Cultivation of Microalgae in Phototrophic, Mixotrophic and Heterotrophic Conditions

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Abstract

Algal growth and production depend on nutrient supply and culture systems. This thesis compares the performance of algal growth in phototrophic, mixotrophic and heterotrophic conditions. *Isochrysis galbana*, a common species in aquaculture was used as a model species to identify the appropriate condition and nutrient requirement to optimise algal growth and bioproduct production. Experiment 1 identifies the source of organic carbon and environmental conditions in the culture of *I. galbana*. Algal growth was inhibited in heterotrophic culture when glucose, glycerol and sodium acetate were separately used as organic carbon. However, algal growth was enhanced by glycerol supplementation in mixotrophic culture compared to phototrophic culture. The optimal mixotrophic condition for algal growth is 50 mM glycerol, 35‰ salinity and a light regime at 100 photons μmol m^{-2} s^{-1} with 12 h light and 12 h dark. Algal production in mixotrophy was higher than in either phototrophy or heterotrophy. Experiment 2 compares nitrogen and phosphorus requirements of *I. galbana* between phototrophic and mixotrophic conditions. Three nitrogen sources (nitrate, ammonium and urea) and one phosphorus source were tested at various concentrations. Better growth performance and nutrient conversion efficiency were achieved in the mixotrophic condition and urea was the preferred nitrogen source for algal growth. The requirements of nitrogen and phosphorus were not different between phototrophic and mixotrophic conditions but algal production and nutrient conversion efficiency in mixotrophy were greater than in phototrophy. Experiment 3 compares the pigment and proximate composition in *I. galbana* under phototrophic and mixotrophic conditions in an attempt to improve algal product quality. The contents of chlorophylls *a* and *c* and carotenoid in mixotrophic culture increased by 60% and the productions of protein, lipid and carbohydrate were also

enhanced compared to those in phototrophic culture. This study indicates that mixotrophic culture promotes pigment and proximate production and the change of fatty acid profile depends on the addition of organic carbon in the culture medium. Experiment 4 examines the interactive effect of nitrogen and organic carbon concentrations on lipid and fatty acid production in *I. galbana*. Urea was used as the source of nitrogen at four levels (0, 12.5, 25 and 50 mg L^{-1}) and each nitrogen level was tested against three levels of organic carbon (0, 25 and 50 mM glycerol). Relatively higher lipid content (>400 mg g^{-1}) and lipid production (344.9 mg L^{-1}) were obtained at 25 or 50 mg urea-N L^{-1} with 50 mM glycerol. This study indicates that manipulation of nitrogen and organic carbon can improve lipid and fatty acid production in *I. galbana*. Overall, this thesis research contributes to the knowledge of using mixotrophic culture to improve algal growth and production.

Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for degree or diploma in any university; and that to the best of my knowledge and belief it does not contain material previously published or written by another person except where due reference is made in the text.

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Yousef A. Alkhamis

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CHAPTER 1

General Introduction

1.1 Usefulness of microalgae

Microalgae are photosynthetic micro-organisms containing chemical components that are important for various applications (Fig.1.1). At present, microalgae are extensively used in aquaculture as live food because they constitute essential nutrients for various aquatic animals including fish and shrimp, bivalve and zooplankton (Hemaiswarya et al. 2011; Muller-Feuga et al. 2003). Moreover, microalgae contain micronutrients, vitamins and bioactive materials that are suitable as a food supplement for human, and some algal products have been used in nutraceutical and pharmaceutical industries for improvement human health (Becker 2004). Microalgae can accumulate a considerable amount of polyunsaturated fatty acids (PUFA) such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) that can be an alternative to the commercial PUFA produced from fish oil (Vazhappilly and Chen 1998; Wen and Chen 2002). The microalgal pigments such as chlorophyll, carotenoid and phycobiliprotein are recognised as antioxidants to prevent heart disease in human and improve fish flesh quality in aquaculture (Becker 2004; Spolaore et al. 2006). Recently, microalgae are considered a potential source of energy in the future to partially substitute fossil fuel for energy supply (Huang et al. 2010; Li and Qin 2005). Comparing with other oil crops, some microalgae contain a high amount of lipid up to 50% of dry weight and are an attractive feedstock for biofuel production (Huang et al. 2010). However, the cost effective production of algal feedstock is a major challenge to restrain the supply of algae for industrial uses. Carbon emissions from factories and organic waste have been explored as a nutrient

supply in algal culture to reduce production cost and environmental pollution (Bhatnagar et al. 2011). However, with an increasing demand of microalgae products, it is essential to develop efficient methods for the production of algae and identify the environmental requirement for mass production of microalgae.

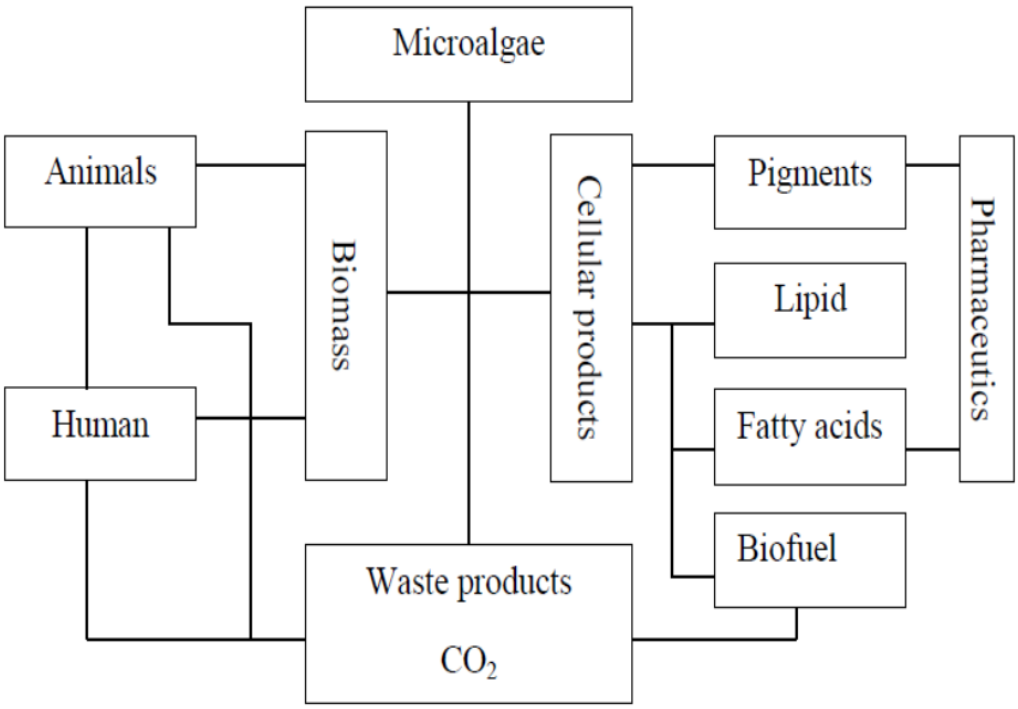

Fig. 1.1 Various applications of microalgal products for human, animals and industries.

1.2 Algal culture conditions: phototrophy, heterotrophic and mixotrophic

Various methods of algal cultivation have been developed since the last century and growing alga has become an important industry. As most microalgae are obligate photoautotrophs, their growth and production require light regardless of natural or artificial sources (Ogbonna and Tanaka 2000). Mass production of microalgae is produced typically in open ponds under sun light but this system has many

disadvantages. The methods for high biomass and productivity of algae in open ponds are not easily transferable between locations as environmental factors are not controllable outdoors (Perez-Garcia et al. 2011). Obtaining mono-culture in outdoor ponds is difficult due to contamination by other microalgae and microorganisms in an open system. Moreover, the volume of culture in open ponds is usually huge leading to harvesting processes to be difficult and costly (Ogbonna and Tanaka 2000). Light is one of the most important factors affecting algal growth because self-shading occurs when cell densities increase and the depth of light penetration decreases. Algae below far below water surface usually do not receive sufficient light and thus reduce growth and productivity (Chen and Chen 2006; Perez-Garcia et al. 2011; Wen and Chen 2003). The algal biomass and productivity in open ponds can reach up to 1 g L^{-1} and 60-100 mg L^{-1} d^{-1}, respectively (Pulz 2001), but a further increase of algal production in ponds needs to overcome the problem of light limitation.

Enclosed photobioreactors are designed to overcome the problem of light limitation in traditional open systems (Tredici 2004). The advantages of this system include a low chance of contamination and environmental factors are controllable (Wen and Chen 2003). Also, final biomass and productivity can reach over 8 g L^{-1} and 2.7 g L^{-1} d^{-1}, respectively (Chen et al. 2011; Pulz 2001), which much higher than in the open pond system. However, the production volume is low and the cost for scaling up and operation is high (Tredici 2004). Although this system minimises algal contamination, the system sterilisation using heat and chemical agents to sterilise the system is also costly (Lee 2001). Although recent technology of photobioreactor manufacture has made great progress in microalgae culture, achieving a high production volume is still costly due to light supply and operation

cost. Due to the drawbacks of algal production in the photoautotrophic system, it is necessary to seek alternative technology without depending on much illumination to achieve sufficient production.

Cultivation of microalgae in a heterotrophic condition is feasible since some microalgae are facultative to grow in dark (Gladue and Maxey 1994; Vazhappilly and Chen 1998). The presence of organic carbon substrates such as sugar and organic acids supplies energy and carbon for the growth of algal cells in dark (Perez-Garcia et al. 2011). In the supply of organic carbon, microalgae grow rapidly and produce high biomass by assimilating organic carbon from the substrate (Chen and Chen 2006; Wen and Chen 2003). In heterotrophic culture, biomass of some microalgae can reach over 40 g L^{-1} (Gladue and Maxey 1994). Moreover, in Japan 50% commercial production of *Chlorella* sp. has been produced heterotrophically in stirred fermenters for industrial uses (Lee 2004). The production cost in a heterotrophic fermenter is low due to the absence of light and the capacity of holding high algal biomass (Wen and Chen 2003). In addition, the quality and quantity of biosynthesis products are high and algal lipids and fatty acids are more suitable for pharmaceutical application due to low contamination (Chen and Chen 2006; Jiang and Chen 1999). However, the heterotrophic system also has disadvantages. Some commercial microalgae cannot grow in total dark or utilise organic carbon without light (Perez-Garcia et al. 2011). Synthesis of some cellular products such as pigments is repressed in the absence of light and the cultures can be potentially contaminated by the growth of heteotrophs especially with the increase of organic carbon load (Shi et al. 2000). Therefore, heterotrophic culture may not be suitable for many species of freshwater algae but little is known on the culture of most marine algal species in a heterotrophic condition.

Mixotrophic culture is another applicable method to enhance microalgal production (Liang et al. 2009; Marquez et al. 1993) where light energy and organic carbon substrate are provided concurrently so that microalgae can simultaneously process photosynthesis and oxidative metabolism (Liu et al. 2009). Under this condition, light limitation is no longer an issue since the cell growth in a mixotrophic conditions usually requires low light intensity and utilisation of organic carbon is more dominant (Ogbonna and Tanaka 2000). Therefore, some microalgae in mixotrophy grow faster than in either heterotrophy or phototrophy.

The growth rate of microalgae in mixotrophic culture is high and in some cases it can exceed the sum of specific growth rates in heterotrophic and photoautotrophic cultures (Chojnacka and Noworyta 2004; Liu et al. 2009). Moreover, the algal biomass and bioproduct productivity in mixotrophic culture are higher than in other culture systems (Kim et al. 2012; Liang et al. 2009; Zhang et al. 2011). Although mixotrophic culture can be efficient in algal culture, only a small group of algal species can utilise organic carbon substrates (Gladue and Maxey 1994; Vazhappilly and Chen 1998; Wood et al. 1999). Due to increasing demand in algal production, mixotrophic culture could be a potential technology for algal production. However, among the commonly used marine algae species in aquaculture, little is known on the potential growth under a mixotrophic condition.

1.3 Importance of *Isochrysis galbana*

There are some phototrophic marine microalgae that are potentially able to grow in heterotrophic or mixotrophic conditions. However, a number of characteristics are considered to make an algal species for commercial use: (1) be able to grow fast and reach high biomass (Becker 2004), (2) possess a small size with a structure of thin or

no cell wall as food for aquatic animals (Guedes and Malcata 2012), (3) contain high nutritional components including pigments, lipid and a high proportion of PUFA (Hemaiswarya et al. 2011), and (4) tolerate the change of environmental conditions (Gladue and Maxey 1994). Unlike other marine microalgae, golden algae such as *Isochrysis* spp have been one of primary foci in research and commercial application due to possessing some attractive characteristics as food for aquatic animals (Marchetti et al. 2012).

Isochrysis is a genus of haptophyte including the species *Isochrysis galbana*, *I. litoralis* and *I. maritima* (Guiry and Guiry 2008; Wikfors and Patterson 1994). *Isochrysis galbana* Tahitian strain (T-Iso) is a common strain that is most widely used as live food in aquaculture (Fidalgo et al. 1998; Tzovenis et al. 2003). More recently, the Tahitian strain of *Isochrysis* (T-ISO) is transferred to a new genus *Tisochrysis lutea* (Bendif et al. 2013). In this thesis, these two names are used interchangeably to comply with the references in previous publications.

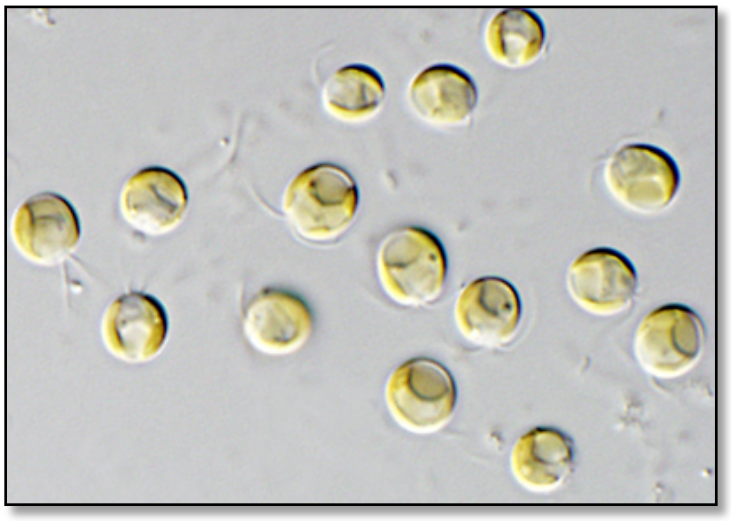

Fig. 1.2 Image of *Isochrisis galbanna* (https://www.google.com.au/search?q=Isochrysis&lr=&hl=en&as_qdr=all&tbm=isch&tbo=u&source=univ&sa=X&ei=u5hBVc-uL8K5mAXljYHIDw&ved=0CI0BEIke&biw=1067&bih=473).

The *I. galbana* is a unicellular alga in an ellipsoid shape with two flagella. Cells are golden brown and are 5-6 µm long and 2.5-3 µm wide without cell wall (Liu et al 2013). This species is mainly used in marine aquaculture and is an essential live food for marine animals including molluscs, crustaceans and zooplankton because it has appropriate cell size for grazers to ingest and digest (Sánchez et al. 2013). In aquaculture, *I. galbana* is easy to culture, grow fast and tolerate fluctuation of environmental conditions (Tzovenis et al. 2003; Van Bergeijk et al. 2010).

The *I. galbana* contains high nutritional products and supplies with necessary nutrients and energy for the growth and survival of many aquatic animals. Protein and lipid constitute 50% and 30% of the cell dry weight, respectively (Fig. 1.3) (Martínez-Fernández et al. 2006). Lipid in algae is characterised with high amounts of long chain polyunsaturated fatty acids (5.75% by dry weight) among which DHA and EPA account for 1.21 % of the dry weight (Lin et al. 2007). Comparing with different algal species, the extracellular and bioactive compounds of *I. galbana* have been demonstrated a most functional effect on animal health (Custódio et al. 2014). In addition, *I. galbana* contains a considerable amount of pigments and carotenoids in particular (da Silva Gorgônio et al. 2013; Mulders et al. 2013). In the future, as *I. galbana* can produce a relatively high amount of lipid, it is recommended to be a potential source of feedstock for biodiesel production (Roopnarain et al. 2014; Sánchez et al. 2013). Therefore, the increasing interest in *I. galbana* is the encouragement to develop a culture protocol to improve production efficiency using heterotrophic and mixotrophic technology. Nevertheless, the knowledge of using the heterotrophic production mode on this alga is still limited. Therefore, this thesis examines the feasibility of using heterotrophic and mixotrophic culture to improve growth and bioproduct production of *I. galbana*. Four research areas were proposed

to assess the feasibility of various culture systems to: (1) identify the appropriate growth mode and suitable environmental conditions for growth, (2) determine the nutrient requirements to optimise algal growth, (3) assess the changes of cell compositions under different nutrient conditions, and (4) refine the nutrient requirements to maximise lipid and fatty acid productions.

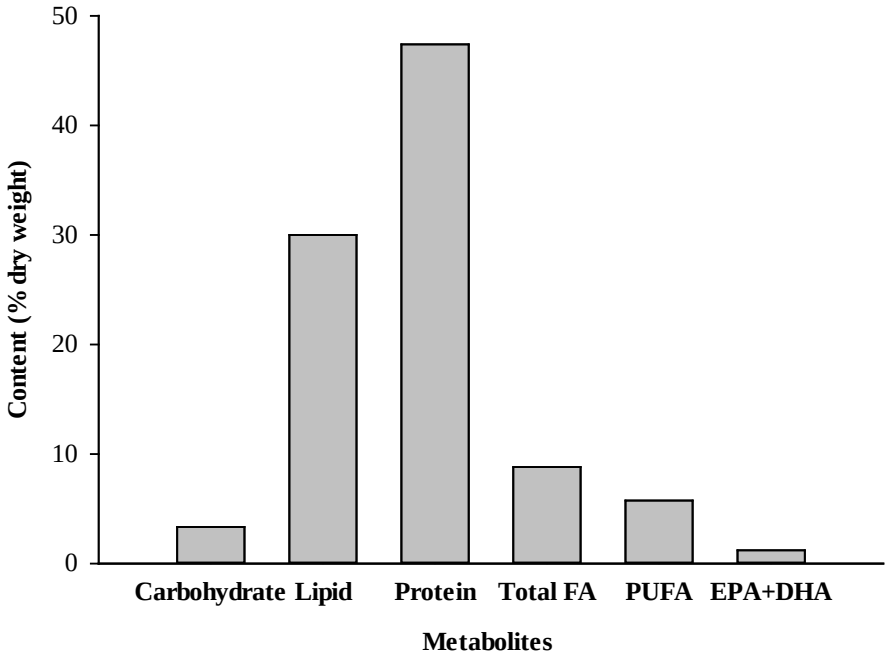

Fig. 1.3 Chemical composition of *Isochrysis galbana* (Martinez-Fernandez et al. 2006)

1.4 Requirement of organic carbon and environmental conditions for *I. galbana*

The environmental requirement for the growth of microalgae differs from phototrophic, heterotrophic and mixotrophic conditions (Lee 2004). The growth requirement of *I. galbana* has been mostly studied in a phototrophic condition (Grima et al. 1994; Lin et al. 2007; Sánchez et al. 2000) but not yet in heterotrophic and mixotrophic conditions. The growth of microalgae in the heterotrophic and

mixotrophic cultures relies on the supply of organic carbon but the ability to assimilate organic carbon from the substrates is species-specific (Perez-Garcia et al. 2011). Therefore, the first step of this study was to investigate the ability of *I. galbana* to grow under heterotrophic and mixotrophic conditions using different organic carbon substrates. Previous studies have shown that the growth rate and production of algae depend on the source of organic carbon (Azma et al. 2011; Cerón García et al. 2005; Fang et al. 2004). Moreover, organic carbon sources, light intensity and salinity were important environmental factors contributing to algal growth in heterotrophic and mixotrophic cultures (Chojnacka and Noworyta 2004; Heredia-Arroyo et al. 2011; Jiang and Chen 1999; Rym et al. 2010; Wen and Chen 2001; Xie et al. 2001). Therefore, it is necessary to study the impact of these factors on algal growth in heterotrophy and mixotrophy before optimising the environmental requirement for algal growth.

Several sources of organic carbon have been used in the cultivation of microalgae in heterotrophic and mixotrophic conditions (Chen and Chen 2006). However, the successful growth of algae in heterotrophic or mixotrophic culture may not only depend on the type but also on the quantity of organic carbon. The information on the influence of organic carbon concentration on the growth of marine algae is limited (Cerón García et al. 2006; Fang et al. 2004; Wen and Chen 2000). The excessive use of organic carbon in heterotrophic and mixotrophic cultures can inhibit algal growth (Chen and Chen 2006). As the ability to assimilate organic carbon differs among algal species (Perez-Garcia et al. 2011), knowledge on the impact of organic carbon source and concentration on algal growth needs to be species-specific.

Light is essential for algae that do not assimilate organic carbon in dark (Ogbonna and Tanaka 2000). However, light limitation is not a critical factor for algae that can facultatively simulate organic carbon as energy for cell metabolism (Cid et al. 1992; Lee 2001). Light intensity and photoperiod determine the amount of light energy for microalgal growth in a phototrophic condition (Wahidin et al. 2013), but it is not clear if light intensity and photoperiod can affect algal growth in a mixotrophic condition. Previous studies have shown that light intensity can affect the growth of cyanobacteria *Spirulina platensis* in a mixotrophic condition (Chojnacka and Noworyta 2004; Vonshak et al. 2000). Therefore, it is necessary to test if light intensity and duration can affect the growth of *I. galbana* in a mixotrophic condition.

Salinity is another important factor that has strong impact on algal growth and metabolite accumulation (Wen and Chen 2003). The level of salinity is highly considered when choosing the growth culture system as it may affect organic uptake from the substrate (Das et al. 2011). Previous studies have shown that each algal species or strain has an optimal salinity range for growth and survival in a phototrophic condition (Jiang and Chen 1999; Wen and Chen 2001). In heterotrophic and mixotrophic conditions, however, bacteria may prevail the culture system due to the presence of organic carbon, and salinity may determine the risk of bacterial contamination in mixotrophic systems particularly in an open culture system (Das et al. 2011; Wang et al. 2014). High salinity is not preferred in a heterotrophic culture system because salinity can cause corrosion to fermenters (de Swaaf et al. 1999; Gladue and Maxey 1994). Therefore, identification of the optimal salinity range is necessary to improve algal growth and production in a heterotrophic or a mixotrophic system. However, the impact of salinity on *I. galbana* growth is not known in a mixotrophic or a heterotrophic condition.

1.5 Nitrogen and phosphorus requirements of *I. galbana* in mixotrophic culture

In cultivation of microalgae, medium formulation is a key factor to achieve high algal density and production and the medium must contain the basic requirements for cell reproduction and metabolite production (Azma et al. 2011). Nitrogen and phosphorus are two major elements to supply nutrients for biosynthesis and cell maintenance (Chen and Chen 2006). Although the requirements of nitrogen and phosphorus of *I. galbana* in a phototrophic condition have been studied (Fidalgo et al. 1998; Liu et al. 2013; Zhang et al. 2001), the nutrient requirements may change in a mixotrophic condition (Wang and Peng 2008). Previous studies indicate that nitrogen and phosphorus are depleted rapidly during the log growing phase under a mixotrophic condition (Liu et al. 2011; Wen and Chen 2000; Xu et al. 2004), but the quantitative requirements of nitrogen and phosphorus are not known for algae in a mixotrophic condition. Therefore, there is a need to test the nitrogen and phosphorus requirements of *I. galbana* under a mixotrophic condition to optimise the nutrient requirement for algal growth.

1.6 Assessment of biochemical composition in *I. galbana*

The cellular compositions of microalgae consist of vital and nutritionally valuable products including pigments, protein, carbohydrate, lipid and fatty acids (Spolaore et al. 2006). Nutrient and environmental factors have an important effect on biosynthesis of algal metabolites (Hu 2004)**.** Manipulation of these factors may increase the content of cellular bioproducts but may also decrease cell growth (Hu 2004). To obtain both high quality and high quantity of algal cells is a challenge in algal cultivation because enhancement of nutrient quality in algal cells may compromise algal growth and productivity. In the past, many efforts have been made to improve algal cellular quality by manipulating environmental conditions (Abu-

Rezq et al. 1999; Renaud et al. 2002; Tzovenis et al. 1997; Zhang et al. 2001). Culturing microalgae in heterotrophic and mixotrophic regimes may improve cell growth but their impact on biochemical composition in algal cells is not clear. Previous studies demonstrate that microalgae can produce high cell biomass in heterotrophic and mixotrophic conditions but there is a conflict on their effects on cellular composition (Cerón García et al. 2006; Cheirsilp and Torpee 2012; Fang et al. 2004; Heredia-Arroyo et al. 2011; Liang et al. 2009; Liu et al. 2009). The feasibility of using heterotrophic or mixotrophic culture as an alternative to phototrophic culture needs to be assessed based on algal growth and cellular products. The cell compositions of *I. galbana* have been studied in phototrophic culture (Fidalgo et al. 1998; Martínez-Fernández et al. 2006), but it is still unknown under heterotrophic and mixotrophic conditions.

1.7 Interactive effects of nitrogen and organic carbon on lipid production

Lipid and fatty acid contents are relatively high in *I. galbana* (Martínez-Fernández et al. 2006) but the overall production of these cellular products is considered unsatisfactory due to infirmity in algal biomass production in a phototrophic condition. Achievement of high lipid productivity depends on the results of cell biomass production and cellular lipid contents (Lv et al. 2010). Therefore, it is important to identify the ideal condition to obtain high biomass containing a high amount of lipid in *I. galbana*. Algal growth and lipid accumulation are regulated by the availability of nutrients in the culture medium (Sánchez et al. 2000). In heterotrophic and mixotrophic conditions, the organic carbon source is a key factor stimulating algal growth. In addition, nitrogen is the major factor that improves algal growth efficiency and stimulates lipid accumulation when nitrogen is low in the environment (Cho et al. 2011; Marchetti et al. 2012). Therefore, to achieve

high algal growth and lipid content in algae, it is very important to identify the suitable balance between organic carbon and nitrogen in the medium as these nutrients can regulate the process for protein and lipid synthesis in algae (Wen and Chen 2003).

Most researchers have studied the effect of organic carbon substrates on algal growth and lipid synthesis, but the dependent effect of organic carbon and nitrogen on biomass production and lipid accumulation is neglected. Changes of fatty acid profiles in algae are related to the change in lipid compositions when a high lipid content is obtained (Chen and Johns 1991; Wen and Chen 2003). A protocol for successful algal culture should produce a high cell growth rate and a large quality of lipid content (Lv et al. 2010). Therefore, understanding the interactive effect of nitrogen and organic carbon on lipid accumulation is needed in *I. galbana* between phototrophic and mixotrophic culture conditions.

1.8 Study objectives

The overall aim of this study is to investigate the feasibility of using heterotrophic and mixotrophic systems to improve the efficiency of marine microalgae culture and enhance algal biomass and production of bioproducts. To achieve this objective, a number of specific aims are proposed to understand:

(1) the requirements of organic carbon and environmental conditions for *I. galbana*;

(2) nitrogen and phosphorus requirements of *I. galbana* in mixotrophic culture;

(3) biochemical composition in *I. galbana* in different culture systems; and

(4) interactive effects of nitrogen and organic carbon on lipid production.

The present thesis consists of four studies to address these objectives. *Isochrysis galbana* was chosen as model species as it is a commonly used algal species in marine aquaculture.

1.8.1 Study 1: Cultivation of *Isochrysis galbana* in phototrophic, heterotrophic, and mixotrophic conditions

The aim of this study is to identify the suitable growth mode among phototrophic, heterotrophic and mixotrophic culture systems. This study investigates the optimal requirement of organic carbon, light energy and salinity for *I. galbana* culture. The results of this study provide the basic knowledge to optimise the algal culture protocol for improvement of *I. galbana* production.

1.8.2 Study 2: Comparison of N and P requirements of *Isochrysis galbana* under phototrophic and mixotrophic conditions

Based on the understanding of the optimal environmental requirements identified in Study 1, the growth and nutrient consumption were compared in *I. galbana* cultures under phototrophic and mixotrophic conditions by growing algae at different sources and concentrations of nitrogen and phosphorus. This study compared the nitrogen and phosphorus requirements of *I. galbana* in phototrophic and mixotrophic conditions. The outcome of this study provides basic information on nitrogen and phosphorus concentrations for cultivation of *I. galbana* in a mixotrophic condition.

1.8.3 Study 3: Comparison of pigment and proximate compositions of *Isochrysis galbana* in phototrophic and mixotrophic cultures

After identifying the optimal environmental and nutrient requirements of *I. galbana* in previous studies, this study assesses the impact of growth condition on bioproduct accumulation in algae. The pigments content and gross composition were compared between phototrophic and mixotrophic conditions. These findings lead to

determination of the feasibility for choosing a growth condition to improve algal cell growth and bioproduct production in *I. galbana* culture.

1.8.4 Study 4: Enhancement of lipid and fatty acids production of *Isochrysis galbana* by manipulation of medium nitrogen and organic carbon

The aim of this study is to understand the nitrogen and organic carbon requirements to achieve high production of lipid and fatty acids. This study particularly tests the interactive effect of nitrogen and organic carbon on lipid and fatty acid accumulation and production of *I. galbana*. The result of this study identifies the optimal nutrient condition to improve lipid and fatty acid production in *I. galbana*.

1.9 Thesis organisation

The present thesis consists of six chapters to illustrate specific research aims, methods and results ultimately contributing to improvement of algal growth and production. Chapter1 presents a general introduction on the importance of microalgae and some key issues in algal production systems. A brief background provides the advantage and disadvantage of phototrophic, heterotrophic and mixotrophic culture systems and the reasons why *Isochrysis galbana* was used as model species to study. The introduction provides background and knowledge gaps in the cultivation of microalgae in heterotrophic and mixotrophic conditions. Four data chapters (2, 3, 4 and 5) represent four major experiments in this study. These chapters are formatted as a manuscript for journal publication. Therefore, it may be noticed some repetitions between chapters in background and methods. Although independent objectives are specified in each chapter, they are all complementary to each other towards the overall thesis objectives stated in this introduction chapter. Within each chapter, the word "study" refers to each respective and sole chapter. All of the studies were performed

by the author of the present thesis under the supervision of the principal supervisor. However, the principal supervisor is listed as a co-author on each manuscript for publication in peer reviewed journals. Chapter 6 is a general discussion reviewing the main results found in each study of the thesis research and providing suggestions for future research.

Chapter 1: General introduction

Chapter 2: Cultivation of *Isochrysis galbana* in phototrophic, heterotrophic, and mixotrophic conditions (published as Alkhamis Y and Qin JG. 2013. Cultivation of *Isochrysis galbana* in phototrophic, heterotrophic and mixotrophic conditions. BioMed Research International (formerly titled Journal of Biomedicine and Biotechnology, vol 2013, Article ID 983465, 9 p.).

Chapter 3: Comparison of N and P requirements of *Isochrysis galbana* under phototrophic and mixotrophic conditions (published as Alkhamis Y and Qin JG. 2014. Comparison of N and P requirements of *Isochrysis galbana* under phototrophic and mixotrophic conditions. Journal of Applied Phycology (DOI: 10.1007/s10811-014-0501-5).

Chapter 4: Comparison of pigment and proximate compositions of *Tisochrysis lutea* in phototrophic and mixotrophic cultures (published as Alkhamis Y, Qin JG. 2015. Comparison of pigment and proximate compositions of *Tisochrysis lutea* in phototrophic and mixotrophic cultures. Journal of Applied Phycology (DOI 10.1007/s10811-015-0599-0).

Chapter 5: Enhancement of lipid and fatty acid production of *Isochrysis galbana* by manipulation of nitrogen and organic carbon in culture medium (submitted to Algal

Research as Alkhamis Y, Qin JG. 2015. Enhancement of lipid and fatty acid production of *Isochrysis galbana* by manipulation of medium nitrogen and organic carbon, under review).

Chapter 6: General discussion, conclusions and future research.

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CHAPTER 2

Cultivation of *Isochrysis galbana* in Phototrophic, Heterotrophic and Mixotrophic Conditions

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2.1 Abstract

This study compared the growth and biomass production of *Isochrysis galbana* under heterotrophic, mixotrophic and phototrophic conditions using different organic carbon sources. The growth of *I. galbana* was inhibited in heterotrophy, but was enhanced in mixotrophy compared to that in phototrophy. Subsequently, the influences of organic carbon and environmental factors (light and salinity) on the growth of *I. galbana* were further investigated. Algal dry weight increased as glycerol concentrations increased from 0 to 200 mM and the highest algal production occurred at 50 mM glycerol. At a range of light intensities of 25-200 µmol photons m^{-2} s^{-1}, the highest algal growth rate occurred at 100 photons µmol m^{-2} s^{-1}. The growth of *I. galbana* was significantly affected by photoperiod, and the maximal dry weight was obtained at 12 h light and 12 h dark. In the salinity test, *I. galbana* could grow in a wide range of salinities from 10 to 65‰, but the 35‰ salinity was optimal. This study suggests that the growth and production of *I. galbana* can be improved using mixotrophic culture at 50 mM glycerol in 35‰ salinity.

Keywords: Organic carbon, Glycerol, Light intensity, Photoperiod, Salinity

2.2 Introduction

Microalgae have been used as live feed in aquaculture, additives in human health food and feedstock for pharmaceutical industries and biofuel production (Muller-Feuga et al. 2003; Perez-Garcia et al. 2011). Because most microalgae are photosynthetic, they are conventionally cultured under sunlight or artificial light with a supply of either carbon dioxide or air. However, algal growth efficiency is restricted by light penetration but aeration may increase the likelihood of

contamination by other species of algae or bacteria. Self-shading occurs concurrently with the increase of algal cell density and this leads to low light penetration, slow algal growth and low production (Chen and Chen 2006).To overcome the challenge of light and aeration-dependent algal growth, the feasibility of using mixotrophic or heterotrophic methods has been explored as an alternative to phototrophic algal culture (Lee 2001). In heterotrophy, algae grow in darkness where cells get energy completely from organic carbon in the media, while in mixotrophy algae can obtain energy from both organic carbon and light. Such a condition is suitable for algal species that cannot grow in complete darkness but require low light or agitation (Perez-Garcia et al. 2011). Growth rate and biomass production for some algae in mixotrophic or heterotrophic conditions can be several times higher than in a photoautotrophic condition alone (Heredia-Arroyo et al. 2011; Wen and Chen 2000b). Moreover, the synthesis of metabolic products such as lipids and pigments are influenced by the quality and quantity of organic carbon (Wen and Chen 2003).

Many species of microalgae are able to grow in both heterotrophic and mixotrophic conditions (Gladue and Maxey 1994; Vazhappilly and Chen 1998). For instant, the marine diatom *Cyclotella cryptica* has a high productivity in heterotrophy than in phototrophy (Pahl et al. 2010). In addition, the growth rate of *Nitzschia laevis* in either a heterotrophic or a mixotrophic condition is higher than that in a phototrophic condition (Wen and Chen 2000b). As an extreme example, the productivity of *Tetraselmis suecia* in a heterotrophic condition can be two times higher than that in a phototrophic condition (Azma et al. 2011). On the other hand, some algae cannot successfully grow in heterotrophy. For example, *Nannochloropsis* sp. grow slowly in heterotrophy (Fang et al. 2004), and *Phaeodactylum tricornutum* does not grow at all in heterotrophy with organic carbon in the media but its growth

is faster in mixotrophy than in phototrophy (Cerón García et al. 2006). Glucose, glycerol and acetate are commonly used as a source of organic carbon in algal culture (Perez-Garcia et al. 2011). However, acetate usually inhibits the growth of marine microalgae (Cerón García et al. 2005; Wood et al. 1999; Xu et al. 2004), but enhances the growth of freshwater algae (Heredia-Arroyo et al. 2011; Orosa et al. 2001). Among marine algae, the growth of *P. tricornutum* is inhibited when the level of glycerol is >100 mM (Cerón García et al. 2006), but *Nannocloropsis* sp. and *Cyclotella* sp. can utilise glycerol efficiently in mixotrophy (Wood et al. 1999). Therefore, there is a need to identify the source and quantity of organic carbon for commercially important algal species in a mixotrophic or heterotrophic culture. Das et al. (2011) showed that the growth of *Nannocloropsis* sp. was higher in 21 mM glycerol than in glucose at the same level of organic carbon. On the other hand, Xu et al. (2004) demonstrated that glucose at 30 mM significantly enhanced the growth of *Nannochloropsis* sp. Similarly, as glucose increased from 10 to 217 mM, the growth of *N. laevis* started to increase, and reached the maximum at 217 mM glucose (Wen and Chen 2000a). The addition of organic carbon can make the growth of algae become independent of CO_2 supply and cut off the cost of aeration in algal culture.

Light intensity and photoperiod are essential to autotrophic algal species that cannot assimilate organic carbon (Lee 2004). However, in mixotrophic algae, both light and organic carbon can serve as the energy source for algae (Lee 2004). In mixotrophic culture, *T. suecia* can reach the maximal density at 17 μmol m^{-2} s^{-1} which is lower than the optimal level in phototrophic culture (Cid et al. 1992). The effect of light intensity on the growth of *Spirulina platensis* is similar under either a phototrophic or a mixotrophic condition, but the inhibitory effect of high light intensity is more pronounced in phototrophic culture (Vonshak et al. 2000). On the

other hand, some algal species and strains in mixotrophic culture can be protected by adding organic carbon and the photoinhibtiory threshold can be increased (Chojnacka and Noworyta 2004).

Algal growth can also be affected by salinity though the salinity impact on growth depends on algal species and the algal products examined (Chen and Chen 2006). For instance, a salinity of 8 g L^{-1} NaCl is optimal for heterotrophic growth of *N. laevis* which is different from the optimal salinity for fatty acid production (Wen and Chen 2001). Das et al. (2011) found that the biomass and lipid content of *Nannochloropsis* sp. was similar at 35 and 50‰ in mixotrophic culture. Furthermore, de Swaaf et al. (1999) also reported that the cell density and lipid content of heterotrophic *Crypthecodinium cohnii* were similar from 17.5 to 28.8‰ salinity. These findings suggest the possibility of using salinity variation to control algal growth and metabolite accumulations (Das et al. 2011; Wood et al. 1999).

Although trophic status can regulate the growth of some algal species, the environmental requirements for algae to achieve maximum growth in phototrophic, mixotrophic and heterotrophic conditions are little known. At present, our knowledge on optimum growth requirements of microalgae in a mixotrophic or heterotrophic condition is limited especially in algal species that have been widely used in aquaculture. In this study, we used *I. galbana* as a representative for many other algae used as live feed in aquaculture to explore the possibility of using organic carbon in the media to improve the production efficiency. Our objectives were to compare the growth potential of *I. galbana* in phototrophic, mixotrophic and heterotrophic conditions and identify the requirements of organic carbon, light regime and salinity in the culture of mixotrophic or heterotrophic algal species. The

use of organic carbon in mixotrophic culture would also reduce the need for carbon dioxide in the culture and facilitate the growth of algal species sensitive to agitation.

2.3 Material and methods

2.3.1 Experimental protocols

This study examined the requirement of environmental conditions and the growth of a haptophyceae marine microalgae *Isochrysis galbana* in the media with organic carbon. The algal specimen was obtained from the Australian National Algae Culture Collection (Hobart, Tasmania) and the basal culture media was made with the f/2 formula in filtered sea water at 35‰ salinity. Prior to the experiment, the culture media were autoclaved at 121 °C for 115 min. Glycerol, glucose and acetate as organic carbon were sterilised in an autoclave at 115 °C for 10 min. Microalgae were cultured in 250 ml sterilised flasks containing 150 ml media and 10% (v/v) algal inoculum. Flasks were illuminated by white cool fluorescent lamps to achieve different levels light intensity. Light intensity was measured at the surface of the media using the Light ProbeMeter^{TM} (Extech Instruments Corp, Nashua, USA). The flasks were placed on an orbital shaker at 100 rpm at 24 ºC. Additional agitation of the culture media was conducted by shaking the flasks twice daily.

Experiment 1: Algal growth in different trophic conditions

The growth response of *I. galbana* was examined in a phototrophic, mixotrophic, and heterotrophic culture, respectively. Glycerol, glucose and acetate were separately used as an organic carbon source in the heterotrophic and mixotrophic cultures. The concentrations of these substrates were adjusted to the same carbon concentration (12 mM) and no additional carbon was added during the experiment. The flasks of phototrophic cultures were incubated in 24 ºC and exposed to continuous light at 50

µmol photons m^{-2} s^{-1} in the phototrophic and mixotrophic cultures. In the heterotrophic culture, flasks were wrapped by foil paper in complete dark. At day 10, cultures were harvested to determine algal biomass by dry weight. Four replicates were used in each treatment.

Experiment 2: Effect of organic carbon levels on algal growth

Based on the result of Experiment 1, glycerol as an organic carbon source was chosen to explore the growth response of *I. galbana* to different levels of glycerol in mixotrophy using similar protocols as in Experiment 1. To explore the optimal concentration of organic carbon, seven concentrations of glycerol were used as organic carbon in the culture media. Algae were grown in flasks containing 150 ml of f/2 media and enriched with different concentrations of glycerol (0, 5, 10, 25, 50, 100 and 200 mM). Algae were cultured at 24 ºC and illuminated with continuous light at an intensity of 50 µmol photons m^{-2} s^{-1}. This experiment lasted 10 days and algal production was determined by dry algal biomass at the end.

Experiment 3: Effect of light and salinity on algal growth

Based on the result of Experiment 2, the effect of light intensity on the growth of *I. galbana* was further tested in a glycerol concentration of 50 mM under mixotrophic culture. Cultures were illuminated with cool white fluorescent light tubes for 24 h a day with five light intensities at 25, 50, 100, 150 and 200 µmol photons m^{-2} s^{-1} in triplicate. Cultures were incubated under a constant temperature at 24 °C and algal density in each flask was measured every two days. All cultures were harvested by day 10 to determine algal biomass in dry weight.

Based on the results of the previous trials, light intensity was set at 50 µmol

photons m^{-2} s^{-1} and glycerol was supplied at 50 mM. Then, the impact of photoperiod on the growth of *I. galbana* was tested at four photoperiods with daily light of 24, 12, 8 and 4 h in both phototrophic and mixotrophic conditions at 24 °C. Algal densities in the flasks of different treatments were quantified every 2 days. Algal biomass was determined at the end of the 10-day experiment.

After the optimal levels of light intensity and photoperiod were obtained, the effect of salinity on the growth of *I. galbana* was tested at five levels of salinity: 10, 20, 35, 50 and 65‰ with four replicates each. Prior to adding nutrients to the seawater, the salinity levels were adjusted by adding sodium chloride or distilled water using a portable refractometer (Extech, RF20). The mixotrophic culture media contained 50 mM glycerol. Cultures were carried out in 250 ml flasks containing 150 ml media and a 10% (v/v) algal inoculation. Flasks were incubated at 24 ºC under daily illumination of 12 h light at a light intensity of 50 µmol m^{-2} s^{-1}. Algal cultures were incubated for 10 days and the algal samples were taken to measure algal density every other day. Algal biomass was determined by harvesting at the end of the experiment by drying algae to a constant weight.

2.3.2 Determination of algal growth and biomass

Algal density and dry biomass were used to determine algal performance. On each sampling day, after a thorough hand mixing, 5 ml of liquid was taken from each algal culture flask using an automatic pipette. The algae were preserved in 5% Lugo's iodine for later numeration. Algal cell density was determined using a hemocytometer on a microscope at 400 × magnification. Each sample was numerated in four replicates and the mean was used as the algal density for each replicate. Biomass production was estimated by measuring algal dry weight at the end of each

experiment. A volume of 100 ml algal cells was centrifuged at 5000 × *g* for 10 min and the algal pellets were washed off with distilled water. Each sample was separately dried in an oven at 65 ºC when the constant weight was reached (Liang et al. 2009; Zhang et al. 2011). The precision of algal weight was measured to the nearest 0.001 mg. Since the algal growth was all determined during the exponential period (1-10 days), the specific growth rate was calculated according to this equation:

$$\mu = (In\ X_2 - In\ X_1)/ (t_2 - t_1)$$

where, X_2 and X_1 are the dry cell weight (g L^{-1}) at time t_2 and t_1 (day), respectively.

2.3.3 Statistical analysis

Data were analysed using the software program SPSS (version18). Experimental results were analysed by one way ANOVA for Experiments 1 and 2, but two-way ANOVA was used for Experiment 3. Multiple comparisons were tested by Tukey *post hoc* analysis when the main treatment effect was significant at $P < 0.05$.

2.4 Results

2.4.1 Algal growth at different trophic conditions

The growth pattern of *I. galbana* is shown in Fig. 2.1A and 2.1B. The growth of *I. galbana* was significantly different between the three growing conditions ($P < 0.05$). The growth pattern was almost the same at the first two days in the phototrophic and mixotrophic cultures. However, the cell density increased exponentially after day 2, indicating that the algae started to use organic carbon for growth. The growth rate of *I. galbana* was significantly higher in the mixotrophic culture than in the phototrophic culture. However, in heterotrophy (Fig. 2.1B), the growth of *I. galbana*

was sustained by all organic carbon substrates in the first 2-4 days, but an overall decline of algal growth was observed after 4 days except that algae in acetate remained relatively unchanged.

In addition, the mixotrophic growth of *I. galbana* was significantly affected by the type of the organic carbon substrates ($P < 0.05$). Glycerol and glucose significantly increased the algal growth ($P < 0.05$) and the maximum algal density occurred in mixotrophy with glycerol while acetate had a negative impact on growth rate. In mixotrophy with either glycerol or glucose, the algal growth rate was faster than in phototrophy alone ($P < 0.05$), but there was no significantly difference in growth between acetate and the phototrophic control ($P > 0.05$).

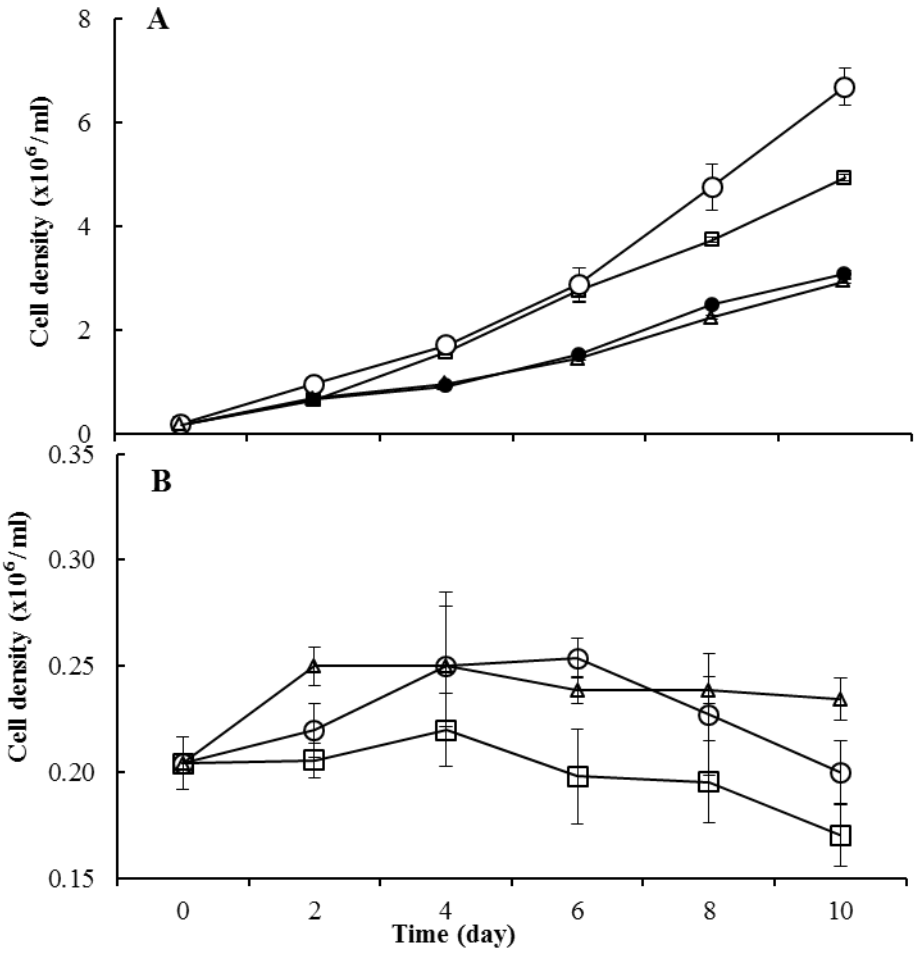

Fig. 2.1 Cell density ($\times 10^6$ ml^{-1}) of *I. galbana* cultured under the mixotrophy (A) and heterotrophy (B) with glucose (□), glycerol (○) and acetate (△), compared with phototrophic control (●). Data are shown as mean ± SE (n = 4).

The algal dry weight and specific growth rate were compared in phototrophy and mixotrophy (Fig.2.2 A and 2.2 B) and significant differences were found ($P < 0.05$) between these treatments. The specific growth rate and dry weight were maximal in mixotrophy with glycerol, being 0.54 h^{-1} and 223.25 mg L^{-1}, respectively, while the specific growth rate and dry algal weight of the phototrophic culture were respectively 0.47 h^{-1} and 106.75mg L^{-1}. However, the specific algal growth rates in phototrophic culture were not significantly different ($P > 0.05$) from those in the mixotrophic culture with glucose or acetate as organic carbon.

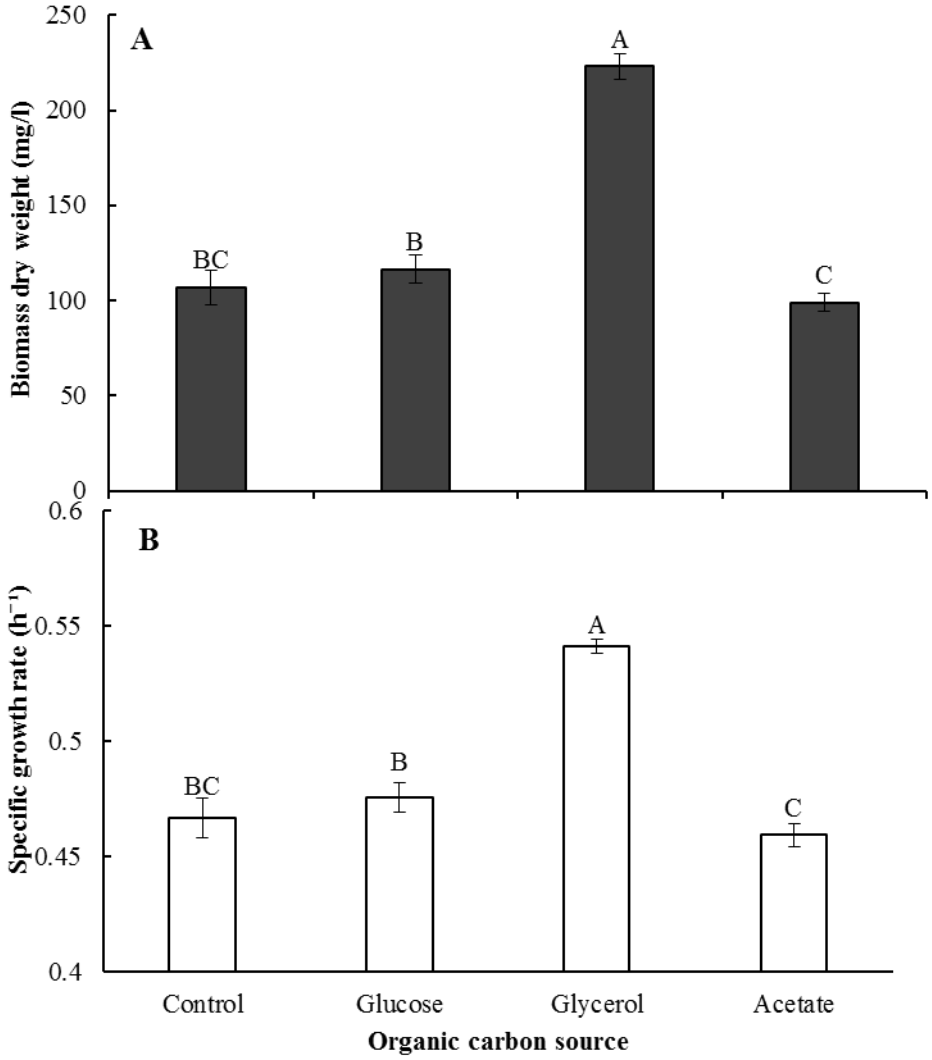

Fig. 2.2 Algal dry weight (A) and specific growth rate (B) of *I. galbana* supplemented with different organic carbon sources. Data are shown as mean ± SE (n = 4).

2.4.2 Effect of organic carbon on algal growth

The growth of *I. galbana* significantly differed ($P < 0.05$) between glycerol concentrations (Fig. 2.3A). Algal dry weight significantly increased ($P < 0.05$) from 106.75 to 231 mg L^{-1} when the glycerol concentrations increased from 0 to 50 mM. The media supplemented with 25 or 50 mM glycerol yielded higher dry weight ($P > 0.05$) than other treatments. However, dry weight decreased when the glycerol

concentration was at 100 mM and over ($P < 0.05$). Similarly, the specific growth rate was significantly affected by the glycerol concentration (Fig. 2.3B). The specific growth rate increased from 0.47 h^{-1} to 0.54 h^{-1} as the cultures were supplemented with different levels of glycerol. However, at high glycerol concentrations 25-100 mM, specific growth rates were not significantly different ($P > 0.05$). A reduction of the specific growth rate occurred at 200 mM glycerol.

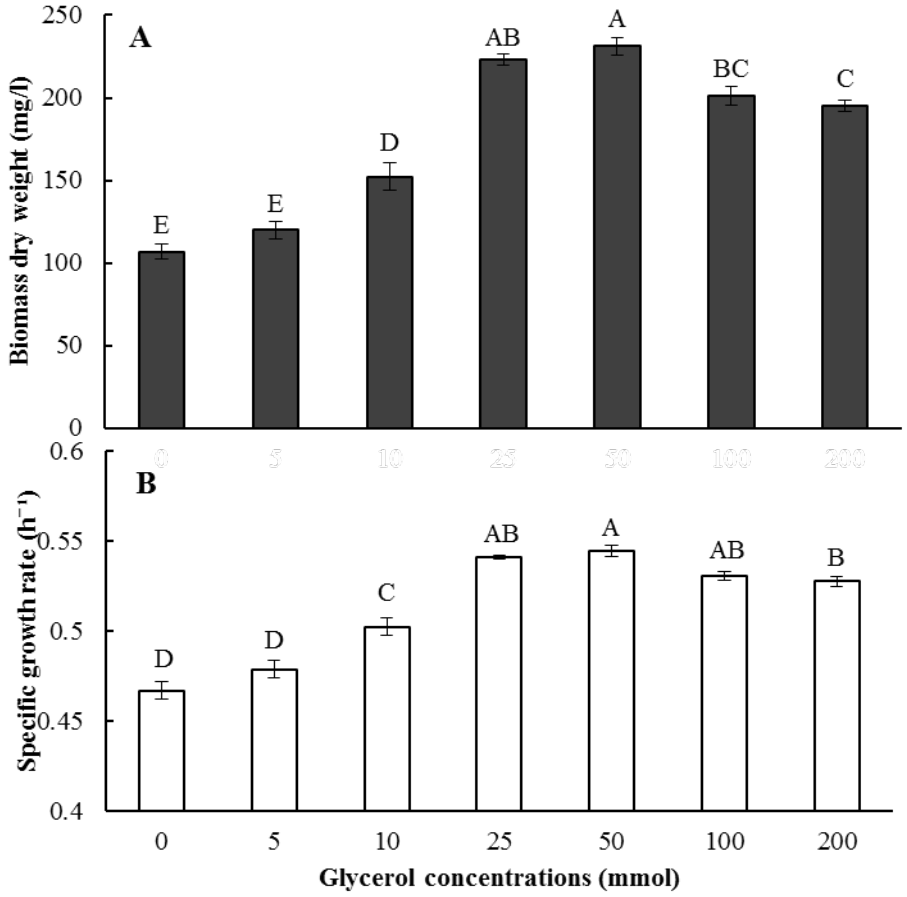

Fig. 2.3 Algal dry weight (A) and specific growth rate (B) of *I. galbana* under mixotrophy with different glycerol concentrations. Data are shown as mean ± SE (n = 4).

2.4.3 Effect of environmental factors on algal growth

2.4.3.1 Light intensity

Two-way ANOVA analysis indicated that the dry biomass of *I. galbana* was significantly affected by both light intensity and trophic conditions ($P < 0.05$). At any light intensities between 25 and 200 µmol photon m^{-2} s^{-1}, the growth of *I. galbana* was faster in mixotrophy than in phototrophy (Fig. 2.4. A). Algal dry weight in phototrophy was not significantly different ($P > 0.05$) in the range of light intensities of 50, 100 and 200 µmol photon m^{-2} s^{-1} whereas algal weight under 25 µmol photon m^{-2} s^{-1} was significantly ($P < 0.05$) less than other light levels. Under mixotrophy, maximum algal production obtained at 100 µmol photon m^{-2} s^{-1} was 245 mg L^{-1} whereas the algal production at 50 µmol photon m^{-2} s^{-1} was 231.25 mg L^{-1}, which was not significantly different ($P > 0.05$). Reduction of mixotrophic cells was observed at 25 and 200 µmol photon m^{-2} s^{-1} indicating that these light intensities are not suitable for algal growth. In sole phototrophy, even though algal growth rates were less than in mixotrophy, light effect was not significant ($P > 0.05$).

The specific growth rates of algae in phototrophic and mixotrophic cultures at various light intensities are shown in Fig. 2.4 B. Algal specific growth rate was faster in mixtrophy than in phototrophy regardless of light intensity ($P < 0.05$). The specific growth rate of algae in phototrophic cultures at light intensities of 50 - 200 µmol photon m^{-2} s^{-1} was not significantly affected by light intensity, which was opposite to the result in mixotrophy. In mixotrophy, algae grew faster at 100 µmol photon m^{-2} s^{-1} than at other light intensities ($P < 0.05$), but there was no difference in algal growth between 50 and 100 µmol photon m^{-2} s^{-1} ($P > 0.05$). A reduction of the specific growth rate was only observed at 200 µmol photon m^{-2} s^{-1} when algae grew mixotrophically.

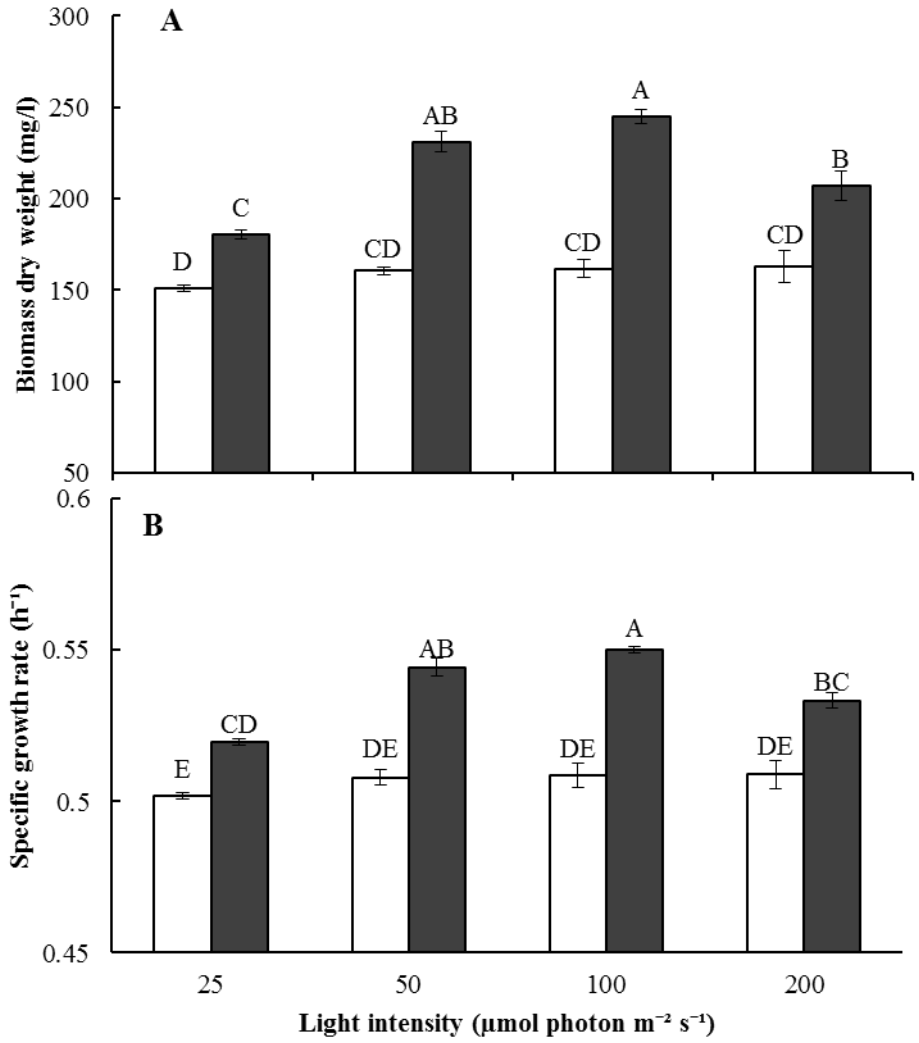

Fig. 2.4 Effect of light intensity on algal dry weight (A) and specific growth rate (B) of *I. galbana* under phototrophic (blank) and mixotrophic (dark) conditions. Data are shown as mean ± SE (n = 4).

2.4.3.2 Photoperiod

Both photoperiod and trophic condition significantly impacted algal growth and production. Also, the interaction between trophic condition and photoperiod was significant ($P < 0.05$). As shown in Fig. 2.5A, the algal biomass in phototrophic cultures was not significantly affected ($P > 0.05$) by photoperiods but it was significantly lower than that in the mixotrophic cultures ($P < 0.05$). In mixotrophy,

there was no significant difference in biomass between 8 and 24 h photoperiods, but algal biomass at the photoperiod of 4 h significantly decreased ($P < 0.05$). Algal biomass (223.25 mg L^{-1}) at the 12 h photoperiod was significantly higher than at any other photoperiods ($P < 0.05$). At the 4 h photoperiod, algal biomass in mixotrophy (133.25 mg L^{-1}) was significantly higher than that in phototrophy at any other photoperiods ($P < 0.05$).

Algal specific growth rate in phototrophy did not differ between any photoperiods ($P > 0.05$, Fig. 2.5B). In mixotrophy, the specific growth rate was not significantly different between the 8 h and 24 h photoperiods while it was significantly higher at the 12 h photoperiod ($P < 0.05$) than that at any other photoperiods. At the 4 h photoperiod, algal grew faster in mixotrophy than that in phototrophy regardless of photoperiods ($P < 0.05$).

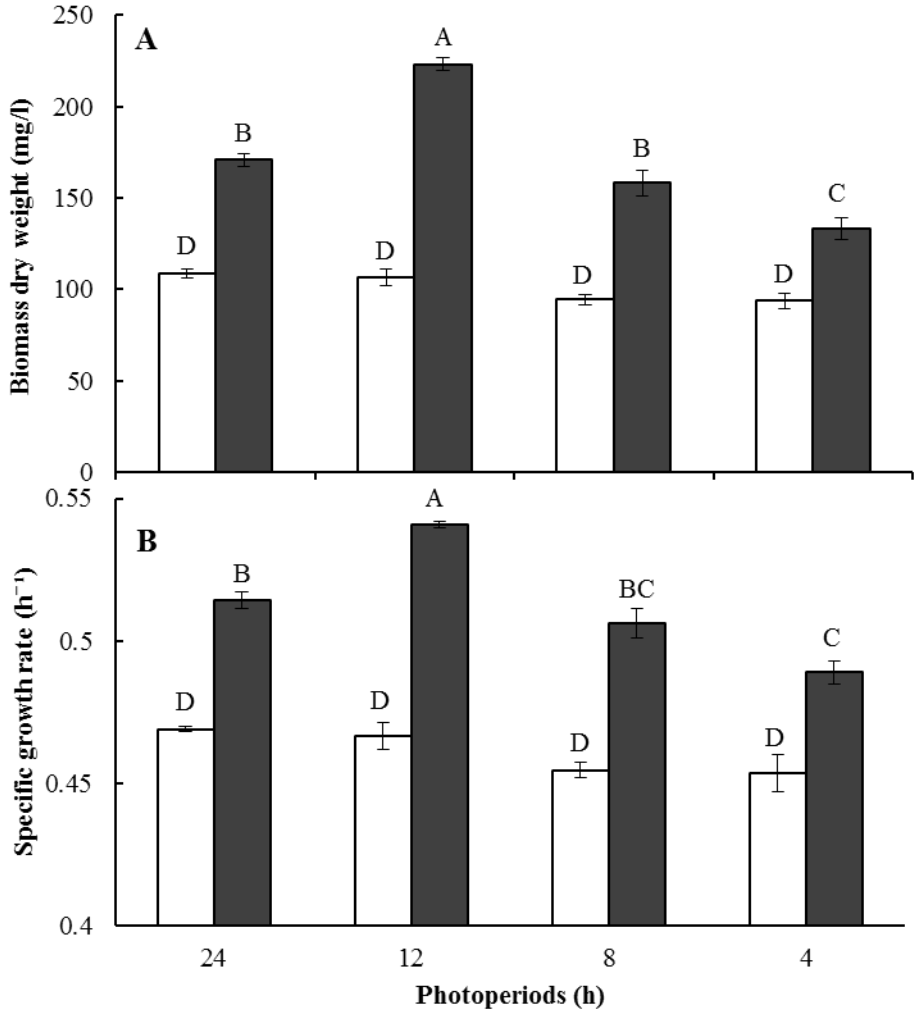

Fig. 2.5 Effect of photoperiod on algal dry weight (A) and specific growth rate (B) of *I. galbana* under phototrophic (blank) and mixotrophic (dark) conditions. Data are shown as mean ± SE (n = 4).

2.4.3.3 Salinity

Salinity and trophic conditions significantly influenced algal biomass production ($P < 0.05$), and the interaction between these two factors was also significant (Fig. 2.6 A). The impact of salinities on algal growth was stronger in mixotrophy than in phototrophy. In mixotrophy, algal biomass significantly ($P < 0.05$) increased as salinity increased from 10 to 65‰. In mixotrophy, the maximum biomass occurred at

35‰, while algal biomass significantly decreased at 50 and 65‰ ($P < 0.05$) though algal biomass at 20 and 65‰ salinities was not significantly different ($P > 0.05$). In contrast, the influence of salinity on biomass production in phototrophic cultures was insignificant. In mixotrophic cultures, lower algal production occurred at 10‰ and higher production at 35‰ salinity. Algal production in mixotrophy was 238.50 mg L^{-1} which was 2 times higher than in phototrophic culture (106.75 mg L^{-1}).

The specific growth rates of algae were significantly affected by salinity in both phototrophic and mixotrophic cultures (Fig. 2.6B). However, the impact of salinity on the specific growth rate in mixotrophy was higher than in phototrophy. When the salinity was 35 – 65‰, there was no significant impact on specific growth rates in phototrophy ($P > 0.05$). Under mixotrophic cultures, however, the specific growth rates were significantly different between 35 and 50‰ and between 50 and 65 ‰. At 10 ‰, the specific growth rate was not significantly different in both trophic conditions. Higher growth rate was obtained at 35‰ salinity for both trophic statuses but it was 18% higher in mixotrophy than in phototrophy ($P > 0.05$).

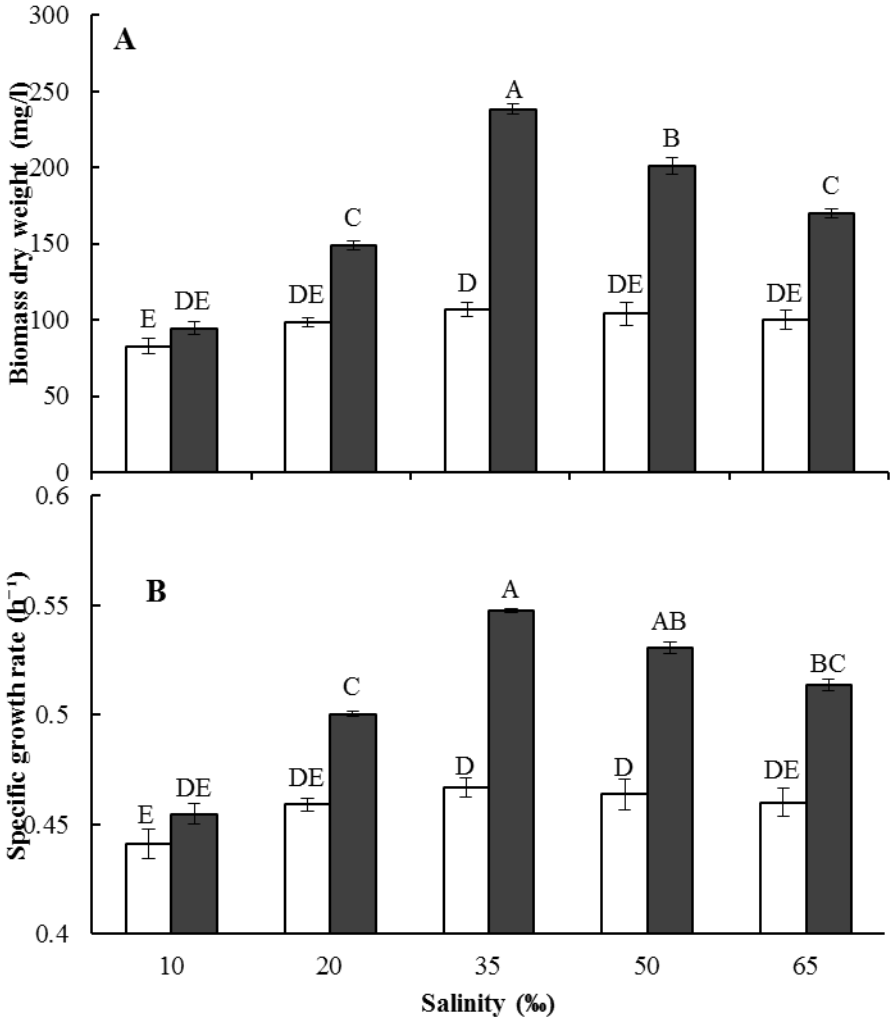

Fig. 2.6 Effect of salinity on algal dry weight (A) and specific growth rate (B) of *I. galbana* under phototrophic (blank) and mixotrophic (dark) conditions. Data are shown as mean ± SE (n = 4).

2.5 Discussion

2.5.1 Algal growth in heterotrophic, mixotrophic and phototrophic conditions

Algal growth can be potentially improved by supplementing organic carbons to the media in heterotrophic or mixotrophic culture (Lee 2001). However, the ability of microalgae to grow in media with organic supplementation depends on algal species and the sources of organic carbon (Azma et al. 2011; Chen and Chen 2006). In this study, the growth of *I. galbana* was inhibited in heterotrophic culture, which agrees with the previous reports on heterotrophic growth of this species (Gladue and Maxey 1994; Vazhappilly and Chen 1998). On the other hand, some algae such as *Nitzschia laevis* and *Chlorella protothecoides* can grow in heterotrophic or mixotrophic culture by achieving 4-5 fold faster growth than in phototrophic culture (Heredia-Arroyo et al. 2010; Wen and Chen 2000b). In the present study, *I. galbana* showed the highest growth rate in the mixotrophic culture when glycerol was the carbon source, and algal dry weight was 2.1 times higher than in the phototrophic condition. Similarly, Liu et al (2009) found that the production of *Phaeodactylum tricornutum* in mixotrophy was 1.6 times higher than in phototrophy, and Das et al. (2011) found that the dry weight of *Nannochloropsis* sp. in mixotrophy was 1.35 times greater than that in phototrophy.

In this study, glycerol was the only carbon source that efficiently promoted the growth of *I. galbana* under the mixotrophic condition, which agrees with Wood et al. (1999) who found that some marine microalgae species grew better in media supplied with glycerol than with glucose or acetate. Moreover, *P. tricornutum* (Cerón García et al. 2006) and *Nannochloropsis* sp. (Das et al. 2011) grow faster in mixotrophy with glycerol as a carbon source than with any other organic carbons. In

other studies, however, glucose could enhance the growth of *Cyclotella cryptica* (Pahl et al. 2010), *Tetraselmis suecica* (Azma et al. 2011) and *Chlorella vulgaris* (Heredia-Arroyo et al. 2011) in heterotrophic culture, but this is at odds with our results. In the present study, *I. galbana* was unable to assimilate acetate which agrees with an early report by Cerón García et al (Cerón García et al. 2005) that *P. tricornutum* could not assimilate acetate, possibly because acetate is toxic to some algal species (Perez-Garcia et al. 2011). Clearly, glycerol is the best carbon source to support the *I. galbana* growth in mixotrophic culture. Overall, growing *I. galbana* in a mixotrophic condition is a promising approach to improve algal production.

2.5.2 Glycerol concentrations

In this study, glycerol concentrations were tested to optimise glycerol supplementation to the culture media. The growth of *I. galbana* increased exponentially with the increase of glycerol concentration from 0 to 50 mM. When glycerol was over 50 mM, a reduction in algal growth was observed, indicating that algal growth is impeded by high glycerol concentrations. However, specific growth rates and algal dry weights at all glycerol concentrations in mixotrophy were higher than those in phototrophy. In another study, Cerón García et al. (2006) found that 100 mM of glycerol was optimal for *P. tricornutum* in mixotrophic culture, but algal growth was inhibited when glycerol content exceeded 100 mM. Similarly, the growth of *Chlorella vulgaris* was improved at a glycerol concentration of 100 mM (Liang et al. 2009). By comparison, a high amount of glycerol at 325 mM enhanced the growth of *C. protothecoides* in heterotrophic culture (O'Grady and Morgan 2011). Our study demonstrates that adding low concentrations of glycerol is sufficient to achieve a high growth rate of *I. galbana*. Thus, the optimum glycerol concentration is considered at 50 mM for cultivation *I. galbana.*

2.5.3 Effect of environmental factors on algal growth

2.5.3.1 Light intensity

Microalgae capable of growing under a mixotrophic condition usually require a low light but can tolerate high light photoinhibition (Cid et al. 1992; Vonshak et al. 2000). In this study, *I. galbana* in mixotrophic culture achieved a high growth rate at light intensities of 25-100 µmol m^{-2} s^{-1} while the maximum biomass production was achieved at 100 µmol m^{-2} s^{-1}. These results agree with Sloth et al. (2006) who found that the growth of *Galdieria sulphuraria* in mixotrophy increased as light intensity increased from 65 to 128 µmol m^{-2} s^{-1} while the highest growth occurred at 100 µmol m^{-2} s^{-1}. A green alga *Platymonas subcordiformis* grew faster in mixotrophic culture at 95 µmol m^{-2} s^{-1}. In our study, the growth of *I. galbana* was not significantly enhanced with the increase of light intensity in phototrophic culture, but Tzovenis et al. (2003) and Marchetti et al. (2012) both reported that the maximal growth of *I. affinis galbana* occurred at a light intensity over 200 µmol m^{-2} s^{-1}. It seems that the light intensity in our study was not optimal for the growth of *I. galbana*.

In this study, a light inhibitory effect occurred in the mixotrophic culture at 200 µmol photon m^{-2} s^{-1}. However, the light inhibitory effect was not observed in the phototrophic culture. In an early study, the inhibitory effect of high light intensity up to 400 µmol m^{-2} s^{-1} was not observed on *I. galbana* when grown in phototrophy (Marchetti et al. 2012; Tzovenis et al. 2003). This implies that under mixotrophy *I. galbana* become sensitive to high light intensity. Moreover, the growth rates of *C. vulgaris* and *Scenedesmus acutus* were inhibited under mixotrophy when the light intensity was >80 µmol m^{-2} s^{-1} and the growth rate was lower than in phototrophy (Ogawa and Aiba 1981). In contrast, *Spirulina platensis* can grow at high light intensity and no light inhibitory influence was observed in mixotrophy while the

growth was inhibited in phototrophy as light intensity increased (Chojnacka and Noworyta 2004; Vonshak et al. 2000). Our study demonstrates that in mixotrophic culture, high light intensity may result in photoinhibition of *I. galbana*, whereas high growth rates can be achieved by culturing algae mixotrophically at a low light, which can reduce algal production costs.

2.5.3.2 Photoperiod

Photoperiod represents the duration that algae can receive light energy (Wahidin et al. 2013). A short photoperiod can stimulate algae to use organic substrates in mixotrophic culture (Ogbonna and Tanaka 2000). In this study, the maximum growth of *I. galbana* occurred in the photoperiod of 12 h light :12 h dark in mixotrophic culture while the algal growth rate reduced when the light period was <12 h, but *I. galbana* grew faster in mixotrophy than in phototrophy regardless of photoperiods, except for full darkness. On the other hand, we found that the phototrophic growth of *I. galbana* was not significantly different at all photoperiods, which may be due to the use of low light intensity 100 µmol m^{-2} s^{-1} in this study. Wahidin et al. (2013) found that the growths of *Nannochloropsis* sp. in both photoperiods of 24:0 h and 12:12 h were not significantly different at a light intensity of 100 µmol m^{-2} s^{-1} whereas the maximum cell density was obtained at the photoperiod 16:8 h. In another study, Tzovenis et al. (2003) reported that the growth of *I. aff. galbana* under a discontinuous light regime was better than continuous one. Our study implies that the mixotrophic system offers advantage to grow *I. galbana* to reduce power cost for algal production. Therefore, the photoperiod of 12 h light to 12 h dark cycle is recommended as a suitable photoperiod for *I. galbana*.

2.5.3.3 Salinity

In an open system of algal culture, salinity fluctuates due to evaporation or rainfall may impact algal growth (Pal et al. 2011). Cultivation of microalgae in hyper salinity or brackish water has some advantages. For instance, Heredia-Arroyo et al. (2011) found that the lipid accumulation increased when *C. vulgaris* grew mixotrophically with 35 g L^{-1} NaCl while Wen and Chen (2001) found that the heterotrophic growth rate of *N. leavis* was higher at a salinity 8 g L^{-1} NaCl. In this study, *I. galbana* was able to grow in a wide range of salinity from 10 to 65‰ under both mixotrophic and phototrophic culture, which agrees with the salinity range of this algae reported by Kaplan *et al*. (1986) who found that *I. galbana* could grow from 5 to 60‰ NaCl. In the present study, the growth of *I. galbana* in phototrophy did not significantly vary from 10 to 65 ‰ salinity, though algal growth reduced when salinity was either above or below 35 ‰ in mixotrophy. In contrast, Das et al. (2011) found that the biomass yield of *Nannocloropsis* sp. in phototrophy decreased by 15% when salinity increased to 50‰ whereas in mixotrophy the biomass yield was not different between 35 and 50‰ salinities. Our study suggests that *I. galbana* can grow well regardless of salinity, which is a value trait for algal culture in a situation where high evaporation may elevate salinity in outdoor culture. Although, mixotrophic cultures granted high growth, *I galbana* seemed to be sensitive to higher salinity in the presence of organic carbon.

2.6 Conclusion

Isochrysis galbana could grow successfully in mixotrophic culture. The optimal glycerol concentration to support the mixotrophic growth of *I. galbana* was 50 mM glycerol. The growth of *I. galbana* under mixotrophic conditions was better than its growth under phototrophic conditions but the growth rate was inhibited in

heterotrophy. The optimal light intensity and photoperiod were 100 µmol photon m^{-2} s^{-1} and 12 h, respectively for *I. galbana* in mixotrophy. This species could tolerate a wide range of salinity in phototrophy, but 35‰ salinity was optimal for algal growth in mixotrophy. The results of this study can be applied in aquaculture to improve algal production efficiency. Further research may include the examination of the effect of the growth condition on the change of biochemical composition of *I. galbana.*

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CHAPTER 3

Comparison of N and P Requirements of *Isochrysis galbana* under Phototrophic and Mixotrophic Conditions

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3.1 Abstract

This study investigated the optimal N and P requirements for the growth of *Isochrysis galbana* under phototrophic and mixotrophic conditions. Algae were cultured in the f/2 basal medium modified with N and P concentrations. In the phototrophic condition, three N forms: sodium nitrate, ammonium sulphate and urea were tested at six N levels (0, 12.5, 25, 50, 100 and 200 mg N L^{-1}) and P was tested at five levels (0, 1.3, 2.6, 5.2 and 10.4 mg P L^{-1}). In the mixotrophic condition, the N or P modified f/2 basal medium was supplemented with 50 mM glycerol as the source of organic carbon. Growth was significantly influenced by the trophic conditions and N sources. The optimal N was 12.5– 200 mg NO_3-N L^{-1}, 12.5–25 mg NH_3-N L^{-1} or 12.5–50 mg urea-N L^{-1}. The optimal P was 1.3–5.2 mg PO_4-P L^{-1} for growth. In all treatments, the algal production in mixotrophy was over twofold higher than that in phototrophy. The maximal algal dry weight (235.7 mg L^{-1}) and nutrient to algal biomass conversion efficiency (21.7 mg mg^{-1}) were obtained in the cultures with urea as the N source under mixotrophy. The requirements of N and P were not different in phototrophy and mixotrophy, but dry weight production and nutrient conversion efficiency in mixotrophy were greater than in phototrophy, indicating that mixotrophic culture is more effective in the production of *I. galbana*.

Keywords: *Isochrysis galbana* . Nitrogen . Phosphorus . Phototrophy . Mixotrophy . Algae

3.2 Introduction

Most marine microalgae are photosynthetic organisms that are the essential food for marine grazers including mollusc, crustacean larvae and zooplankton due to the proper nutritional value and digestibility of microalgae (Gladue and Maxey 1994).

With the increasing demand of seafood from aquaculture, hatcheries require a large quantity of live algae to feed marine larvae in their early developmental stage (Borowitzka 1997). To a certain extent, live microalgae are indispensable as a diet for major aquatic animals in aquaculture as alternative feed to live algae usually gives poor growth and survival for marine larvae (Hemaiswarya et al. 2011). Thus, the supply of adequate microalgae with high nutrition quality is a challenge for a marine hatchery (Azma et al. 2011). Currently, the cultivation of microalgae in a phototrophic system is the dominant protocol to supply live algae in aquaculture, but algal productivity in such a system is low due to self-shading and light limitation (Wang et al. 2014). A high algal growth rate and high cell density can be achieved by the manipulation of algal growing conditions (Li et al. 2014). Under a heterotrophic or mixotrophic conditions, organic carbon substrates such as sugar and alcohol play an important role to supply energy and carbon for algal growth (Chen and Chen 2006). Heterotrophic and mixotrophic growth have been reported as a useful approach to boost production for some microalgae species with no light or low light supply (Andrade and Costa 2007; Andruleviciute et al. 2013; Cheirsilp and Torpee 2012). Consequently, the addition of organic carbon to the culture medium has been used to increase algal production and improve algal nutrition as live food for marine larvae. For example, in the culture of *Nannochloropsis* sp., the addition of glycerol to the media can increase dry weight production by 40 % and the lipid content by 30 % compared with the solely phototrophic culture (Das et al. 2011).

Besides carbon and light supply, microalgae also require other nutrients for growth and cell division. Nitrogen and phosphorus are the two fundamental elements required in algal culture media. Microalgae can utilise nitrogen in different forms such as nitrate, ammonium and urea, and the preferred nitrogen source is alga species

specific (Perez-Garcia et al. 2011). While most microalgae prefer nitrate and urea, ammonium is considered an inconvenient source of nitrogen since the assimilation of ammonium causes pH to drop and the acidic condition may lead to the decline of algal growth (Kim et al. 2013a; Wen and Chen 2001; Yongmanitchai and Ward 1991). The effect of nitrogen source on the uptake rate depends on culture conditions. For instance, the growth rate of *Phaeodactylum tricornutum* was lower in ammonium than in nitrate or urea in a phototrophic condition (Yongmanitchai and Ward 1991). However, Cerón García et al. (2000) found that the maximal growth of *P. tricornutum* occurred in ammonium chloride when the culture medium was supplemented with organic carbon as glycerol. In contrast, Combres et al. (1994) found that the uptake rate of ammonium by *Scenedesmus obliquus* was lower in a mixotrophic condition than in a phototrophic condition. Moreover, the requirement of phosphorus differs between trophic conditions. For instance, *Chlorella pyrenoidosa* require less phosphate in the presence of glucose than without glucose (Qu et al. 2008). Therefore, there is a need to further explore the difference of nutrient requirements in algae between phototrophic and mixotrophic conditions.

The golden brown flagellate *Isochrysis galbana* is a common species used as a live food in aquaculture because of its rapid growth in mass culture (Liu et al. 2013). This species is preferred by most marine larvae due to cell size, nutrient content and digestibility (Wikfors and Patterson 1994). In addition, its proximate composition contains a high level of polyunsaturated fatty acids, particularly docosahexaenoic acid (DHA) (Liu et al. 2013). This species is able to grow mixotrophically with high growth rate and biomass production (Alkhamis and Qin 2013). The requirements of nitrogen and phosphorus for the growth of *I. galbana* have been studied only under phototrophic conditions (Fidalgo et al. 1998; Liu et al. 2013). However, the

requirements of nitrogen and phosphorus by *I. galbana* under a mixotrophic condition are unknown. This study aimed to compare the responses of *I. galbana* to different nutrient sources, nitrogen concentrations and phosphorus concentrations in phototrophic and mixotrophic conditions. The optimisation of nutrient requirements of *I. galbana* in mixotrophic conditions will contribute to the improvement of algal growth efficiency and the mass production of this commonly used species in aquaculture and other industrial uses.

3.3 Materials and methods

3.3.1 Microalgae and culture conditions

The marine microalga *I. galbana* (CS-22) was obtained from the Australian National Algae Culture Collection (Hobart, Tasmania). Algal cultures were carried out in natural seawater (35‰) enriched with the basal f/2 nutrients (Guillard and Ryther 1962) with variations of N and P concentrations. Prior to the experiment, the culture media were autoclaved at 121 °C for 15 min. Glycerol as a source of organic carbon was sterilised in an autoclave at 115 °C for 10 min and was only supplemented to the mixotrophic cultures. All cultures were carried out in 250 mL sterilised flasks containing 100 mL medium and 10 % (v/v) algal inoculum. The flasks were placed on an orbital shaker at 100 rpm at 24 °C under a daily illumination of 12 h light at 50 μmol photons m^{-2} s^{-1} measured at the surface of the media using a Light ProbeMeter (Extech Instruments Corp, USA). Illumination was provided with white cool fluorescent lamps.

3.3.2 Experimental design

Three nitrogen sources: nitrate as $NaNO_3$, ammonium as $(NH_4)_2SO_4$ and urea (CH_4N_2O) at six concentrations 0,12.5, 25, 50, 100 and 200 mg N L^{-1} were tested in

the phototrophic condition with three replicates. Except for nitrogen, other nutrients were added as the same as in the f/2 medium. The environmental and nutrient conditions for the mixotrophic algal culture were identical to the phototrophic conditions except that 50 mM glycerol was supplemented to each treatment as organic carbon. The dissolved organic carbon in the original seawater was not measured, but the zero glycerol addition in the phototrophic medium was used as the control to the mixotrophic medium added with 50 mM glycerol. All experiments with different nitrogen sources lasted 10 days when the stationary phase of growth was reached.

Based on the results of the nitrogen experiments, urea was identified as the optimal source of nitrogen, and 12.5 mg urea- N L^{-1} was the optimal level of nitrogen for *I. galbana* growth in the trial for testing phosphorus requirement. Five levels of P as sodium di-hydrogen orthophosphate (NaH_2PO_4) at 0, 1.3, 2.6, 5.2 and 10.4 mg P L^{-1} were used in the media contained 12.5 mg urea-N L^{-1}. In the mixotrophic condition, 50 mM glycerol was supplemented to each P treatment as the source of organic carbon. Three replicates were used in both phototrophic and mixotrophic cultures, and each experiment lasted 10 days when the stationary phase of algal growth was reached.

3.3.3 Determination of algal growth

Algal cell density was quantified by taking 2 mL sample from each flask every 2 days during the experimental period. The absorbance was measured at 680 nm. The regression between algal cell densities and optical densities was assessed by measuring the optical density of a series of diluted algal samples with known cell densities counted on a microscope with a haemocytometer. The optical densities

(OD) at 680 nm were plotted versus known cell densities to determine the linear relation (Fig. 3.1). The algal cell density (Y) was calculated according to the linear Eq. (1) with the optical density (x) at 680 nm.

$$Y = 1163x - 0.0025, (R^2 = 0.98, P < 0.05) \quad (1)$$

Algal production was determined by measuring the total dry weight as the algal production at the end of each experiment. A volume of 100 mL culture was centrifuged at 5000×g for 10 min, and the algal pellets were washed with distilled water and dried in an oven at 65 °C until the constant weight. The algal weight was measured to the nearest 0.001 mg. The nutrient conversion efficiency ($Y_{X/N}$) was calculated using the amount of biomass production and nutrient reduction (Eq. 2) (Doran 1995).

$$Y_{X/N} = (dx/dt)/(ds/dt) \quad (2)$$

where dx is the change of biomass and ds is the change of nutrient concentration in the substrate concentrations during time t.

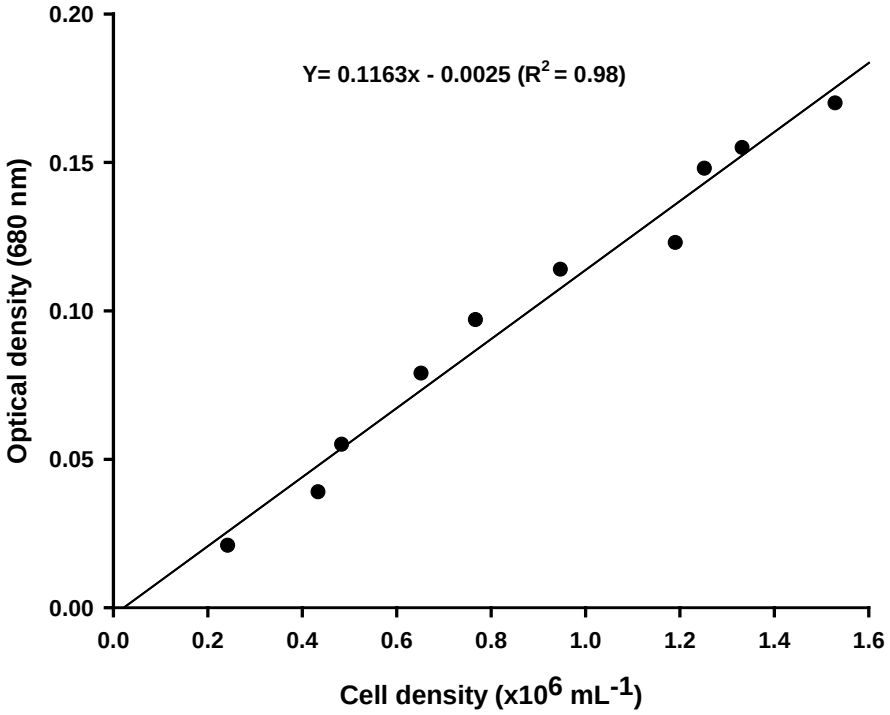

Fig. 3.1 The standard curve of linear regression between algal cell densities and optical densities at 680 nm

3.3.4 Determining nutrient concentrations

The utilisation rate of each nutrient was determined from the samples which were cultured in the phototrophic and mixotrophic conditions. The initial nutrient concentrations were the optimal N 12.5 mg N L^{-1} and optimal P concentration (2.6 mg P L^{-1}) determined from the previous trials. A sufficient volume of the culture media was taken every 2 days to measure residuals of N and P, and the samples were filtered through GF/C filters and kept in a −20 °C freezer until analysis. The residual of N as nitrate and ammonia and P as phosphate was measured photometrically using nutrient test kits (Aquaspex Water testing products, Blackwood, SA, Australia). The residuals of urea were analysed by the decomposition of total nitrogen in the sample into nitrogen monoxide, then the total nitrogen concentration was detected using a Shimadzu Total Organic Carbon and Total Nitrogen Analyser (TOC

VCSH/CSN+TNM-1, Shimadzu, Japan) by a chemiluminescence gas analysis.

3.3.5 Statistical analysis

The experimental design to test the effect of nitrogen on algal growth included three factors (nitrogen sources, concentrations and trophic conditions). Significant differences between means of each variable were tested by three-way ANOVA. To detect the treatment effects, this test was followed by MANOVA unless the interaction effects were found significant ($P < 0.05$). In the phosphorus trial, the differences between the means of the algal density, algal production and nutrient conversion efficiency were tested by two-way ANOVA with P concentration and trophic conditions as two fixed factors. Multiple comparisons were tested by Tukey post hoc analysis when the main treatment effect was significant at $P < 0.05$. Data were analysed using SPSS (version 18).

3.4 Results

3.4.1 Effect of nitrogen sources and nitrogen concentrations

The growth of *I. galbana* was tested in six N concentrations (0, 12.5, 25, 50, 100 and 200 mg N L^{-1}) of each nitrate, ammonium and urea under both phototrophic and mixotrophic conditions. Each of the three treatment factors nitrogen sources, nitrogen concentration and trophic conditions had significant ($P < 0.05$) impact on the cell density of *I. galbana* (Table 3.1). The interaction effect between these factors was also significantly different ($P <0.05$).

Table 3.1 Summary of the ANOVA table for testing the effect of nitrogen concentration, nitrogen source and trophic conditions on algal growth.

Source	*SS*	*DF*	*MS*	*F*	*P*
Concentrations (C)	55.36	4	13.84	861.65	0.01
Sources (S)	50.70	2	25.35	1578.05	0.01
Trophic conditions (T)	76.42	1	76.42	4757.86	0.01
C × S	45.90	8	5.74	357.24	0.01
C × T	5.13	4	1.28	79.85	0.01
S × T	2.50	2	1.25	77.52	0.01
C × S × T	7.81	8	0.98	60.78	0.01

The effect of N concentration and trophic conditions on algal growth with N source is presented in Fig. 3.2. As algal growth was not detectable in the phototrophic or mixotrophic treatments without N, these growth rates were removed from the analysis. The effect of N concentrations on growth was not significantly different between trophic conditions when nitrate was the N source ($P > 0.05$). The alga was able to grow in a wide range of nitrate concentrations, but there were no increases in cell densities in both mixotrophic and phototrophic cultures at nitrate concentrations of 12.5 to 200 mg NO_3-N L^{-1}. The impact of N concentration on cell density in the phototrophic and mixotrophic conditions was significant when N was supplied as ammonium or urea ($P < 0.05$). The algal cell density in mixotrophy was enhanced when the N concentration was 12.5 to 25 mg NH_4-N L^{-1} ($P < 0.05$, Fig. 3.2) compared with phototrophy, though algal densities were not significantly different between the N concentrations of 12.5 and 25 mg NH_4-N L^{-1} ($P > 0.05$). When ammonium was increased to 50 mg NH_4-N L^{-1}, algal densities sharply

decreased and algal growth was much inhibited when the N concentration was >100 mg NH_4-N L^{-1} in both photo and mixotrophic conditions. Algal cell density in mixotrophy was higher than that in phototrophy when urea was the N source ($P < 0.05$). However, the effect of urea concentration on cell density was not significant at N concentrations between 12.5 and 50 mg urea-N L^{-1} in both trophic conditions ($P > 0.05$). Cell density significantly decreased when the urea concentration increased from 100 to 200 mg urea-N L^{-1} ($P< 0.05$). Among all N treatments, the maximal algal density occurred when nitrogen was in the range of 12.5–50 mg urea-N L^{-1} in both phototrophic and mixotrophic conditions.

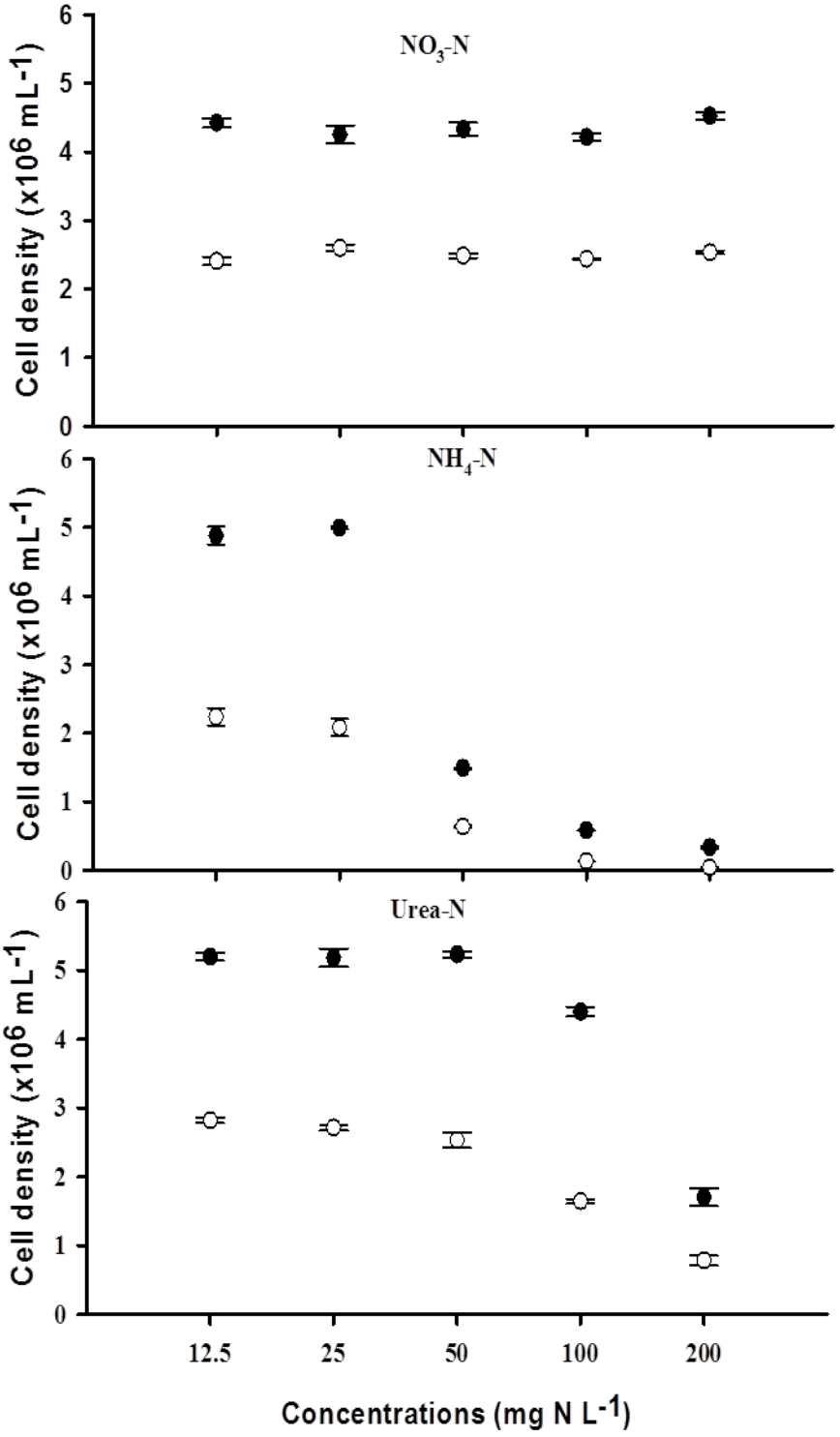

Fig. 3.2 The effect of nitrogen concentrations and sources (nitrate, ammonium and urea) on the final algal cell densities under phototrophic (white circle) and mixotrophic (black circle) conditions (data shown as mean ± SE, *n*=3)

3.4.2 Effect of phosphorus concentrations

The final cell densities at the end of the trial were significantly affected by phosphorus concentrations and trophic conditions ($P < 0.05$, Fig. 3.3). However, P concentrations did not significantly affect growth in phototrophic cultures. The final algal densities in the culture of 0–5.2 mg P L^{-1} were not significantly different in the phototrophic condition ($P > 0.05$). However, P enrichments increased algal density in the mixotrophic culture compared with the control ($P < 0.05$), but there were no significant differences in density when phosphorus was >1.3 mg P L^{-1} ($P > 0.05$). The maximum cell density reached 5.8×10^6 cells mL^{-1} in the mixotrophic culture with P addition, while the minimum cell density was 3.8×10^6 cells mL^{-1} in cultures without P addition. Despite the same P additions in both trophic conditions, the final cell densities in mixotrophic conditions were significantly greater than those in phototrophic conditions ($P < 0.05$).

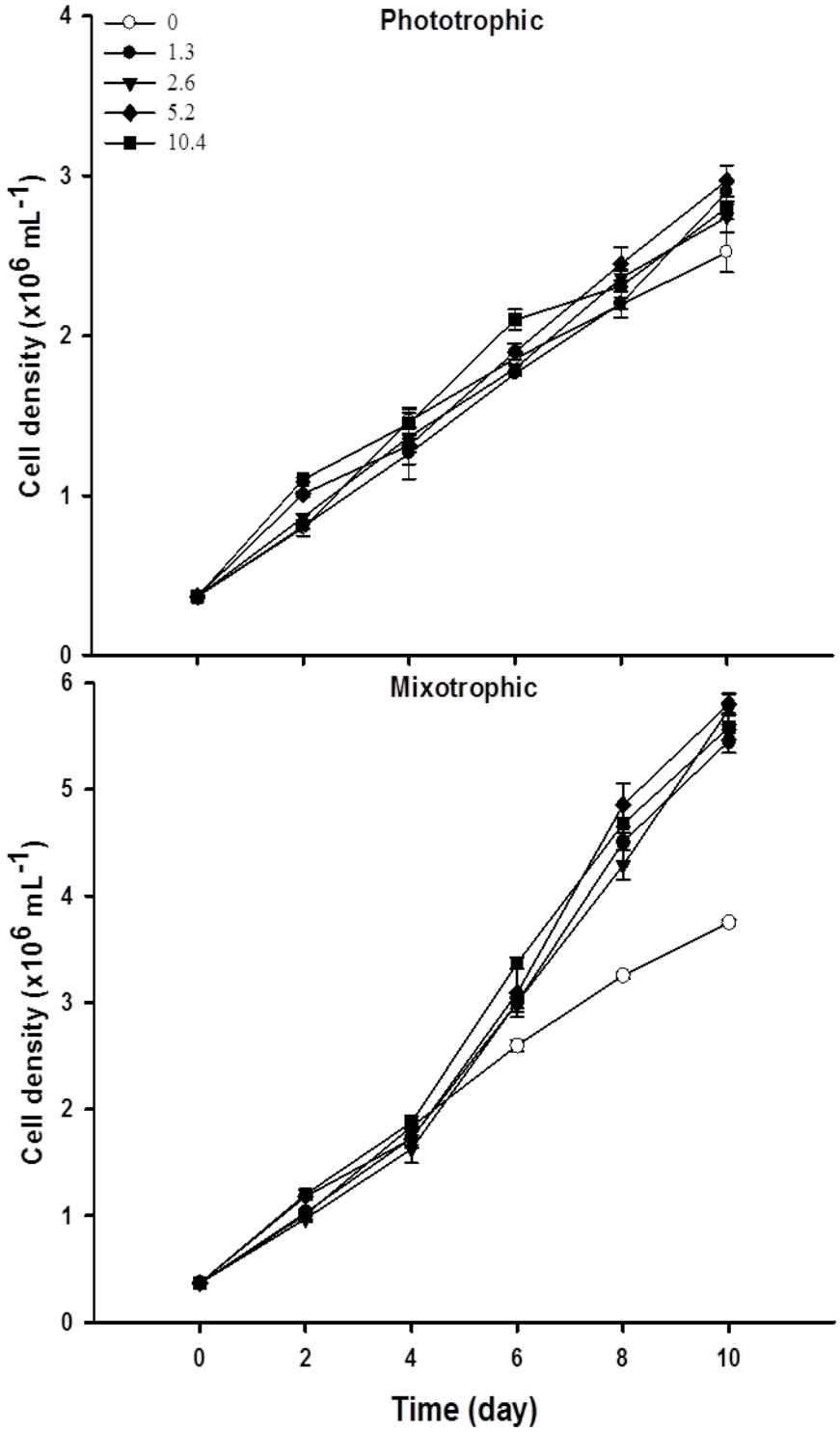

Fig. 3.3 The effect of phosphorus concentrations (0, 1.3, 2.6, 5.2 and 10.4 mg L^{-1}) on algal cell density in phototrophic and mixotrophic conditions (data shown as mean ± SE, *n*=3)

3.4.3 Algal production and nutrient conversion efficiency

Algal production and nutrient conversion efficiency are presented in Fig. 3.4. These parameters were significantly affected ($P < 0.05$) by N sources and trophic conditions. Among the three N sources, the algal production in the culture with urea was significantly higher than that with ammonium or nitrate as the N source. In addition, production was promoted in the mixotrophic condition, and the maximum algal dry weight in mixotrophy (235.7 mg L^{-1}) was two times higher than that in phototrophy (115.3 mg L^{-1}). The values of nutrient conversion efficiency to algal biomass based between N sources were significantly different ($P < 0.05$) in both trophic conditions. The nutrient to biomass conversion efficiency (Fig. 3.4) in mixotrophy with urea and ammonium was 21.7 and 18.8 mg mg^{-1}, respectively, which was significantly higher than that with nitrate ($P < 0.05$). While in phototrophy, the nutrient conversion efficiency in the urea treatment was 14.85 mg mg^{-1}, which was significantly higher than that in the nitrate (11.97 mg mg^{-1}) or ammonium (9.92 mg mg^{-1}) treatments ($P < 0.05$).

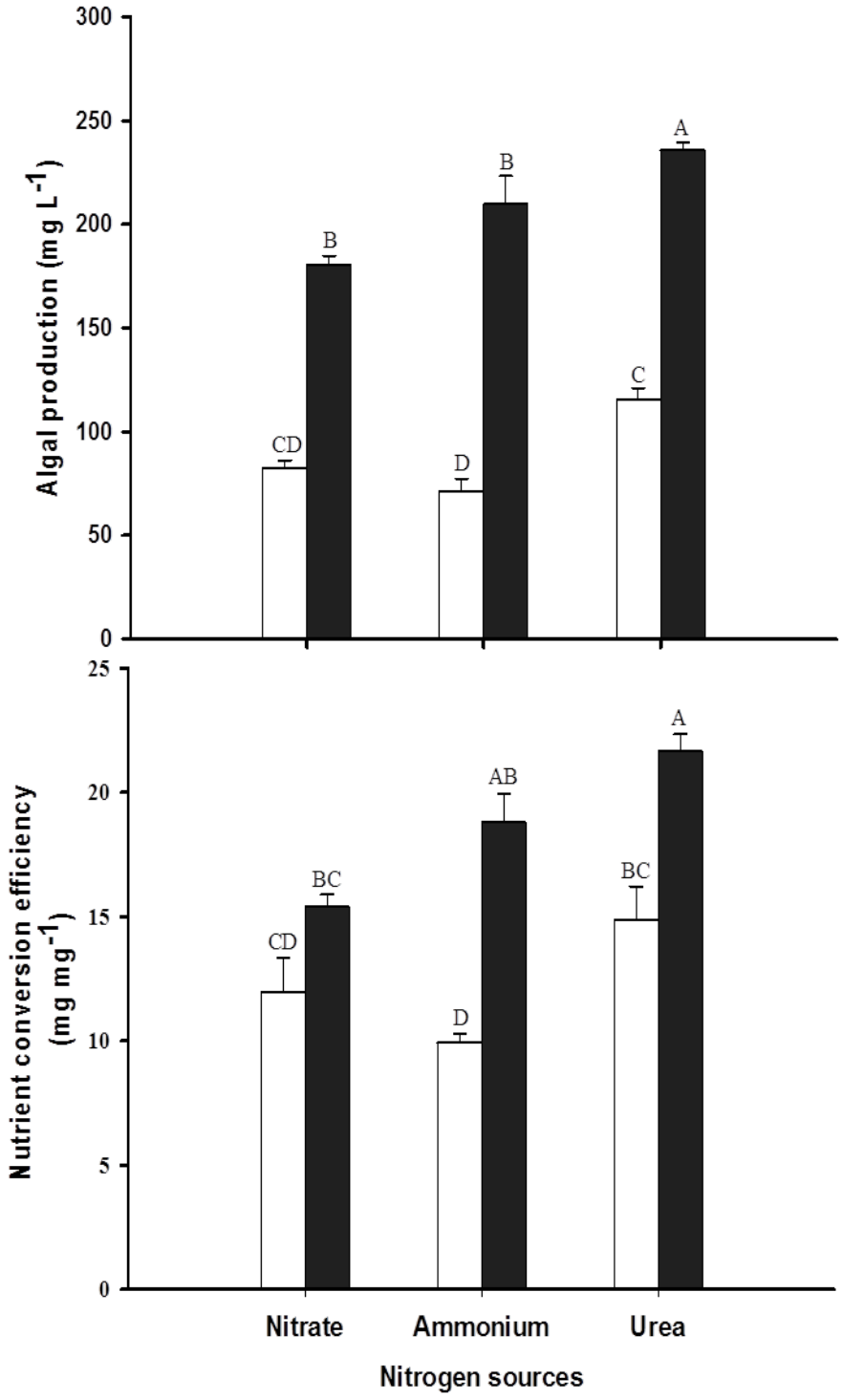

Fig. 3.4 Algal production and nutrient conversion efficiency to algal biomass in different nitrogen sources under phototrophic (white bar) and mixotrophic (dark bar) conditions (data shown as mean ± SE, *n*=3)

3.4.4 Nutrient depletion

The reduction of nitrate, ammonium and urea concentration in the culture media over time is shown in Fig. 3.5. The reduction rates of the three nitrogen sources in the mixotrophic condition were faster than that in the phototrophic condition. At the end of trial, 56 % nitrate, 58 % ammonium and 62 % urea were removed from the substrates in phototrophic culture, but 93 % nitrate, 90 % ammonium and 87 % urea were depleted from the substrates in the mixotrophic culture. Phosphorus depletion rate was also higher in the mixotrophic condition than that in the phototrophic condition.

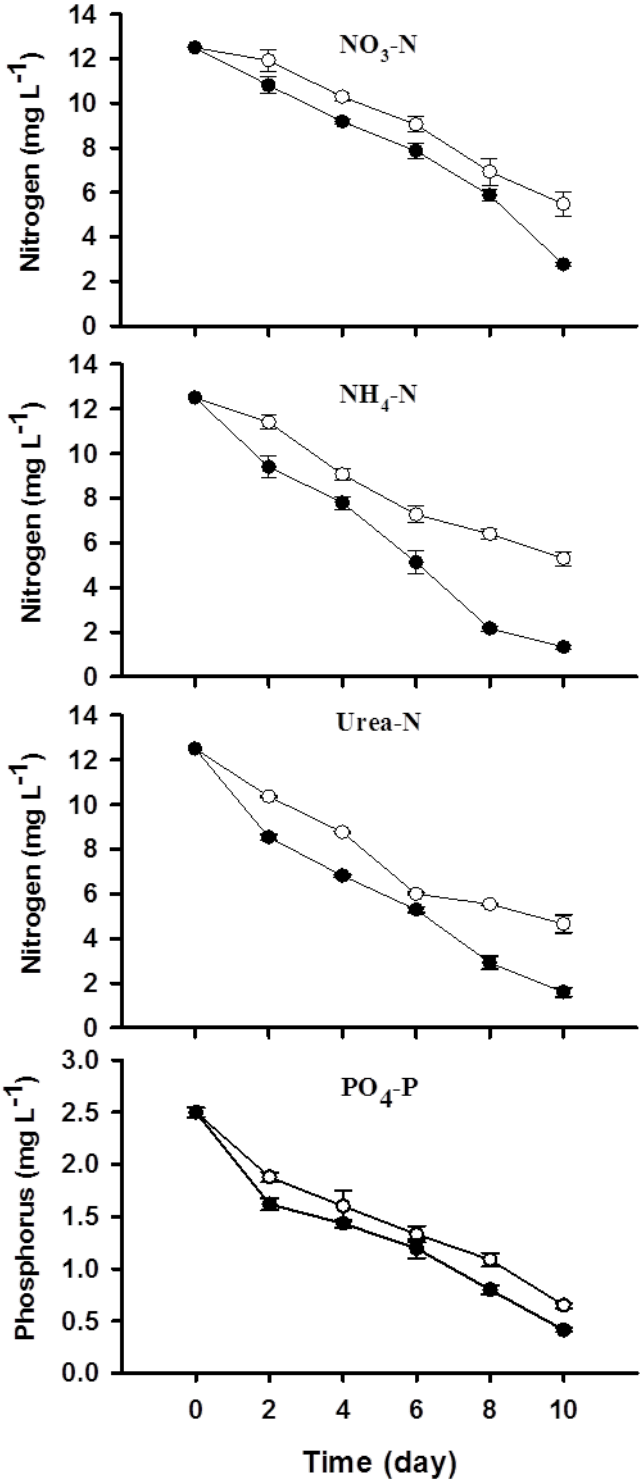

Fig. 3.5 Reduction of nitrate, ammonium, urea and phosphorus in phototrophic (white circle) and mixotrophic (black circle) conditions (data shown as mean ± SE, *n*=3)

3.5 Discussion

This study compared the N and P requirements of *I. galbana* in phototrophic and mixotrophic conditions with three N sources over a broad range of N concentrations. Algal cell abundance in all mixotrophic cultures was higher than in the phototrophic cultures regardless of N source. Although the dissolved organic matter was not measured in the original seawater, the significantly higher algal abundance in the mixotrophic medium compared with that in the phototrophic medium without organic carbon addition suggests that the added organic carbon has enhanced algal growth. Similarly, enhanced growth in mixotrophic culture compared to phototrophic culture has been reported in other marine species such as *Nannochloropsis* sp. (Xu et al. 2004), *P. tricornutum* (Cerón Garcıa et al. 2006), *Dunaliella salina* (Wan et al. 2011) and freshwater species such as *Chlorella vulgaris* (Heredia- Arroyo et al. 2011) and *Scenedesmus* sp. (Andruleviciute et al. 2013). The fast growth in mixotrophy is possibly due to the supply of both light and organic carbon as energy sources (Wang et al. 2014).

Soluble nitrogen is an essential nutrient for the growth of *I. galbana* as well as for other phytoplankton species (Grobbelaar 2004). In this study, the growth of *I. galbana* depended on N concentrations, but the algae were able to utilise nitrate, ammonium and urea as the sole of nitrogen source to support growth in both phototrophic and mixotrophic conditions. The effects of these nitrogen compounds were also studied on the phototrophic growth *I. galbana* in phototrophic conditions (Feng et al. 2011; Liu et al. 2013) and on the heterotrophic growth of *Nitzschia laevis* and *Tetraselmis suecica* where organic carbon was supplied in the substrate (Azma et al. 2011; Cao et al. 2008). In these studies, nitrate and urea were identified as the most appropriate nitrogen sources for algal growth, but ammonium was least

effective. Urea as a source of organic N plays a dual role in algal nutrition as it is metabolised into ammonia and carbon dioxide through hydrolysis. In the present study, the impacts of these three N sources on growth were comparable between phototrophic and mixotrophic conditions, but growth was N concentration dependent, except for the nitrate nitrogen. Similarly, comparable growth rates were also found in *Cyclotella cryptica* in heterotrophic culture when different N sources were used (Pahl et al. 2012).

In this study, although the growth of *I. galbana* in the mixotrophic cultures was faster than that in the phototrophic culture, the cell densities were not different between nitrate concentrations from 12.5 to 200 mg N L^{-1} in both trophic conditions. Nitrate can stimulate the growth of *Chlorella protothecoides* and *N. laevis* at a broad range of concentrations from 14 to 560 mg NO_3-N L^{-1} (Shi et al. 2000; Wen and Chen 2001). Liu et al. (2013) found that the cell density of *I. galbana* was enhanced when the culture medium was enriched with nitrate from 6.5 to 200 mg N L^{-1}. However, we found that the impact of the trophic condition on growth depended on nitrogen concentrations. The growth of *I. galbana* was enhanced more in the mixotrophic condition than in the phototrophic condition when the N concentration was < 50 mg N-NH_4 or < 100 mg N-urea L^{-1}. However, the advantage of fast growth disappeared in the mixotrophic condition when the ammonium and urea concentrations exceeded these threshold values. This phenomenon was previously observed in the heterotrophic growth of *C. cryptica* when the ammonium or urea concentrations were 25–300 mg N L^{-1}, but the growth advantage in heterotrophic conditions disappeared when the N concentration exceeded 25 mg NH_4- N or 150 mg urea-N L^{-1} (Pahl et al. 2012). The negative impact of ammonia on cell density at high concentrations is possibly due to its toxic effect on growth (Källqvist and

Svenson 2003).

The growth of *I. galbana* was affected by phosphorus, but the impact of P concentration was much less than nitrogen. In this study, *I. galbana* could grow phototrophically and mixotrophically in medium without P addition. The algal abundance was not significantly affected by P concentrations from 0 to 10.4 mg P L^{-1} in phototrophy. The P requirement in microalgae is species dependent in phototrophic culture. For instance, Yongmanitchai and Ward (1991) and Kim et al. (2012) found that *P. tricornutum* and *D. salina* showed the same growth pattern at P concentrations of 8.9–88.9 and 0.77– 12.40 mg P L^{-1}, respectively. In addition, we found that the P concentration in the range of 1.3–10.4 mg P L^{-1} had little effect on mixotrophic growth of *I. galbana*. Interestingly, when phosphate was not added to the mixotrophic culture medium, the cell density was significantly lower than that in the cultures with P additions. However, this P-dependent growth did not happen in the phototrophic culture. According to Martínez et al. (1997), *S. obliquus* could grow in a P-free medium depending on the internal reserve P content such as polyphosphate. In our study, it is likely that algae depleted P reserves faster in mixotrophy than in phototrophy, and the cell density significantly declined in the mixotrophic culture without P addition.

Although the optimal N and P concentrations for the growth of *I. galbana* were not different between phototrophic and mixotrophic conditions, algae in the mixotrophic culture utilised nutrient faster than in the phototrophic culture. The depletion rates of N and P in mixotrophy were two times faster than in phototrophy, which is similar to the growth of *Chlorella sorokiniana* where nutrients are depleted two times faster in mixotrophy than in phototrophy (Kim et al. 2013b). Moreover, we

found that the nutrient conversion efficiency to biomass production was higher in mixotrophy than in phototrophy. In other studies, the conversion efficiency of nutrients in the substrate into the biomass of *N. laevis* and *Spirulina* sp. was also increased when algae were cultured mixotrophically (Chojnacka and Zielińska 2012; Wen and Chen 2000). In this study, the maximal value of nutrient conversion efficiency and biomass production were achieved when the alga was cultured mixotrophically with urea as the N source. Urea is a source of organic nitrogen and supports fast growth either in phototrophic and mixotrophic conditions (Perez-Garcia et al. 2011). Feng et al. (2011) demonstrated that urea is a superior N source to produce maximal cell density and dry weight of *Isochrysis zhangjiangensis*. Although the required nitrogen concentration for algal growth was not different between trophic conditions in the present study, the change of N source and trophic conditions could improve growth suggesting that the mixotrophic mode is a feasible process to grow *I. galbana* with urea as the recommended N source.

In conclusion, the optimal N and P requirements for the growth of *I. galbana* was studied under phototrophic and mixotrophic conditions. Growth was enhanced in mixotrophy compared with that in phototrophy, but the growth advantage disappeared when the N concentrations exceeded 50 mg NH_4-N or 100 mg urea-N L^{-1}. The P requirements for the growth of *I. galbana* were similar between phototrophic and mixotrophic conditions. Algal production and the efficiency of nutrient conversion to biomass were enhanced when the algae were cultivated mixotrophically. This study shows that the algae grow faster mixotrophically than phototrophically, while the requirements for N and P concentrations are similar between the two trophic conditions. Urea is recommended as the N source for *I. galbana* at 12.5–50 mg urea-N L^{-1}.

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CHAPTER 4

Comparison of Pigment and Proximate Compositions of *Tisochrysis lutea* in Phototrophic and Mixotrophic Cultures

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4.1 Abstract

Pigment contents and proximate compositions are important traits to evaluate the nutritional value of microalgae. The environmental condition and nutrient availability in the culture medium are hypothesised to regulate algal colour, nutritional composition and production. This study aimed to compare the pigment and proximate compositions in *Tisochrysis lutea* under phototrophic and mixotrophic conditions in an attempt to improve algal product quality. Algae were grown in the phototrophic and mixotrophic conditions at 50 μmol photons m^{-2} s^{-1} for 12 h daily, but 50 mM glycerol was added as a carbon source in the mixotrophic culture. The pigment contents in algae significantly increased in the mixotrophic condition, and the contents of chlorophylls *a* and *c* and carotenoids increased nearly 60 % compared with those in the phototrophic condition. The contents of saturated and polyunsaturated fatty acids in algae were significantly higher while the content of monounsaturated fatty acids was significantly lower in the mixotrophic condition than those in the phototrophic condition. Although the contents of protein, lipid and carbohydrate in *T. lutea* were not different, their overall production was significantly enhanced in the mixotrophic condition compared to the phototrophic condition. This study indicates that mixotrophic culture promotes pigment and proximate production and the change of fatty acid profile in algae depends on the addition of organic carbon to the culture medium.

Keywords Protein . Lipid . Carbohydrate . Pigment . Mixotrophy . Phototrophy . Fatty acids

4.2 Introduction

Microalgae have the ability to convert light energy to chemical energy through photosynthesis and produce a variety of bioproducts for industrial applications. Microalgae can synthesise two main categories of pigments including primary pigments such as chlorophylls *a*, *b* and *c* and auxiliary pigments such as carotenoids (Spolaore et al. 2006). Pigments have multiple functions contributing to improvement of growth and health in aquatic animals in aquaculture and poultry (Becker 2004). Carotenoids are a primary source for a variety of pigment products including antioxidants and provitamin A that are immune regulators and are important to the growth and survival of animals (Domínguez et al. 2005; Ip et al. 2004). Shahidi et al. (1998) reported that fish fed a high level of carotenoids show improved immunity and resistance to bacterial and fungal infections. Carotenoids are commonly used as an additive to aquaculture feed to improve the colour of animal skin and flesh (Becker 2004; Tanoi et al. 2006). Also, carotenoids are important for human health and can potentially prevent cancer and cardiovascular diseases (Jalal et al. 2013). Algal pigments are an acceptable ingredient in healthy food and drug industries (Kumar et al. 2014). In most microalgae, chlorophylls account for 0.5–1.5% of the dry weight while the amount of carotenoids is about 0.1–0.2 % dry weight (Becker 2004). The variations of chlorophylls and carotenoids can be affected by the available nutrients and light spectra and intensity (Jalal et al. 2013; Wen and Chen 2008).

In addition, algae can accumulate protein, lipid and carbohydrate though the relative amount of these compounds varies among algal species. Algal cells usually contain high protein up to 15–52 % of dry weight when grown at the optimal condition (Muller-Feuga et al. 2003). Lipids in algae account for 4–70 % of dry

weight (Huerlimann et al. 2010; Renaud et al. 1999) and among which, polyunsaturated fatty acids (PUFA) account for 30–50 % of the total lipid (Jiang and Chen 2000). In addition, microalgae contain carbohydrates around 5–12 % of dry weight (Muller-Feuga et al. 2003).

Algal growth, pigment synthesis and nutrient composition can be regulated by environmental factors including light, temperature, pH, salinity and nutrients in the culture media (Chen and Chen 2006; Hu 2004; Pal et al. 2011). Therefore, manipulations of these factors can influence the rate of synthesis and accumulation of cellular organic compounds (Perez-Garcia et al. 2011). For instance, nitrogen deficiency and low light intensity can decrease the amount of chlorophylls but increase carotenoids in algae (Hu 2004; Jalal et al. 2013). Algae can be induced to synthesise more protein in the optimal growth condition (Das et al. 2011) while the accumulations of lipids and carbohydrates are enhanced under suboptimal conditions. Huang et al. (2013) reported that the lipid contents of *Tetraselmis subcordiformis*, *Nannochloropsis oculata* and *Pavlova viridis* significantly increased as the nitrogen supplementation decreased. However, under limited nitrogen, *Dunaliella* sp. and *Tetraselmis suecica* can change metabolic activity and increase carbohydrate synthesis instead of lipid storage (Hu 2004).

The cellular components determine the nutritional value of microalgae as food for aquaculture animals (Renaud et al. 2002). Thus, the supply of adequate microalgae with high nutrition quality is essential to the success of animal production in marine hatchery (Azma et al. 2011). The major challenge in aquaculture is the quantitative and qualitative supply of live food to feed animals (Muller-Feuga et al. 2003). Currently, the dominant protocol of algal culture still relies on the

phototrophic system, but the low algal productivity constrains the yield due to self-shading and light limitation (Wang and Peng 2014). However, the change of heterotrophic and mixotrophic growth conditions is a useful approach to boost the biomass and secondary metabolite productivity in some microalgal species (Andrade and Costa 2007; Andruleviciute et al. 2013; Cheirsilp and Torpee 2012). For instance, the content of chlorophylls and carotenoids in *Haematococcus pluvialis* and *Phaeodactylum tricornutum* in mixotrophic culture increased by 3–5 times compared with those in phototrophic culture (Cerón Garcıa et al. 2006; Orosa et al. 2001). Under a mixotrophic condition, the organic carbon substrate such as sugar and alcohol plays an important role to provide energy and carbon to enhance algal growth in a low light condition (Chen and Chen 2006; Wang and Peng 2014).

Several organic carbon substrates are supplied to the culture medium in a mixotrophic condition to increase algal cell density and production of bioproducts. Particularly, glycerol is a useful organic carbon source to increase algal growth and stimulate the accumulation of specific cellular products (Perez-Garcia et al. 2011). For instance, glycerol can stimulate the cell growth and pigment production of a diatom *P. tricornutum* (Cerón Garcıa et al. 2005) and *Chlamydomonas acidophila* (Cuaresma et al. 2010). Moreover, Lewitus et al. (1991) reported that the photosynthetic system structure of a marine cryptophyte *Pyrenomonas salina* was affected by glycerol addition as evidenced by the change of thylakoid number and the particle size in the photosynthetic system.

The lipid and fatty acid compositions of algae in mixotrophic culture differ from those in phototrophic culture. In *Chlorella vulgaris*, the lipid content in mixotrophic culture is seven times higher than that in phototrophic culture (Heredia-Arroyo et al.

2011). In *Chlorella pyrenoidosa*, the proportions of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) in mixotrophy are higher than those in phototrophy (Rai et al. 2013). However, the enhancement of lipid in algae under mixotrophy is species-specific (Li et al. 2014). Fang et al. (2004) reported that *Nannochloropsis* sp. produced more eicosapentaenoic acid (EPA, 20:5 n-3) in phototrophy while the diatom *Nitzschia laevis* produced more EPA in mixotrophy (Yan et al. 2000). In mixotrophic culture, *C. vulgaris* yielded high lipid and carbohydrate (Kong et al. 20 13), but *Chlorella sorokiniana* produced high lipid and protein (Wang et al. 2011). Clearly, the use of mixotrophic culture can change not only the biomass production but also the chemical composition. However, for some commonly used algae in aquaculture, our understanding on the impact of mixotrophy on algae cellular compositions and the nutrient quality is limited.

Tisochrysis lutea (previously known as the Tahitian strain of *Isochrysis*, T-Iso, Bendif et al. 2013) is commonly used as live food in aquaculture due to its fast growth and high content of docosahexaenoic acid (DHA, C22:6 n-3) (Liu et al. 2013). It has been reported that this species can grow in a mixotrophic condition with high growth rate and biomass production (Alkhamis and Qin 2013; Babuskin et al. 2014), but the effect of culture conditions on the synthesis and accumulation of bioproducts in *T. lutea* is unknown. This study aimed to compare the impact of phototrophic and mixotrophic conditions on photosynthetic pigments and proximate compositions in *T. lutea*. The results would illustrate the advantage of manipulating the environmental conditions to improve cellular compositions and nutritional value of microalgae for potential uses in aquaculture and other industrial applications.

4.3 Materials and methods

4.3.1 Microalgae and culture condition

The marine microalga *Tisochrysis lutea* was obtained from the Australian National Algae Culture Collection (Hobart, Tasmania). Culture medium was prepared with the f/2 medium modified with urea as the sole nitrogen source at 25 mg N L^{-1}. Cultures were carried out in 2-L Erlenmeyer flasks containing 1.8 L of seawater (35 ‰) and enriched with the f/2 medium nutrients. Flasks were sterilised in an autoclaved at 121 °C for 15 min and left to cool prior to inoculation with 10 % (v/v) of exponential cells. Flasks were assigned to two groups of nutrient treatments: (1) the mixotrophic cultures were supplemented with organic carbon, and (2) phototrophic cultures were used as the control. Pure glycerol (Merck 99 % pure- GA 010, Chem-Supply, Australia) was used as organic carbon substrate and was sterilised in an autoclave at 115 °C for 10 min and supplemented to the mixotrophic culture at 50 mM. Air flowed to the culture flask through a 0.2-μm filter to provide aeration and mixing in each flask. The growth condition for the phototrophic and mixotrophic cultures was 24 °C and 50 μmol photons m^{-2} s^{-1} of daily illumination for 12 h. Illumination was provided with white cool fluorescent lamps, and its intensity was measured at the surface of the media using the Light ProbeMeter (Extech Instruments Corp, USA).

Algae were harvested from the cultures on day 10 at the beginning of the stationary growth phase and then were centrifuged at 5000×g for 10 min. The algal pellets were washed twice with 0.5 M ammonium formate and distilled water to remove extra salt on the algal surface. Each sample was separately treated in a freeze-drier and kept in at a −20 °C freezer until analysis.

4.3.2 Pigments

Pigments were determined and expressed on a dry weight basis. Algal samples were homogenized in 90 % acetone and incubated in dark at 4 °C for 24 h. The extracted samples were centrifuged at 5000×g for 10 min. The concentrations of chlorophylls *a* and *c* and total carotenoid (mg g^{-1} DW) were determined according to the method of Parsons et al. (1984).

4.3.3 Lipid and fatty acids

Total lipids were quantified gravimetrically after solvent extraction as described by Bligh and Dyer (1959). A 100-mg sample of lyophilised algal biomass was extracted with a solvent mixture of chloroform and methanol (1:2 v/v). The lower part of the liquid layer after the extraction phase was dried under nitrogen prior to weighing. Lipids obtained from the previous step were transesterified to fatty acid methyl esters (FAME) using sulphuric acid in methanol for 3 h at 70 °C and then extracted using n-heptane. Subsequently, samples were analysed on a GC chromatograph (Hewlett-Packard 6890, USA) equipped with a 30-m capillary column (50 mm × 0.32 mm BPX-70, SGC Pty Ltd, Australia) and a flame ionisation detector. Helium was used as a carrier gas and injected at a rate of 1.5 mL min^{-1} at a split ratio of 20:1. The temperatures of injector and detector were programed at 250 and 300 °C, respectively. The oven temperature was adjusted initially at 140 °C and stepwise increased at 5 °C min^{-1} to 220 °C. To identify and quantify unknown FAME, the outputs chromatographic from GC were compared with those of commercial lipid standards (Nu-ChekPrep Inc) using the Hewlett- Packard Chemstation data system.

4.3.4 Protein

Crude protein composition was determined according to the method of Dumas (Nielsen 2010) on a rapid N Rapid N III nitrogen analyser (Elementar Americas, USA). Samples of 100-mg freeze-dried algae were combusted at 940 °C by injecting pure oxygen. The gases produced in the combustion process were reduced to N_2 and analysed with a detector. Then, the value of total nitrogen was multiplied by a factor of 6.25 to quantify the protein content.

4.3.5 Carbohydrate

The content of total carbohydrate was analysed using the colorimetric method (Dubois et al. 1956). Soluble carbohydrate was extracted with addition of 0.5 M sulphuric aids and phenol to 25 mg of freeze-dried algal samples and then incubated at 90 °C for 5 min. The absorbance of mixtures was measured at 490 nm. The carbohydrate content was quantified according to the glucose standard (Sigma Aldrich, Cat. No. G7528).

4.3.6 Statistical analysis

The study experiment was run with three replicates for each treatment, and data were analysed statistically by the SPSS software (version 20). Significant differences between means of each variable were tested by one-way ANOVA. Multiple comparisons were made with the Tukey HSD procedure in post hoc analysis when the main treatment effect was significant at $P < 0.05$.

4.4 Results

4.4.1 Pigments

The content of cellular pigments was determined at the end of the trial when the cultures reached the stationery phase. The effect of growth conditions on the

accumulation of photosynthetic pigments and accessory pigments is shown in Fig. 4.1. Chlorophylls *a* and *c* and total carotenoids were the main pigments detected, and their concentrations between the phototrophic and mixotrophic conditions were significantly different ($P < 0.05$). Algae in the mixotrophic condition supported with 50 mM glycerol contained significantly higher chlorophyll *a* (18.9 mg g^{-1}) than in the phototrophic condition (7.8 mg g^{-1}, $P < 0.05$). Similarly, algae in the mixotrophic culture showed significantly higher carotenoid (11.5 mg g^{-1}) than those in the phototrophic culture (4.8 mg g^{-1}, $P < 0.05$). However, the *T. lutea* cells produced a small amount of chlorophyll *c*, but its content was significantly enhanced in the mixotrophic condition (0.51 mg g^{-1}) compared with those in the phototrophic condition (0.30 mg g^{-1}, $P < 0.05$).

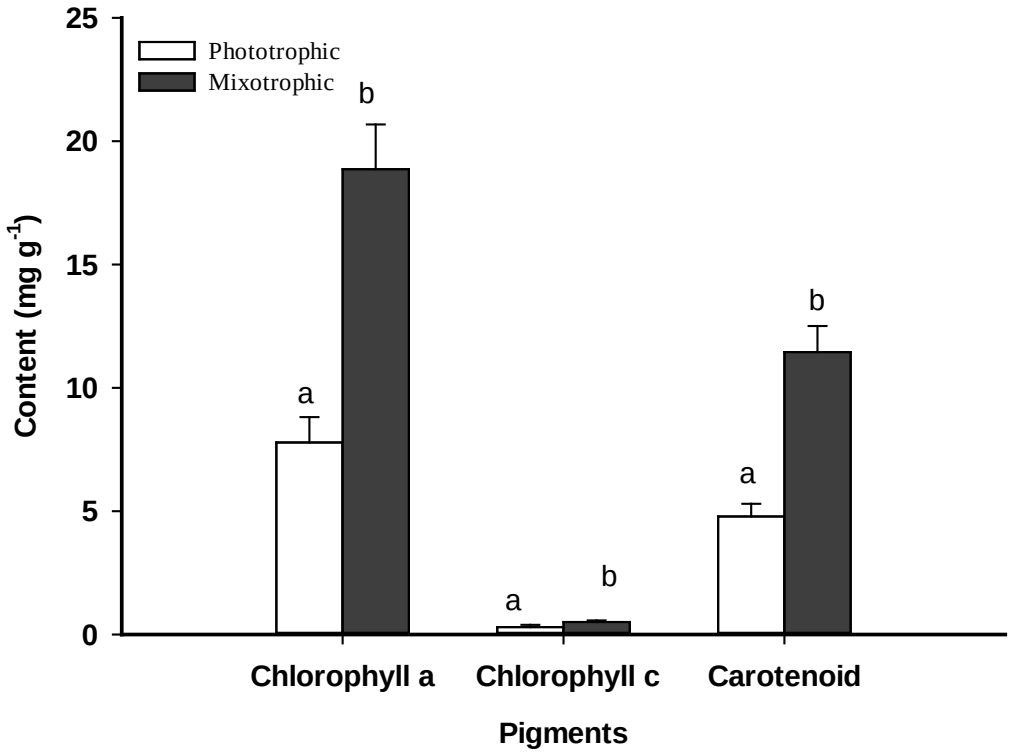

Fig. 4.1 The pigment compositions of *Tisochrysis lutea* under phototrophic and mixotrophic conditions. Data are presented as mean ± SE ($n = 3$)

4.4.2 Proximate biochemical composition

Table 4.1 shows the cellular content and production of *T. lutea* cultured in the phototrophic and mixotrophic conditions. In phototrophy, the contents of crude protein (36.4 %), lipid (22 %) and carbohydrate (9.4 %) were not significantly different ($P > 0.05$) from those of protein (41.7 %), lipid (19.3 %) and carbohydrate (7.5 %) in mixotrophy. However, the yield of protein (350 mg L^{-1}), lipid (162.1 mg L^{-1}) and carbohydrate (63 mg L^{-1}) in the mixotrophic culture were significantly higher than those (139.1 mg protein L^{-1}, 83.6 mg lipid L^{-1}, and 37.5 mg carbohydrate L^{-1}) in the phototrophic culture ($P < 0.05$). The magnitude of yield increase in mixotrophic culture was 2.5-fold in protein, 2.0-fold in lipid and 1.8-fold in carbohydrate compared with those in the phototrophic culture.

Table 4.1 Proximate content and production of *Tisochrysis lutea* under phototrophic and mixotrophic conditions.

	Content (% DW)		Production (mg DW l^{-1})	
	Phototrophy	Mixotrophy	Phototrophy	Mixotrophy
Protein	36.7 ± 1.2^{A}	41.7 ± 2.9^{A}	139.1 ± 4.4^{a}	350.0 ± 2.4^{b}
Lipids	22.0 ± 1.6^{A}	19.3 ± 1.9^{A}	83.6 ± 2.8^{a}	162.1 ± 0.9^{b}
Carbohydrate	9.4 ± 1.9^{A}	7.5 ± 1.2^{A}	35.7 ± 7.2^{a}	63.0 ± 1.8^{b}

Data are presented as mean ± SE ($n = 3$)

4.4.3 Fatty acid profiles

The fatty acid profiles of *T. lutea* grown under the phototrophic and mixotrophic conditions are presented in Fig. 4.2. The SFA (35.4 %) and PUFA (29.1 %) of algae in the mixotrophic culture were significantly higher than those (31.8 % SFA and 26.1 % PUFA) in the phototrophic culture ($P < 0.05$). On the other hand, the MUFA (32.6

%) of algae in the mixotrophic condition was significantly lower ($P < 0.05$) than that (39 %) in the phototrophic condition ($P < 0.05$).

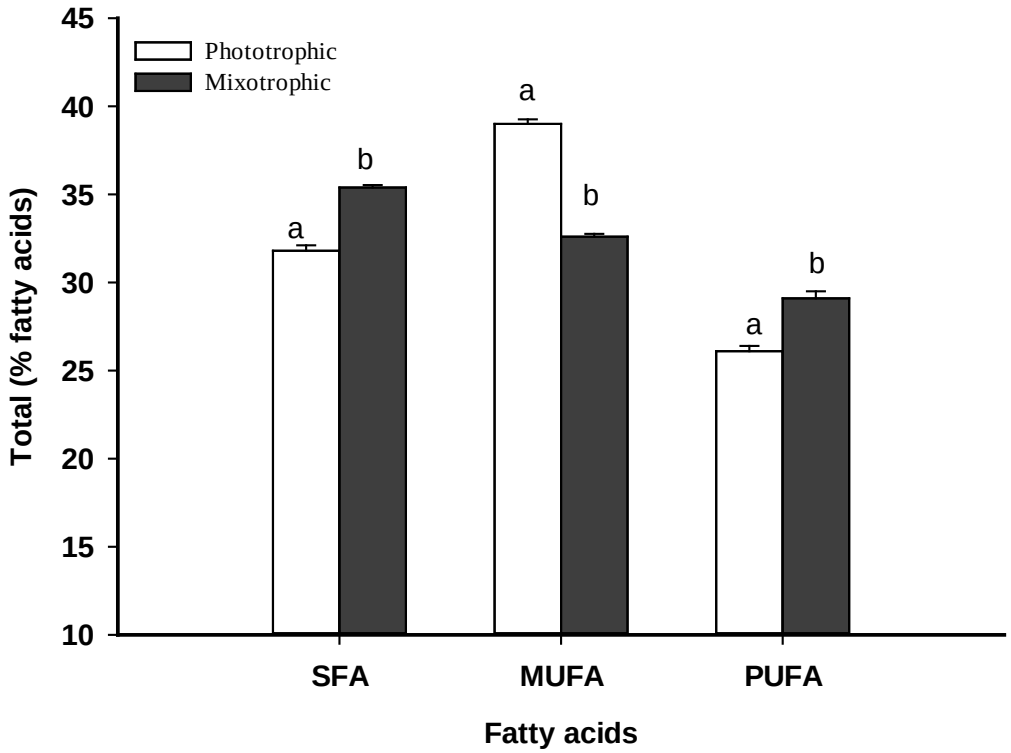

Fig. 4.2 The variation on total percentage of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids of *Tisochrysis lutea* grown under the phototrophic and mixotrophic conditions

The major fatty acid profiles under both trophic conditions are listed in Table 4.2. Saturated fatty acids mainly consisted of myristic (14:0) and palmitic acid (16:0), whereas the percentage of myristic acid in mixotrophy (24.8 %) was significantly higher ($P < 0.05$) than that in phototrophy (21.2 %). In contrast, the pentadecylic acid (15:0, 0.9 %) in the phototrophic culture was significantly lower than that (0.6 %) in the mixotrophic culture ($P < 0.05$). There were five fatty acids belonging to MUFA. The contents of palmitoleic (16:1n-7) and eicosenoic acid

(20:1) fatty acids were 6.6 and 20.4 %, respectively, in the phototrophic culture and were significantly higher than those (6.3 and 13.7 %, respectively) in the mixotrophic culture ($P < 0.05$). However, the contents of 14:1 (0.7 %) and 18:1n-9 (10 %) fatty acids of algae in the mixotrophic culture were significantly higher than those (0.5 and 9.3 %, respectively) in the phototrophic culture ($P < 0.05$).

In PUFA, linoleic acid (18:2 n-6, 6.9 %) and linolenic acid (18:3 n-3, 11.0 %) in mixotrophy were significantly higher ($P < 0.05$) than those in phototrophy (4.0 and 9.5 %, respectively). The algae in phototrophic and mixotrophic cultures had a small amount of EPA which was not significantly different between two cultures. However, DHA in the phototrophic culture (10.1 %) was significantly higher than that in the mixotrophic culture (8.6 %, $P < 0.05$). In addition, the fatty acid profile of algae in mixotrophy showed a higher content of the total n-6 PUFA than that in phototrophy ($P < 0.05$). The value of the PUFA n3/n6 ratio was lower (2.3 %) in the mixotrophic culture than that in the phototrophic culture (3.4 %).

Table 4.2 The variation on fatty acid profile of *Tisochrysis lutea* under phototrophic and mixotrophic conditions.

Fatty acids (%)	Phototrophy	Mixotrophy
SFA		
14:0	$21.2^{a} \pm 0.25$	$24.8^{b} \pm 0.08$
15:0	$0.9^{a} \pm 0.05$	$0.6^{b} \pm 0.02$
16:0	$9.2^{a} \pm 0.30$	$9.6^{a} \pm 0.06$
∑SFA	$31.8^{a} \pm 0.32$	$35.4^{b} \pm 0.13$
MUFA		
14:1	$0.5^{a} \pm 0.02$	$0.7^{b} \pm 0.01$
16:1n-7	$6.6^{a} \pm 0.13$	$6.3^{b} \pm 0.03$
18:1n-9	$9.3^{a} \pm 0.10$	$10.0^{b} \pm 0.07$
18:1n-7	$1.6^{a} \pm 0.01$	$1.5^{a} \pm 0.01$
20:1	$20.4^{a} \pm 0.19$	$13.7^{b} \pm 0.17$
∑MUFA	$39.0^{a} \pm 0.26$	$32.6^{b} \pm 0.16$
PUFA		
18:2n-6	$4.0^{a} \pm 0.06$	$6.9^{b} \pm 0.04$
18:3n-3	$9.5^{a} \pm 0.25$	$11.0^{b} \pm 0.12$
20:5 n-3 EPA	$0.5^{a} \pm 0.01$	$0.6^{a} \pm 0.01$
22:5n-6	$1.5^{a} \pm 0.05$	$1.6^{a} \pm 0.02$
22:6 n-3 DHA	$10.1^{a} \pm 0.22$	$8.6^{b} \pm 0.16$
∑PUFA	$26.1^{a} \pm 0.30$	$29.1^{b} \pm 0.40$
Sum *n*-3	$20.1^{a} \pm 0.29$	$20.2^{a} \pm 0.04$
Sum *n*-6	$6.0^{a} \pm 0.13$	$8.9^{b} \pm 0.06$
n-3/n-6	$3.4^{a} \pm 0.08$	$2.3^{b} \pm 0.01$

Data are presented as mean ± SE (n = 3)

4.5 Discussion

The composition of algal cellular products can be altered by the manipulation of growth conditions (Kong et al. 2013). The photosynthetic pigments chlorophyll *a* and *c* and carotenoids are the main pigments synthesised by *T. lutea* (Mulders et al. 2013). In the present study, the mixotrophic condition significantly stimulated the synthesis of chlorophylls *a* and *c* and total carotenoids. In contrast, Liu et al. (2009) found that the contents of chlorophyll *a* and carotenoids of *P. triconutum* in mixotrophy enriched with glycerol were reduced compared with those in phototrophy, but the content of chlorophyll *c* was not significantly different between the phototrophic and mixotrophic conditions. The impact of mixotrophic condition on the pigment composition depends on species and the source of organic carbon in the media. For example, in the mixotrophic condition with glucose as organic carbon, *Chlorella zofingiensis* (Ip et al. 2004), *Botryococcus braunii* (Wan et al. 2011) and *Nannochloropsis* sp. (Cheirsilp and Torpee 2012) contained less chlorophylls than in the phototrophic condition. However, the contents of chlorophylls and carotenoids in the blue-green alga *Arthrospira (Spirulina) platensis* were similar between phototrophic and mixotrophic conditions when glucose was the organic carbon source (Marquez et al. 1993). In the green algae *Nannochloropsis* sp. and the diatom *P. tricornutum*, the reduction of photosynthesis was concomitant with an increase in respiration and a reduction of chlorophylls and carotenoids under both heterotrophic and mixotrophic conditions using glucose and glycerol as organic carbon (Lin et al. 2007; Fang et al. 2004). However, in this study, the use of glycerol as the source of organic carbon, promoted the contents of chlorophylls *a* and *c* and total carotenoids. Similarly, *P. tricornutum* contained 2.62 % chlorophylls and 0.45 % carotenoids in mixotrophic culture with glycerol, which was 1 and 3 times higher than in

phototrophic culture, respectively (Cerón Garcıa et al. 2006). In another study, Bhatnagar et al. (2011) found that the chlorophyll *a* and *b* contents in *Chlamydomonas globosa* increased in mixotrophic culture using glycerol and acetate as organic carbon, while these pigment contents were reduced in the presence of methanol as organic carbon. However, neither glycerol nor glucose stimulated *C. vulgaris* to produce more chlorophylls *a* and *b* and carotenoids (Kong et al. 2013). Nevertheless, the use of 50 mM glycerol as organic carbon in this study stimulated the pigment production in *T. lutea*. These results indicate that organic carbon can impact the accumulation of photosynthetic pigments in algae, but the outcome depends on algal taxonomy and the type of organic carbon.

Protein, lipid and carbohydrate are the main nutritional components in microalgae, but the relative content of these constituents can be changed by manipulating the environmental conditions (Perez-Garcia et al. 2011). The chemical composition of *T. lutea* is mainly characterised by a high percentage of crude protein in the range of 30–48 % DW (Martínez- Fernández et al. 2006; Renaud et al. 2002). In this study, *T. lutea* had a high content of crude protein (36.7–41.7 %), but this was not significantly different between the phototrophic and mixotrophic cultures. Likewise, the lipid and carbohydrate contents were not significantly affected by the culture conditions. It is possible that nutrient content and algal metabolism are species-specific. In another study, the protein content increased by 11 % and carbohydrate reduced by 10 % in *C. sorokiniana* in mixotrophy compared with phototrophy (Kumar et al. 2014). In contrast, *C. vulgaris* grown mixotrophically accumulated more lipids and carbohydrate, rather than increasing protein (Kong et al. 2013). In the present study, the lipid content in *T. lutea* was not significantly different between trophic conditions. A similar result was reported by Babuskin et al.

(2014) who found that the lipid content of *T. lutea* under the mixotrophic culture (30 %) was not different from that under the phototrophic culture (32 %). It seems that the cellular content of major nutrient constituents in some algae does not easily change with the environmental conditions.

Despite the relative unchanged nutrient contents in algal cells in this study, the mixotrophic culture significantly increased the production of protein, lipid and carbohydrate compared with the phototrophic culture because the mixotrophic culture produced high algal biomass. For a commercial application, the bioproduct productivity is a more critical factor than the cellular content (Cheirsilp and Torpee 2012). From this perspective, our study demonstrates that the application of the mixotrophic growth mode can lead to high biomass and pigment production in algae.

The fatty acid profile showed that the sum of MUFA and SFA accounted for nearly 70 %, while PUFA accounted for about 25 % of the total fatty acids. These results are in accordance with the study of Custódio et al. (2014) who found that the fatty acid profile of *T. lutea* was dominant in MUFA (36.1 %) and SFA (34.7). However, the MUFA was significantly decreased while SFA and PUFA were increased under the mixotrophic condition. In a recent study, Babuskin et al. (2014) reported that in mixotrophic culture, *T. lutea* altered its fatty acid profile to increase the SFA with the decrease of MUFA and PUFA. On the other hand, the change of the growth condition in *C. pyrenoidosa* from the phototrophic to mixotrophic condition led to the increase of SFA and MUSA from 15 and 21 % to 37 and 35 %, respectively, but PUFA decreased from 57 to 24 % (Rai et al. 2013).

Polyunsaturated fatty acids are the most important component in the nutrition for most aquatic animals (Hemaiswarya et al. 2011). The use of the mixotrophic regime

can maximise the content of PUFAs in algal cells (Cerón Garcıa et al. 2006). In the present study, the total amount of PUFA was enhanced while the content of DHA was reduced in the mixotrophic culture. The decrease of the DHA in the mixotrophic condition was also observed in *T. lutea* (Babuskin et al. 2014). This study demonstrates that the fatty acid compositions in *T. lutea* can be altered by the change of trophic condition. In addition, despite the low n3/n6 ratio (2.3:1) in *T. lutea* in mixotrophy, the n3/n6 ratio of 2-5 is recommend in live food for mariculture (Lin et al. 2007; Sánchez et al. 2000), suggesting that the n3/n6 ratio of *T. lutea* in mixotrophy is suitable to feed marine animals.

As *T. lutea* is a common live feed for marine animals, this study indicates that the use of glycerol in the mixotrophic culture is a potential approach to increase pigment contents in algae, which has important implication for improving animal health and product quality in aquaculture. This present study may shed light on altering pigment composition and enhancing pigment contents in other microalgae by supplying organic carbon to the culture media. Hence, the mixotrophic algal culture is a promising tactic to increase the production of proximate nutrients and pigments in microalgae.

In conclusion, the pigment and chemical composition of *T. lutea* were assessed in phototrophic and mixotrophic cultures. In mixotrophy, the addition of glycerol to the culture medium significantly increased the content of chlorophylls and carotenoids, and also increased the overall algal biomass production. The contents of protein, lipid and carbohydrate were not significantly affected by the growth conditions, but their production was significantly increased under the mixotrophic condition. Algae in the mixotrophic condition could accumulate more saturated and polyunsaturated

fatty acids. Thus, the mixotrophic condition is a promising technology in algal culture to improve the cellular content and bioproduct yield.

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CHAPTER 5

Enhancement of Lipid and Fatty acid Production in *Isochrysis galbana* by Manipulation of Medium Nitrogen and Organic Carbon

This chapter has been submitted to Algal Research as:

Alkhamis Y, Qin JG. 2015. Enhancement of lipid and fatty acid production in *Isochrysis galbana* by manipulation of medium nitrogen and organic carbon. (under review).

5.1 Abstract

This study tests the impact of nitrogen and organic carbon on the content and production of lipid and fatty acids in *Isochrysis galbana* in mixotrophic culture. The algae were cultured in the f/2 algal medium, but urea-N was used as the source of nitrogen at four levels (0, 12.5, 25 and 50 mg L^{-1}) and each nitrogen level was tested against three levels of glycerol as organic carbon (0, 25 and 50 mM glycerol). Lipid content and production were significantly influenced by the concentration changes of both nitrogen and organic carbon. The relatively higher lipid content (>400 mg g^{-1}) and lipid production (344.9 mg L^{-1}) were obtained at 25 or 50 mg urea-N L^{-1} with 50 mM glycerol supplementation. This study indicates that manipulation of nitrogen and organic carbon is an efficient strategy to improve lipid and fatty acid production in *I. galbana*.

Keywords Organic carbon, Nitrogen, Lipids, Fatty acids, *Isochrysis galbana*, DHA

5.2 Introduction

Microalgae are an essential food for zooplankton and other invertebrate larvae in marine fish and mollusk hatcheries due to their suitability, edibility and nutrient composition to grazers. Lipid and fatty acids are the main nutritional components required for growth and survival of aquatic animals (Martínez-Fernández et al. 2006). Furthermore, polyunsaturated fatty acids (PUFA) such as eicosapentanenoic acid (EPA) and docosahexaenoic acid (DHA) are important for human health and especially beneficial to the human cardiological system (Wen and Chen 2003). Microalgae also can produce a high content of lipid that is potentially a feedstock for biofuel production (Bellou et al. 2014). As the demand for lipid and fatty acids from

algae are increasingly high, it is necessary to identify the conditions that favour the production of lipid in algae culture (Hemaiswarya et al. 2011; Wang et al. 2014; Wen and Chen 2003).

Isochrysis galbana is a species of golden algae commonly used to feed animals in aquaculture because it is rich in PUFA (Lin et al. 2007). The lipid in *I. galbana* can be potentially utilised for commercial applications as an alternative source of lipid, PUFA and DHA in particular (Liu et al. 2013; Sánchez et al. 2013). However, there is a desire to increase the content of lipid and fatty acid in algal cells to achieve commercial feasibility (Huang et al. 2013). Algal growth and the accumulation of bioproducts are regulated by nutrient availability and environmental conditions (Hu 2004). In microalgae, lipid synthesis is usually stimulated under a stress condition such as nutrient deficiency (Pal et al. 2011; Selvakumar and Umadevi 2014). Nitrogen is an important nutrient responsible for regulating algal growth and lipid biosynthesis. For instance, although microalgae grow fast at sufficient nitrogen supply, nitrogen deficiency stimulates lipid accumulation but reduces algal growth (Lv et al. 2010). Nevertheless, the reduction of nitrogen in the culture medium is not a sustainable strategy to boost lipid production because high lipid content in algal cells cannot compensate the reduction of low biomass production under nitrogen limitation in the medium (Mairet et al. 2011). In *I. galbana* culture, among various sources of nitrogen such as nitrate, ammonium and urea, the best nitrogen source for the algal growth is urea in a mixotrophic condition (Alkhamis and Qin 2014), but it is not clear on the dependent effect of organic carbon and urea on algal growth.

In a phototrophic condition, CO_2 is the main source of carbon for photosynthesis in algae to regulate cell growth and lipid synthesis (Chiu et al. 2009; Yoo et al. 2010). However, algal growth and lipid production can be limited by inorganic

carbon and light penetration when algal cells reach a high density (Das et al. 2011; Li et al. 2014; Wang et al. 2014). In order to overcome low algal production in phototrophic culture, the mixotrophic system has been used in the culture of various algal species where algae can use both inorganic and organic carbon towards biomass production (Wang et al. 2014). The use of organic carbon such as glucose and glycerol can effectively boost algal growth and lipid synthesis. For instance, glycerol stimulates the cell growth of *Chlorella pyrenoidosa* and *Haematococcus* sp. and increases biomass production of both species by over three-fold and lipid content by 30% (Andruleviciute et al. 2013; Rai et al. 2013). In our past research, glycerol supplementation enhanced the growth and biomass production of *I. galbana* but the lipid content was not significantly changed by glycerol supplementation (Alkhamis and Qin 2013).

The influence of organic carbon on algae growth depends on the availability of nitrogen because these elements are the principal constituent in the metabolic products (Pagnanelli et al. 2014). In algae, assimilation of nitrogen to amino acids is associated with the availability of carbon skeleton and energy reserve in the form ATP and NADH through carbon metabolism (Perez-Garcia et al. 2011). In mixotrophic culture, the relative amount of carbon to nitrogen can regulate lipid productivity through controlling the metabolic pathways in protein and lipid synthesis (Wen and Chen 2003). Either nitrogen or carbon limitation can impede cell growth and lipid synthesis (Picardo et al. 2013). However, in mixotrophic culture, the increase of organic carbon concentration at a given level of nitrogen can stimulate cell growth but not lipid accumulation in algal cells (Babuskin et al. 2014; Heredia-Arroyo et al. 2011). The effect of organic carbon concentration on stimulation of lipid accumulation can be related to nitrogen concentration in the

medium (Chen and Johns 1991). According to Li et al. (2015) when the C/N ratio increased to 92.7:1, the lipid content of *C. vulgaris* increased by two-fold compared with the control without organic carbon supplementation.

Despite the enhancement of organic carbon on algal lipid and fatty acids in *I. galbana* (Alkhamis and Qin 2013), little is known on the dependent effect of organic carbon and nitrogen on lipid and fatty acid synthesis in algae. Therefore, this study aims to investigate the interactive effect between organic carbon and nitrogen concentration on lipid and fatty acid production in *I. galbana*. As urea was reported as the best nitrogen source (Alkhamis and Qin 2014) and glycerol as the best source of organic carbon for the growth of *I. galbana* (Alkhamis and Qin 2013), this study further explored the optimal combination of urea and glycerol concentrations in the culture of *I. galbana* in an attempt to maximise the lipid and fatty acid production in algal culture.

5.3 Materials and methods

5.3.1 Culture condition

The marine microalga *Isochrysis galbana* was obtained from the Australian National Algae Culture Collection (Hobart, Tasmania). The algal stock was cultured in the f/2 medium (Guillard and Ryther 1962) before its inoculation into the culture flasks. To study the effect of nitrogen and organic carbon on lipid and fatty acids synthesis, the experimental design included four levels of nitrogen (0, 12.5, 25, and 50 mg urea-N L^{-1}) and three levels of organic carbon (0, 25, 50 mM glycerol) in triplicate. The f/2 formula was the basal culture medium, but urea was the sole nitrogen source and pure glycerol (Merck 99% pure-GA 010, Chem-Supply, Australia) was used as organic carbon. Cultures were carried out in 2-L Erlenmeyer

flasks containing 1.8-L seawater (35‰) enriched with the f/2 medium. Flasks were sterilised in an autoclaved at 121 °C for 15 min and left to cool to the room temperature prior to inoculation with 10% (v/v) of algal cells in the exponential growth phase. Glycerol was sterilised separately in an autoclave at 115 °C for 10 min and then added to the culture medium with designated concentrations. The air was compressed to the culture flasks through a 0.2-µm filter to provide aeration in each flask. Cultures were incubated at 24 ºC and 50 µmol m^{-2} s^{-1} of daily illumination for 12 h. Illumination was provided with white cool fluorescent lamps and its intensity was measured at the surface of the culture medium using the Light ProbeMeter™ (Extech Instruments Corp, Nashua, USA). The experiment lasted 10 days until the stationary phase was reached.

5.3.2 Determine total organic carbon and total nitrogen

As urea could release nitrogen as well as organic carbon into culture medium, the total dissolved organic carbon (TOC) and nitrogen (TN) were measured at the beginning and at the end of the experiment using the standard analytical methods (APHA 1998). Samples were injected into a catalyst packed combustion tube at a furnace temperature of 720°C, causing the decomposition of the TN in the sample into nitrogen monoxide. Then, nitrogen monoxide was taken by the carrier gas through a cooling and dehumidifying process and the final TN concentration was detected using a Shimadzu total organic carbon and total nitrogen analyser (TOC-VCSH/CSN+TNM-1, Shimadzu, Japan). TOC was measured by acidification of the sample in the sample tube and CO_2 was removed by purging with CO_2-free air and then the final concentration of TOC was also measured by the Shimadzu analyser.

5.3.3 Determine algal dry weight

Algae were harvested from the culture on day 10 at the beginning of the stationary growth phase and then were centrifuged at 5000× *g* for 10 min. The algal pellets were washed twice with 0.5 M ammonium formate and distilled water to remove extra salt on the algal surface. Each sample was separately treated in a freeze drier and kept in a -20°C freezer until analysis.

5.3.4 Lipid and fatty acids analysis

Total lipids were quantified gravimetrically after solvent extraction as described by Bligh and Dyer (1959). A sample of 100 mg of lyophilised algal biomass was extracted by the solvent mixture of chloroform and methanol (1:2 v/v). The lower part of the liquid layer after the extraction phase was dried under a nitrogen flow prior to weighing. Lipids obtained from the previous step were transesterified to fatty acid methyl esters (FAME) using sulfuric acid in methanol for 3 h at 70 °C and then extracted using n-heptane. Subsequently, samples were analysed on a GC chromatograph (Hewlett-Packard 6890, CA, USA) equipped with a 30-m capillary column (50 mm × 0.32 mm BPX-70, SGC Pty Ltd, Victoria, Australia) and a flame ionisation detector. Helium was used as a carrier gas and injected at a rate of 1.5 ml min^{-1} at a split ratio of 20:1. The temperatures of the injector and detector were programed at 250 and 300 °C, respectively. The oven temperature was adjusted initially at 140 °C and stepwise increased at 5°C min^{-1} to 220 °C. To identify and quantify of unknown FAME, the outputs chromatographic from GC were compared with those of commercial lipid standards (Nu-ChekPrep Inc) using the Hewlett-Packard Chemstation data system.

5.3.5 Statistical analysis

The results were presented as mean ± and standard error (SE). The data analysis was conducted using the SPSS software (version 20) and significant differences of means between treatments were tested by two-way ANOVA. When the main treatment effect was significant at $P < 0.05$, multiple comparisons were made with the Tukey HSD procedure in post hoc analysis.

5.4 Results

5.4.1 Nitrogen and organic carbon utilisation

The initial and final concentrations of nitrogen and carbon in the culture medium were measured as total nitrogen (TN) and total organic carbon (TOC) (Table 5.1). Algal inoculation to the experimental vessels provided a small amount of nitrogen and organic carbon into the cultures as a nutrient source particularly in the culture without provision of either nitrogen or organic carbon. Regardless of nitrogen concentrations in the treatment, the percent utilisation of TN in all treatments was high in the cultures supplied with glycerol. Algae consumed 13.6% TOC in the cultures containing 12.5 mg L^{-1} urea-N and 25 mM glycerol, and 15.6% TOC in the cultures containing 50 mg L^{-1} urea-N and 50 mM glycerol. In contrast, algae only consumed 7.5 – 11.7% TOC in the rest of nitrogen and glycerol combinations. In the absence of glycerol supplementation, algae consumed <10% TOC in the cultures with any levels of nitrogen supplementation.

Table 5.1 Nutrient consumption by *I. gabana* culture at different nitrogen and organic carbon concentrations. Total nitrogen (TN) and total organic carbon (TOC) are presented as mean ± SE (n = 3).

	N (mg urea-N L^{-1})											
	0			12.5			25			50		
C (mM glycerol)	0	25	50	0	25	50	0	25	50	0	25	50
Initial TN (mg L^{-1})	2.8 ± 0.03	3.0 ± 7.2	3.2 ± 2.1	17.1 ± 0.1	17.4 ± 0.2	17.4 ± 0.1	32.4 ± 0.2	32.2 ± 0.7	32.4 ± 0.2	64.1 ± 0.2	64.1 ± 0.9	62.1 ± 0.3
Final TN (mg L^{-1})	1.3 ± 0.1	0.2 ± 0.9	0.5 ± 3.5	4.8 ± 1.8	2.2 ± 0.1	2.3 ± 0.2	19.2 ± 0.4	10.4 ± 1.6	3.4 ± 0.9	35.2 ± 1.4	22.9 ± 1.9	23.3 ± 1.2
utilisation %	53.6	93.3	84.4	71.9	87.4	86.8	40.7	67.7	89.5	45.1	64.3	62.5
Initial TOC(mg L^{-1})	9.6 ± 0.1	1185 ± 3.2	2260± 8.0	18.5 ± 0.2	1207.5 ± 3.5	2255.8 ± 9.7	22.8 ± 0.1	1196.8 ± 7.5	2308.3 ± 3.7	32.5 ± 0.7	1170 ± 7.4	2313.5 ± 6.4
Final TOC (mg L^{-1})	8.7 ± 0.8	1145 ± 7.5	2232 ± 2.8	17.9 ± 0.3	1042.7 ± 1.3	2086 ± 3.6	20.8 ± 1.4	1015.3 ± 8.1	2038.7 ± 4.1	31.1 ± 0.2	986.7 ± 4.9	2111 ± 9.3
utilisation %	9.4	3.3	1.2	3.2	13.6	7.5	8.7	15.2	11.7	4.3	15.6	8.8

5.4.2 Impact of nitrogen and organic carbon on lipid content and production in algae

The cellular lipid content was not significantly ($P > 0.05$) affected by organic carbon alone but the interactive effect between nitrogen and organic carbon was significant on lipid content in algae (Table 5.2). Without nitrogen supplementation, the algal lipid content was significantly higher in the cultures with 0 and 25 mM glycerol than that in the culture with 50 mM glycerol ($P < 0.05$), but no significant difference was detected between the culture with 0 and 25 mM glycerol ($P > 0.05$, Fig. 5.1A). At 12.5 and 50 mg urea-N L^{-1}, the lipid contents were not significantly different regardless of the levels of glycerol concentration ($P > 0.05$). At 25 mg urea-N L^{-1}, the lipid content was similar at 0 and 25 mM glycerol but higher than at 50 mM glycerol ($P < 0.05$). In cultures without glycerol or with 25 mM glycerol, algae contained higher lipid in the medium without nitrogen addition than with nitrogen additions ($P < 0.05$), and algae contained higher lipid in the culture containing 50 mg urea-N L^{-1} than in the culture containing 12.5 mg urea-N L^{-1} ($P < 0.05$). In contrast, algae at 50 mM glycerol contained higher lipid at 25 or 50 mg urea-N L^{-1} than at 0 or 12.5 mg urea-N L^{-1} ($P < 0.05$).

Lipid production in Fig. 5.1B measured the total amount of lipid content multiplying by the total biomass at harvest. The impact of nitrogen on lipid production depended on organic carbon concentrations in the medium ($P = 0.01$, Table 5.2). The algal lipid production in the cultures without nitrogen supplementation was not different regardless of glycerol concentrations ($P > 0.05$). At 12.5 or 25 urea-N L^{-1}, the lipid production was highest at 50 mM glycerol but lowest at 0 urea-N L^{-1}. When the nitrogen concentration was at 50 urea-N L^{-1}, the

algal lipid production at 25 and 50 mM glycerol was significantly higher than in the culture without glycerol addition ($P < 0.05$). Without glycerol addition, the lipid production in algae increased from 0 to 12.5 urea-N L^{-1}, but no further increase was observed when the nitrogen concentration was beyond 25 urea-N L^{-1} ($P > 0.05$). As the glycerol concentration increased to 25 mM glycerol, the lipid production of algae significantly increased from each increment of 0, 12.5, 25 and 50 urea-N L^{-1} ($P < 0.05$). At 50 mM glycerol, the lipid production peaked at 25 mg urea-N L^{-1} ($P < 0.05$) and no further increase was observed when nitrogen reached 50 mg urea-N L^{-1} ($P > 0.05$).

Table 5.2 Summary of the ANOVA table for testing the effect of nitrogen and organic carbon concentration on lipid accumulation and production of *I. galbana*.

Source	*SS*	*DF*	*MS*	*F*	*P*
lipid content					
Nitrogen (N)	501.9	3	167.3	37.6	0.01
Carbon (C)	9.1	2	4.5	1.0	0.38
N × C	331.7	6	55.3	12.4	0.01
lipid production					
Nitrogen (N)	287743.6	3	95914.5	1009.9	0.01
Carbon (C)	176839.5	2	88419.8	930.9	0.01
N × C	73408.4	6	12234.7	128.8	0.01
SFA					
Nitrogen (N)	27119.6	3	9039.9	62.9	0.01
Carbon (C)	2395.4	2	1197.7	8.3	0.01
N × C	1557.6	6	259.6	1.8	0.14
MUFA					
Nitrogen (N)	11629.2	3	3876.4	51.1	0.01
Carbon (C)	1007.7	2	503.9	6.6	0.01
N × C	886.0	6	147.7	1.9	0.11
PUFA					
Nitrogen (N)	1062.8	3	354.3	8.7	0.01
Carbon (C)	221.4	2	110.7	2.7	0.09
N × C	1002.7	6	167.1	4.1	0.01
DHA					
Nitrogen (N)	99.5	3	33.2	10.9	0.01
Carbon (C)	83.1	2	41.6	13.7	0.01
N × C	88.4	6	14.7	4.9	0.01
DHA production					
Nitrogen (N)	730.1	3	243.4	176.3	0.01
Carbon (C)	503.5	2	251.8	182.4	0.01
N × C	187.8	6	31.3	22.7	0.01

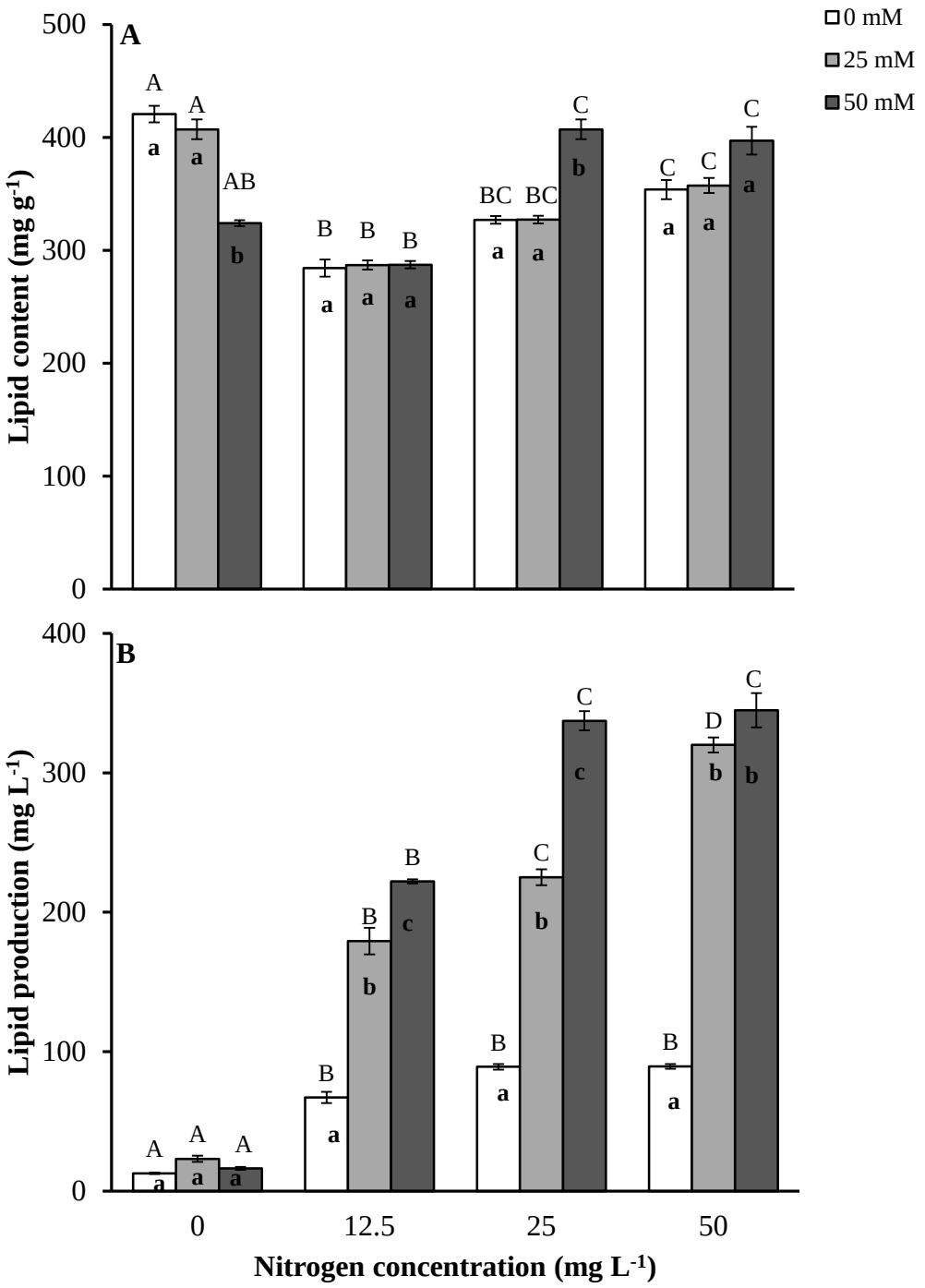

Fig. 5.1 Lipid content (A) and production (B) of *I. galbana* cultured at different combinations of nitrogen and organic carbon concentration (data shown as mean ± SE, *n* =3). Different capital letters above the bars indicate significant difference between treatments at different levels of nitrogen ($P < 0.05$). Different small letters inside the bars indicate significant difference between treatments at the same of nitrogen ($P < 0.05$)

5.4.3 Impact of nitrogen and organic carbon on fatty acid contents in algae

The compositions of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) in *I. galbana* at different nitrogen and organic carbon concentrations are shown in Fig. 5.2. The two-way ANOVA on SFA and MUFA showed no significant interaction between nitrogen and organic carbon ($P > 0.05$). The increase of nitrogen concentration reduced the SFA and MUFA contents in algae, but the supplementation of glycerol increased the SFA and MUFA contents in algae ($P < 0.05$). The impact of nitrogen on the PUFA content (Fig. 5.3C) depended on glycerol concentrations ($P < 0.05$). At 0, 12.5 and 50 mg urea-N L^{-1}, the PUFA contents were not significantly different regardless of glycerol concentrations ($P > 0.05$). However, at 25 mg urea-N L^{-1}, the PUFA at 25 mM glycerol was higher than that at 0 and 50 mM glycerol ($P < 0.05$), but no significant difference between 0 and 50 mM glycerol ($P > 0.05$). In the culture without glycerol, the PUFA was significantly higher at 50 mg urea-N L^{-1} than at other nitrogen concentrations. At 25 mM glycerol, the PUFA reached the maximal level at 25 mg urea-N L^{-1} while it was similar between 0 and 50 mg urea-N L^{-1} ($P > 0.05$). The PUFA content was not significantly different between nitrogen concentrations in cultures with 50 mM glycerol ($P > 0.05$).

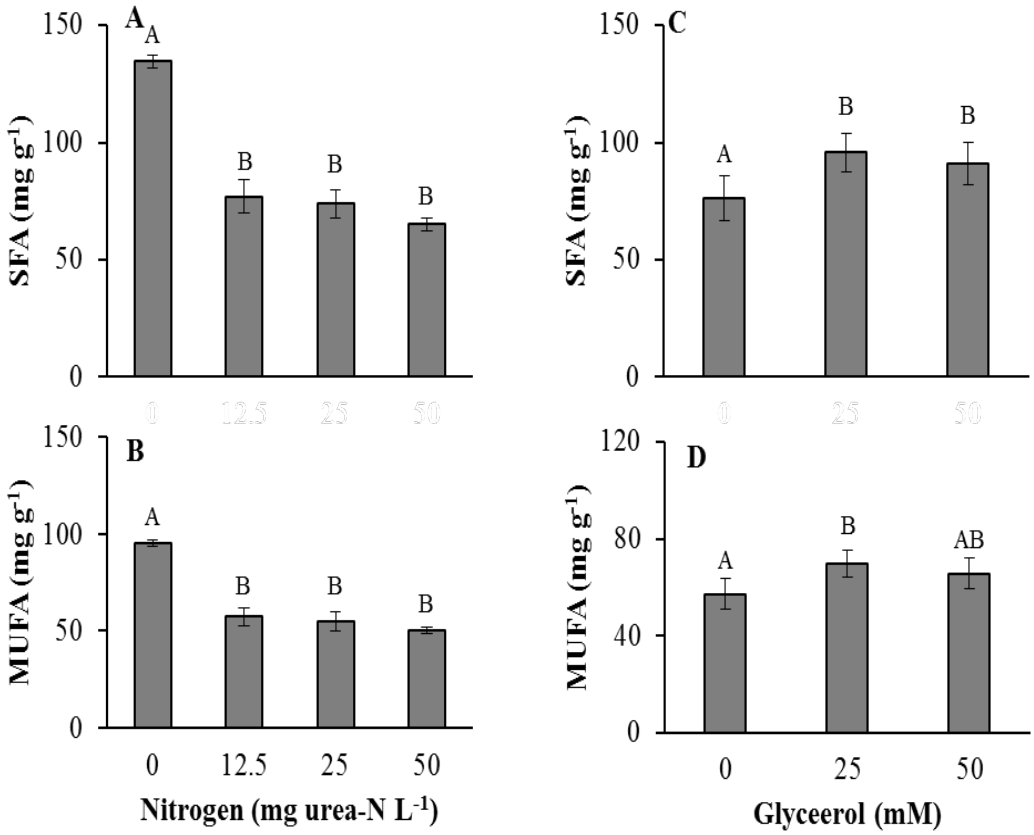

Fig. 5.2 SFA and MUFA content of *I. galbana* cultured at different nitrogen (A and B) and organic carbon (C and D) concentration (data shown as mean ± SE, $n = 3$). Different capital letters above the bars indicate significant difference between treatments at different levels of nitrogen ($P < 0.05$)

5.4.4 Impact of nitrogen and organic carbon on DHA content and production in algae

The DHA content in *I. galbana* was significantly affected by the interactive effect of nitrogen and glycerol ($P < 0.01$, Fig. 5.3A). At 0, 12.5 and 50 mg urea-N l^{-1}, the DHA content was similar among 0, 12.5 and 50 mM glycerol concentrations ($P > 0.05$), but at 25 mg urea-N L^{-1} the DHA content in algae was significantly higher at 25 or 50 mM glycerol than at 0 mM glycerol ($P < 0.05$). When the level of glycerol reached 25 and 50 mM, the DHA content in algae was higher at 25 mg urea-N L^{-1} than at other nitrogen concentrations ($P < 0.05$).

The DHA production in algae varied with nitrogen and glycerol concentrations (Fig. 5.3B). In cultures without nitrogen supplementation, no significant difference was detected regardless of glycerol concentrations ($P > 0.05$). However, at 12.5, 25 and 50 mg urea-N L^{-1}, the supplementation of glycerol at 25 or 50 mM increased DHA production ($P < 0.05$). Without glycerol supplementation, the DHA production was not significantly affected by the increase of nitrogen from 0 to 50 mg urea L^{-1} ($P > 0.05$). At 25 and 50 mM glycerol the DHA production increased when the nitrogen concentration increased from 0 to 25 mg urea-N L^{-1} ($P < 0.05$), but no further significant increase was observed when the nitrogen concentration was beyond 25 mg urea-N L^{-1} ($P > 0.05$).

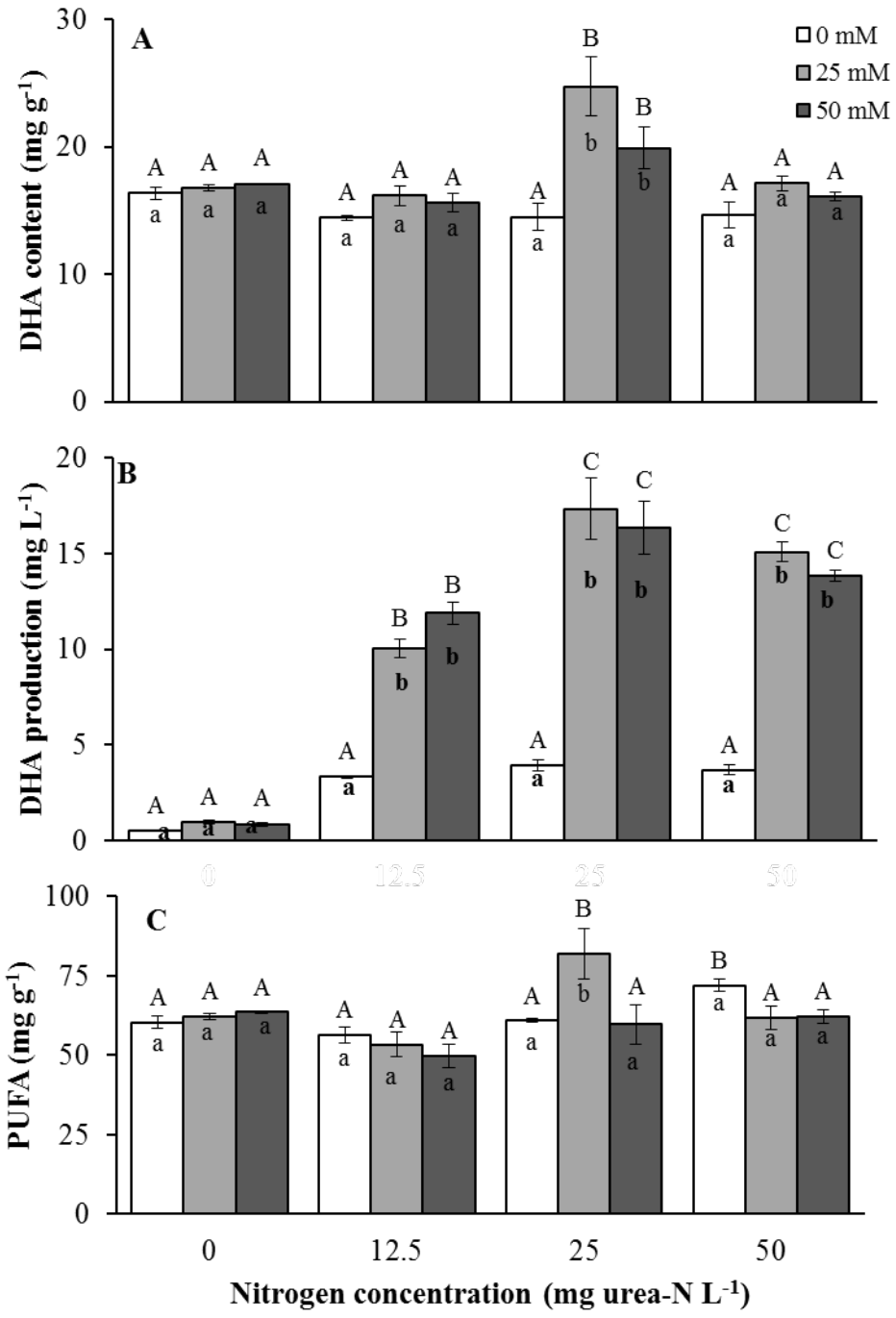

Fig. 5.3 The DHA content (A) and production (B), and the PUFA content (C) of *I. galbana* culture at different combinations of nitrogen and organic carbon concentration (data shown as mean ± SE, $n = 3$). Different capital letters above the bars indicate significant difference between treatments at different levels of nitrogen ($P < 0.05$). Different small letters inside the bars indicate significant difference between treatments at the same of nitrogen ($P < 0.05$)

5.5 Discussion

Nitrogen and organic carbon are important constitutes of lipids and their availability in culture medium may regulate the efficiency of lipid productivity in algae (Babuskin et al. 2014; Liu et al. 2013). Supplementation of organic carbon such as glycerol and glucose to the culture of *I. galbana* can enhance algal cell growth, but it did not influence lipid content in algae (Alkhamis and Qin 2015; Babuskin et al. 2014). In the present study, the nitrogen and organic carbon concentrations in the culture medium concurrently influenced lipid accumulation in *I. galbana*. Microalgae under an unfavorable condition especially nitrogen deficiency accumulate more lipid as energy storage (Huang et al. 2013; Pal et al. 2011). However, the remarkable increase of algal lipid content in this study was found both in the treatment without nitrogen addition but supplemented with 0 or 25 mM glycerol and in the treatment of high nitrogen supply (25 and 50 mg urea-N L^{-1}) supplemented with organic carbon at 50 mM glycerol. In contrast, at moderate nitrogen concentrations (12.5 and 25 urea-N L^{-1}) the algal lipid content was not dependent on glycerol supplementation from 0 to 50 mM. This finding is in accordance with the result of Estévez-Landazábal et al. (2013) who found that the regulation of algal lipid content depended on both glycerol and nitrogen concentrations and the high lipid content occurred in the combination of 250 mg nitrate-N L^{-1} and 100 mM glycerol in *Chlorella vulgaris*. In our study, the lipid content reached 400-420 mg g^{-1}, which is the highest content ever reported in *I. galbana*, and the maximal lipid content in this species is 220 - 320 mg g^{-1} in other studies (Babuskin et al. 2014; Brown et al. 1998; Martínez-Fernández et al. 2006). In the present study, when *I. glabana* produced the maximum amount of lipid in the best nitrogen and carbon combination (i.e., 50 mM glycerol and 25-50 urea-N L^{-1}),

algae consumed 9-12% of TOC in the presence of glycerol, but consumed 63-90% of nitrogen in the culture medium. Similarly, Li et al. (2015) reported that lipid accumulation in *C. vulgaris* increased in the condition when the culture medium became nitrogen deficiency and excess organic carbon (glucose) was available in the medium.

In the present study, although the high lipid content was found in algae without nitrogen supplementation, lipid production was low. When nitrogen increased from 12.5, 25 to 50 mg urea-N L^{-1}, lipid production significantly increased with the increase of glycerol from 0 to 50 mM. Similarly, in another study, the maximum lipid production occurred when nitrogen increased from 70 to 250 mg nitrate-N L^{-1}, and glycerol increased from 10 to 100 mM in *C. vulgaris* (Estévez-Landazábal et al. 2013). In order to achieve a satisfactory lipid production, algae should contain a high level of lipid content and maintain a high growth rate to ensure a high lipid yield (Lv et al. 2010). In the present study, the highest lipid production (344.9 mg L^{-1}) was achieved in the culture condition of 25-50 mg urea-N L^{-1} and glycerol at 50 mM glycerol. In previous studies, organic carbon in the culture medium can enhance algal lipid production but did not change the lipid content in algae (Babuskin et al. 2014; Cheirsilp and Torpee 2012; Heredia-Arroyo et al. 2011). However, the present study indicates that organic carbon substrates as glycerol can enhance both lipid content and lipid production in algae with sufficient urea available in the environment.

The study on the impact of nutrients on the change of fatty acid compositions in algae is rarely reported (Alkhamis and Qin 2015). The present study shows that SFA and MUFA contents were affected by the level of organic carbon in the medium but the content of PUFA in algae was concurrently regulated by both organic carbon and nitrogen in the medium. In other studies, the SFA and MUFA contents, but not

PUFA, in *Nannochloris* sp. and *Chlorella pyrenoidosa* could be increased by manipulation of organic carbon concentrations (Andruleviciute et al. 2013; Rai et al. 2013). However, the present study demonstrates that maximal PUFA (81.9 mg g^{-1}) can be achieved through adjusting nitrogen to 25 mg urea-N L^{-1} and glycerol to 25 mM in a mixotrophic condition, which is much higher than what reported in *I. galbana* (57.5 mg g^{-1}) by Martínez-Fernández et al. (2006) under a phototrophic condition.

As an major species of PUFA in algae, DHA is an important component being attractive in commercial applications such as aquaculture and pharmaceutical industry (Liu et al. 2013) and *I. galbana* is a potential source of DHA production in microalgae though its low productivity hinders further expansion at a commercial scale (Lin et al. 2007; Poisson and Ergan 2001). In the present study, the manipulation of organic carbon and nitrogen concentrations under a mixotrophic condition influenced DHA synthesis and its proportion to the other fatty acid species in algae. The highest DHA content in *I. galbana* reached 24.8 mg g^{-1} (10.2% of total fatty acids) under the culture condition of 25 mg urea-N L^{-1} and 25 mM glycerol. By comparison, the highest DHA content ever reported in *I. galbana* 17 mg g^{-1} of algae (or 17.5 % total fatty acids) was found under a phototrophic condition in a bubble column bioreactor (Liu et al. 2013). Babuskin et al. (2014) reported that organic carbon reduces the DHA content in *I. galbana* but still enhances the DHA yield due to high biomass production. Similarly, the present study demonstrates that the increase of nitrogen and organic carbon concentrations in the medium did not proportionally increase DHA content in algae, but increased the overall DHA production. Under a condition of 25 mg urea-N L^{-1} with 25 or 50 mM glycerol, the DHA production in algae reached maximum (ca.17.3 mg L^{-1}). Therefore, the

manipulation of organic carbon and nitrogen in the culture medium can improve not only DHA composition in *I. galbana* but also the DHA yield.

In summary, manipulation of organic carbon and nitrogen concentrations in the culture medium significantly influenced lipid content, lipid production and fatty acid composition in *I. galbana*. The culture with 25-50 mg urea-N L^{-1} with 50 mM glycerol is the optimal condition for lipid production in *I. galbana*. The impact of nitrogen on the composition of SFA and MUFA is independent of organic carbon, but the composition of PUFA is significantly affected by the interactive effect of nitrogen and carbon. The optimal balance for satisfactory DHA content and production were determined at 25 mg urea-N L^{-1} and 25 mM glycerol. This study suggests that manipulation of glycerol and urea concentrations in the culture medium is essential to achieve a satisfactory lipid and fatty acid production.

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CHAPTER 6

General Discussion, Conclusions and Future Research

6.1 General discussion

Microalgae are an essential source of feed for some marine animals and also serve as food supplements for human health (Spolaore et al. 2006). The cellular products in algae are useful materials for many applications in nutritional and pharmaceutical industries. In addition, microalgae have a potential of becoming a feedstock for biofuel production to supply renewable energy (Huang et al. 2010; Spolaore et al. 2006). The majority of algal biomass is produced in phototrophic systems and for this reason biotechnological application is facing a challenge due to low light penetration to the algal culture system (Azma et al. 2011). To become competitive and economically viable, microalgae production systems therefore need improvement to meet the requirement of industrial application. There has been intensive research in an attempt to remove the technical bottleneck limiting algal production by using heterotrophic and mixotrophic algal culture systems. However, there is no comprehensive research to identify the requirements for marine microalgae to grow under mixotrophic and heterotrophic conditions. This thesis addresses the characteristics of algal growth in different trophic systems, environmental and nutrient requirements for optimal algal growth, conditions to increase the production of pigments in algae, and the interactive effect of organic carbon and nitrogen on cellular bioproducts in mixotrophic culture. As a common species of algae in aquaculture, *Isoochrysis galbana* was used as a model species to explore the optimal conditions for the production of algal biomass and bioproducts including pigments, fatty acids, lipid and protein in phototraphic, heterotrophic and

mixotrophic systems.

6.1.1 Identification of the optimal culture system and environmental conditions for algal growth

The ability of microalgae to grow in heterotrophic and mixotrophic conditions varies among algal species (Azma et al. 2011; Chen and Chen 2006). For a given species of algae, the heterotrophic growth potential is regulated by the supply of organic carbon and environmental conditions such as light and salinity (Rym et al. 2010; Wen and Chen 2001). Chapter 2 firstly examined the influence of organic carbon substrates on cell growth and biomass production of *I. galbana* in phototrophic, heterotrophic and mixotrophic systems. In this study, the heterotrophic condition was not appropriate for *I. galbana* as algal growth was inhibited in all cultures with organic carbon in dark. Similarly, *I. galbanna* failed to grow in heterotrophic conditions in other studies regardless of the source of organic carbon (Gladue and Maxey 1994; Vazhappilly and Chen 1998). Interestingly, *I. galbana* showed the highest growth rate in mixotrophic culture and algal dry weight was 2.1 times higher than that in the phototrophic condition when glycerol was supplied as organic carbon. This is in an agreement with Wood et al. (1999) who found that some species of marine microalgae grew faster in the media supplied with glycerol than with glucose or acetate. Likewise, the production of *Phaeodactylum tricornutum* and *Nannochloropsis* sp. in mixotrophy were also higher than that in phototrophy (Das et al. 2011; Liu et al. 2009). This result implies that glycerol is the only source of organic carbon that can efficiently promote the growth of *I. galbana* in the mixotrophic condition. Furthermore, this study indicates that the concentration of glycerol is crucial for the mixotrophic growth of *I. galbana*. The growth of *I. galbana* increased exponentially from 0 to 50 mM glycerol, but a

reduction in algal growth was observed when glycerol was over 50 mM, indicating that algal growth is impeded by extremely high glycerol concentrations. In another study, the growth of *P. tricornutum* in mixotrophic culture was enhanced by the increase of glycerol concentration but algal growth was inhibited when the glycerol content exceeded 100 mM (Cerón García et al. 2006). Thus, this result suggests that the level of glycerol concentration is critical to achieve a high growth rate of *I. galbana*.

This study revealed that light intensity also affected the growth rate and biomass production of *I. galbana* in mixotrophic culture. In the range of light intensities tested (25–200 μmol m^{-2} s^{-1}), the maximum biomass production was achieved at 100 μmol m^{-2} s^{-1}, suggesting that a low level of light intensity is required to obtain even higher algal production. In another study, Sloth et al. (2006) found that the growth of *Galdieria sulphuraria* in mixotrophy increased as the light intensity increased from 65 to 128 μmol m^{-2} s^{-1} while the highest growth rate occurred at 100 μmol m^{-2} s^{-1}. According to other studies (Cid et al. 1992; Vonshak et al. 2000), microalgae under mixotrophic conditions usually require low light but can tolerate high light before photoinhibition occurs. However, the light inhibitory effect in the present study was not observed in the phototrophic culture but occurred in the mixotrophic culture at 200 µmol photon m^{-2} s^{-1}. In another study, no light inhibitory effect was observed when *I. galbana* grew at 400 µmol m^{-2} s^{-1} in phototrophy (Tzovenis et al. 2003). This phenomenon suggests that high light illumination should be avoided in mixotrophic culture.

Besides the requirement of light intensity in mixotrophic culture, photoperiods represent the requirement of light duration that algae can receive (Wahidin et al. 2013). In the present study, the effect of photoperiod on algal growth was more

pronounced in mixotrophic culture than in phototrophic culture. The maximum growth of *I. galbana* occurred in the photoperiod of 12 h light: 12 h dark in mixotrophic culture while the algal growth rate reduced when the light period was less than 12 h. Tzovenis et al. (2003) reported that the growth of *I. galbana* under a discontinuous light regime was faster than that under a continuous light supply. Therefore, the photoperiod of 12 h light to 12 h dark cycle is recommended in the mixotrophic culture of *I. galbana*.

Salinity is another important factor that has strong impact on algal growth and metabolite production (Wen and Chen 2003). Therefore, this study explored the optimal salinity for growing *I. galbanna*. We found that *I. galbana* could tolerate salinity variation from 10 to 65‰, though algal growth decreased when salinity was either above or below 35‰ in mixotrophy. In contrast, Das et al. (2011) found that the biomass yield of *Nannochloropsis* sp. in phototrophy decreased by 15% when salinity was 50‰ whereas the biomass yield was not different between 35 and 50‰ salinities in mixotrophy. Our study suggests that *I. galbana* can grow well in the presence of organic carbon in mixotrophy regardless of salinity, which is a valuable trait for algal culture in a situation where elevated salinity may occur when evaporation is high.

6.1.2 Nitrogen and phosphorus requirements

Nitrogen and phosphorus are essential elements for algal growth but their requirements in algae may greatly differ between phototrophic and mixotrophic conditions (Fang et al. 2004; Liu et al. 2011; Wen and Chen 2000). This study tested the nitrogen and phosphorus requirements of *I. galbana* under a mixotrophic condition in an attempt to optimise the nutrient supply for algal growth. In Chapter 3, algal growth rates and nutrient conversion efficiency were compared between

phototrophic and mixotrophic conditions. In this study, the impacts of the three nitrogen sources (nitrate, ammonium and urea) at different concentrations (0-200 mg N L^{-1}) on algal growth were compared between phototrophic and mixotrophic conditions. The three nitrogen sources all sustained algal growth in both phototrophic and mixotrophic conditions but the mixotrophic culture produced more algal cells and higher biomass production than the other two cultures. The level of optimal nitrogen concentration varied with nitrogen sources. Nitrate supported algal growth at all concentrations tested, but the fast growing advantage of algae disappeared in the mixotrophic culture when the nitrogen concentration exceeded 50 mg ammonium-N L^{-1} or 100 mg urea-N L^{-1}. This similar growth pattern was also found in *Cyclotella cryptica* when different nitrogen sources were used in a mixotrophic culture (Pahl et al. 2012).

The present study revealed that the requirement of phosphorus was not different between phototrophic and mixotrophic conditions. In contrast, Qu et al. (2008) found that *Chlorella pyrenoidosa* required less phosphorus in a heterotrophic condition than in a phototrophic condition. Based on the result of this study, *I. galbana* showed fast nitrogen depletion in mixotrophy than in phototrophy and algae can effectively convert nitrogen and phosphorus from the media to algal cells in mixotrophic conditions. Similarly, the conversion efficiency of nutrients from the substrate into the biomass of *Nitzschia laevis* and *Spirulina* sp. was higher when algae were cultured in a mixotrophic condition than in a phototrohic condition (Chojnacka and Zielińska 2012; Wen and Chen 2000). Thus, although the requirements of nitrogen and phosphorus were not different between phototrophy and mixotrophy, the mixotrophic culture is more effective for biomass production in *I. galbana*.

6.1.3 Variation of algal cellular contents in mixotrophic culture

Manipulations of nutrient concentrations and environmental factors can influence the rate of synthesis and accumulation of cellular organic compounds in algae (Perez-Garcia et al. 2011) but may reduce algal growth (Hu 2004). The change from a phototrophic to mixotrophic conditions is a useful approach to vary the biomass and metabolite composition in some microalgal species (Andrade and Costa 2007; Andruleviciute et al. 2013; Cheirsilp and Torpee 2012). However, because the response of metabolite synthesis to nutrient supply varies among algal species, the optimal growth condition for the production of a given set of cellular compounds in algae should be established on a species-by-species basis. In Chapter 4, the impact of growth conditions on accumulation of pigments and cellular compounds was examined. Previous studies indicate that the presence of glucose and glycerol as organic carbon in a mixotrophic condition can suppress the synthesis of photosynthetic pigments (Cheirsilp and Torpee 2012; Ip et al. 2004; Liu et al. 2009). However, in the present study, the pigments content in algae significantly increased up to 60% in the mixotrophic condition compared with those in the phototrophic condition. The proximate composition of protein, lipid and carbohydrate in algae were not different between phototrophic and mixotrophic conditions but the overall production of proximate compounds was significantly enhanced in the mixotrophic condition compared to the phototrophic condition. This result is consistent with the findings in previous studies by Babuskin et al. (2014), and Cheirsilp and Torpee (2012) who demonstrated that the synthesis of bioproducts in *I. galbana* and *Nannochloropsis* sp. was not affected by the change of growing conditions but the production of bioproducts was higher in the mixotrophic culture than in the phototrophic culture because the mixotrophic culture produced more algal biomass.

This study suggests that mixotrophic culture is better than phototrophic culture as the former promotes more pigment and proximate production.

6.1.4 Identification of optimal N and C combinations for the production of lipids and fatty acids

As lipids and fatty acids are important constituents in algae (Spolaore et al. 2006), there is a desire to improve their content and production in algal culture. Based on the result of Chapter 4, the manipulation of organic carbon in mixotrophic culture could improve the overall production of lipids and fatty acids but the cellular contents were relatively unchanged. In a commercial operation, satisfactory productivity of algal cellular products relies on the combination of high biomass production and high cellular contents of desirable bioproducts (Lv et al. 2010). Nitrogen is an important nutrient for regulating algal growth and lipid synthesis. The use of organic carbon such as glucose and glycerol can also effectively boost algal growth and lipid synthesis. Therefore, to achieve a high algal growth rate and lipid content in mixotrophy requires identification of the balance between organic carbon and nitrogen in the culture medium.

Chapter 5 studied the interactive effect of nitrogen and organic carbon on lipid and fatty acid accumulation and production in *I. galbana.* In this chapter, *I. galbana* cultured in the medim without nitrogen supplementation yileled high lipid content but low lipid production due to low produciton of algal biomass. Similarly, in previous studies, nitrogen deficiency stimulated lipid accumulation but reduced algal growth (Lv et al. 2010; Pal et al. 2011; Selvakumar and Umadevi 2014). However, in the present study, the impact of nitrogen concentration on lipid content and lipid production depended on the concentration of organic carbon. The high lipid content and production occurred in the algae grown in 25 and 50 mg urea-N L^{-1}

supplemented with organic carbon at 50 mM glycerol. This finding is in accordance with the result of Estévez-Landazábal et al. (2013) who found that lipid accmulation and production in *Chlorella vulgaris* depended on both glycerol and nitrogen concentrations, and high lipid content and lipid production occurred in the combination of 250 mg nitrate-N L^{-1} and 100 mM glycerol.

This study has also demonstrated that among fatty acid classes in algae, only PUFA was regulated by both organic carbon and nitrogen in the medium and the maximal PUFA (81.9 mg g^{-1}) content was obtained in the combination of 25 mg urea-N L^{-1} and glycerol to 25 mM. In other studies, the SFA and MUFA contents, but not PUFA in *Nannochloris* sp. and *Chlorella pyrenoidosa* were increased by manipulating organic carbon concentrations (Andruleviciute et al. 2013; Rai et al. 2013). In another study, organic carbon reduced DHA content in *I. galbana* but still enhanced the overall DHA yield due to high biomass production (Babuskin et al. 2014). The present study indicates that the manipulation of organic carbon and nitrogen concentrations can influence DHA content and its percentage to other fatty acids in algae. The highest DHA content in *I. galbana* reached 24.8 mg g^{-1} (10.2% of total fatty acids) under the culture condition of 25 mg urea-N L^{-1} and 25 mM glycerol, which is about 1.4 times higher than that in other nitrogen and glycerol combinations (0, 12.5 and 50 mg urea-N L^{-1} with 0 and 50 mM glycerol).

6.2 Conclusions and recommendations

To improve the efficiency of marine microalgae culture and enhance biomass and bioproduct production, this thesis investigated the feasibility of culturing algae in heterotrophic and mixotrophic conditions. The following conclusions can be drawn for improvement algal culture.

1. The growth of *I. galbana* was inhibited in the heterotrophic condition but enhanced in the mixotrophic condition. The growth of algae in mixotrophic culture is faster than in phototrophic culture. The recommended condition for *I. galbanna* growth is using glycerol as the source of organic carbon at 50 mM. The environmental conditions should be set at 35‰ salinity with a light intensity of 100 photons μmol m^{-2} s^{-1} and a photoperiod of 12 h light and 12 h dark.

2. The phosphorus and nitrogen forms and concentrations play a significant role in algal growth. The conversion efficiency of nutrients from media to algal biomass is affected by the growth condition. It is recommended that for optimal growth and nutrient conversion efficiency, the algal culture medium be supplied with urea as a source of nitrogen at 50 mg urea-N L^{-1} and phosphorus at 1.3 mg PO_4-P L^{-1}.

3. The cellular composition of algae is a crucial measure of algal quality in commercial application and can be altered by nutrient manipulation in the medium. The pigment content and metabolite production in *I. galbana* in mixotrophic culture were higher than in phototrophic culture. Therefore, it is recommended that the efficient production of pigments (e.g., chlorophyll *a* and *c* and carotenoids) and the production of proximate compounds (e.g., protein, carbohydrate and lipid) in *I. galbana* can be achieved under the mixotrophic condition with 50 mM glycerol as organic carbon.

4. Nitrogen and organic carbon can concurrently regulate algal cell growth and lipid accumulation. The high lipid content occurs in nitrogen deficiency but the high algal cell growth rate occurs in the medium with organic carbon supplementation. Therefore, it is recommended that sufficient lipid and fatty acid production be offered in the combination of nitrogen at 50 mg urea-N L^{-1} and organic carbon at 50 mM glycerol.

6.3 Future research

The outcomes of this research provide a solid foundation to improve growth and production of cultivated marine microalgae and contribute new knowledge to our understanding on algal growth in mixotrophic systems. Nevertheless, some questions are still outstanding and future research should be focused on the following issues:

1. Although glycerol as a source of organic carbon can stimulate algal growth, achieving the maximal level of algal growth and production is still a challenge. As the mixotrophic condition depends on the supply of inorganic and organic carbon, it is not clear the interactive effect of inorganic carbon and organic carbon on algal growth and nutrient assimilation in a mixotrophic condition. Due to facility limitation and workload overflow, the current study did not test the impact of inorganic carbon source on *I. galbana* in mixotrophic condition. This is an important issue because we need to understand the balance between photosynthesis and respiration to achieve a higher growth rate and biomass production. The further research in this area will improve production efficiency by providing appropriate amount of inorganic carbon to reach the potential of algal growth in a mixotrophic situation.

2. Microalgae produce a variety of pigments and valuable metabolite products that are possibly applicable to pharmaceutical application and functional food for human. To be used in a commercial application, it is important to increase volumetric production of cellular bioproducts. Therefore, it is worth further investigating the impact of organic carbon substrate on algal cellular products and accumulation of bioactive materials at a large scale.

3. The use of organic carbon substrate can increase the risk of bacterial contamination. Due to working at a small scale in a laboratory condition, contamination did not occur in this study. At a large scale, the environmental condition is difficult to control and algal culture may be susceptible to heterotroph invasion. Future research should be focused on understanding the growth dynamics of heterotrophic organisms and their abundance control when organic carbon is used in the substrate in large-sale facilities.

4. Efficient production of lipids and fatty acids may also depend on phosphorus availability. Future research should identify the optimal combination of nitrogen, phosphorus and carbon for algal growth in mixotrophic conditions. As identification of the requirement of multiple nutrients is a complex process, it is recommended to use a surface response methodology to optimise the multiple nutrient requirements in algal culture.

6.4 References

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