in the world. In European countries, especially Austria, Spain, France and Italy, market share of diesel-based cars has exceeded 50% since 2006 (Neste Oil 2006). Based on the current consumption of about 11.6 million tons of crude oil per day, it is expected that the existing resources can only suffice for a rather short time (Vasudevan and Briggs 2008). Analysing global oil depletion, the UK Energy Research Centre concluded that a peak of conventional oil production would be reached between 2020 and 2030 when the easily available resources will be used up (Sorrell et al. 2009). However, new oil and gas reserves have constantly been found. The most exciting discovery is that new geological surveys show that as much as a fifth of the world's undiscovered but exploitable gas and oil reserves lie under the Arctic ice (McCarthy 2008). In this situation, potential oil and gas refining will increase fossil fuel reserves. The use of fossil fuels can result in increased emissions of carbon dioxide (CO_2) to the atmosphere (Naustdalslid 2011), causing the risk of an exponential rise of the greenhouse effect, which can result in all kinds of catastrophes to our planet Earth and its inhabitants (Kumssa and Jones 2010). Anthropogenic climate change could increase the probability of large spatial scale extreme weather events, like heat waves and floods (Phelan et al. 2010).

To confront global climate changes, biofuel (biodiesel, bioethanol, biogas, etc.), as a renewable and alternative energy source, is being developed and put into practice. Currently, microalgae-based biofuels have received much attention. There are several reasons why microalgae comprise one of the most promising biofuel feedstocks (Greenwell et al. 2010). First, microalgae have high photosynthetic efficiency and can grow very rapidly. Chisti (2008) found that microalgae could produce much more oil than other tested plant materials (e.g. soybean, corn, rapeseed, palm, sunflower) per unit growing area per year. Second, microalgae can be cultivated without occupying farmland (Aikins et al. 2009), and thus could reduce possible damage to the agricultural ecosystem and traditional food webs. Third, freshwater is not essential for microalgae, nutrients can be supplied from wastewater (Mulbry et al. 2009) and CO_2 from combustion gas (Wang et al. 2008) during cultivation. Fourth, microalgae can be collected very quickly, obviously accelerating the biodiesel production process (Avagyan 2008). Fifth, the property of uniform cell structure, without bark, stems, branches or leaves, makes the commercial production of microalgae attractive (Avagyan 2008), and the operation and control of reproduction conditions much more practical. Finally, the physical and fuel properties (e.g. density, viscosity, acid value, heating value, etc.) of biodiesel from microalgal oil in general are comparable to those of fossil fuel diesel (Miao and Wu 2006).

Recently, microalgae production for biofuel extraction has enjoyed a surge in popularity and become a

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major research interest in the energy field. Although microalgae are not yet produced at a large scale for bulk applications, recent advances – particularly in methods of system biology, genetic engineering and biorefining – present opportunities to develop this process in a sustainable and economical way within the next 10–15 years (Wijffels and Barbosa 2010). Along with the development of microalgae production technologies, commercial microalgae farming corporations at a relevant scale, such as Earthrise, Cyanotech and Oilgae, are emerging. Hence, identifying the relative sustainable impacts, including the environmental, economic, social and cultural impacts, is essential for a corporation in order to minimise potential risks associated with microalgae farming practices.

Purpose and perspective

This paper is an exploratory study and the purpose of the research is to map the environmental, economic, social and cultural risks of microalgae production for biofuel use from a sustainability perspective (Figure 1), using risk and environmental impact analyses. Identifying the core environmental, economical, social and cultural impacts associated with microalgae production is a first step in supporting development of a sustainable biofuel industry. This is especially important for the following reasons: today more than ever before, unpredictable environmental issues strongly bound with economical, social and cultural impacts of the energy sector dominate the international agenda (Demain 2009); and relative analyses can highlight any unsustainable aspects of microalgae production, so that they can be forecast in advance and either mitigated or eliminated.

There is fragmented published information available on the sustainability concerns and risks related to microalgae production. Ketola (2011) suggested some potential environmental, social, cultural and economical impacts of algae use when analysing the interrelations between food, energy and water production. From a sustainability perspective, this paper explores the potential risks and challenges related with microalgae biomass production for biofuel use, systematically and explicitly, including the environmental (water, land, biodiversity and greenhouse gases), economical, social and cultural dimensions. Subsequently, recommendations concerning government policies, company behaviours and public participation are suggested in order to minimise the potential risks.

Potential risks and challenges

Environmental effects

Water

During microalgae cultivation, a water resource is indispensable, together with its management in terms of water utilisation, downstream water and groundwater.

Demands on water for commercial microalgae biomass production can present tremendous challenges for microalgae cultivation, especially in water-constrained regions. Without feasible water-use planning for microalgae production processes, water cannot be used effectively or could even be squandered. It is reasonable to anticipate the need to manage and/or recycle water, as this will profoundly affect the scalability and sustainability of microalgae production. For instance, land-based microalgae cultivation systems will suffer from significant water losses as a result of unpredictable evaporation (Kovacevic and Wesseler 2010), during which water is removed but the salts in the water are left behind (Qin 2005). Consequently, sooner or later, microalgae growth will be reduced due to salt accumulation in the cultivation system. In this situation, it is necessary to calculate the amount of water needed to replenish the system (freshwater or low salt water), and then periodically add more water into the system.

Microalgae biomass contains considerable amounts of protein (Becker 2007), and on the basis of biomass composition, the quantity of nitrogen (N) required as fertiliser is estimated to be 8–16 tons N/ha (Sialve et al. 2009; Markou and Georgakakis 2011), which means that microalgae production involves enormous amounts of N fertiliser. Systems' discharge is inevitably the most highly regulated and possibly the most toxic component of any industrial process because it releases wastes into the environment, and microalgae production is no exception. The widespread use of large amounts of fertiliser for microalgae cultivation can lead to direct and indirect releases of reactive N into the environment. Because of the limited N efficiency, especially at increasing rates of fertiliser application, N releases can be very large. Releases can be in the form of nitrates to the groundwater, ammonia (NH_3), nitrous oxide (N_2O) and other nitrogen oxide emissions to the atmosphere (Erisman et al. 2009). Another issue is the potential risk that the whole system might fail since it is subject to environmental variables, such as climate

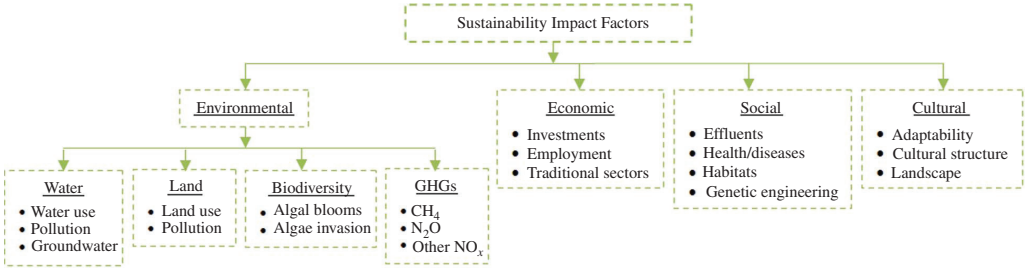

Figure 1. A framework for sustainability impact analysis.

conditions (García-González et al. 2003). For example, the National Aeronautics and Space Administration is developing an Offshore Membrane Enclosure for Growing Algae project, which is a closed system designed to grow algae in plastic bags in an open ocean system. Susceptible to weather conditions, ocean waves and tides, this system risks failure, as it requires high-quality plastic bags, which currently have a lifespan of only 2 years. Once the system fails, microalgae may die, and the high concentration of dead organic matter would start to decay. Undoubtedly, the decay process would consume dissolved oxygen in the water, resulting in hypoxic conditions (Hearn and Robson 2000), so that the system could become a secondary pollution source.

In practice, in order to reduce the use of fertilisers, wastewater rich in N and phosphorus (P) could be used as cultivation media (Markou and Georgakakis 2011), and at the same time the microalgae could be used to decrease the inorganic and organic loads of such wastewater, thus providing a method for wastewater treatment (Aslan and Kapdan 2006; Shi et al. 2007; Ayhan 2010). In this situation, compared to agriculture-based biofuel species, microalgae can significantly improve water quality since limited amounts of fertiliser, which are easily dissolved in rainwater or runoff, need to be applied to a microalgae system. However, in a microalgae cultivation system, chemicals and disinfectants are often widely used for pest prevention, system cleaning and the disinfection of culture equipment. Likewise, during biomass harvest, chemical and metal flocculants, such as alum, ferric chloride and lime (Schenk et al. 2008), are also widely used. The chemicals, especially toxic chemicals, will not be fully used up or degraded during algal biomass accumulation. As a result, receiving waterways will be contaminated and even damaged, and the local residents will suffer the effects.

Like almost all systems of agriculture and urbanisation, microalgae production will permeate groundwater. Large surface areas of concrete will interrupt the original hydrological cycle so that biological activity in the soil below will be reduced. In this situation, rainwater infiltration shortage can cause inefficient aquifer recharge (Lee and Lee 2000), which is crucial to the water purification, and can lead to a decline in the water table of an aquifer (Squeo et al. 2006). Another existing problem is that an aquifer may not be recharged effectively if water used in the whole process of algal culture is mainly extracted from ground sources but released into surface waters after use.

Land

Microalgae production systems have much lower land-quality requirements than other bioenergy activities, such as biofuel crop production. This makes huge areas of land accessible (Schenk et al. 2008), such as arid, semiarid or saline soils, infertile farmland, polluted land and other land of low economic value. However, land design and planning with accurate first-hand data and scientific predictability is essential, and lack of such data would lead to risks, such as soil pollution, soil erosion, land-use overexpansion, increased pressure on farmland, land-use efficiency reductions, etc.

The significant issues connected with the lack of supplementary facilities may not be fully anticipated in advance during system design. For example, heavy rain or flooding may lead to high-nutrient and high-biomass overflow if no rainfall diversion facility is established, which will result in soil pollution. Moreover, site preparation for pipelines and production facilities involves the removal of rocks and earth. This will change soil structure, physical and chemical properties, and consequently could cause soil erosion, compaction or hardening and even increased geological hazards.

To meet the feedstock demand for additional microalgae production and commercial profit, some areas may be inappropriately designated. This could indirectly result in land-use overexpansion for biomass production from existing agro-forestland and farmland, when consumers of the feedstock, such as food markets, do not plan to decrease their feedstock demand. Microalgae production on land requires space for large-scale facility construction (Bruton et al. 2009), which may lead to issues of occupancy of land resources and poor land-use efficiency. For instance, open systems with integrated wastewater treatment sometimes require additional ponds for microalgae screening or sediment settlement. This can be seriously restrictive in land-constrained areas where competition for land use is intense, since regional land with low current economic and ecological value may be scarce.

There is no denying that a cultivation plant can be established on unproductive land, but if rainwater storage is insufficient because of the lack of adequate precipitation, pipeline installation is inevitably required in order to obtain sufficient water to maintain algal production. This situation will intensify, especially when the plant is remote. Another concern is transportation and its mode, for example truck, railway, ship or pipeline (Kumar et al. 2006). Undoubtedly, transport emissions are directly linked to transport distance and the mode of transport (Gold and Seuring 2011).

Biodiversity

The term biodiversity is used to refer to the richness of the life forms and their associations in a biome (Suneetha 2010). In a balanced system, weather, predators, diseases and availability of food sources all affect species presence and population size (Benton 2001). Water contamination and the presence of alien invasive species will threaten the development and stability of biodiversity in any system.

A microalgae culture facility, whether a closed or open system, can be established in a water-rich delta, a desert or in the open sea. In an open system, the use of large quantities of fertiliser for microalgae cultivation can lead to direct and indirect releases of reactive N species into the environment. Moreover, in microalgae culture systems chemicals and disinfectants are widely used for pest prevention,

water treatment, and cleaning and disinfection of equipment. Moreover, during biomass harvest, flocculants are also essential. As a result, the downstream discharge of residual chemical nutrients can lead to net increases in nutrient levels in the receiving water body. Although further environmental effects have not yet been fully known assessed, eutrophication resulting from nutrient imbalance in water will give rise to toxic algal blooms and fish kills in the natural environment (Glibert et al. 2005; Estrada et al. 2009).

There are over 100,000 microalgal species, and only a handful have been well studied and adopted for widespread cultivation in the aquaculture and food industry. Creating pure microalgae culture is difficult, and involves the limitation or exclusion of natural and native species. Any exotic or potentially invasive microalgae species from system wastewater that are released into the natural environment will threaten the integrity of local and regional ecosystems, since downstream water may contain non-harvested microalgal cells. The movement or drift of microalgae carries risks for wild species, and may result in biological invasions. Through species competition, large-scale microalgal reproduction will threaten the safety of native species, and could even cause a biological disaster – species extinction (Fritts and Rodda 1998).

Greenhouse gases

Microalgae can fix CO_2 from different sources (Wang et al. 2008): (1) from the atmosphere, (2) from industrial exhaust gases (e.g. flue gas and flaring gas) and (3) from soluble carbonates (e.g. $NaHCO_3$ and Na_2CO_3). The CO_2 captured in microalgae biomass can reduce levels in the atmosphere, but a low level of CO_2 can limit microalgae growth (Chelf et al. 1993; Wang et al. 2008). In practice, extra CO_2 from combustion gases is often transferred into the culture system as a carbon source (Chisti 2007; Wang et al. 2008). When passing through the culture system, some CO_2 will be dissolved in water, which will reduce the pH. However, if the microalgae take up sufficient CO_2, then the pH will increase. The corresponding impacts caused by changing pH are unexpected and uncertain, and requires further research.

Another risk is greenhouse gas releases for microalgae systems [mainly CO_2, methane (CH_4) and N_2O] and NH_3 release. At night and on cloudy/rainy days, microalgae consume oxygen in respiration, causing anaerobic zones in the culture water, leading to the emission of CH_4 and N_2O. The emissions of N_2O generally range between 1% and 3% of the fertiliser added (IPCC 2006), whereas for NH_3 they are 0.6–9% of the fertiliser (Erisman et al. 2009). CH_4 and N_2O, respectively, possess 21- and 310-fold the global warming potential of CO_2 (IPCC 2006), while the released ammonia can lead to toxicity (Sialve et al. 2009). Moreover, if the culture system fails, dead microalgae biomass will be broken down through the action of anaerobic microorganisms. This denitrification process will increase emissions of CO_2, CH_4 and especially N_2O (Tortosa et al. 2011). All three of these gases make significant contributions to global warming (IPCC 2006; Liu et al. 2010).

Economic impacts

Theoretically, microalgae can produce between 10 and 100 times more oil per acre than 'traditional' crops, but such capacities have not been effectively commercially realised (Greenwell et al. 2010). The challenge remains to decrease and even close the cost gap between microalgae-based biofuels and fossil oil. The most important cost of microalgae production is the capital cost, which is usually highest in the start-up phase. Taking algal biodiesel as an example, capital costs can account for 49.9% of the total cost (Kovacevic and Wesseler 2010). Furthermore, operation and maintenance costs are substantial, and fertiliser is the largest contributor to the cost of algal biomass production, for example fertiliser constitutes nearly half of the overall cost of *Spirulina* cultivation (Venkataraman et al. 1982). As a result, enormous investments are required. In this situation, the biofuel price will usually stay high, which will possibly make algal biofuel production uncompetitive compared to the current price of fossil diesel (about US$3.9 per gallon in late 2011). The cost data reported in existing publications range from US$15 per barrel (Green Car 2006) to several hundred dollars per barrel (Pimentel et al. 2009), depending on climate, species, growing systems and other conditions. Confronted with this situation, investors will be cautious when planning to establish a new microalgae project, and compare and weigh the influences on cost parameters, such as biomass yield, lipid content, capital required, operational costs, energy use and other factors.

Hence, microalgae-based biofuel production may compete with other traditional microalgae uses such as cosmetics and chemicals (Spolaore et al. 2006), which may lead to a depression or shrinkage in the original industries.

Social aspects

The shortage of reliable information, including information on health and safety issues, production transparency and concerns for environmental sustainability, has been taken as the key factor in a loss of confidence in the microalgae industry by communities. Effluents contain some toxins stemming from fertilisers for algal growth, disinfectants for system cleaning and flocculants for biomass harvest. Once these substances are discharged into the environment without any treatment, they will exert a detrimental effect on the health of plants, animals and people. If the system fails, the toxic matter can reside in co-products and/or by-products, such as animal feed (Sheih et al. 2009). Once they are absorbed into the body by chemical accumulation through the food chain, over time, animals and people will suffer.

Recently, there have been some breakthroughs in identifying relevant bioenergy genes and pathways in

microalgae, and genetic modification techniques have been developed to engineer some strains (Beer et al. 2009). The application of genetically modified organisms requires a transparency of the knowledgebase on the risks and benefits concerned, since it is subject to strict safety precautions (Tamis et al. 2009). In theory, there is potential for the large-scale application of genetically modified algae, but concerns have been raised about potential effects that may not be immediately apparent or known (Greenwell et al. 2010). Besides the general protest against the application of modified organisms, safety requirements will be demanded, both for economic and energetic output due to measures such as disinfectant use in the culture system, and measures to prevent the introduction of the genetically modified species to the environment.

Another public health risk is that in an open-culture system some microalgae species may provide habitats particularly favourable for mosquito reproduction (Marten 2007), and mosquitoes attached to surface biomass might become a medium to transmit diseases, such as malaria. According to statistical data from the World Health Organization (WHO) (2008), half of the world's population is at risk of malaria, and an estimated 250 million cases led to nearly 1 million deaths in 2006. In this situation, pesticide use is a last resort, but should be used with caution since it will cause new pollution to the environment. What is more, attention needs to be paid to the impacts on wildlife habitats subjected to the changed ecology and microclimate during the large-scale construction. For example, animals may accidentally fall into a cultivating pond since they may think the green surface is a broad grassland.

Cultural impact

The idea that microalgae can be used as feedstock for food, forage, renewable chemicals (Eriksen 2008) and many other products (e.g. cosmetics, vitamins) has been widely accepted around the world. However, large-scale microalgae culture technology will only begin to attract researchers after an energy crisis. People may have the misconception of microalgae becoming a booming industry. Recent studies on microalgae cultivation have been premature and based on pilot-scale studies. Consequently, it is essential to verify the feasibility of the algal oil concept before projects can be scaled up. In industrialised countries, microalgae culture technology is accessible, while people in developing countries may hold the opinion that traditional microalgae use is the best choice, as microalgae biomass is a very new biofuel type to them. Therefore, it is difficult for such people to adapt biomass fuel in their daily life as an alternative to conventional fossil fuels. As a result, it requires considerable time to set up microalgae production in different cultures.

Along with increasing automation, the necessary work force will be small. For instance, GreenFuel Technologies Corporation laid off 19 people, almost about half of its staff, 2 years ago (LaMonica 2009). Personnel changes in the workforce cannot be beneficial, but are corrosive, the existing cultural structures of an organisation as an enterprise culture are subject to the size and nature of the organisation's workforce and external environment. Confronted with this situation, the employees will be affected by requirements for consistent and efficient performance, team cohesiveness and strong company alignment towards goal achievement.

Moreover, nowadays people attach more importance to the aesthetics of their surrounding environment. During microgalae system construction and facility establishment, the landscape may be damaged by a series of mechanical activities. Furthermore, the potentially irrational layout of production facilities will also affect landscape coordination and harmony.

Conclusions

From a sustainability perspective, the potential risks and challenges associated with the production of microalgae for biofuel use have been mapped out in this paper, systematically and explicitly, including environmental, economical, social and cultural dimensions, using knowledge from risk analysis and environmental impact analysis. These risks are summarised in Table 1.

Recommendations

Undeniably, the environmental, economical, social and cultural benefits involved in microalgae mass production have the potential to make significant contributions to a sustainable industry. However, in order to develop and commercialise a sustainable product in the long term, the associated risks, which commonly exist in production processes, must be known and addressed through relevant measures. Efficient government policies, proactive company behaviours and positive public participation will play an important role in tackling, or even eliminating, potential risks associated with the algal biofuel industry. Only in this way, can the algal biofuel industry enjoy prosperity in a sustainable manner.

From a regulation and policy standpoint, there are several mandatory measures that can advance algal industry development sustainably. Primarily, the roles and responsibilities within government agencies must be clarified in order to make sure that microalgae production can be regulated to meet the demands of the marketplace. Second, a life cycle analysis at the biofuel product design phase must be conducted to foresee any potential risks. Then, contracts for environmental impact statements must be reinforced and a regulatory industry roadmap developed. Furthermore, international regulations, guidelines and standards for sustainable microalgae production must be brought in and adopted. Moreover, sub-industry collaboration has to be encouraged so that investment capital can be collected more easily and the advantages of such technologies can become effective. Last, but not least, the government must use all kinds of media to put propaganda

Table 1. Potential sustainability risks and challenges of microalgae production for biofuel use.

Environmental dimension	Economic dimension	Social dimension	Cultural dimension
Water resource abuse Damage to waterways Groundwater may not be recharged effectively Cause soil pollution, land-use overexpansion, land service expectancy decrease and soil erosion Detrimental effect on local ecosystem, causing eutrophication, algal blooms, fish kills and biological invasion Greenhouse gases, for example NO_x, CH_4 and NH_3 emissions	Start-up phase is expensive Requires enormous investments Might cause depression or shrink in the traditional algal industry	Local farm animals, wildlife and people might suffer from water pollution Safety of genetically modified algae may not be immediately apparent or known Diseases (e.g. malaria) spread Animal habitat interruptions	It requires time for people to adapt to and accept unconventional microalgae utilisation Personnel changes in workforce might harm existing cultural structures Landscape aesthetics might be affected

activities into practice, covering environmental, economic, social and cultural aspects.

As for the company entity, the following issues must be proactively addressed. First, before establishment of an algal industry, explicit water and land-use planning must be carried out in an environmentally friendly manner. Second, in order to minimise damage to aquatic ecosystems, the relative measures must be put into effect to treat wastewater, maintain downstream water quality and minimise groundwater infiltration. Third, the periodicity and volumes of recycled water must be anticipated in advance, and non-recycled water with a high salt content must be disposed of appropriately. Fourth, a careful assessment of potential risks prior to the introduction of new species must be conducted to prevent habitat damage, ecological invasion, disease spread, etc. Fifth, in the long term, higher-value co-products or by-products must be developed in order to improve the economic competitive position of microalgae production. Finally, the transparency of process inputs and outputs must be introduced.

From the public perspective, public participation will highly influence the sustainable production of the algal industry. Public perception of participation in microalgae industry as an environmentally responsible steward will help facilitate its establishment and development. The effective communications and activities between the government and company will help people to adopt a more positive attitude towards the algal biofuel companies.

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Nutrient removal and biodiesel production by integration of freshwater algae cultivation with piggery wastewater treatment

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ABSTRACT

An integrated approach, which combined freshwater microalgae *Chlorella zofingiensis* cultivation with piggery wastewater treatment, was investigated in the present study. The characteristics of algal growth, lipid and biodiesel production, and nutrient removal were examined by using tubular bubble column photobioreactors to cultivate *C. zofingiensis* in piggery wastewater with six different concentrations. Pollutants in piggery wastewater were efficiently removed among all the treatments. The specific growth rate and biomass productivity were different among all the cultures. As the initial nutrient concentration increased, the lipid content of *C. zofingiensis* decreased. The differences in lipid and biodiesel productivity of *C. zofingiensis* among all the treatments mainly resulted from the differences in biomass productivity. It is worthy of note that the diluted piggery wastewater with 1900 mg L^{-1} COD provided an optimal nutrient concentration for *C. zofingiensis* cultivation, where the advantageous nutrient removal and the highest productivities of biomass, lipid and biodiesel were presented.

1. Introduction

Nutrient pollution by animal wastewaters is becoming a more and more severe problem if disposed inappropriately. This not only has substantially negative impacts on agricultural development, but also can affect improvement in the quality of the environment and people's lives (Zhu et al., 2011). Biological treatment of these wastewaters is a preferable solution to agricultural nutrient management in an effort to search for sustainable development. Additionally, issues associated with greenhouse gas (GHG) emissions and fossil fuel depletion are strongly bound, resulting from the large amounts of GHGs emitted into the atmosphere from the industry and transportation sectors. The development of renewable and sustainable energy is a promising approach to avoid further aggravation of the energy crisis and global climate changes and to increase the energy reserves of different countries (Lam and Lee, 2012). Under these scenarios, using animal wastewater that is rich in nitrogen and phosphorus as a nutrient source to cultivate microalgae represents one of the best future measures (Chen et al., 2012). Compared to agriculture-based biofuel plants, microalgae cultivated in wastewaters

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can consume significantly less freshwater and improve water quality. This is because no or limited chemical fertilizers, which are easily dissolved in rainwater or runoff, need to be applied to the system (Zhu and Ketola, 2012). In addition, microalgae are rich in lipids, starch and protein, which can be utilized as a non-food-based feedstock for biofuels (mainly in the form of biodiesel, bioethanol and biogas) and chemical production (Chen et al., 2012).

Currently, there is a lot of on-going research on the treatment of industrial, municipal and agricultural wastewaters by microalgae culture systems (Zhang et al., 2012; Ji et al., 2013; Samori et al., 2013). It was found that when cultivating *Arthrospira platensis* in olive-oil mill wastewater the maximum removal of chemical oxygen demand (COD) was 73.18%, while phenols, phosphorus and nitrates in some runs were completely removed (Markou et al., 2012). Ruiz-Marin et al. (2010) compared two species of microalgae growing as immobilized and free-cells to test their abilities to remove total nitrogen (TN) and total phosphate (TP) in batch cultures with urban wastewater. Kothari et al. (2012) found that *Chlorella pyrenoidosa* could remove about 80–85% TP and 60–80% of TN from dairy wastewater. Nonetheless, there are a small number of publications available on the potential of the combination of microalgae cultivation in poultry wastewater, such as piggery wastewater, with microalgae-based biofuels production. This is especially important since the microalgal biofuel applications appear to be strongly economically convenient only in conjunction with wastewater treatment (Samori et al., 2013). In addition, it is necessary to dilute the original poultry wastewater when the nutrient concentrations are high, but the optimal dilution ratio for biofuel-targeted microalgae production is still unknown.

The aim of this study was to measure wastewater pollutant removal and microalgal biomass accumulation for value-added energy applications. Thus, an integrated approach, which combined freshwater microalgae *Chlorella zofingiensis* cultivation with piggery wastewater treatment, was investigated in this manuscript. To summarize, the objectives of our study were: 1) to determine an optimal dilution of piggery wastewater for algal cultivation, 2) to reveal relevant nutrient removal abilities and 3) to specify the productivities of biomass, lipids and biodiesel.

2. Material and methods

2.1. Microalgae strain and pre-culture conditions

The microalgae *Chlorella zofingiensis* was obtained from a lab in the Guangzhou Institute of Energy Conversion of the Chinese Academy and grown in a BG11 medium (Rippka et al., 1979), consisting of: (1) the following solid ingredients: $NaNO_3$ (1.5 g L^{-1}), $K_2HPO_4 \bullet 3H_2O$ (40 mg L^{-1}), $MgSO_4 \bullet 7H_2O$ (75 mg L^{-1}), $CaCl_2 \bullet 2H_2O$ (36 mg L^{-1}), Na_2CO_3 (20 mg L^{-1}) and $FeCl_3 \bullet 6H_2O$ (3.15 mg L^{-1}) and (2) the following chemicals: citric acid (6 mg L^{-1}) and 1 mL per liter of trace elements solution consisting of H_3BO_3 (2.86 mg L^{-1}), $MnCl_2 \bullet 4H_2O$ (1.81 mg L^{-1}), $ZnSO_4 \bullet 7H_2O$ (0.22 mg L^{-1}), $Na_2MoO_4 \bullet 2H_2O$ (0.39 mg L^{-1}), $CuSO_4 \bullet 5H_2O$ (0.08 mg L^{-1}), $Co(NO_3)_2 \bullet 6H_2O$ (0.05 mg L^{-1}) and concentrated H_2SO_4 (1 mL). The initial pH of the medium was adjusted to 6.8. The seed culture was grown in a 1.37-L tubular bubble column photobioreactor (tbcPBR, height ~70 cm and internal diameter ~5.0 cm) containing 800 mL of the medium, aerated with compressed air with 5–6% CO_2, a surrounding temperature of 25 ± 1 °C and illuminated with cool white fluorescent lamps on one side of the photobioreactors (light intensity of 230 ± 20 μmol m^{-2} s^{-1}) continuously.

2.2. Culture of Chlorella zofingiensis with piggery wastewater

Piggery wastewater from a private farm with about 100 swine near the laboratory was used as a substrate to cultivate *C. zofingiensis*. Pre-treatment was carried out by sedimentation and filtration with a filter cloth to remove large, non-soluble particulate solids. After filtration the substrate was autoclaved for 20 min at 121 °C, after which the liquid was stored at 4 °C for 2 days for settling any visible particulate solids and the supernatant was used for microalgae growth studies. The characteristics and features of the raw and autoclaved wastewater are summarized in Table 1.

The autoclaved supernatant was diluted with distilled water to five different concentrations at a level of 2500, 1900, 1300, 800 and 400 mg L^{-1} COD. The undiluted autoclaved supernatant (control group) and the five dilutions were used accordingly as media for algal cultivation. Subsequently, a volume of 720 mL of piggery wastewater with the different COD concentrations mentioned above were introduced into tbcPBRs, after which the pH values of the media were adjusted according to pre-determined values. Afterward, a volume of 80 mL of seed microalgae suspension with an optical density (OD_{680}) of 2.952 was introduced into each tbcPBR. The culture conditions were identical to those described in Section 2.1. All treatments including a control group were carried out in duplicates. In all cases, microalgae were grown for 10 days.

2.3. Analytical procedures

2.3.1. Sampling and nutrients analysis

A volume of 3 mL microalgae suspension was collected every day from each tbcPBR for nutrient removal analysis starting from inoculation. The samples were first centrifuged at 1811.16 × g for 10 min, after which the supernatants were filtered using a 0.45 μm nylon membrane filter. Then, the filtrates were appropriately diluted and analyzed for COD, TN and TP following the Hach DR 2700 Spectrophotometer

Table 1 – Characteristics of original and autoclaved piggery wastewater used in the experiments (means ± sd).

Parameter	Original concentration	Autoclaved concentration
pH	6.1 ± 0.1	6.2 ± 0.0
Suspended Solid (mg L^{-1})	492 ± 31	366 ± 23
COD (mg L^{-1})	3700 ± 51	3500 ± 63
TN (mg N L^{-1})	162.0 ± 8.0	148.0 ± 4.0
TP (mg PO_4^{-3} –P L^{-1})	209.0 ± 5.5	156.0 ± 8.0

Manual (Hach, 2008). The percentage removal was obtained using the following expression:

$$\text{Percentage removal } W\% = 100\% \times (C_o - C_i)/C_o \quad (1)$$

where C_o and C_i are defined as the mean values of nutrient concentration at initial time t_0 and time t_i, respectively.

2.3.2. Determination of microalgae growth

A correlation between the optical density of *C. zofingiensis* at 680 nm and the dried biomass was pre-determined. OD_{680} was measured every day using spectrophotometer (Jingke 722N, Shanghai, China). The correlation is shown below:

$$\text{Dry weight}(g\,L^{-1}) = 0.3387 \times OD_{680},\ R^2 = 0.9913 \quad (2)$$

The specific growth rate μ in exponential phase of algal growth was measured by using Eq. (3) (Issarapayup et al., 2009):

$$\mu(day^{-1}) = \ln(N_2/N_1)/(t_2 - t_1) \quad (3)$$

where N_1 and N_2 are defined as dry biomass ($g\,L^{-1}$) at time t_1 and t_2, respectively. The biomass productivity (P) was calculated according to the following formula:

$$P = (DW_i - DW_0)/(t_i - t_0) \quad (4)$$

where DW_i and DW_0 are dry biomass ($g\,L^{-1}$) at time t_i and t_0 (initial time), respectively.

After *C. zofingiensis* grew for 10 days, microalgae cells from each tbcPBR were collected and centrifuged at 5032.08 × g and 4 °C for 15 min. Supernatants were decanted and cell pellets were washed with distilled water and then freeze-dried under −80 °C. The dried microalgae biomass was collected and sealed in empty containers for lipid extraction and fatty acid methyl ester (FAME) content analysis.

2.3.3. Lipid extraction

A modified method by Bigogno et al. (2002) was applied to quantify the amount of total lipid content. 100–150 mg of freeze-dried samples of *C. zofingiensis* were extracted with 2 mL of methanol containing 10% dimethyl sulfoxide (DMSO) in a water bath shaker at 45 °C for 45 min. The mixture was centrifuged at 1811.16 × g for 10 min, after which the supernatant was collected and the leftover was re-extracted twice following the same procedures. Then, the leftover was extracted with a 4-mL mixture of hexane and ether (1:1, v/v) in a water bath shaker at 45 °C for 60 min. The mixture was centrifuged at 1811.16 × g for 10 min, after which the supernatant was collected and the leftover was re-extracted twice following the same procedures. All the supernatants were incorporated and 6 mL of distilled water was added to the combined extracts, so as to form a ratio of methanol with 10% DMSO, diethyl ether, hexane and distilled water of 1:1:1:1 (v/v/v/v). The organic phases containing lipids were combined into a pre-weighed glass tube and evaporated to dryness under the protection of nitrogen. Then, the lipids were freeze-dried at −80 °C for 24 h. Thereafter, the total lipids were measured gravimetrically and lipid content was expressed as a % of dry weight.

2.3.4. FAME content analysis

The content of FAME was analyzed following a one step extraction-transesterification method by Indarti et al. (2005), after suitable modifications. Transesterification was performed using 20 mg of freeze-dried samples of *C. zofingiensis*, 2.5 mL of methanol with 2 wt.% of concentrated sulfuric acid (H_2SO_4) as the catalyst. The reaction was carried out in a water bath shaker at 80 °C for 2.5 h. On completion of the reaction, the mixtures were cooled down to room temperature, after which 1 mL of distilled water and 2 mL of hexane were added into the mixture. After the formation of two phases, the upper phase containing FAME was transferred to a clean, 10 mL bottle and dried with water-free Na_2SO_4. Until now, the sample was ready for the FAME composition and content analysis, which was carried out by gas chromatograph with a FID detector (Shimadzu GC-2010, Japan). The injector and detector temperatures were set at 300 and 280 °C, respectively. The temperature gradient was programmed from 130 to 180 °C with an increase of 10 °C/min followed by a rise to 210 °C with 2 °C/min and then the temperature was fixed at 210 °C for 3 min. The compounds detected were identified and quantified using the NIST Mass Spectral Search Program.

All the experiments were carried out in duplicate and average values were reported. Results were performed with EXCEL (Microsoft Office Enterprise, 2007) and SPSS 11.5 for Windows (SPSS Inc., 2007) and analysis of variance (ANOVA) was determined wherever applicable.

3. Results

3.1. Microalgal growth

The growth characteristics of *C. zofingiensis* under six nutrient concentration levels within ten days were investigated as shown in Fig. 1. These curves illustrate all characteristic growth phases in a batch culture of microalgae, except the decaying phase (also called lysis phase), which is not evident. The lag time for *C. zofingiensis* cultivated in the media with a

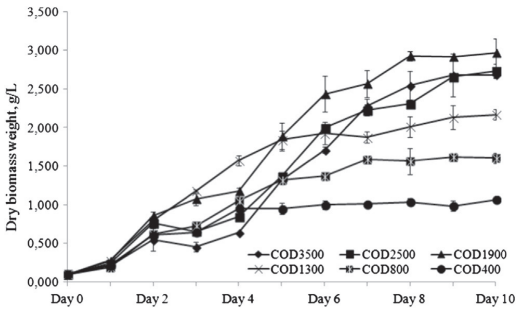

Fig. 1 – Growth curves for *C. zofingiensis* grown under six nutrient concentration levels within ten days (means ± sd). The initial nutrient concentration of six cultures was as follows (unit, mg L^{-1}): COD 3500, TN 148 and TP 156; COD 2500, TN 106 and TP 111; COD 1900, TN 80 and TP 85; COD 1300, TN 55 and TP 58; COD 800, TN 34 and TP 36; COD 400, TN 17 and TP 18. The COD concentration was used to present the initial nutrient concentration in all cases in this paper.

COD concentration of 400, 800, 1300 and 1900 mg L^{-1} was about one day with earlier pending exponential and stationary phases. Nevertheless, the culture with 2500 and 3500 mg L^{-1} COD witnessed the fluctuating lag phase, which lasted for about four days. Afterward, the algal growth moved into the exponential phase where biomass weights were significantly ($P < 0.05$) increased on day five. By the end of experiment, there were significant ($P < 0.05$) differences between the biomass weight and the initial nutrient concentration among all the treatments.

After the lag phase, the microalgae grown in the media with 800 and 400 mg L^{-1} COD witnessed a rapid growth with a specific growth rate μ of around 0.5 day^{-1} and a doubling time of around 1.4 days (Table 2). The μ of culture in 3500, 2500, 1900 and 1300 mg L^{-1} COD piggery wastewater lay at 0.287, 0.322, 0.340 and 0.431 day^{-1}, respectively. The final biomass increase and biomass productivity in the piggery wastewater treatment with initial COD concentration at 1900 mg L^{-1} were the highest, correspondingly reaching 2.962 ± 0.192 g L^{-1} and 296.16 ± 19.16 mg L^{-1} day^{-1}, while the culture in 800 and 400 mg L^{-1} COD piggery wastewater showed the lowest biomass increase (1.603 ± 0.063 and 1.063 ± 0.011 g L^{-1}, respectively) and biomass productivities (160.34 ± 6.32 and 106.28 ± 1.15 mg L^{-1} day^{-1}, respectively). The biomass productivity in this study was higher than that in the research by Min et al. (2011) who achieved 34.6 mg L^{-1} day^{-1} biomass when they cultivated *Chlorella* sp. in a pilot-scale photobioreactor using centrate wastewater. The results also showed that there were significant ($P < 0.05$) differences between the initial nutrient concentration and the specific growth rate, doubling time and biomass productivity among all the treatments.

3.2. Nutrient removal

The variation in COD, TN and TP removal with time in different initial nutrient concentrations of piggery wastewater for the ten-day batch culture is exhibited in Fig. 2.

The COD contaminants were dramatically decreased within 2 days in all experiments. Later on, the removals in the treatment with initial COD concentrations of 1900, 1300, 800 and 400 mg L^{-1} were slowed down and stabilized until the end of the experiment, while the culture in 2500 and 3500 mg L^{-1} COD witnessed an obvious drop in COD reduction in the third day and then a continuous raise to a steady removal level. In total, when the test came to an end, 74.29%, 78.18%, 79.84%, 76.46%, 65.81% and 67.25% of COD were accordingly removed from the 3500, 2500, 1900, 1300, 800 and 400 mg L^{-1} COD cultures.

The tendency for TN removal in all treatments was similar to that for COD removal in this study. The 2500 and 3500 mg L^{-1} COD media witnessed TN and COD removal decrease on day three, followed by a subsequent increase to a stable stage until the end of the experiment. Within the ten-day cultivation, most of the TN (82.70%) in the 1900 mg L^{-1} COD culture could be reduced, while the 400 mg L^{-1} COD medium could only remove 68.96% TN, following the 800, 1300, 3500, and 2500 mg L^{-1} COD media with 70.88%, 77.81%, 78.72% and 81.03% TN removal, respectively.

From Fig. 2c, it can be seen that the consumption of TP in this study increased as the initial nutrient concentration decreased, while the COD and TN removals were not influenced by the initial nutrient concentration. During the lag phase, there was no evident fluctuation in TP removal in all cultures. TP removal in the cultures of 1900, 1300, 800 and 400 mg L^{-1} COD occurred at more than 90% even within five-day cultivation, and the ultimate removal could reach 98.17%, 98.62%, 99.44% and 100%, respectively. In contrast, TP removal in 3500 and 2500 mg L^{-1} COD cultures was stably raised until the end of experiment, correspondingly arriving at 85.00% and 89.23%. The ten-day removals of COD, TN and TP were significantly ($P < 0.05$) different among all the cultures.

3.3. Lipid content and productivity

The lipid content of *C. zofingiensis* in six different cultures was measured and is shown in Fig. 3. The lipid accumulation was affected by the initial nutrient concentration. The highest microalgal lipid accumulation occurred in the treatment with the initial COD concentration at 400 and 800 mg L^{-1}, respectively reaching 45.81% and 42.16% of the dry weight. As the initial nutrient concentration increased, the lipid content of *C. zofingiensis* decreased from 45.81% to 33.91% (3500 mg L^{-1} COD medium) of the dry weight.

Moreover, the chemical composition of *C. zofingiensis* cultured in different nutrient loadings of piggery wastewater was measured to unveil the effects of nutrient conditions on algal ingredients. The microalgal biomass from diluted piggery wastewater with different nutrient concentrations was collected at the end of the experiment. The sugar, protein and lipid contents of the algal biomass were determined (Fig. 3). There was no evident difference in sugar percentage among

Table 2 – Growth parameters of *C. zofingiensis* in tbcPBRs under six nutrient concentration levels within ten days (means ± sd).

Initial nutrient concentration (mg L^{-1} COD)	Specific growth rate μ (day^{-1})	Doubling time (days)	Biomass increase (g L^{-1})	Biomass productivity (mg L^{-1} day^{-1})
3500	0.287 ± 0.000	2.42 ± 0.00	2.646 ± 0.046	267.81 ± 4.55
2500	0.322 ± 0.017	2.15 ± 0.02	2.733 ± 0.091	273.33 ± 9.10
1900	0.340 ± 0.001	2.04 ± 0.01	2.962 ± 0.192	296.16 ± 19.16
1300	0.431 ± 0.023	1.61 ± 0.08	2.166 ± 0.067	216.63 ± 6.71
800	0.487 ± 0.006	1.42 ± 0.02	1.603 ± 0.063	160.34 ± 6.32
400	0.492 ± 0.034	1.41 ± 0.10	1.063 ± 0.011	106.28 ± 1.15

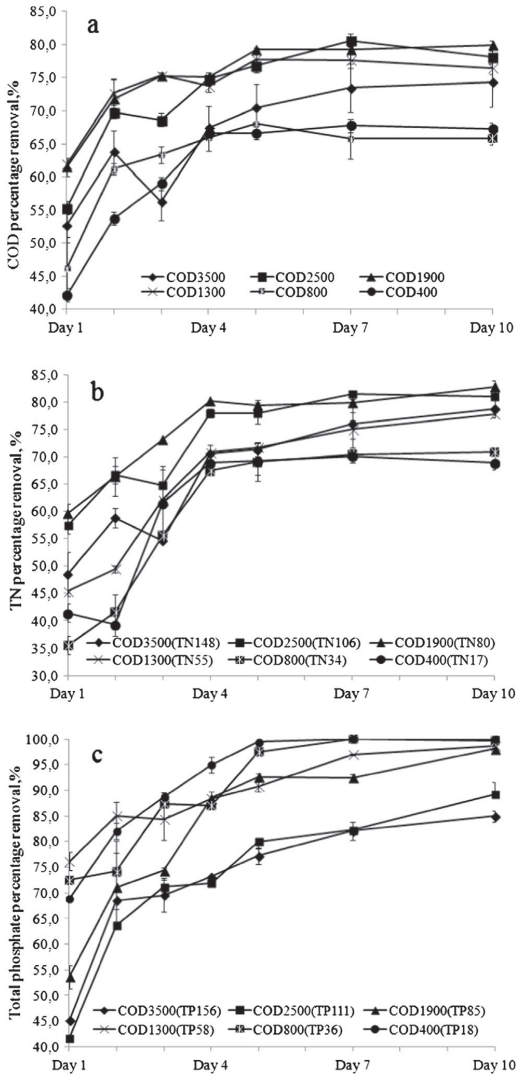

Fig. 2 – Nutrient removals by *C. zofingiensis* grown under six nutrient concentration levels within ten days (means ± sd). (a) COD removal profile; (b) TN removal profile; (c) TP removal profile.

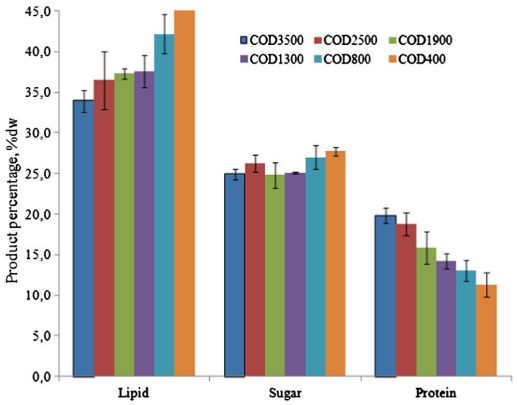

Fig. 3 – – Chemical contents of *C. zofingiensis* biomass which was cultured in different nutrient concentrations of piggery wastewater within ten days (means ± sd).

the algae cultured in different nutrient concentrations. The sugar contents in this study (24.81%–27.76%) were higher than those found by Huo et al. (2012), who achieved around 18% sugar of *C. zofingiensis* grown in bench-scale outdoor ponds using winter dairy wastewater. As shown in Fig. 3, as the initial nutrient concentration increased, the lipid content decreased ($P < 0.05$, $R = 91.3\%$), but the protein content increased from 11.29% to 19.82% ($P < 0.05$, $R = 98.2\%$). This trend for change in the protein content in algal cells was in agreement with the research conducted by Chen et al. (2012), who cultivated *Chlorella* sp. with diluted animal waste.

The lipid productivities of *C. zofingiensis* in six cultures were calculated as shown in Fig. 4. The results indicated that the lipid productivities ranged from 48.69 to 110.56 mg L^{-1} day^{-1} in the relationship: 1900 mg L^{-1} COD culture (110.56) > 2500 mg L^{-1} COD culture (99.71) > 3500 mg L^{-1} COD culture (90.81) > 1300 mg L^{-1} COD culture (80.98) > 800 mg L^{-1} COD culture (67.59) > 400 mg L^{-1} COD culture (48.69). In this study the differences in lipid productivity of *C. zofingiensis* among different treatments mainly resulted from the differences in their biomass, since the deviations in lipid contents of *C. zofingiensis* within different treatments were not large in contrast. The lipid contents of *C. zofingiensis* in 800 and 400 mg L^{-1} COD culture were high, but their biomass productivities were limited, resulting in relatively low lipid productivities.

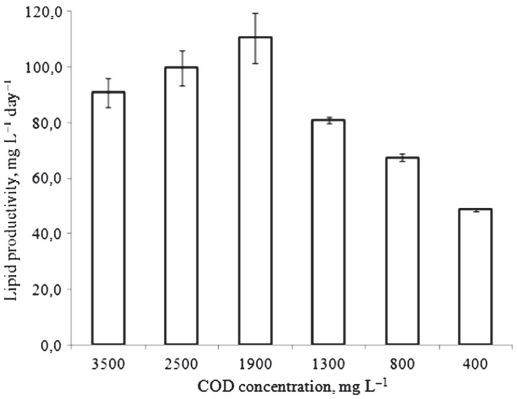

Fig. 4 – Lipid productivity of *C. zofingiensis* grown under six nutrient concentration levels within ten days (means ± sd).

3.4. FAME composition and biodiesel productivity

Fatty acid methyl esters, which are obtained from vegetable oils and animal fat through a transesterification process, are the chemical ingredients of biodiesel. Not all lipids can be converted to FAME (Li et al., 2011), for example, glycolipids and phospholipids. Therefore, measurement of fatty acid in algal biomass is an important method for evaluating the amount of lipids suitable for biodiesel conversion. In the present study, the FAME compositions of *C. zofingiensis* mainly consisted of C16:0 (palmitic acid methyl ester), C18:2 (octadecadienoic acid methyl ester) and C18:3 (octadecatrienoic acid methyl ester), as shown in Table 3.

The highest FAME yield appeared in 400 mg L^{-1} COD culture where 11.15 g-biodiesel/100 g-dry weight, with C18:0 as the most abundant fatty acid, was derived from *C. zofingiensis*, while the lowest yield occurred in 3500 mg L^{-1} COD medium (8.80% of dry weight). Apart from that, FAME yields with 9.97%, 10.18%, 10.01% and 10.84% of dry weight were respectively achieved in 2500, 1900, 1300 and 800 mg L^{-1} COD cultures. The FAME yields among all cultures were significantly ($P < 0.05$) different, accordingly to statistical analysis. These findings were in accordance with research by Li et al. (2011), who cultivated *Chlorella* sp. in autoclaved and raw municipal wastewater and achieved the FAME yields with 9.98% and 11.04% of dry weight, respectively. After multiplying the FAME yield with relative biomass productivity, the according biodiesel productivity in this study was obtained, ranging from 11.85 to 30.14 mg L^{-1} day^{-1} in the following sequence: 1900 mg L^{-1} COD culture (30.14) > 2500 mg L^{-1} COD culture (27.26) > 3500 mg L^{-1} COD culture (23.56) > 1300 mg L^{-1} COD culture (21.67) > 800 mg L^{-1} COD culture (17.38) > 400 mg L^{-1} COD culture (11.85). Combining Table 3 with Fig. 3, it was not difficult to find that only about one third to one forth of lipids could be converted into biodiesel. This was because some lipid types such as chlorophyll, glycolipid and phospholipid were not efficient ingredients for biodiesel production and thus would not be converted into biodiesel.

4. Discussion

4.1. Algal growth

Like other microorganisms, microalgae growth can undergo four growth phases: lag, exponential, stationary, and lysis (Li et al., 2011). In this study, the lack of a visible lysis phase (Fig. 1) was because the cultivation period was short. Research by Moazami et al. (2012) showed that the lysis stage would appear after about 18 days. The microalgae cultivated in the media with the COD concentration of 400, 800, 1300 and 1900 mg L^{-1} had a short lag and exponential stage, and their stationary phase came earlier until the end of cultivation. However, the exponential growth in the culture with 2500 and 3500 mg L^{-1} COD lasted for three to four days, following a fluctuating lag phase, which lasted for about 4 days. In the lag state, algal cells needed to adapt to the new environment with wastewater nutrient sources that were different from those the BG 11 medium contained. Meanwhile, the low algal cell concentration in the beginning of cultivation might cause a lack of self-shading (Li et al., 2011). Thus, it was likely that cells at such a low concentration received too much light, thus resulting in photoinhibition (Feng et al., 2012).

4.2. Nutrient removal

The high percentage removals of COD, TN and TP were achieved among all the treatments (Fig. 2). In this study the COD removal was not correlated with the initial COD concentration. This finding was, however, in contrast to the finding by Travieso et al. (2006). They used the settled and diluted wastewater to grow *Chlorella vulgaris* and found that COD removals increased from 20.6% to 88.0% as the initial COD concentration decreased from 1000 to 250 mg L^{-1}. The fluctuation in COD and TN levels in 2500 and 3500 mg L^{-1} COD cultures might result from nutrient uptake by new cells and nutrients decomposed and released from insoluble organic

Table 3 – Summary of FAME profile for *C. zofingiensis* cultivated in piggery wastewater with different nutrient concentrations within ten days.

FAME composition		COD3500	COD2500	COD1900	COD1300	COD800	COD400
Saturated fatty acids (% of total FAME)	C16:0	31.87	27.50	19.73	33.24	33.58	23.87
	C18:0	0.00	7.33	17.66	17.38	11.35	33.81
	C24:0	1.22	0.77	7.73	0.00	0.00	0.00
	Subtotal	33.09	35.60	45.11	50.61	44.92	57.69
Monoenoic fatty acids (% of total FAME)	C16:1	4.16	4.90	5.42	4.06	5.57	8.73
	C20:1	0.60	0.19	0.81	0.13	0.32	3.13
	C22:1	1.64	2.61	0.00	0.95	1.29	6.86
	C24:1	4.28	6.79	5.46	4.98	5.14	5.75
	Subtotal	10.68	14.49	11.68	10.13	12.32	24.46
Polyenoic fatty acids (% of total FAME)	C18:2	36.45	32.91	26.89	23.91	28.71	10.22
	C18:3	16.68	15.12	14.82	13.73	18.08	3.67
	C20:2	3.10	1.89	1.50	1.62	1.67	3.96
	Subtotal	56.23	49.91	43.20	39.25	48.46	17.85
C16–C18 (% of total FAME)		89.17	87.75	84.51	92.31	97.28	80.31
Total (% of dw)		8.80	9.97	10.18	10.01	10.84	11.15
Biodiesel productivity (mg L^{-1} day^{-1})		23.56	27.26	30.14	21.67	17.38	11.85

matters (Min et al., 2011). Previous study has reported that microalgae can assimilate NH_4–N, nitrate and simple organic nitrogen such as urea, acetic acid and amino acids in wastewater (Barsanti and Gualtieri, 2006; Su et al., 2011; Huo et al., 2012). This was because the microalgal cell required nitrogen for protein, nucleic acid and phospholipid synthesis. Thus, microalgae growth is believed to be essential for nitrogen removal via uptake, decay and sedimentation (Zimmo et al., 2003). Surprisingly, phosphate removal in the present study was relatively high, while the removal in other studies was lower (70.3 ± 11.4%) for *C. pyrenoidosa* (Su et al., 2011). The consumed phosphorus in piggery wastewater was mainly used and assimilated for the synthesis of *C. zofingiensis*. The pH value in this study slowly increased from 6.2 to 7.4 resulting from the photosynthesis of microalgae and the reduction of the organic pollutant. The increase of pH in cultures could help contribute to the precipitation of phosphorus and the increase of phosphate adsorption on microalgal cells (Ruiz-Marin et al., 2010) and thus the decrease of phosphorus levels in cultures occurred.

In this study the piggery wastewater was sterilized by autoclave before the introduction of *C. zofingiensis*. During the experiment, it was found that all the cultures were not polluted by other microalgae species and protozoa. However, the compressed air with 5–6% CO_2 used in this study was not sterilized, for example, with a membrane filter. Thus, the culture system might contain bacteria via air introduction, which could also contribute to the degradability of pollutants (Chen et al., 2012). Several previous studies reported that the interaction of algae and other microorganisms, such as bacteria, in the culture system could contribute to wastewater nutrient removal (Bordel et al., 2009; Su et al., 2011; Zhang et al., 2012). In microalgal–bacterial culture system, microalgae can provide O_2, which is necessary for aerobic bacteria to biodegrade organic pollutants and consume in turn the CO_2 that is produced by bacteria through respiration (Munoz and Guieysse, 2006). Microalgae can improve bacterial activity by releasing certain extracellular compounds (Liu et al., 2012), while bacterial growth can enhance microalgal metabolism by reducing O_2 concentration in the medium, by releasing growth-promoting factors (Gonzalez and Bashan, 2000), or by degrading larger compounds for the algal cell to easily assimilate. However, microalgae may detrimentally affect bacterial activity by increasing the pH or culture temperature, or by releasing inhibitory metabolites (Gonzalez and Bashan, 2000), while some bacteria can damage the microalgae by releasing soluble cellulose enzyme (Zhang et al., 2012). Meanwhile, some bacteria can also adhere to the inner-wall of the cell to form a bacteria film, which will inhibit microalgae photosynthetic growth by hindering the transmission of light (Zhang et al., 2012). Thus, the interactions between bacteria and algae are complicated and will be influenced by environmental conditions.

4.3. Lipid and biodiesel production

As shown in Fig. 3, the lipid content of *C. zofingiensis* decreased as the initial nutrient concentration increased. The fact that the cells with lower initial nutrient concentrations accumulated more lipids might be because the lower biomass concentration in 400 and 800 mg L^{-1} COD cultures could enable more cells to receive a higher light intensity, which activated and promoted cellular lipid storage. This phenomenon is in accordance with previous studies by Rodolfi et al. (2009) and Feng et al. (2012), who proved that lipid accumulation (mainly triacylglycerols) can be raised by increasing light intensity. In the later period of this experiment TP deficiency or limitation occurred in the 400 and 800 mg L^{-1} COD cultures could help enhance the synthetic rate of lipid accumulation and at the same time inhibit protein synthesis (Feng et al., 2011). Khozin-Goldberg and Cohen (2006) also suggested that lipid storage in *Monodus subterraneus* could be increased by P starvation.

Unlike other species like *Botryococcus braunii*, which mainly contain long-chain hydrocarbons for fatty acids (Banerjee et al., 2002), in our study *C. zofingiensis* was found to have shorter carbon chains for fatty acids. They mainly contain 16–18 carbons (Table 3), which are ideal for biodiesel conversion (Huang et al., 2010). The C16:0, C16:1 (palmitoleic acid methyl ester), C18:0 (stearic acid methyl ester), C18:2 and C18:3 of *C. zofingiensis* in 3500, 2500, 1900, 1300, 800 and 400 mg L^{-1} COD cultures represented a major portion of fatty acid methyl esters compositions, accounting for a total of 89.17%, 87.75%, 84.51%, 92.31%, 97.28% and 80.31%, respectively. Previous study has reported that lipid content and fatty acid composition were greatly affected by culturing conditions, growth period, and environmental situations (Petkov and Garcia, 2007; Li et al., 2011) and thus it was not surprising to observe the different FAME profiles in this study. The unsaturated fatty acid methyl esters for C16:1, C18:2 and C18:3 in cultures with 3500, 2500, 1900, and 800 mg L^{-1} COD were predominant in the FAME profile, accordingly accounting for 57.30%, 52.92%, 47.12% and 52.36%, while the counterpart in the 1300 and 400 mg L^{-1} COD cultures shared 41.70% and 22.62% of the total FAME. It is worthy of note that the pour point of biodiesel can be reduced when higher compositions of unsaturated fatty acid methyl esters increase (Abdelmalik et al., 2011), thus making the use of algal biodiesel in countries with cold climates like Nordic nations more feasible.

4.4. Methods for future scale-up

The extensive experiment showed that without sterilizing the piggery wastewater, the system was subject to contamination by a local algae species called *Scenedesmus* sp. and protozoa such as rotifer. Thus, the piggery wastewater used in this study needed to be sterilized before the introduction of *C. zofingiensis*. Nonetheless, pre-treatment by autoclave is energy-intensive and complex for real operation, contributing to the main bottleneck of the amplification of cultivating microalgae in wastewaters in practice. In this respect, it is necessary to search for an easier and cheaper method for wastewater sterilization before the introduction of algae. Amazingly, in the extensive experiment we found that using sodium hypochlorite (NaClO) was an effective solution to pre-treating piggery wastewater. The piggery wastewater could be pre-treated with 10% NaClO at a ratio of 0.1 mL per 1 L substrate for 12 h in a dark environment, after which the liquid was exposed to sunlight for 12 h and then was stored at 4 °C for one day to settle out any visible particulate solids and to prepare the supernatant for microalgae growth studies. The extensive experiment results showed that no obvious

difference existed in the performances in algal growth, lipid and FAME contents, and nutrient removal when the piggery wastewater was pre-treated by NaClO or autoclaving. Previous study has reported that 73.18% COD removal was achieved when *Arthrospira (Spirulina) platensis* was cultivated in 25% olive oil mill wastewater pre-treated by 12.5 g L^{-1} NaClO (Markou et al., 2012). In addition, we found that the cost of NaClO as a pre-treatment method was only around 0.05 USD per 1 kg of *C. zofingiensis* biomass.

Like most of feeding modes in the previous studies (Lam and Lee, 2012; Tastan et al., 2012; Samori et al., 2013), this study was based on batch operation, which lasted for ten days. This is time-consuming, causing a limited capacity and ability to treat piggery wastewaters in practice. Thus, it is necessary to design a semi-continuous feeding operation and find out its efficiency and stability in an attempt to investigate the feasibility of growing microalgae as a practical approach. During the extensive experiment, the semi-continuous operation was first carried out in batch mode for six days and then in semi-continuous mode with 50% of algae culture solution harvested and the same amount of fresh wastewater replenished every 36 h for a period of nine days. The wastewater used in batch and semi-continuous operation was 1900 mg L^{-1} COD culture. The results showed that the daily biomass productivity during semi-continuous operation verified the robustness of the culture strategy, since the average biomass concentration was achieved at 1.971 g L^{-1}, with a net productivity of 1.314 g L^{-1} day^{-1}, which was higher than the results in the batch culture as shown in Table 2. As shown in Fig. 2, *C. zofingiensis* can successfully remove wastewater nutrients, and even in the beginning phase (day one and two), the reduction was evident. Based on this observation, it can be extrapolated that most of the wastewater nutrients during the semi-continuous feeding operation can be removed.

Therefore, on the basis of the observation from our extensive experiments, it is greatly possible to amplify the cultivation of *C. zofingiensis* in piggery wastewater in practice. In an attempt to improve the economics of algal biodiesel production, a proposed scale-up scheme for *C. zofingiensis* production using piggery wastewater is designed as shown in Fig. 5. Additionally, except biodiesel, biogas and fertilizer, as shown in Fig. 5, value added products including bio-ethanol, high-value protein, cosmetics, etc. can be further produced through algae biomass production.

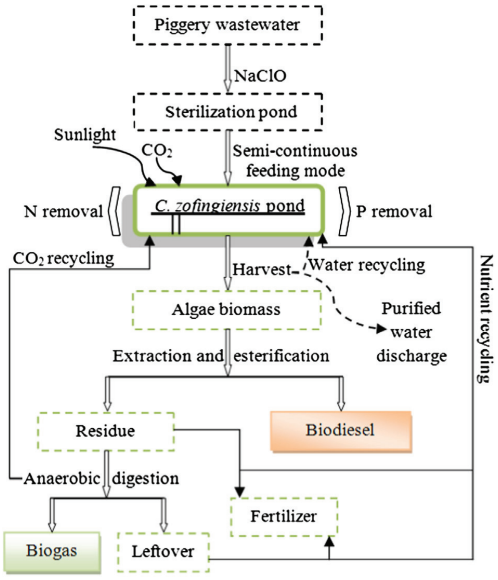

Fig. 5 – Proposed scale-up scheme for *C. zofingiensis*-based biofuel production using piggery wastewater. Although this image is meant to illustrate the connections between each process, the model also eludes to the physical layout of the integrated approach, as the algal ponds must be located close to the pig farm.

5. Conclusions

Pollutants in piggery wastewater media with six nutrient concentration levels were efficiently utilized by *C. zofingiensis* cultivated in tbcPBRs for 10 days with COD, TN and TP removal ranging from 65.81% to 79.84%, from 68.96% to 82.70% and from 85.00% to 100%, respectively. The specific growth rate of microalgae cultivated in cultures ranged from 0.287 to 0.492 day^{-1} with a doubling time from 2.42 to 1.41 days. The lipid accumulation was affected by initial nutrient concentration, ranging from 33.91% to 45.81%. The lipid and biodiesel productivity ranged from 48.69 to 110.56 mg L^{-1} day^{-1} and from 11.85 to 30.14 mg L^{-1} day^{-1}, respectively. It is worthy of note that the highest biomass productivity was 296.16 mg L^{-1} day^{-1}, the highest lipid productivity was 110.56 mg L^{-1} day^{-1} and the highest biodiesel productivity was 30.14 mg L^{-1} day^{-1} when cultivating *C. zofingiensis* in the 1900 mg L^{-1} COD culture.

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Recycling harvest water to cultivate *Chlorella zofingiensis* under nutrient limitation for biodiesel production

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HIGHLIGHTS

- Nutrient-limited cultures had much higher lipid contents than nutrient-full cultures.
- N- and P-limited culture observed the highest FAME yield at 10.95% of dry weight.
- N-limited culture and P-limited culture shared the highest biodiesel productivities.
- Harvest water could be recycled twice at a degree of 100% to re-produce algae.

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ABSTRACT

Harvest water recycling for *Chlorella zofingiensis* re-cultivation under nutrient limitation was investigated. Using 100% harvest water, four cultures were prepared: Full medium, P-limited medium, N-limited medium and N- and P-limited medium, while another full medium was also prepared using 50% harvest water. The results showed that the specific growth rate and biomass productivity ranged from 0.289 to 0.403 day^{-1} and 86.30 to 266.66 mg L^{-1} day^{-1}, respectively. Nutrient-limited cultures witnessed much higher lipid content (41.21–46.21% of dry weight) than nutrient-full cultures (26% of dry weight). The N- and P-limited medium observed the highest FAME yield at 10.95% of dry weight, while the N-limited culture and P-limited culture shared the highest biodiesel productivity at 20.66 and 19.91 mg L^{-1} day^{-1}, respectively. The experiment on harvest water recycling times demonstrated that 100% of the harvest water could be recycled twice with the addition of sufficient nutrients.

1. Introduction

There has been the increasing interest in seeking renewable and alternative energy for sustainable development. Microalgae, with a much higher oil yield per unit area than terrestrial oily crops, are a promising biodiesel feedstock with the potential to displace fossil fuels and meet the world's energy demands without threatening food supplies (Lakaniemi, 2012). The advantages of microalgae as a biodiesel feedstock are many, but the most amazing one is that it has the potential to integrate microalgae cultivation in wastewater with microalgae-based biodiesel production.

Using wastewater to cultivate microalgae has been studied as an alternative solution for wastewater treatment for more than 50 years. The technology of using microalgae in wastewater treatment is based on natural ecosystems, and thus it is environmentally friendly (Zamani et al., 2012). Algae can be used to clean polluted water because nutrients (mainly nitrogen and phosphorus) in wastewater can be absorbed and incorporated into microalgal cells and thus removed. Previous studies show that algae cultivated in wastewaters can effectively reduce nutrients (Cho et al., 2011; Zhang et al., 2012; Zhu et al., 2013a). Lim et al., (2010) investigated the potential application of *Chlorella vulgaris* for bioremediation of textile wastewater using high rate algae ponds and found that 44.4–45.1% ammonia (NH_4–N), 33.1–33.3% phosphate (PO_4–P) and 38.3–62.3% chemical oxygen demand (COD) were reduced within 12 days. Within 10 days, the highest nitrogen (N) and phosphorus (P) removal efficiencies at 91.0% and 93.5%, respectively, were obtained by Su et al., (2012) who used an algal–bacterial culture to treat domestic wastewater and accumulate biomass simultaneously.

In an effort to search for sustainability in water usage, the integration of microalgae production with wastewater treatment, scale-up potential and harvest water recycling are the three key challenges that should be explored to support microalgae-based biodiesel industry. When cultivating *Chlorella zofingiensis* with piggery wastewater, the results showed that piggery wastewater

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diluted with 1900 mg L^{-1} COD provided an optimal nutrient concentration for microalgal growth, where the advantageous nutrient removal and the highest productivities of biomass, lipid and biodiesel were presented (Zhu et al., 2013a). The results also showed that it is greatly possible to amplify the cultivation of *C. zofingiensis* in piggery wastewater for nutrient removal and biodiesel production (Zhu et al., 2013b). The harvest water recycling for *C. zofingiensis* re-cultivation will be investigated in detail in this work. All of the above studies can help contribute to speed up the process for the commercialization of cultivating *C. zofingiensis* in piggery wastewater for biodiesel production.

During microalgae production, biomass harvest from effluent is a necessary step to improve the quality of the treated wastewater and avoid the outflow of biomass, which can potentially be utilized to produce biodiesel (Zamani et al., 2012), leaving a large volume of harvest water especially when the biomass concentration is low. Without any detailed experimental exhibition available, many previous studies have recommended that harvest water should be recycled into the microalgae system not only for water reuse but also for nutrient recovery (Chowdhury et al., 2012; Lam and Lee, 2012; Resurreccion et al., 2012; Zhu and Ketola, 2012; Guieysse et al., 2013). Yang et al., (2011) found that the calculated water footprint without the recirculation of harvest water during microalgae-based biodiesel production was as high as 3.726 *t* water kg^{-1} biodiesel, while recycling harvest water could respectively reduce the water and nutrients usage by 84% and 55%. Nonetheless, researchers still know little about the feasibility of recycling harvest water to re-cultivate microalgae on the basis of experimental data. For instance, biomass and lipid productivities, and fatty acid composition still require investigation. In addition, researchers still do not know how many times the harvest water can be recycled at a degree of 100%. After recycling several times, the harvest water is susceptible to contamination by fungus and bacteria (Lam and Lee, 2012; Zhu and Ketola, 2012). Meanwhile, some metabolites released during microalgae growth might be inhibitive for microalgal production when harvest water is circulated into the cultivation system several times.

Previous studies have reported that N or P deficiency or limitation can promote lipid accumulation (Devi and Mohan, 2012; Feng et al., 2012; Roleda et al., 2013; Zhu et al., 2013a). Considering that nutrient contents might be low in the harvest water, a certain amount of chemical nutrients should be added. In this work freshwater microalgae *C. zofingiensis* was cultivated under nutrient limitation using harvest water from the production of *C. zofingiensis* in piggery wastewater to prepare the media. To summarize, two objectives of this study were employed: (1) To find out the productivity of biomass and lipids, and the composition of fatty acids when recycling harvest water for *C. zofingiensis* re-cultivation under nutrient limitation conditions, and (2) to unveil to what extent the harvest water can be circulated effectively at a degree of 100%.

2. Methods

2.1. Microalgae strain and pre-culture conditions

C. zofingiensis was obtained from a lab in the Guangzhou Institute of Energy Conversion of the Chinese Academy. And it was grown in a BG11 medium (Rippka et al., 1979), consisting of: (1) The following solid chemicals: $NaNO_3$ (1.5 g L^{-1}), $K_2HPO_4{\cdot}3H_2O$ (40 mg L^{-1}), Na_2CO_3 (20 mg L^{-1}), $CaCl_2{\cdot}2H_2O$ (36 mg L^{-1}), $MgSO_4{\cdot}7H_2O$ (75 mg L^{-1}) and $FeCl_3{\cdot}6H_2O$ (3.15 mg L^{-1}) and (2) the following ingredients: Citric acid (6 mg L^{-1}) and 1 mL L^{-1} of trace elements solution consisting of $MnCl_2{\cdot}4H_2O$ (1.81 mg L^{-1}), H_3BO_3 (2.86 mg L^{-1}), $Na_2MoO_4{\cdot}2H_2O$ (0.39 mg L^{-1}), $CuSO_4{\cdot}5H_2O$ (0.08 mg L^{-1}), $Co(NO_3)_2{\cdot}6H_2O$ (0.05 mg L^{-1}), $ZnSO_4{\cdot}7H_2O$ (0.22 mg L^{-1}) and concentrated H_2SO_4 (1 mL). The initial pH of the medium was adjusted to 6.8. The seed culture was grown in a 1.37-L tubular bubble column photobioreactor (tbcPBR), which has a height of 70 cm, an internal diameter of 5 cm and a thickness of 1.5 mm. The culture, with 800 mL of the medium, was cultivated under indoor conditions with a surrounding temperature of 25 ± 1 °C, aerated with compressed air with 5–6% CO_2 and continuously illuminated with cool white fluorescent lamps on one side of the photobioreactor (light intensity of 230 ± 20 μmol m^{-2} s^{-1}).

2.2. Composition of original piggery wastewater and harvest water

The original piggery wastewater was from a private farm (about 100 swine) near the laboratory. Pretreatment was carried out by sedimentation and filtration with a filter cloth to remove large, non-soluble particulate solids. After filtration the substrate was autoclaved for 20 min at 121 °C, after which the liquid was stored at 4 °C for 2 days to allow any visible particulate solids to settle. The supernatant and its five media diluted at different ratios were used to cultivate *C. zofingiensis*. After 10 days' growth, microalgae suspension was centrifuged at 3000 rpm for 10 min and the centrate was collected as harvest water (Zhu et al., 2013a). The characteristics and features of the original autoclaved wastewater and its harvest water are summarized in Table 1.

2.3. Experimental design

2.3.1. Recycling harvest water to re-grow algae under nutrient limitation

In order to investigate harvest water recycling for the re-production of microalgal biomass, 100% and 50% of harvest water were used as the cultivation media. Several studies have reported that N or P deficiency can improve lipid accumulation and transformation (Khozin-Goldberg and Cohen, 2006; Devi and Mohan, 2012; Feng et al., 2012; Roleda et al., 2013). In practice, after microalgae collection, the harvest water might lack N or P. The authors also designed the nutrient-limited conditions to cultivate *C. zofingiensis* using harvest water. In this study, a certain amount of chemical nutrients was added following Table 2. After preparation of the media the N and P concentrations of cultures were exhibited in Table 2, where full media with 100% and 50% recycling harvest water had equal N and P concentrations with 82 mg N L^{-1} and 81.5 mg PO_4^{-3}–P L^{-1}. The cultivation period lasted for 8 days in these tests.

2.3.2. Harvest water recycling times

In order to find out how many times the harvest water can be recycled, the 100% harvest water with the full medium (initial nutrient concentration at 82 mg N L^{-1} and 81.5 mg PO_4^{-3}–P L^{-1}) was recycled for three runs. The 1st and 2nd run lasted for 8 days, while the 3rd run lasted for 6 days. In each run microalgae growth

Table 1
Characteristics of autoclaved original wastewater and harvest water used in the experiments (mean ± SD).

Parameter	Autoclaved concentration	Harvest water concentration
pH	6.2 ± 0.0	7.4 ± 0.0
Suspended Solid (mg L^{-1})	366 ± 23	37 ± 8
COD (mg L^{-1})	3500 ± 63	435 ± 11
TN (mg N L^{-1})	148.0 ± 4.0	16.0 ± 0.7
TP (mg PO_4^{-3}–P L^{-1})	156.0 ± 8.0	7.0 ± 0.1

Table 2
The amount of urea [$CO(NH_2)_2$] and KH_2PO_4 added in the media and the final N and P concentrations.

Culture	Urea (mg L^{-1})	KH_2PO_4 (mg L^{-1})	TN (mg N L^{-1})	TP (mg PO_4^{-3}–P L^{-1})
100% Harvest water N+P+	141.4	74.5	82.0	81.5
100% Harvest water N+P−	141.4	–	82.0	7.0
100% Harvest water N−P+	–	74.5	16.0	81.5
100% Harvest water N−P−	–	–	16.0	7.0
50% Harvest water N+P+	–	–	82.0	81.5

Note: N+P+, N+P−, N−P+, and N−P− means full medium, P-limited medium, N-limited medium, and both N- and P- limited medium, respectively. 50% harvest water means half of the medium was from the original piggery wastewater and the other half was from the harvest water. – means no nutrients needed to be added.

conditions including the optical density (OD_{680}) variation were observed every day.

2.4. Cultivating C. zofingiensis

The above experiments were performed in tbcPBRs, which were the same as the ones described in Section 2.1. A volume of 720 mL of the above media (Table 2) was accordingly introduced into each tbcPBR, after which the pH values of the media were adjusted according to pre-determined values. Afterwards, a volume of 80 mL of seed microalgae suspension with OD_{680} of 3.150 was introduced into each tbcPBR. The indoor conditions involved were identical to those described in Section 2.1. All the experiments were carried out in duplicate.

2.5. Analytical procedures

2.5.1. Water analysis

All water analysis involved followed the Hach DR 2700 Spectrophotometer Manual (Hach. Procedure Manual, 2008), after the samples were firstly centrifuged at 3000 rpm for 10 min and then the supernatants were filtered using a 0.45 μm nylon membrane filter.

2.5.2. Determination of microalgae growth

The optical density of *C. zofingiensis* at 680 nm was determined every day using a spectrophotometer (Jingke 722 N, Shanghai, China). A linear relationship between the dried biomass and the OD_{680} was pre-determined, as shown below:

$$\text{Dry weight}(gL^{-1}) = 0.3401 \times OD_{680}, R^2 = 0.9876 \quad (1)$$

The specific growth rate (μ) in the exponential phase of algal growth was measured. The expression followed Eq. (2) (Issarapayup et al., 2009) below:

$$\mu(day^{-1}) = \ln(dw_2/dw_1)/(t_2 - t_1) \quad (2)$$

where dw_1 and dw_2 represent dry biomass (g L^{-1}) at time t_1 and t_2, respectively. The biomass productivity (P_{bm}) was determined using the following expression:

$$P_{bm}(mg\ L^{-1}day^{-1}) = (dw_i - dw_0)/(t_i - t_0) \quad (3)$$

where dw_i and dw_0 represent dry biomass (g L^{-1}) at time t_i and t_0 (initial time), respectively. The lipid productivity (P_{lp}) was determined using the following expression:

$$P_{lp}(mg\ L^{-1}day^{-1}) = P_{bm} \times C_{lp}/100 \quad (4)$$

where C_{lp} represents lipid content (% of dw). The biodiesel productivity (P_{bd}) was determined using the following expression:

$$P_{bd}(mg\ L^{-1}day^{-1}) = P_{bm} \times C_{FAME}/100 \quad (5)$$

where C_{FAME} represents FAME content (% of dw).

Microalgae cells from each tbcPBR were collected after *C. zofingiensis* grew for 8 days. The samples were centrifuged at 5000 rpm and 4 °C for 15 min. Supernatants were decanted and cell solids were washed carefully with distilled water, after which the cell solids were freeze-dried at −80 °C. The dried microalgae biomass was sealed in empty containers for lipid extraction and analysis of fatty acids methyl esters (FAME).

2.5.3. Lipid extraction

The quantification of the amount of total lipid content was measured according to a modified method by Bigogno et al., (2002). 100–150 mg of freeze-dried samples were weighed and then extracted with 2 mL of methanol containing 10% dimethyl sulfoxide (DMSO). The extraction took place in a water bath shaker at 45 °C for 45 min. The mixture was centrifuged at 3000 rpm for 10 min, after which the supernatant was collected. The leftover was re-extracted twice following the same procedures as mentioned above. Then, the leftover was extracted using a 4-mL mixture of hexane and ether (1:1, v/v). The extraction took place in a water bath shaker at 45 °C for 60 min. The mixture was centrifuged at 3000 rpm for 10 min, after which the supernatant was collected. The leftover was re-extracted twice following the same procedures as mentioned above. All the supernatants were incorporated and 6 mL of distilled water was added to the combined extracts. Thus, the ratio of methanol with 10% DMSO, diethyl ether, hexane and distilled water was 1:1:1:1 (v/v/v/v). The organic phases containing lipids were collected and combined into a pre-weighed glass tube. Afterwards, the samples were evaporated to dryness under the protection of nitrogen. Then, the lipids were freeze-dried at −80 °C for 24 h. Finally, the lipids were measured gravimetrically as a % of the dry weight.

2.5.4. FAME content analysis

A one step extraction–transesterification method by Indarti et al., (2005) was applied to analyze the content of the FAME after suitable modifications. 20 mg of freeze-dried samples of *C. zofingiensis* were weighed. Transesterification was performed using 2.5 mL of methanol with 2 wt.% of concentrated sulfuric acid (H_2SO_4) as the catalyst. The transesterification took place in a water bath shaker at 80 °C for 2.5 h. When the reaction was completed, the mixtures were cooled down to room temperature. Then, 1 mL of distilled water and 2 mL of hexane were added into the mixture to form two phases. The upper phase, which contained FAME, was transferred to a clean, 10 mL bottle and dried with water-free Na_2SO_4. At his point the sample was prepared well for the analysis of the FAME composition, which was carried out by a gas chromatograph with a FID detector (Shimadzu GC-2010, Japan). The temperatures of the injector and detector were set at 300 and 280 °C, respectively. The temperature gradient was programmed from 130 to 180 °C with an increase of 10 °C min^{-1} followed by a rise to 210 °C with 2 °C min^{-1} and then the temperature was fixed at 210 °C for 3 min. The compounds detected were identified and quantified using the NIST Mass Spectral Search Program.

All the experiments were carried out in duplicate and average values were reported. Results were performed with EXCEL and SPSS 11.5 for Windows.

3. Results and discussion

3.1. Harvest water recycling for C. zofingiensis re-production under nutrient limitation

3.1.1. Microalgal growth and biomass productivity

To evaluate the feasibility of the harvest water recycling on the production of microalgal biomass, 100% and 50% harvest water were recycled and used to cultivate *C. zofingiensis*. The media with full nutrients and nutrient limitation media were designed. By replenishing urea and/or KH_2PO_4, four treatments using 100% of the harvest water as the media were set up: 100% harvest water N+P+ culture (full medium), 100% harvest water N+P− culture (P limitation), 100% harvest water N−P+ culture (N limitation) and 100% harvest water N−P− culture (N and P limitation). One treatment using 50% harvest water and 50% fresh autoclaved piggery wastewater as the incorporated medium was also designed, called 50% harvest water N+P+ culture (full medium). The algal growth characteristics of *C. zofingiensis* cultivated in these five media for 8 days are shown in Fig. 1.

The results showed that *C. zofingiensis* in all treatments with 100% or 50% harvest water could grow well. The lag phase of algal growth was missing or shortened in all treatments. This was might because the harvest water still contained some un-harvested algal cells (Park et al., 2011; Lam and Lee, 2012), which could accelerate the growth of algae. Another reason might be that the un-harvested algal cells had already adapted to the wastewater environment, so once the nutrients were available, the microalgae could utilize the nutrients immediately. From the beginning to day 5, *C. zofingiensis* in the 100% harvest water N+P+ medium, 100% harvest water N+P− medium and 100% harvest water N−P+medium shared a similar growth tendency. From day 5 onwards, *C. zofingiensis* in the 100% harvest water N+P+ medium continued to grow robustly, while the growth rate of *C. zofingiensis* in the 100% harvest water N+P− medium and 100% harvest water N−P+ medium slowed down due to N or P limitation. The most robust growth of *C. zofingiensis* occurred in the 50% harvest water N+P+ medium, although the growth was obviously slow in the beginning. Due to the limitation of both N and P, *C. zofingiensis* produced in 100% harvest water N−P− medium demonstrated the weakest growth and came to a stable level within a short time.

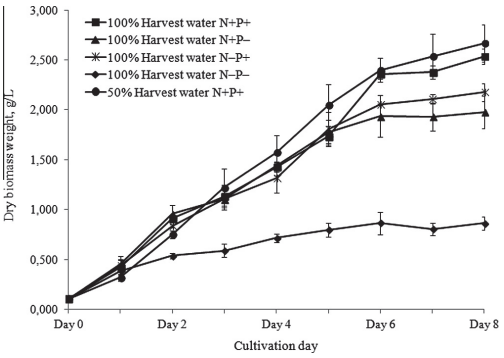

Fig. 1. The growth of *C. zofingiensis* when recycling harvest water as the media with different nutrient conditions (mean ± SD).

C. zofingiensis in the full media with 50% harvest water had the highest specific growth rate μ of 0.403 day^{-1} with the shortest doubling time of 1.72 days (Table 3). The μ of algae cultivated in the 100% harvest water N+P+ culture and the 100% harvest water N−P− culture had similar μ values of 0.345 and 0.334 day^{-1}, respectively. Algae in the N-limited culture and the P-limited culture had the lowest μ of 0.289 and 0.307 day^{-1}, respectively. The μ observed in this study was almost in agreement with the findings by Zhu et al., (2013b), who grew *C. zofingiensis* in piggery wastewater for 10 days and obtained μ of 0.320–0.340 day^{-1}. By the end of this test, the biomass productivities of algae ranged from 86.30 to 266.66 mg L^{-1} day^{-1} in the relationship: 50% harvest water N+P+ culture (266.66) > 100% harvest water N+P+ culture (253.48) > 100% harvest water N−P+ culture (217.58) > 100% harvest water N+P− culture (197.73) > 100% harvest water N−P− culture (86.30). It is easy to determine that the biomass productivity in the full medium was higher than that in the nutrient-limited cultures. This observation was in accordance with research by Mujtaba et al., (2012), who cultivated *Chlorella vulgaris* in bubble-column photobioreactors for 10 days and achieved the biomass productivity of 128 and 197 mg L^{-1} day^{-1} under N-depleted and N-rich conditions, respectively.

3.1.2. Lipid content and productivity

The lipid content and productivity of *C. zofingiensis* when recycling the harvest water at a degree of 100% or 50% under N- and/or P-limitation are exhibited in Fig. 2.

The lipid contents of *C. zofingiensis* cultured in nutrient limited conditions were obviously higher than those in cultures with full nutrients, since nutrient limitation favors lipid accumulation and transformation (Widjaja et al., 2009; Hodaifa et al., 2012; Roleda et al., 2013). Ito et al., (2013) found that in nitrogen deficient conditions amino acids in algal cells decreased to 1/20 the original amount, or less, while the quantities of neutral lipids increased greatly. In addition, Devi and Mohan (2012) suggested that the stored carbohydrates during growth phase might have channeled towards the formation of triacylglycerides (TAGs). The highest lipid content was observed in the 100% harvest water N−P− culture at 46.21%. The 100% harvest water N+P− culture (42.71%) witnessed a slightly higher lipid content than the 100% harvest water N−P+ culture (41.21%). Using 50% and 100% harvest water to produce algal biomass under full nutrient conditions, the algal lipid content appeared to be no difference, reaching around 26% of the dry

Table 3
Growth parameters of *C. zofingiensis* when recycling the harvest water at a degree of 100% or 50% under different nutrient conditions (mean ± SD).

Recycling classification	Specific growth rate μ (day^{-1})[a]	Doubling time (days)	Dry weight (g L^{-1})	Biomass productivity (mg L^{-1} day^{-1})
100% Harvest water N+P+	0.345 ± 0.038	2.01 ± 0.22	2.535 ± 0.078	253.48 ± 7.76
100% Harvest water N+P−	0.289 ± 0.013	2.39 ± 0.11	1.977 ± 0.162	197.73 ± 16.19
100% Harvest water N−P+	0.307 ± 0.025	2.26 ± 0.18	2.176 ± 0.088	217.58 ± 8.81
100% Harvest water N−P−	0.334 ± 0.025	2.08 ± 0.16	0.863 ± 0.066	86.30 ± 6.61
50% Harvest water N+P+	0.403 ± 0.033	1.72 ± 0.14	2.667 ± 0.188	266.66 ± 18.82

[a] The μ in full media and N or P limitation medium was determined according to Eq. (2) during the time period day 1 to day 6, while 100% Harvest water N−P− medium was measured according to Eq. (2) during the time period day 1 to day 2.

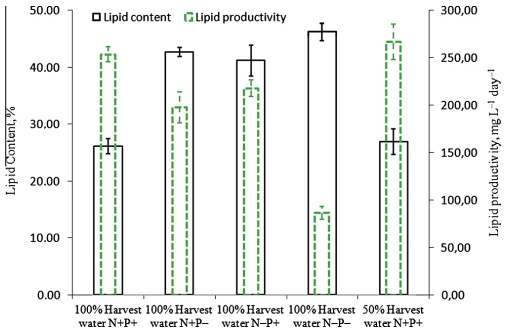

Fig. 2. Lipid content and productivity of *C. zofingiensis* when recycling the harvest water at a degree of 100% or 50% under different nutrient conditions (mean ± SD).

weight. This percentage was within the reported range of Chlorella for lipid content (26–32%) under regular conditions (Chisti, 2007).

Influenced by the biomass productivities, the sequence of lipid productivities in cultures was not identical to that of the lipid contents. The 100% harvest water N−P− culture enjoyed the highest lipid content, but the relative lipid productivity was the lowest due to the limited biomass productivity. The lipid yields ranged from 39.88 to 89.65 mg L^{-1} day^{-1} in the following order: 100% harvest water N−P+ culture (89.65) > 100% harvest water N+P− culture (84.44) > 50% harvest water N+P+ culture (71.73) > 100% harvest water N+P+ culture (66.34) > 100% harvest water N−P− culture (39.88). Feng et al., (2012) also designed three treatments (N+P+ culture, N+P− culture and N−P+ culture) to grow *C. zofingiensis* using fresh water as the solvent and obtained the lipid productivities at 68.1, 44.7 and 87.1 mg L^{-1} day^{-1} from the N+P+ culture, N+P− culture and N−P+ culture, respectively. In their study, the N+P+ culture witnessed higher lipid productivity than the N+P− culture since the P deficiency culture had a much lower biomass concentration and biomass productivity.

3.1.3. FAME composition and biodiesel productivity

Different algal species have different contents and compositions of fatty acids, which are also influenced by cultivation conditions (Cho et al., 2011). In this study there were 10 types of fatty acids from the algae in all of the treatments (Table 4). The main FAME ingredients of *C. zofingiensis* were C16:0 (palmitic acid methyl ester), C18:2 (octadecadienoic acid methyl ester) and C18:3 (octadecatrienoic acid methyl ester). Long chain fatty acids (C22:0, C22:1 and C24:0) were detected in small quantities in all cultures.

In the present study, the FAMEs containing 16 or 18 carbons (C16:0, C18:0, C16:1, C18:2 and C18:3) occupied from 81.95% (100% harvest water N−P− culture) to 94.76% (50% harvest water N+P+ culture) of the total FAMEs, which could improve the quality of biodiesel (Huang et al., 2010). Similar results were obtained by Cho et al., (2011), who grew *Chlorella* sp. in municipal wastewater and achieved high percentages of FAMEs with C16 and C18, ranging from 79.9% to 87.1% of the total fatty acids. In this study the cultures with nutrient limitation shared more FAMEs with C16 and C18, more saturated fatty acids methyl esters and less unsaturated fatty acids methyl esters than the cultures with full nutrients.

The FAME yields ranged from 6.16% to 10.95% of the dry cell weight in the relationship: 100% harvest water N−P− culture (10.95) > 100% harvest water N+P− culture (10.07) > 100% harvest water N−P+ culture (9.49) > 100% harvest water N+P+ culture (6.72) > 50% harvest water N+P+ culture (6.16). The FAME yields in this study were much lower than the 15–30% of the dry weight for FAME yields achieved by Cho et al., (2011). Du et al., (2012) cultivated *Chlorella vulgaris* using recycled aqueous phase nutrients from hydrothermal carbonization process and achieved 9.7–11.2% of FAME yields. Additionally, the FAME yields of nutrient-limited cultures during 8-day cultivation in this study were in line with the findings by Zhu et al., (2013a,b), who cultivated *C. zofingiensis* indoors for 10 days in cultures with COD concentrations $\leqslant$2500 mg L^{-1}. However, the FAME yields of nutrient-full cultures were much lower than those in the studies by Zhu et al., (2013a,b).

Combining Table 4 with Fig. 2, it is not difficult to see that only about one third or one fourth of lipids can be converted into biodiesel. This is because some lipid types such as chlorophyll, glycolipid and phospholipid cannot be converted into biodiesel (Zhu et al., 2013a). The lipid leftovers together with the residuals, which are rich in proteins and carbohydrates (Zhu et al., 2013a), can be used for biogas production via anaerobic digestion to sustain microalgal biodiesel production. According to the calculation by Sialve et al., (2009), the theoretical methane yields for lipids, proteins and carbohydrates are 1.014, 0.851 and 0.415 L g^{-1}, respectively. The residues after anaerobic digestion can be supplied as the nutrient sources for the re-cultivation of algae or can be sold as fertilizers (Zhu et al., 2013a). Affected by the biomass productivities, the 100% harvest water N−P− culture, which observed the highest FAME yields, shared the lowest biodiesel productivity at 9.45 mg L^{-1} day^{-1}. However, importantly, no additional nutrient

Table 4
Summary of FAME profile for *C. zofingiensis* when recycling the harvest water at a degree of 100% or 50% under different nutrient conditions.

FAME composition		100% Harvest water N+P+	100% Harvest water N+P−	100% Harvest water N−P+	100% Harvest water N−P−	50% Harvest water N+P+
Saturated fatty acids (% of total FAME)	C16:0	31.88	41.35	42.34	38.93	32.92
	C18:0	3.94	6.73	7.14	7.15	5.06
	C24:0	3.80	1.15	4.42	0.61	2.98
	Subtotal	39.63	49.23	53.90	46.69	40.97
Monoenoic fatty acids (% of total FAME)	C16:1	4.42	1.45	0.45	5.58	4.40
	C20:1	2.90	3.93	5.20	0.31	4.40
	C22:1	4.33	1.33	1.47	1.08	1.69
	C24:1	2.89	2.83	3.91	2.52	4.48
	Subtotal	14.55	9.54	11.03	9.49	14.96
Polyenoic fatty acids (% of total FAME)	C18:2	30.67	27.25	21.68	29.21	24.85
	C18:3	11.33	13.29	12.84	13.89	14.72
	C20:2	3.82	0.70	0.54	0.72	4.50
	Subtotal	45.82	41.24	35.07	43.82	44.07
C16–C18 (% of total FAME)		82.25	90.06	84.46	94.76	81.95
Total (% of dw)		6.72	10.07	9.49	10.95	6.16
Biodiesel productivity (mg L^{-1} day^{-1})		17.03	19.91	20.66	9.45	16.42

needs to be added into the culture system with N and P limitation, which will save costs. The biodiesel productivities for the 100% harvest water N−P+ culture, 100% harvest water N+P− culture, 100% harvest water N+P+ culture and 50% harvest water N+P+ culture were 20.66, 19.91, 17.03, and 16.41 mg L^{-1} day^{-1}, respectively. Thus, it is indicated that the biodiesel productivities can be raised and the costs of nutrient supply can be reduced under N or P limitation, compared to cultures with full nutrients.

3.2. Microalgal growth during three runs for harvest water recycling

Except the synthesis of lipids, sugar and protein during algal growth, microalgae can also produce some metabolites including amino acids, nucleic acids, polysaccharides, polypeptide, vitamins, organic phosphoric acid, toxins and volatile substances (Zhang et al., 2012). Some of these ingredients, such as some toxins, can be absorbed by algae (Valderrama et al., 2002), while some of them are harmful and inhibitive for algae species. These harmful metabolites can be accumulated in the culture system when circulating harvest water over and over again and thus the growth of biomass will be affected and even inhibited. Another inhibitory contributor might be the susceptibility to contamination by fungus and bacteria (Lam and Lee, 2012). In order to find out to what extent the harvest water can be recycled, 100% of the harvest water with full medium (N+P+) was recycled three times to re-grow *C. zofingiensis*. The growth of *C. zofingiensis* during these three runs was measured (Fig. 3).

As shown in Fig. 3, without any pre-treatment or purification process of the harvest water, the algae could grow rapidly when circulating the harvest water for the 1st and 2nd time. The algae could withstand metabolites and possible contaminants in the culture. Thanks to the un-harvest algal cells (Park et al., 2011), the growth rate of *C. zofingiensis* at the beginning of 4 days in the 1st run was faster than that in the original autoclaved piggery wastewater. From day 5 onwards, the speed slowed down, but there was no obvious inhibition phenomena observed. As to the 2nd run, a robust growth of algae was witnessed in the beginning, but there was a visible inhibition phenomenon in the later stages of the cultivation. As to the 3rd run, the algae could survive throughout the experiment. Nonetheless, there was a significant reduction of algal growth in the 3rd run. The analytical results showed that at the end of the 3rd run the TN and TP concentrations were 50.0 and 42.0 mg L^{-1}, respectively. In other words, the inhibition phenomenon was evident even under the conditions with sufficient nutrients. With the assistance of microscopic analysis, no protozoa such as rotifer or any other algae species was observed. Thus, the culture might contain some inhibitive factors, which could be some harmful metabolites, salinity stress due to evaporation, and/or inhibitory bacteria. The continuous accumulation of harmful metabolites might form concentration stress, which hinders the growth of algae. Nevertheless, which kind of algal metabolite might be inhibitive still needs to be further investigated. In addition, the salinity increase in harvest water recycled 3 times might begin to affect the growth of algae in a detrimental manner (Mata et al., 2010). Finally, inhibitory bacteria reproduction might also be a contributor. Zhang et al., (2012) reported that some bacteria could damage the microalgae by releasing soluble cellulose enzyme or toxic substances, while some bacteria could also adhere to the inner-wall of the cell which would inhibit photosynthesis by hindering the transmission of light. Fergola et al., (2007) suggested that *Vibrio*, *Flavobacterium*, *Pseudomonas*, and *Alteromonas* could excrete extracellular substances to kill algal cells. Thus, the biomass productivity in the 3rd run was not satisfactory. On the basis of the above observation, the authors can come to the conclusion that harvest water could be 100% recycled twice to prepare a full nutrient medium to re-grow *C. zofingiensis*.

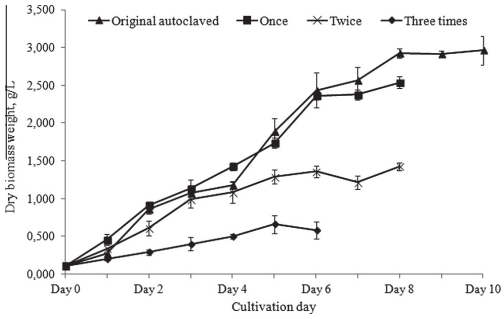

Fig. 3. Growth performance changes of *C. zofingiensis* with harvest water recycling times in a full medium (mean ± SD).

4. Conclusion

Harvest water from *C. zofingiensis* production could be recycled to re-grow *C. zofingiensis* with biomass productivities ranging from 86.30 to 266.66 mg L^{-1} day^{-1}. The lipid contents of the 100% harvest water N+P− culture and 100% harvest water N−P+ culture were 42.71% and 41.21% of dry weight, respectively. The 100% harvest water N−P− culture witnessed the highest FAME yield at 10.95% of dry weight, while nutrient-limited cultures observed the highest biodiesel productivities at around 20 mg L^{-1} day^{-1}. Harvest water from algae production could be 100% recycled twice with the addition of sufficient nutrients.

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Scale-up potential of cultivating *Chlorella zofingiensis* in piggery wastewater for biodiesel production

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HIGHLIGHTS

- Using NaClO was an effective way to pretreat wastewater for algae growth.
- *C. zofingiensis* was able to adapt and grow well outdoors.
- 1.314 g L^{-1} day^{-1} of biomass was achieved stably under semi-continuous operation.
- It is greatly possible to amplify *C. zofingiensis* production using piggery wastewater.

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ABSTRACT

Scale-up potential of cultivating *Chlorella zofingiensis* in piggery wastewater for simultaneous wastewater treatment and biodiesel production was tested. The cultivation of *C. zofingiensis* with autoclaved wastewater and NaClO-pretreated wastewater, cultivation of algae indoors and outdoors, and stability of semi-continuous feeding operation were examined. The results showed that *C. zofingiensis* cultivated in piggery wastewater pretreated by autoclaving and NaClO had no evident difference in the performance of nutrient removal, algal growth and biodiesel production. The outdoor cultivation experiments indicated that *C. zofingiensis* was able to adapt and grow well outdoors. The semi-continuous feeding operation by replacing 50% of algae culture with fresh wastewater every 1.5 days could provide a stable net biomass productivity of 1.314 g L^{-1} day^{-1}. These findings in this study can prove that it is greatly possible to amplify the cultivation of *C. zofingiensis* in piggery wastewater for nutrient removal and biodiesel production.

1. Introduction

The world population is expanding, creating a demand for alternative and renewable sources to fossil oil. Bioenergy, which is considered carbon-neutral and renewable, can contribute to the sequestration of carbon dioxide and help satisfy the ever-increasing demand for energy consumption (Jeon et al., 2012). Biodiesel, the mixture of fatty acid methyl esters (FAMEs), has recently received a great deal of attention due to the advantages related to its renewability and biodegradability (Chen et al., 2012). Biodiesel can be generated from edible or non-edible plant oils, animal fats, and waste cooking oils. In the US and Europe biodiesel from soybean or rapeseed has been enjoying a surge in popularity (Feng et al., 2011). However, the demand of biodiesel in market far exceeds the accessibility of these kinds of feedstock. In addition, the feasibility of producing biodiesel from edible oils may conflict with current energy policies, and the growth of oil crops may compete with food crop cultivation for lands (Zhu and Ketola, 2012). Thereby, a major insight into the quest for new sources of biodiesel can be definitely offered by microalgae. Microalgae are photosynthetic microorganisms that can efficiently use CO_2 (carbon source), light (energy source) and water to synthesize carbon-rich lipids (Khan et al., 2009), which can be converted into biodiesel via transesterification process. Importantly, growth of microalgae to generate biodiesel will not compromise crop-based production of food, fodder and other products (Chisti, 2007), since a lot of unfertile lands can be used to cultivate microalgae. Other advantages of the use of microalgae to produce biodiesel lie in, such as, high photosynthetic yields, high lipid content, easy harvest, and freshwater and fertilizer saving by using nutrient-rich wastewaters.

Among researches associated with microalgae production for biodiesel, the use of wastewaters to cultivate microalgae has got plenty of interest and has been considered as a promising solution. Chinnasamy et al. (2010) grew a consortium of 15 native algal

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isolates in a wastewater containing 85–90% carpet industry effluents with 10–15% municipal sewage, and found that maximum biomass and lipids were produced and more than 96% nutrients were removed in 72 h at 15 °C. The removals of chemical oxygen demand (COD), total nitrogen (TN), and total phosphorus (TP) at 70%, 61%, and 61% were respectively obtained in the study by Min et al. (2011) who employed a locally isolated microalgae strain *Chlorella* sp. to treat municipal wastewater using a pilot-scale photobioreactor (PBR). Kothari et al. (2012) tested an integrated approach for wastewater treatment and biodiesel production by introducing *Chlorella pyrenoidosa* into dairy wastewater, attaining the expected results with about 80–85% P and 60–80% N removal. However, most of the researches are based on the lab or pilot scale and thus microalgae are not yet commercially produced on a large scale for bulk application (Zhu and Ketola, 2012). Still, the wastewaters used usually need to be sterilized by autoclave before the introduction of microalgae species, which is energy-intensive and complex for real operation. Moreover, most of feeding modes in the previous studies are based on batch operation which is time-consuming, causing a limited capacity and ability to treat wastewaters. All of these can contribute to the bottlenecks of the amplification of cultivating microalgae species in wastewaters in practice, showing that this apparently promising approach is still in its infancy.

In this work freshwater microalgae *Chlorella zofingiensis* was cultivated using piggery wastewater as medium. In order to simply investigate its scale-up potential, three objectives of this study were employed: (1) to compare the cultivation of *C. zofingiensis* in supernatant piggery wastewater respectively pretreated by autoclaving and sodium hypochlorite (NaClO), (2) to compare the cultivation of *C. zofingiensis* under indoor and outdoor conditions, and (3) to investigate stability and biomass productivity under semi-continuous feeding operation.

2. Methods

2.1. Pure microalgae strain and pre-culture conditions

The microalgae *C. zofingiensis* was preserved in the lab in Guangzhou Institute of Energy Conversion of Chinese Academy and grown in BG11 medium (Rippka et al., 1979), consisting of: (1) the following solid ingredients: $NaNO_3$ (1.5 g L^{-1}), $K_2HPO_4{\cdot}3H_2O$ (40 mg L^{-1}), $MgSO_4{\cdot}7H_2O$ (75 mg L^{-1}), $CaCl_2{\cdot}2H_2O$ (36 mg L^{-1}), Na_2CO_3 (20 mg L^{-1}), $FeCl_3{\cdot}6H_2O$ (3.15 mg L^{-1}), and (2) the following chemicals: citric acid (6 mg L^{-1}) and 1 mL per liter of trace elements solution[1] consisted of H_3BO_3 (2.86 mg L^{-1}), $MnCl_2{\cdot}4H_2O$ (1.81 mg L^{-1}), $ZnSO_4{\cdot}7H_2O$ (0.22 mg L^{-1}), $Na_2MoO_4{\cdot}2H_2O$ (0.39 mg L^{-1}), $CuSO_4{\cdot}5H_2O$ (0.08 mg L^{-1}), $Co(NO_3)_2{\cdot}6H_2O$ (0.05 mg L^{-1}) and concentrated H_2SO_4 (1 mL). The initial pH of the medium was adjusted to 6.8. The seed culture was grown in the 1.37 L tubular bubble column photobioreactors (tbcPBRs, height 70 cm and internal diameter 5.0 cm) containing 800 mL of medium, aerated with compressed air with 5–6% CO_2, surrounding temperature of 25 ± 1 °C and illuminated with cool white fluorescent lamps at the single side of the tbcPBRs (light intensity of 230 ± 20 μmol m^{-2} s^{-1}) continuously.

2.2. Composition and pretreatment of piggery wastewater

The experiments were carried out using piggery wastewater from a private farm (about 100 swine) near the laboratory as a substrate. Pretreatment was carried out by sedimentation and filtration with filter cloth to remove large non-soluble particulate solids. After filtration the substrate was divided into two equal portions. One portion was autoclaved for 20 min at 121 °C, after which the liquid was stored at 4 °C for 2 days to settle out any visible particulate solids and the supernatant was used for microalgae growth studies. The other was pretreated with 10% NaClO at ratio of 0.1 mL per 1 L substrate for 12 h under dark environment, after which the liquid was exposed to sunlight for 12 h (light intensity of 1530 ± 219 μmol m^{-2} s^{-1}) and then was stored at 4 °C for 1 day to settle out any visible particulate solids to prepare the supernatant for microalgae growth studies. Previous study showed that a ratio of original piggery wastewater at 0.54-fold with 1900 mg/L COD was the optimal nutrient concentration for the cultivation of *C. zofingiensis* (Zhu et al., unpublished information). On the basis of this observation, both the autoclaved and NaClO-pretreated supernatant wastewater used for all treatments were diluted to 1900 mg/L COD for microalgal cultivation. The characteristics and features of the autoclaved and NaClO-pretreated wastewater before dilution are summarized in Table 1.

Table 1
Characteristics of autoclaved and NaClO-pretreated piggery wastewater before dilution in the experiments (means ± SD).

Parameter	Autoclaved concentration	NaClO-pretreated concentration
pH	6.2 ± 0.0	6.2 ± 0.0
Suspended solid (mg L^{-1})	366 ± 23	118 ± 11
COD (mg L^{-1})	3500 ± 63	3460 ± 51
TN (mg N L^{-1})	148.0 ± 4.0	139.0 ± 6.0
TP (mg PO_4^{-3}–P L^{-1})	156.0 ± 8.0	146.5 ± 1.5

2.3. Experimental design

There were three relative experimental designs in this paper. (1) The effect of piggery wastewater pretreatment on microalgal growth, nutrients removal and biodiesel productivity were studied under indoor culture conditions. The target was to find out if the easier pretreatment by NaClO could obtain ideal results, thus accelerating scale-up process. The growth of *C. zofingiensis* in this test would last for 10 days. (2) The microalgal growth and biodiesel productivity were researched under indoor and outdoor conditions by using autoclaved supernatant wastewater, with the objective of providing outdoor cultivation data for the possible amplification. The growth of *C. zofingiensis* in this run would last for 10 days as well. (3) The efficiency and stability of semi-continuous feeding operation were tested under indoor conditions, aiming at investigating the feasibility of growing microalgae as a practical approach to remove nutrients and accumulate biomass. The semi-continuous operation was first carried out in batch mode for 6 days and then in semi-continuous mode with 50% of algae culture solution harvested and the same amount of autoclaved supernatant wastewater replenished every 36 h for a period of 9 days.

2.4. Cultivating C. zofingiensis

The above experiments were performed in tbcPBRs whose properties were the same as the ones described in Section 2.1. A volume of 720 mL of pretreated supernatant piggery wastewater was accordingly introduced into each tbcPBR, after which the pH values of the media were adjusted according to pre-determined values. Afterwards, a volume of 80 mL of seed microalgae suspension with optical density (OD_{680}) of 3.0 were, respectively introduced into each tbcPBR. The indoor conditions involved were identical to those described in Section 2.1. To create the outdoor conditions for *C. zofingiensis* cultivation, two tbcPBRs were located under the roof of the lab, continuously aerated with compressed air

[1] The concentration of trace elements solution was reported as stock concentration.

with 5–6% CO_2, surrounding temperature of 29.4 ± 3.9 °C and sunlight intensity of 842 ± 778 μmol m^{-2} s^{-1} (8 am to 6 pm during the day). All the experiments were carried out in duplicate.

2.5. Analytical procedures

2.5.1. Sampling and nutrients analysis

A volume of 3 mL microalgae suspension was collected every day from each tbcPBR for nutrient removal analysis starting from inoculation. The samples were first centrifuged at 3000 rpm for 10 min, after which the supernatants were filtered using the 0.45 μm nylon membrane filter. Then, the filtrates were appropriately diluted and analyzed for COD, TN, and TP following the Hach DR 2700 Spectrophotometer Manual (Hach, 2008). The percentage removal was obtained by using the following expression:

$$\text{Percentage removal } W\% = 100\% \times (C_o - C_i)/C_o \quad (1)$$

where C_o and C_i are defined as the mean values of nutrient concentration at initial time and time t_i, respectively.

2.5.2. Determination of microalgae growth

A correlation between the optical density of *C. zofingiensis* at 680 nm and dried biomass was pre-determined. OD_{680} was measured every day using spectrophotometer (Jingke 722 N, Shanghai, China). The correlation is shown below:

$$\text{Dry weight } (g\,L^{-1}) = 0.3387 \times OD_{680}, R^2 = 0.9913 \quad (2)$$

The specific growth rate (μ) in exponential phase of algal growth was measured by using Eq. (3) (Issarapayup et al., 2009):

$$\mu\ (day^{-1}) = \ln\ (N_2/N_1)/(t_2 - t_1) \quad (3)$$

where N_1 and N_2 are defined as dry biomass (g L^{-1}) at time t_1 and t_2, respectively. The biomass productivity (P) was calculated according to the following formula:

$$P = (dw_i - dw_0)/(t_i - t_0) \quad (4)$$

where dw_i and dw_0 are dry biomass (g L^{-1}) at time t_i and t_0 (initial time), respectively.

After the *C. zofingiensis* grew for 10 days, microalgae cells from each tbcPBR were collected and centrifuged at 5000 rpm, 4 °C for 15 min. Supernatants were decanted and cell pellets were washed with distilled water and then freeze-dried under −80 °C. The dried microalgae biomass were collected and sealed in an empty container for lipid extraction and fatty acid methyl ester (FAME) content analysis.

2.5.3. Lipid extraction

A modified method of Bigogno et al. (2002) was applied to quantify the amount of total lipid content. 100–150 mg freeze-dried samples of *C. zofingiensis* were extracted with 2 mL methanol containing 10% dimethyl sulfoxide (DMSO) in a water bath shaker at 45 °C for 45 min. The mixture was centrifuged at 3000 rpm for 10 min, after which the supernatant was collected and leftover was re-extracted twice following the same procedures. Then, the leftover was extracted with 4 mL mixture of hexane and ether (1:1, v/v) in a water bath shaker at 45 °C for 60 min. The mixture was centrifuged at 3000 rpm for 10 min, after which the supernatant was collected and leftover was re-extracted twice following the same procedures. All the supernatants were incorporated and 6 mL distilled water was added to the combined extracts, so as to form a ratio of methanol with 10% DMSO, diethyl ether, hexane and distilled water of 1:1:1:1 (v/v/v/v). The organic phases containing lipids were combined into a pre-weighed glass tube and evaporated to dryness under the protection of nitrogen. Then, the lipids were freeze-dried under −80 °C for 24 h. Thereafter, the total lipids were measured gravimetrically, and then lipid content was expressed as % of dry weight.

2.5.4. FAME content analysis

The content of FAME was analyzed following a one step extraction-transesterification method by Indarti et al. (2005), after suitable modifications. Transesterification was performed using 20 mg of freeze-dried samples of *C. zofingiensis*, and 2.5 mL methanol with 2 wt.% of concentrated sulfuric acid (H_2SO_4) as the catalyst. The reaction was carried out in a water bath shaker at 80 °C for 2.5 h. On completion of the reaction, the mixtures were cooled down to room temperature, after which 1 mL distilled water and 2 mL hexane were added into the mixture. After the formation of two phases, the upper phase containing FAME was transferred to a clean, 10 mL bottle and dried with water-free Na_2SO_4. Until now, the sample was ready for the FAME composition and content analysis which was carried out by a gas chromatograph with FID detector (Shimadzu GC-2010, Japan). The injector and detector temperatures were set at 300 and 280 °C, respectively. The temperature gradient was programmed from 130 to 180 °C with an increase of 10 °C/min followed by a rise to 210 °C with 2 °C/min, and then the temperature was fixed at 210 °C for 3 min. The compounds detected were identified and quantified using NIST Mass Spectral Search Program.

All the experiments were carried out in duplicate and average values were reported. Results were performed with EXCEL (Microsoft Office Enterprise, 2007) and SPSS 11.5 for Windows (SPSS Inc., 2007).

3. Results and discussion

3.1. Nutrient removal in indoor cultivation of C. zofingiensis

Nutrient removal comparison of the cultivation of *C. zofingiensis* with autoclaved medium and NaClO-pretreated medium under indoor conditions was measured as shown in Fig. 1.

From Fig. 1, it was not tough to find that there was no obvious difference in nutrient removals when culturing *C. zofingiensis* with autoclaved medium and NaClO-pretreated medium. The nutrient removals continuously climbed within the first 4 days, slowed down afterwards, and stabilized from Day 5 on. The phenomenon that the nutrient removal was high in the beginning of algal growth in this study was in accordance with the research by Li et al. (2011), who grew *Chlorella* sp. in the highly concentrated municipal wastewater and discovered that most of the nutrients were removed within the first 3 days. After 10-day cultivation, *C. zofingiensis* grown in autoclaved piggery wastewater could remove 79.84% COD, 82.70% TN and 98.17% TP, while, using NaClO-pretreated piggery wastewater to culture *C. zofingiensis*, 78.29% COD, 84.49% TN and 95.26% TP were reduced. Previous study reported that the maximum of COD removal with 73.18% was achieved when *Arthrospira (Sprirulina) platensis* was cultivated in 25% olive oil mill wastewater pretreated by 12.5 g L^{-1} NaClO (Markou et al., 2012). Kong et al. (2010) cultivated *Chlamydomonas reinhardtii* in municipal wastewater using biocoil photobioreactor, and found that approximately 83.00% of the N and 14.45% of the P were removed from the centrate wastewater without any dilution. Surprisingly, the TP removal in this present study was evidently high. The reason might be apart from phosphorus uptake the increase of pH to around 7.4 in the treatment cultures at the end of this experiment could help contribute to the precipitation of phosphorus and the increase of phosphate adsorption on microalgal cells (Ruiz-Marin et al., 2010).

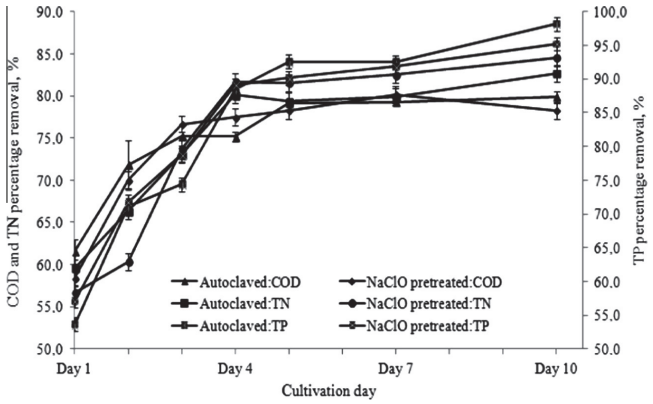

Fig. 1. Nutrient removals by *C. zofingiensis* grown in piggery wastewater pretreated by autoclave and NaClO under indoor conditions (means ± SD).

3.2. C. zofingiensis cultivation under indoor and outdoor conditions

3.2.1. Algal growth characteristic

The growth characteristics (Fig. 2) of *C. zofingiensis* grown under indoor and outdoor conditions treating piggery wastewater illustrated that the lag phase was not obvious, only lasting for one day until Day 1. After that, *C. zofingiensis* stepped into exponential phase which was not evident as well but still visible. From Day 8 on, the relatively stationary phase appeared.

Under indoor situation, there was no evident difference in growth of microalgae cultivated in autoclaved piggery wastewater and NaClO-pretreated piggery wastewater, and their final biomass productivities were 296.16 and 285.96 mg L^{-1} day^{-1}, respectively. The use of NaClO for pretreatment could slightly improve the growth of *C. zofingiensis* from Day 3 to Day 8, which was in agreement with the findings by Mutanda et al. (2011) who suggested that the growth rate of the *Chlorella* spp. was enhanced in post-chlorinated wastewater supplemented with 5 mM $NaNO_3$ and the maximal biomass productivity was achieved. The use of NaClO could decrease turbidity (Table 1), and thus improved the photosynthetic potential of the microalgae, resulting in faster growth of algal cells (Borowitzka et al., 1998). Markou et al. (2012) used different concentrations of NaClO to pretreat olive-oil mill wastewater before the cultivation of *Arthrospira (Spirulina) platensis*, and achieved the maximum biomass productivity of 121 mg L^{-1} day^{-1}. The specific growth rate μ of microalgae grown in autoclaved culture was a little higher than that in NaClO-pretreated culture, coming up to 0.340 and 0.337 day^{-1} with a doubling time of 2.04 and 2.06 days, respectively (Table 2).

Amazingly, *C. zofingiensis* could survive and adapt well outdoors, but was observed to grow a little more slowly under outdoor environment. Currently, most of the studies related to algae production are designed for indoor culture under a controllable environment which is pretty easy to pursue. Nonetheless, in an effort to search for the potential of commercial production, outdoor culture with easy access to sunlight is the most viable solution, since it can reduce the energy input and thus improve its cost effectiveness (Lam and Lee, 2012). As illustrated in Fig. 3, under outdoor conditions the change of weather, temperature and light intensity was inconsistent. During 10-day experiments under uncontrolled environment, the outdoor air temperature and light intensity (on the basis of single measurement) varied from 21.5 to 34.5 °C, and from

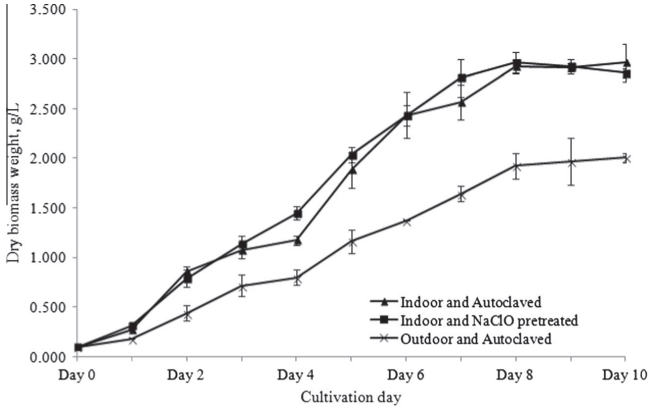

Fig. 2. Growth curves for *C. zofingiensis* grown under indoor and outdoor conditions with different pretreatments (means ± SD).

Table 2
Growth parameters of *C. zofingiensis* in tbcPBRs under indoor and outdoor conditions with different pretreatments (means ± SD).[a]

Culturing condition	Specific growth rate μ day^{-1})[b]	Doubling time (days)	Biomass productivity (mg L^{-1} day^{-1})
Indoor and autoclaved	0.340 ± 0.001	2.04 ± 0.01	296.16 ± 19.16
Indoor and NaClO-pretreated	0.320 ± 0.004	2.17 ± 0.02	285.96 ± 4.93
Outdoor and autoclaved	0.337 ± 0.013	2.06 ± 0.08	200.58 ± 4.60

[a] Data were represented as mean ± standard deviation of duplicates.
[b] μ was determined according to Eq. (3) during the time period Day 1 to Day 8 (t_1 = 1 and t_2 = 8).

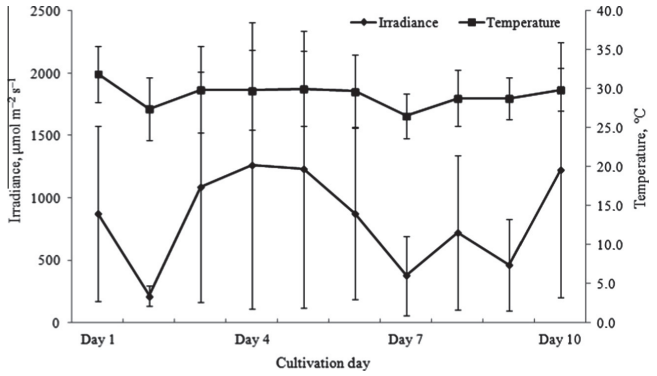

Fig. 3. The fluctuation of irradiance and air temperature of each day (8:00 am to 6:00 pm) under outdoor conditions (means ± SD). The temperature and irradiance were measured daily at 8 am, 10 am, 12 pm, 14 pm, 16 pm and 18 pm, and the mean values and standard deviations were reported.

115 to 2046 μmol m^{-2} s^{-1}, respectively. The μ of *C. zofingiensis* cultivated outdoors was 0.337 day^{-1} with a doubling time of 2.06 days, and the final biomass productivity arrived at 200.58 mg L^{-1} day^{-1}.

3.2.2. Lipid content and productivity

This study demonstrated that *C. zofingiensis* cultivated outdoors with the autoclaved piggery wastewater could achieve the highest lipid content of 34.82% in comparison with algae cultivated indoors using autoclaved piggery wastewater and NaClO-pretreated piggery wastewater (Fig. 4). The lipid content of algae cultivated outdoors was a little higher than the 31.8% lipid content of *C. zofingiensis* cultured in bench-scale outdoor ponds by Huo et al. (2012), who used acetic acid to regulate pH of dairy wastewater during cultivation. In spite of the highest lipid content, the outdoor algae had the lowest lipid productivity (69.83 mg L^{-1} day^{-1}), which was impacted by the lowest biomass obtained under outdoor conditions. The lipid content of *C. zofingiensis* cultured with autoclaved medium and NaClO-pretreated medium under indoor conditions was similar, reaching 33.33% and 32.99%, respectively. Influenced by biomass productivity, *C. zofingiensis* in NaClO-pretreated piggery wastewater observed a little lower lipid productivity of 94.33 mg L^{-1} day^{-1} than that in autoclaved medium (98.71 mg L^{-1} day^{-1}). Feng et al. (2011) also achieved the highest lipid content of 42% and the lipid productivity of 147 mg L^{-1} d^{-1} by cultivating *Chlorella vulgaris* in autoclaved artificial wastewater under indoor conditions.

3.2.3. FAME content analysis

Biodiesel is a mixture of fatty acid alkyl esters produced by a transesterification process where triacylglycerols react with methanol or ethanol, a mono-alcohol (Ehimen et al., 2010). The FAME composition of *C. zofingiensis* cultivated under indoor and outdoor conditions with different pretreatments for ten days was exhibited in Table 3.

As it could be easily found from Table 3, there were 9 or 10 FAMEs within the algal biodiesel composition, and the most abundant composition was C18:2 (octadecadienoic acid methyl ester) with the content ranging from 22.94% to 36.05%. This finding was partially in accordance with the observation by Lam and Lee (2012), who achieved the highest C18:2 at around 44% of the total FAMEs when using organic fertilizer to cultivate *Chlorella vulgaris*. The main composition of FAME in all cases was C16:0 (palmitic acid methyl ester), C16:1 (palmitoleic acid methyl ester), C18:0 (stearic acid methyl ester), C18:2 and C18:3 (octadecatrienoic acid methyl ester), which accounted for 71.79–84.97% of total FAME. The fatty acids with 16–18 carbon atoms are considered as the ideal ingredients for biodiesel production, since the properties such as density, viscosity, flash point and heating value can be enhanced, and thus the quality of biodiesel can be improved (Xu et al., 2006). There was no obvious difference in the FAME profile of *C. zofingiensis* under indoor operation. The proportion of unsaturated fatty acid methyl esters from outdoor *C. zofingiensis* was higher than that from indoor algae. This could decrease the pour point of biodiesel, in that it was found that the more the unsaturation, the harder it becomes for the FAME molecules to crystallize (O'Brien, 2004). This makes sense for car users to drive in cold climate conditions.

Some lipid forms such as chlorophyll, glycolipid and phospholipid are not efficient ingredients for FAME transformation. In this present study, the FAME yields of indoor *C. zofingiensis* were close, with the achievement of 11.15 and 10.18 g-biodiesel/100 g-dry weight for the algae grown in autoclaved medium and NaClO-pretreated medium, respectively. These findings were comparable to 9.98 g-biodiesel/100 g-dry weight which was obtained by Li et al. (2011), who cultivated *Chlorella* sp. in autoclaved municipal wastewater under indoor conditions. The FAME yield of algae cultivated

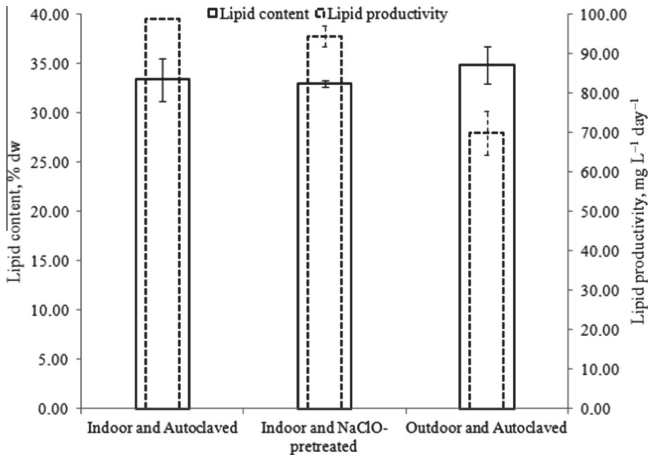

Fig. 4. Lipid content and productivity of *C. zofingiensis* grown under indoor and outdoor conditions with different pretreatments (means ± SD).

Table 3
Summary of FAME profile for *C. zofingiensis* cultivated under indoor and outdoor conditions with different pretreatments.

FAME composition		Indoor and autoclaved	Indoor and NaClO-pretreated	Outdoor and autoclaved
Saturated fatty acids (% of total FAME)	C16:0	19.73	18.76	9.93
	C18:0	17.66	21.64	10.31
	C24:0	7.73	6.01	15.20
	Subtotal	45.11	46.41	35.43
Monoenoic fatty acids (% of total FAME)	C16:1	5.42	6.01	6.62
	C20:1	0.81	0.60	0.60
	C22:1	0.00	2.63	3.34
	C24:1	5.46	3.73	5.71
	Subtotal	11.68	12.97	16.27
Polyenoic fatty acids (% of total FAME)	C18:2	26.89	22.94	36.05
	C18:3	14.82	15.62	8.89
	C20:2	1.50	2.06	3.36
	Subtotal	43.20	40.62	48.30
C16–C18 (% of total FAME)		84.51	84.97	71.79
Total (% of dw)		10.18	10.15	9.19

outdoors was witnessed a little lower than that of indoor algae, reaching up to 9.19 g-biodiesel/100 g-dry weight. This observation under outdoor operation can help verify the potential to amplify the production of *C. zofingiensis*-based biodiesel by the improvement of its cost effectiveness, since biodiesel productivity under outdoor conditions is competitive due to less energy input.

3.3. C. zofingiensis cell growth under semi-continuous operation

Considering that the daily process ability is important for wastewater treatment plants, it is necessary to find out the efficiency and stability of semi-continuous feeding operation in an attempt to investigate the feasibility of growing microalgae as a practical approach. The semi-continuous feeding operation after 6-day batch cultivation was illustrated in Fig. 5. In batch culture, the algal growth went through the visible lag phase and exponential phase. When the biomass accumulation came to 2.448 g L^{-1} at Day 6, the semi-continuous mode lasting for 9 day was operated by replacing 50% of algae culture solution with the same amount of fresh autoclaved supernatant wastewater every 36 h.

As demonstrated in Fig. 5, the daily biomass productivity during 9-day semi-continuous operation verified the robustness of the culture strategy in this study, where the biomass maintained at between 1.706 and 2.022 g L^{-1} with an obvious decrease within the first and second run (Day 7.5 and 9). When the biomass accumulation came to steady level from Day 10.5, the average biomass concentration was achieved at 1.971 g L^{-1} with a net productivity of 1.314 g L^{-1} day^{-1}, which was quite higher than the results in batch culture as shown in Table 2. Two main reasons might account for the high biomass in semi-continuous operation. One was that algae at Day 6 was still at exponential phase, which could accelerate the growth of algae when the fresh nutrients were available, while the other was that the harvest of the high density of algae and supplementation of fresh wastewater could minimize the self-shading effect (Li et al., 2011) which could reduce the photosynthesis efficiency.

3.4. Further discussion of scale-up potential

3.4.1. C. zofingiensis production has potential to be amplified

From technical point of view, there are several advantages involved with potential scale-up of *C. zofingiensis* biomass production for biodiesel conversion. Firstly, no matter whether the piggery wastewater was pretreated by NaClO or autoclaving, the

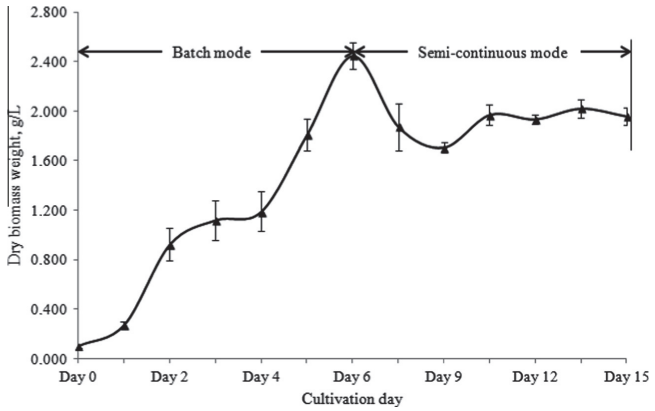

Fig. 5. Growth curve of *C. zofingiensis* during semi-continuous operation process under indoor conditions (means ± SD).

performances in algal growth (Fig. 2 and Table 2) and nutrient removal (Fig. 1) showed no obvious difference. In practice, this makes sense since NaClO sterilization is an easier and cheaper way for wastewater pretreatment before the introduction of algae. During the experiments, it was found that without sterilizing the wastewater, the system was easily polluted by a local algae species called *Scenedesmus* sp. and protozoa such as rotifer. Secondly, *C. zofingiensis* was able to adapt and grow well under uncontrolled conditions. In this present study, experiments were carried out in the southern China, where typical subtropical monsoon climate conditions are represented and the change of weather, temperature and light intensity was inconsistent (Fig. 3). The success in outdoor production would be an added advantage for amplifying *C. zofingiensis* production in practice since outdoor culture can easily access to sunlight and thus its cost-effectiveness can be improved (Lam and Lee, 2012). Finally, the semi-continuous feeding operation in this study could provide efficient and stable performance in biomass production. The retention time for wastewater under the semi-continuous operation is shorter than that under batch mode, which can improve the daily process ability in wastewater treatment plants. Assuming that (1) the retention time for wastewater to be treated in the plant is 36 h, (2) the flow column of treated wastewater is Q m^3 day^{-1}, (3) no harvest water is recycled in the system, and (4) the height of pond is H m, it can be simply calculated that the area S of pond with 1.5Q/H m^2 is required.

Environmentally, environmental merits via wastewater pollutant reduction should also be concerned and calculated as a beneficial value in practice. This study demonstrated that *C. zofingiensis* could effectively reduce nutrients (Fig. 1), which could improve environmental credits. During 10-day cultivation, *C. zofingiensis* could approximately remove wastewater nutrient COD at 80%, TN at 80%, and TP at 92%. Even in the beginning phase (Day 1 and 2), the reduction was evident. Based on this observation, it can be extrapolated that most of the wastewater nutrients during the semi-continuous feeding operation can be removed. In addition, limited or no fertilizer, which is easily dissolved in rainwater or runoff (Vietor et al., 2010), needs to be applied to a microalgae system when employing wastewater as the nutrient source (Zhu and Ketola, 2012). And this can also be one added environmental advantage for algae-based wastewater treatment method.

From the economic perspective, the scale-up combination of *C. zofingiensis* cultivation in piggery wastewater with algal biodiesel production can enhance the cost-effectiveness of this integrated approach. First, wastewater can replace the use of fertilizer, which, undoubtedly, can save nutrient costs. Previous study showed that to produce 1 kg of *C. vulgaris* biomass the total cost of nutrients for organic and inorganic fertilizer was 2.5–3 USD and 60–85 USD, respectively (Lam and Lee, 2012). Second, NaClO is a common industrial chemical, the cost of which as a pretreatment method was only around 0.05 USD per 1 kg of *C. zofingiensis* biomass in this study. Finally, biomass usage can improve the economics of this technology. As shown in Table 3, the biodiesel has advantageous composition predominating with 16–18 carbon atoms, which can improve biodiesel performance (Xu et al., 2006). Probably, the lipid content of algae under semi-continuous operation would not be high since the lipid accumulation would usually happen under nutrient deficiency or limitation (Rodolfi et al., 2009). But sugar, protein and lipid are the three main ingredients of algal cells (Abreu et al., 2012; Huo et al., 2012). Thus, except biodiesel valued added products including bio-ethanol, biogas, high-value protein, fertilizer etc. (Li et al., 2008; Zheng et al., 2012) can be further produced through algae biomass production.

3.4.2. Main uncertainty issues

Although it is greatly possible to amplify the cultivation of *C. zofingiensis* using piggery wastewater for nutrient removal and biodiesel production, some uncertainty issues might contribute to the constraints of this process and thus should be further researched. The main uncertainties include as follows:

(1) *Geographical variation:* Weather, temperature and light intensity, which are changeable geographically, contribute to critical factors for microalgae growth. In this study, *C. zofingiensis* showed robust growth performance when the outdoor air temperature and light intensity varied from 21.5 °C to 34.5 °C, and from 115 to 2046 μmol m^{-2} s^{-1}, respectively. In general, microalgae grow faster with high solar radiation and temperature under a certain range (Yang et al., 2011), but at the same time evaporation rate will increase dramatically. Huo et al. (2012) found that by regulation of pH in dairy wastewater, *C. zofingiensis* could grow well in bench-scale outdoor ponds in winter, when air temperature varied from 6.2 °C to 20.8 °C. However, it is difficult to expect the results when the outdoor temperature and/or irradiance increase or decrease drastically.

(2) *System stability and productivity:* The ability to resist the introduced organic shock loads should be further verified since the discharge of wastewater might fluctuate

considerably. Thus, the biomass productivity might be affected. Moreover, the productivity of biomass under long-term semi-continuous operation should be further investigated as well.

(3) *Pollution:* Although wastewater can be sterilized beforehand using NaClO, there is no guarantee that during long-term semi-continuous operation the system (especially the open system) will not be contaminated by other algae species, protozoa, or bacteria. And the pollution control will be demanding and thus costly.

(4) *Costs:* There are several beneficial indications through *C. zofingiensis* scale-up for biodiesel production, but costs are the main contributing constraints for this practice. The construction costs for open ponds system and PBRs are 100,000 and 1,000,000 USD per hectare, respectively (Carlsson et al., 2007). In addition, operating costs including energy input, cleaning, sterilization, maintenance and chemicals consumption are tough to be expected and will account for large proportion of the total costs.

(5) *Policy implications:* The likeliest influences on scale-up practice will depend on government policy towards carbon dioxide emissions and future research as well. A substantial investment and subsidies are required to make this practice a reality.

4. Conclusion

Pollutants in autoclaved wastewater and NaClO-pretreated wastewater were efficiently utilized by *C. zofingiensis* cultivated indoors. *C. zofingiensis* was able to adapt and grow well outdoors. The FAME yield of *C. zofingiensis* grown in autoclaved medium and NaClO-pretreated medium reached 10.18% and 10.15% of dw respectively, while the counterpart for outdoor algae was 9.19% of dw. The semi-continuous feeding operation could provide a net stable productivity of 1.314 g L^{-1} day^{-1}. These findings in this study could serve as a foundation for further scale-up trials using piggery wastewater for *C. zofingiensis* biomass and biodiesel production.

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