The influence of plant and animal hormones on growth and accumulation of pigments and fatty acids in the microalgae *Scenedesmus quadricauda* (CPCC-158) and the duckweed *Lemna minor* (CPCC-490).

by

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Abstract

The production of biofuels and high commercial value biochemical commodities from microalgae may be considered “third generation biofuel”. One of the promising ways to improve the feasibility of third generation of biofuel and bioproduct economy is the integration of biomass production on the basis of maximum energy and nutrient recycling. Integration of the biomass production also offers significant reduction of environmental emissions due to the energy- and nutrient-recycling strategies. Interest in the use of wastewaters of different origins for the industrial production of microalgae biomass for biofuels and bioproducts has stimulated the studies on the influence of a variety of compounds in wastewaters on microalgae physiology. In this study the influence of four phytohormones (brassinolide, BL; 24-epi-brassinolide, EBL; 3-indole acetic acid, IAA, and abscisic acid, ABB) singularly and in some combinations, and two animal sex hormones (17β-estradiol, E2 and 17,20β-dihydroxy-4-pregnen-3-one, 17,20β-P), on the physiology of *Scenedesmus quadricauda* was tested. The influence of 17β-estradiol on physiology of *S. quadricauda* and the Duckweed *Lemna minor* grown in three different media (Bolt Basal Medium, natural Fishery Wastewater, and Reconstituted Fishery Wastewater) was also investigated. All the hormones tested had positive stimulatory effects on the growth and biosynthetic activity of *S. quadricauda*. Phytohormones stimulated algae growth, and biosynthesis of chlorophyll-a, carotenoids, and lipids when the co-influence of the solvent (DMSO) was taken in account. Among the phytohormones the most powerful inducers of growth, chlorophyll-a, and carotenoid biosynthesis were EBL and IAA, while the phytohormone effect on fatty acids (FAs) biosynthesis followed the order of: ABA ≈ IAA > EBL > BL. Both the quantities of FAs and their profiles depended on the phytohormone type and the specific concentrations tested. FAs profiles also were dependent on the time of harvesting. Synergistic increases in *S. quadricauda* biomass production, chlorophyll-a accumulation, total carotenoid accumulation, as well as total and major neutral lipid (triacylglyceride) accumulation were

observed when EBL and IAA were assessed in combined-hormones trials. The stimulatory effect was maximum - 1.7-, 2.7-, and 3.3-fold greater for chlorophyll-a, total carotenoids, and fatty acids, respectively - compared with single hormone treatments. Both animal steroids tested, E2 and 17,20β-P, demonstrated positive stimulatory effects on *S. quadricauda* cell growth, biomass production, chlorophyll-a, carotenoid, and lipid biosynthesis. E2 was a more effective promoter of all the parameters tested compared to 17,20β-P with one exception: chlorophyll-a biosynthesis was more affected by 17,20β-P. Moreover, the lag phase of algal growth was clearly shorter in the E2 trial resulting in enhancement of both, biomass yield and the rate of biomass production. Both animal steroids stimulated significant increases in the bioaccumulation of major, minor, and total FAs. However, the lowest 17,20β-P concentrations of 1 and 5 ng/L, did not increase total FAs accumulation. A comparison of E2 performance in three types of growth media (BBM, FWW and RFWW) revealed clear differences in the ability of E2 to induce growth and biosynthesis of both *S. quadricauda* and *L. minor*. The most effective E2 concentrations were also evidently different for the algae and the plant when they were grown in the same type of medium. Our results highlight the need for further investigations of the relationships between physicochemical parameters of growth media, the particular organism to be cultured, and any additional stressor or chemical inducer that may be applied to stimulate biomass production and biosynthesis of the molecules of interest. Our results also highlight the possibility that hormones could be used as a tool for algal biosynthesis and, particularly, for fatty acids profile manipulation in the biofuel, pharmaceutical and cosmetic industries.

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Abbreviations

ABA	Abscisic Acid
ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
ARA	Arachidonic Acid
ATP	Adenosine Triphosphate
BBM	Bold's Basal Medium
BDP	Corresponded percent of biodegradation
BFA	Branched-chain fatty acids
BL	Brassinolide
BOD	Biological Oxygen Demand
BRs	Brassinosteroids
CAIA	Canadian Aquaculture Industry Agency
CBEA	Canadian BioEnergy Association
CCM	An Active CO2-concentrating Mechanism
CI	Combination Index
CK	Cytokinin
CMAF	Canadian Model Aquaculture Farm
COD	Chemical Oxygen Demand
CPCC	Canadian Phycological Culture Centre
CRFA	Canadian Renewable Fuels Association
DEFRA	Department of Energy, Food and Rural Affairs, UK
DFO	Department of Fisheries and Oceans

DHA	Docosahexaenoic Acid
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DOE	Department of Energy, USA
dcw	dry cell weight
E2	17β-estradiol
EBL	24-epibrassinolide
EC	Environmental Canada
EC50	50% Effective Concentration
EERE	Energy Efficiency & Renewable Energy
EIA IEO	Energy Information Administration, International Energy Outlook
EPA	Eicosapentaenoic Acid
ETBE	Ethyl Tertiary Butyl Ether
FAs	Fatty Asids
FAMEs	Fatty Acid Methyl Esters
FWW	Fishery Waste Water
GA	Gibberellic Acid or Gibberellin
GLA	γ-linolenic Acid
HPC	Heterotrophic Plate Counts
IAA	3-indoleacetic acid
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IEA	International Energy Agency
LCEA	Life Cycle Energy Assessment

LCI	Limiting Carbon dioxide Inducible gene
LED	Light Emitting Diode
LOEC	Lowest Effective Concentration
Mbp	Millions of Base Pairs
MCAL	Manitoba Chemical Analysis Laboratory
MFC	Microbial Fuel Cell
MSDS	Material Safety Data Sheet
NFE	Nitrogen Free Extract
NOEC	Not Effective Concentration
NSERC	Natural Science and Engineering Research Council, Canada
OECD	Organization for Economic Co-operation and Development, USA
PAR	Photosynthetically Active Radiation
PHA	Polihydroxyalkanoates
PhBR	Photobioreactor
PSA	Photosynthetic Apparatus
PUFA	Polyunsaturated Fatty Acids
QA	Quality Assurance
QC	Quality Control
RFWW	Reconstituted Fishery Wastewater
RNA	Ribonucleic Acid
RSM	Response Surface Methodology
RuBisCO	Ribulose-1,5-bisphosphate Carboxylase/Oxygenase
SA	Salicylic acid

SD	Standard Deviation
SeMet	L-selenomethionine
SGR	Specific Growth Rate
TAG	Triacylglyceride
TC	Total Carotenoids
TDM	Total Dry Matter
TDP	Total Dissolved Phosphorus
TDS	Total Dissolved Solids
TSS	Total Suspended Solids
w/w	weight/weight,
wwt	wet mass weight
UTEX	University of Texas
UV	Ultra Violet radiation

Chapter 1

Literature Review and Project Objectives

1.0 Preface

This review will focus on the most important challenges and the newest achievements in the field of industrial production of microalgal biomass for renewable biofuels, valuable bioproducts for food and animal feed. It will introduce an approach biomass production that integrates microalgae production with aquaponics in a single industrial unit that maximizes energy- and nutrient recycling, and gives an opportunity of using naturally occurring phyto- and fish hormones in microalgae production units.

1.1 Introduction

Without any doubt, the expansion of the global population to over 7 billion will increase the demand for food, fuel, and energy. Together with significant increases in atmospheric carbon dioxide (CO_2) concentrations and the resulting global, greenhouse gas-mediated climate change, our species now faces one of the greatest challenges to our survival (Parry, 2007). Petroleum, which is partially derived from ancient algae deposits, is a limited resource that will eventually run out or become too expensive to recover (Schindler and Zitel, 2008; EIA IEO, 2009). The production of agricultural biomass for the pharmaceutical and animal feed industries is suffering because the growing demand for biomass has resulted in significantly increased costs. Survival of our civilization will depend on innovative technologies that can provide sustainable and renewable sources of non-crop related biomass for biofuels, pharmaceuticals, and animal feed.

First-generation biofuels (bioethanol and biodiesel from grain and oil seeds, respectively) have proven to be marginally viable economically, due to large fluctuations in commodity prices. Additionally, the use of food/feed crop sources of fuels has resulted in a fierce global “food/feed versus fuel” debate (DEFTA, 2008). Second-generation (lignocellulosic) biofuels, based on decomposition of lignocellulosic biomass to sugar monomers, is not dependent on food crop production, but this technology is not yet

competitive economically with either petroleum-based fuels or even with first generation biofuels.

Moreover, one of the many lessons learned from first and second-generation biofuel production is that production of biofuels alone is still not economically viable (Benemann, 2010). Fuels are high-volume, low-value commodities. For viable industrial-scale production, biofuels must be produced in large amounts with low production costs, and sold at prices that are competitive with petroleum-derived fuels. The production of high-value, low volume co-products is essential for the economic success of companies producing biomass-based fuels.

The disadvantages of crop-related and lignocellulosic biofuel production stimulated the search for alternative biomass for biofuel and other bioproducts. The employment of microalgae for production of biofuels and bioproducts is one of the most promising alternative sources of biomass. Biofuel production from microalgae may be considered as "third generation biofuels".

Microalgae are among the most diverse organisms on our planet. Beside high growth rates, microalgae can synthesize an astonishing variety of molecules that have great economic value. The list of the commercially used molecules includes anti-oxidants (such as astaxanthin), vitamins, proteins, and oils (such as omega-3 fatty acids) that can be used for the production of cosmetics, pharmaceuticals, or biofuels (Olaizola, 2000; Adarme-Vega, 2012). Not all the lipids synthesized by microalgae cells are useful for biofuel production. The primary substrates for production of biodiesel are neutral lipids, and particularly triglycerides (TAGs). TAG biosynthesis is species specific and greatly depends on growth conditions. The remainder of the biomass could have other uses, including other energy products such as methanol from gasification of microalgal biomass residues, or ethanol from fermentation of the carbohydrates. Alternatively, proteins can be extracted from the residual microalgal biomass, or it may be used as animal feed. Other lipids also have potential applications as fuel feed-stocks, but would require additional treatments and/or something other than simple trans-esterification as a part of the production pathway. Investigation of TAG biosynthesis and accumulation in the microalgae under various stress

conditions could be regarded as a topical problem.

Another very promising source of biomass for biofuels and valuable co-products is Duckweed, (Family: Lemnaceae), an aquatic plant with the highest growth rate of all vascular plants (Landolt and Kandeler, 1987). Duckweed produces large amounts of molecules of commercial interest (Xu et al, 2011). The most valuable bio-products that Duckweed can produce starch (40-70 % of cell dry weight, dcw) for bioethanol or biobutanol production (Bergmann et al., 2000), proteins (20-45 % of dcw) for poultry and livestock feed (Haustein et al., 1990), and lipids (3-8 % of dcw) with high percent of omega-3 fatty acids (Davis and Arnold, 2000). Importantly, the Duckweed production does not require great investment. Beside the low-costs of indoor biomass production, these approaches also provide recycling of external and internal industrial emissions, enabling concomitant reduction in atmospheric CO_2, waste-heat, and remediation of wastewaters. The residual of microalgae and plant biomass or whole plant biomass (Duckweed) may be used fresh or in a form of pellets as fish feed or feed-supplement ensuring local, fresh, enriched with proteins and carotenoids source of feed.

Despite the intensive research efforts and remarkable amount of academic, government, and industrial institutions involved in biofuels research in recent decades, the industrial-scale production of algae and Duckweed, especially in cold climates, still faces a number of serious challenges. Canada is one of the world leaders in research and commercial innovations in the lignocellulosic-biofuels industry (Logen Ink, Enerkem Inc.; Nexerra Systems Corp., etc.), yet industrial production of microalgal biomass remains underdeveloped. Thus, the development of new technologies and novel approaches for scale-up production of microalgae in cold climate conditions is vital to the economic sustainability of Canada, and leadership in this industry.

One of the promising ways to improve the economic feasibility of third-generation biofuels and bioproducts is integration of the biomass production into one industrial unit on a basis of maximum energy- and nutrient recycling, supported by modeling work and Life Cycle Analyses. Integration of the

biomass production also offers significant reduction of environmental emissions due to the improved energy- and nutrient-recycling system, and due to the use of industrial flue-gas CO_2 and wastewater as the sources of nutrients.

The use of different types of wastewater and industrial CO_2 for microalgae biomass production has been studied in recent decades. For example, a cost-effective method for the improved wastewater application to grow microalgae has been shown to enable the scale-up production of microalgae in Canada's cold climate (McGinn et al., 2012). The use of wastewaters in closed indoor reactors makes it easier to monitor nutrient loading and contaminations.

Beside the use of wastewater, factors that can enhance the production of single-cell oils (TAGs), starch, and other valuable biomolecules by microalgae include, but are not restricted to, altered ambient temperature, nutrients depletion, and light spectrum. A relatively novel approach of improving microalgae biomass production, and/or stimulating neutral lipid accumulation that has gained attention in recent decades is the use of plant and/or animal hormones. Swine hormones in agricultural wastewater have been shown to have a positive effect on algae growth and lipid yield (Zhong et al., 2013). Phytohormones are known to enhance plant growth, organs differentiation and stress tolerance (Osborne and McManus, 2005; Peleg and Blumwald, 2011; Davies, 2013; Vidhyasekaran, 2015). Microalgae also contain essentially all known phytohormones (Stirk and Van Staden, 1996; Kiseleva et al., 2012; Romanenko et al., 2015), but not much is studied about their effects on algal physio logy and stress tolerance.

In recent decades the attention of numerous investigators has been focused on the feasibility of the industrial-scale of microalgae production. This question is highly imperious in a view of indoor biomass production in cold climates. Current knowledge on the positive influence of wastewaters and hormones on microalgae metabolism supports an entirely novel technology that integrates aquaponics, and microalgal biomass productions, where the algae, plants, and fish share space, nutrients, and light, in one industrial unit. Only the combined efforts of science (investigation), industry (implementation), and

government (legislation) can enable the feasibility of industrial establishment of third generation of biofuels and expedite commercialization of microalgae and Duckweed production under Canadian conditions, positively impacting both the economy and the environment.

This review is focused on the most important challenges and the newest achievements in the field of industrial biomass production for renewable biofuels, valuable bioproducts, and food and animal feed summarizing third generation of the production.

1.2 Industrial biomass production for biofuels and bioproducts

1.2.1 From first- to next-generation biofuels

The biofuel industry, as a part of the overall bio-economy, has been recognized since the 1970s when the Organization for Economic Co-operation and Development (OECD) International Energy Agency (IEA) established the IEA-Bioenergy Organization. To address to the suitability of different types of feedstock and their conversion processes for biofuels production, the term of "drop-in biofuels" was introduced. Biologically synthesized fatty acids suitable for use as a power source can be classified as: "green gasoline" (C4 to C6 molecules), biojet fuels (C8 to C12 molecules), and biodiesel equivalents (C14 to C18 molecules). However, the development of "drop-in" fuels based on the carbon chains of fatty acids derived from TAGs (regardless the feedstock used for production), presents an additional technical challenge. Petroleum-derived fuels like gasoline, aviation fuels, and diesel, consist of complex mixtures of alkanes, alkenes, and cyclic aromatic compounds with a range of carbon lengths that are similar to, but not identical with bio-synthesized fatty acid molecules. Moreover, unsaturated fatty acid methyl esters (biodiesel) derived from C16/C18 TAGs have cloud-points (the temperature at which precipitation begins to occur) that are unsuitable for cold temperatures, especially those that occur in northern countries in winter, and at high altitudes. These major challenges can be overcome by subjecting the C16/C18 fatty acids to hydrothermal cracking. Other technological resolutions include: the conversion of bioethanol to ethyl tertiary butyl ether (ETBE) which can be blended with petroleum-derived gasoline (Malca and

Freire, 2006; Petrus and Noordermeer, 2006); genetic engineering of microalgae cells by the cloning and expression of bacterial genes responsible for biosynthesis of branched fatty acids (BFAs) which have physical and chemical properties that are desirable for fuel molecules (Machida and Suzuki, 2017). Developming of other technologies, which offer production of more seutable for biofuels microalgal TAGs are in high demand.

Microalgae biomass as a feedstock for third-generation biofuels has some profound advantages compared to first- and second-generation feedstocks (Figure 1). Microalgae biomass is not-food-related and does not require requires high amount of pesticides and fertilizers and agricultural land as it does of first-generation feedstock.

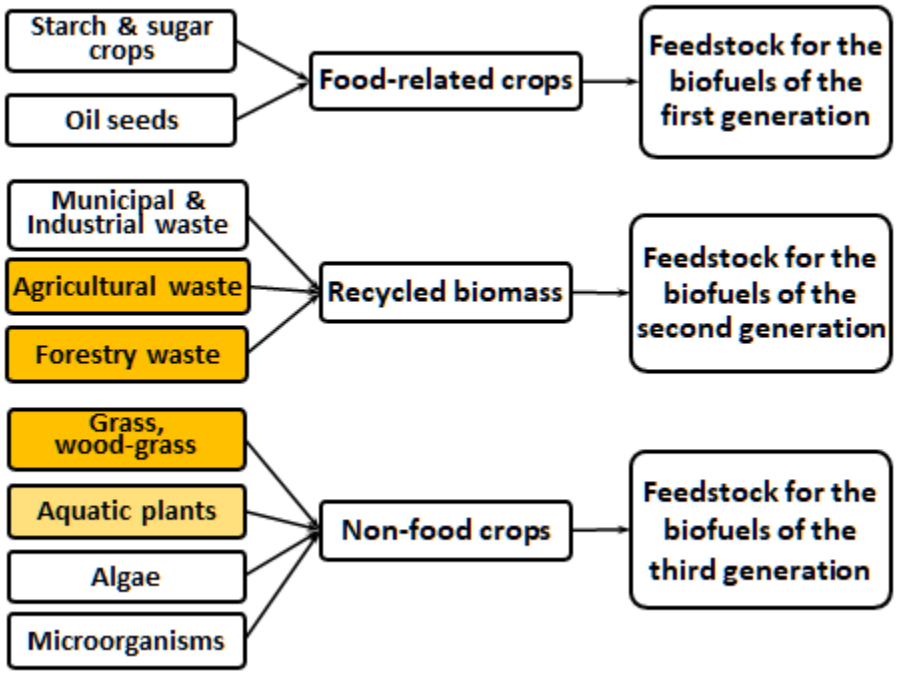


Figure 1. Classification of biomass with the consideration of the biofuel generations. The lignocellulose related biomass is in orange color and the biomass with low % of lignocellulose is in light-orange (designed by Kozlova T.A.)

Third-generation biofuels from microalgae and other microorganisms has some advantages over second-generation biofuels. Microalgal biomass does not require extensive and expensive pre-treatment

consisting of chemical, thermo-chemical, or physico-chemical processing followed by enzymatic hydrolysis as the second=generation feedstock does The biomass production cycles of microalgae is much shorter compared to woody biomass feedstocks which take several years to reach harvesting density (Chin, 2009, Moon et al., 2010). In fact, microalgae and aquatic plants, such as Duckweed and water hyacinth, have high rates of production, and require low inputs (Figure 1). Microalgae are single cell photosynthetic microorganisms that can use light and CO_2 for growth (An et al., 2003; James et al., 2011). Some microalgae can grow in the dark (Perez-Garcia et al., 2011) in the presence of organic carbon sources such as sugars, like glucose, or organic acids, like acetate (Bibeau, 2009). Many microalgae can have acceptable for the industrial scale specific growth rates and biomass productivities (up to 8.5 g/L/day dcw), with high oil (neutral lipid = TAGs) loading (up to 85% of dcw) (Table 1) that enable them to provide yields of biofuel production up to 9000 gallons per acre per year in open pond systems (Naik et al., 2010; Jarvis et al., 2013). The yield of biofuels could be increased, theoretically, to about 270,000 gallons per acre per year (empirically calculated, see Table 2). To achieve this value, new technologies have to be developed to enhance both biomass production and biosynthesis of valuable molecules. , Microalgae can synthesize an astonishing variety of molecules that have a great economic value, including anti-oxidants such as astaxanthin and β-carotene, vitamins, proteins, and oils (e.g., omega-3 fatty acid) that can be used for the production of cosmetics, pharmaceuticals or biofuels (Olaizola, 2000; Adarme-Vega, 2012). Moreover, microalgae have high CO_2 fixation rates and can grow on waste streams taking their abundant nutrient as food. Thus, microalgae industrial production can remediate environmental impact of CO_2 emission and wastewater effluents.

Table 1. Biomass and oil production on current commercially cultivated algae species (maximum known values)

Species	Biomass, (mg/L/day) *	SGR, (h) **	Total TAGs (% dcw) ***	Substrate	References
Chlorella sorokiniana (pyrenoidosa)	355	1.7	85	wastewater	*(***)Nigam et al., 2011 **Makarevičienė et al., 2011
Chlorella vulgaris	860	1.5	32	--	*Widjaja et al., 2009 ***Metting, 1996
Scenedesmus quadricauda	1260	0.8-1.2	56-80	CO_2	*Goswami, 2011 **Makarevičienė et al., 2011 ***Li et al., 2011
Scenedesmus obliquues	8000	1.9	55	swine wastewater	*Kim et al., 2010
Nannochloropsis sp	800	0.7	68	wastewater	***Metting, 1996
Botryococcus braunii	346	0.56	75	wastewater	*Sydney et al., 2011 **Orpez et al., 2009 ***Metting, 1996
Spirulina (Arthrospira platensis)	3100	0.26	20	--	*(**)Leema et al., 2010 ***Yilmaz, 2012
Dunaliella salina	2300	0.88	55	CO_2	**Spolaore et al., 2006 *** Tornabene et al., 1980

Note: SGR, specific growth rate; dcw, dry cell weight

Table 2. Calculations of oil yields for different biomass production system, with percent of intracellular oil.

Outdoor production				
Oil Yield (gal/acre/year)	**Biomass Production, (g/L/day)**	**Oil Content, (%)**	**Species**	**References**
1,500**	~0.1	30	*Chlorella sp*	Sapphire Inc., Cellana LLC
2,000**	~0.1	60	*Chlorella sorokiniana*	Wan et al., 2011
5,000	0.38	30	*Chlorella sp*	empirical
10,000	0.38	60	*Chlorella vulgaris*	empirical
Indoor production				
2,770	0.1	60	*Scenedesmus sp.*	McGinn et al., 2012
4,550(5,000*)	0.35 (0.36)	30(32)	*Scenedesmus sp.* *Nannochloropsis sp*	Rodolfi et al., 2009
10,000*	0.36	60	*Nannochloropsis sp*	Rodolfi et al., 2009
104,085	8	30	*Botryococcus braunii*	An et al., 2003
277,380	8	80	*Chlorella pirenoidosa*	~max empirical

Note: *Numbers are calculated from the data of Rodolfi et al., 2009. **Oil yield that has been proven in outdoor industrial-scale growth facilities; Note that some data have no indication of how they were measured (as wet weight or dry mass). This bias can significantly alter the calculations.

The practical efforts of the recent decades in bringing microalgae production to the industrial scale demonstrated that production of algae biomass for biofuels only could not reach its acceptable cost efficacy. It is vital for the industrial sector to achieve enhanced yield of high-value biomolecules from the same biomass which was grown for biofuels production (Briens and Piskorz, 2008; Benemann, 2010). Carotenoids are among the most valuable products derived from microalgae. They have broad applications in the production of food, animal feeds, dietary supplements and pharmaceuticals. Particularly, the greatly beneficial and protective role of the non-photosynthetic carotenoid astaxanthin has been recognized for human and animals health and its industrial production is a high priority internationally.

Another fast growing feedstock for biofuels, bio-products and animal feed as such Duckweed biomass has been gained more attention in recent decades. Duckweed has been farmed as a feed source for animals including farmed fish for years (Leng et al., 1995; Hanexakowski et al., 1995). Beside high concentrations of starch, protein and lipid, Duckweed is also capable of producing high levels of dextrose, a form of glucose (10 % per gram of biomass), which can be easily converted to biofuel (Barett et al., 2012; Cui and Cheng, 2014; Yu et al, 2014).

1.2.2 Industrial-scale microalgae biomass production

General goals of commercialization of microalgae production could be described as finding way(s) of 1) increasing of biomass and valuable molecules yield, while 2) decreasing the cost of production. Enhanced yield depends on optimization of growth conditions, improving the species/strains used for production, and improving bioreactors, harvesting, and conversion technologies. Integration of biomass production based on maximum recycling of water and industrial wastes such as flue-gas CO_2, wastewaters, and industrial heat greatly decreases the cost of production. Each of these strategies of reducing the cost has multifunctional role in biomass production, which will be discussed further.

1.2.2.1 Microalgal biofuels

The use of microalgae for biofuel production has been a high priority by governments, scientific and engineering societies, and industrial stockholders of many countries around the globe, including Canada. However, there are some substantial obstacles on the way of bringing the microalgae biofuel production to industrial level. The problems can be classified as technological (insufficient quantity and quality of microalgal biomass and production/conversion facilities), economical (high cost of production), and communicative (insufficient coordination of the research, expertise, and industrial efforts in the field). In attempt to coordinate the existing research programs, knowledge and the efforts of industrial units in biofuel sector of the Canadian economy, the Canadian BioFuel Research Network (BioFuelNet, 2011-2015), funded by the Canadian federal government through the NSERC Networks Centers of Excellence program. The general objective of BioFuelNet was "to use a stage-gate approach to facilitate development of biofuels and valuable co-products and to create a realistic roadmap (over a 10-20 year timeline) for the rapid deployment of these technologies to produce 25 % of the fuels and a substantial quantity of the chemicals used in Canada" (BioFuelNet, 2011).

However, to date, the demand in microalgal biomass price reduction is still very high and is estimated as 4-5 fold for biomass contained 60%, w/w of oil (Chisti, 2007) and up to 20-50 fold for biomass contained 30-40%, w/w of oil (Ruiz et al., 2016). It should be noted though that these numbers are estimated without consideration of the cost of co-products and/or application of recycling strategy.

In cold climat such as Canadian, only indoor industrial-scale production of microalgal biomass is possible. PhBRs have been used successfully, when housed within greenhouses and provided with artificial lighting (Roquette, Germany). Air Hydroponic Technology Ltd. of UK went further establishing a novel technology of combining the microalgae production with hydroponic plants cultivation. They embedded medium-size raceways for algae production into greenhouses, passing the hydroponic wastewaters to the raceways for maximum nutrients recycling.

Certainly, the development of new technologies and novel approaches of industrial integration for the algae scale-up production in cold climate is vital to Canadian economy and leadership in the industry. The most important microalgal commodidies beside lipids for biofuels are carotenoids.

1.2.2.2 Carotenoids

Carotenoids are natural anti-oxidants with many high-value industrial applications in the production of animal feed, as well as human dietary supplements and drugs. Carotenoids are fat-soluble pigments produced by all photosynthetic organisms and many heterotrophic bacteria and fungi (Martin et al., 2008; Lemoine and Schoefs, 2010). In plants and algae, carotenoids play an essential role as accessory pigments of the photosynthetic apparatus (PSA) (Grunewald et al., 2001) but in two distinct ways. Primary carotenoids such as β-carotene and lutein function as accessory pigments in the photosystems, as structural components of light harvesting complexes in chloroplasts, as well as photoprotective agents being directly involved in photosynthesis II stage. Secondary carotenoids (non-photosynthesic), such as astaxanthin, canthaxanthin, and zeoxanthin, accumulate in large quantities in lipid bodies outside the chloroplasts, after cells are subjected to stress conditions (Ye et al., 2009). The role of secondary carotenoids in algal cells is not fully understood. They could function as photoprotective filters and as antioxidants preventing accumulation of oxygen radicals (Di Mascio et al., 1991). Non-photosynthetic carotenoids do not participate directly in photosynthesis, but play a role in shielding the photosynthetic apparatus from excessive photosynthetically active radiation (PAR) (Ben-Amotz et al., 1989; Hagen et al., 1994). It has been demonstrated that many non-photosynthetic carotenoids act as strong antioxidants when consumed by animals (Lignell and Inborr, 2000). Animals metabolize dietary carotenoids to produce compounds such as retinal, the light-absorbing prosthetic group of visual opsins, or retinoic acid, a critical morphogen in vertebrate development (Guerin et al., 2003).

In recent decades, astaxanthin has been identified and recognized as a non-photosynthetic carotenoid that is greatly beneficial for human and animal health (Maiani et al., 2009; Fiedor and Burda,

2014). Astaxanthin is used as a coloration-feed supplement (Inborr, 1998; Bjerkeng et al., 1999; Ye et al., 2006) for salmon, crabs, and shrimp, and provides chicken eggs with greater nutritional value (as well as creating eggs with more strongly coloured yolks). It also has been shown that astaxanthin-enriched feed increases survival, success in reproduction, and general wellness of animals (Guerin et al., 2003). The anti-oxidation properties of astaxanthin are nearly 500-times greater than that of vitamin-E (Guerin et al., 2003).

Dietary consumption of astaxanthin has been shown to benefit the immune system, eyes, joints, bones, skin, as well as the cardiovascular nervous systems of human and animals (Nishino et al., 2002; Guerin et al., 2003; Kim et al., 2010). The greatest amount of astaxanthin is produced by a microalga species *H. pluvialis*. AstaFactor Ltd. of Hawaii and Algaetech Inc. of Israel have reported yields of 40 g and 50 g of astaxanthin per kilogram (kg) of dry biomass, respectively. Astaxanthin is synthesized from a precursor, β-carotene, with conversion efficiencies between 40% and 60% (Chumpolkulwong et al., 1997). Importantly, synthesis and accumulation of carotenoids and in particular astaxanthin tightly connects to lipids biosynthesis. Induction of astaxanthin biosynthesis in *H. pluvialis* and other microalgae is accompanied by an intense TAG accumulation (Grunewald et al., 2001; Zhekisheva et al., 2002; Lemoine and Schefs, 2010). However, the inhibition of astaxanthin biosynthesis does not lead to a decrease of TAG accumulation (Zhekisheva et al., 2005). This may be due to the fact that TAG synthesis precedes carotenoid synthesis: only after the lipid depots are formed and carotenogenic enzymes are synthesized on its surface, the biochemical equilibrium in the cells shifts toward carotenoids production.

Another valuable for the industrial production carotenoid is lutein. Lutein demand in United States is estimated to be nearly $150,000,000 per year (Fernández-Sevilla et al., 2010) with the number growing in recent years. Lutein content in *S. quadricauda* can be as high as 0.54% dcw when temperature induction (44 ^{o}C) is applied (Sa´nchez et al., 2008a), while total carotenoids induced by $FeSO_4$ can reach 45 ng/ml (El-Sayed, 2010). Under certain growth conditions *S. quadricauda* carotenoid content is very unusual,

especially in lutein, reaching a very high concentration of 500 mg of lutein per 100 grams of dry weight (or 18 kg/m^2/year), more than 5-times the lutein concentration, obtained from the petals of marigold (Martinez et al., 1999).

Lutein has a surplus value as an important ingredient in feed additives for fishery, pigs, and poultry industries improving meet/flesh quality, coloration, and bringing additional health benefits to the fed animals and, consequently, to humans (Sun et al., 2016). Lutein products have already been introduced into the pharmaceutical industry for production of infant formulas and vitamin supplements with high anti-oxidative properties (Guedes et al., 2011).

1.2.2.3 Other commodities

Microalgae protein, including commercially produced enzymes such as Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) are important high-value commodities for pharmaceutical and agricultural (animal feed) industries (Colla et al., 2007). Compared with other microalgal species, *Scenedesmus sp*. synthesizes elevated amounts of RuBisCO, and distinctive bioavailable selenium in the form of the amino-acid, seleomethonine (Kim et al., 2007; Makarevičienė et al., 2011). Some unusual proteins, such as radial spoke proteins harvested from flagellated algae *Chlamidomonas sp*. and *Haematococcus sp.* (Taylor et al., 2001), play an important role in human health research (Yang et al., 2006). Importantly, algae protein from residual biomass can be utilised after the extraction of lipids and/or carotenoids, which lowers the cost of downstream processing (Vardon et al., 2012; Laurens et al., 2015). For example, residual *H. pluvialis* biomass contains 20-25 mg/g dcw digestible protein (Boussiba and Vonshak, 1991; Panis and Carreon, 2016). Protein concentrations in algae cells vary among species. One of the highest concentrations of protein, 70 % of dcw, was reported in *Spirulina sp,* with most of its protein being digestible (Becker, 2007). Commercial species for protein production, such as *Chlorella vulgaris, S. obliquus*, *Dunaliella salina,* and *Spirulina sp,* contain between 50 and 70 mg/g dcw protein (Um and Kim, 2009; Sydney *et al*., 2010). The protein content of Duckweed is also high, easily digestible, and

varies by species and growth conditions, ranging from 15 to 45 % of dcw (Landolt and Kandeler, 1987; Piotrowska, 2005). These values place the protein content of dry Duckweed biomass between alfalfa meal (20 %) and soybean meal (41.7 %), and suggest that this high protein content could generate post-fermentation residues with high protein concentrations, which could be valuable as an animal feed (Hillman and Culley, 1978).

Non-biofuel oils from microalgae are important commodities, which are in high demand in pharmaceutical, cosmetic and agricultural industries. Omega-3 FAs, the so-called group of polyunsaturated fatty acids (PUFAs) which includes docosahexaenoic acid (DHA: C22:6), eicosapentaenoic acid (EPA: C20:5), arachidonic acid (ARA: C20:4) and γ-linolenic acid (GLA: C18:3) is a group of industrially produced microalgae oils. The commercial microalgae for PUFA production are: *Crypthecodinium cohnii*, *Nannochloropsis* and *Schizochytrium* being rich feedstocks for DHA, EPA (Kyle, 2001; Priyadarshani and Rath, 2012; Collins et al., 2014); *Parietochloris incisa* and *Mortierella alpina* for ARA (Bigogno et al., 2002; Sakuradani et al., 2009), *Spirulina platensis* and *P. incisa* for GLA (Sajilata, 2008; Iskandarov et al., 2011). A great potential for industrial scale omega-3 FAs production is also demonstrated in *Chlorella sorokineana*, *Chl. vulgaris* and *Scenedesmus sp* (Converti et al., 2009; Adarme-Vega et al., 2012, 2014; Salama et al., 2013). Omega-3 fatty acids provide substantial health benefits and have a high market price as dietary supplements. However the solitary production of this co-product from microalgae biomass still is not economically viable (Li et al., 2009).To date, omega-3 fatty acids are typically extracted from fatty fish, putting additional pressure on the global fish stocks (Adarme-Vega, 2012).

Numerous studies have demonstrated high loading of various vitamins in microalgae species. Besides having the highest known concentration of astaxanthin, *Haematococcus* cells synthesize a broad variety of vitamins (C, E, B1, B2, B3, B5, B6, B7, and B12), which gives this microalgal species an exceptional status for industrial production (Boussiba and Vonshak, 1991; Metting, 1996; Chekanov et

al., 2014).

Commercial algae species for vitamin extraction are: *Spirulina sp* (A > B2 > B6 ≥ K > B1 > B12), *D. salina* (A > C > E), *Ch.* vulgaris, *Ch. sorokineana and S. quadricauda* (B12 > folates > B1 ≥ A > B2 > B7, C, B5, K), *Euglena gracilis* (C, E, B6, B2) (Pratt and Johnson, 1977; El Baz et al., 2002; Colla *et al*., 2007; Ogbonda et al., 2007; Li et al., 2008; Avagyan, 2008; Kumunda and Sarada, 2016).

In addition, some microalgae species contain unique compounds, such as the pigment scytonemin and the biopolymer sporopollenin which are highly valuable in cosmetic industry for UV-screening; antioxidants (e.g., polyphenols, tocopherols), antimicrobial, antifungal, antiviral agents, toxins and bio-available selenium with great benefits for animal and human health (Becker 2004; Challouf et al., 2011; Colla *et al*., 2007; Krittaphol et al., 2011). For instance, *S. quadricauda* biomass contains great amounts of L-selenomethionine (SeMet), which is a highly digestible and health-beneficial form of selenium (Krittaphol et al., 2011). Umysová et al. (2009) reported that wild-type *S. quadricauda* accumulated 300 mg/kgSeMet in the presence of 50 mg/Lof selenite (Skřivan et al., 2010). Authors suggested that the algae tolerance mechanism is an internal way to detoxify Se inside the cell which was demonstrated in studies conducted on *S. quadricauda* (Skřivan, 2010) and *Ch. sorokiniana* (Gojkovic et al., 2014). Considering its elevated ability for lipid accumulation and easiness to grow on wastewaters, *S. quadricauda* is on the priority list for industrial scale production (Table 1).

1.2.3 Commercial Duckweed production for biofuels and animal feed

The first industrial-scale production of Duckweed was initiated in 1989 in Bangladesh by a non-governmental organization based in Columbia, Maryland. The PRISM Group developed farming systems for Duckweed growing plants on hospital wastewater and tested its value as a fish feed (Skillicon et al., 1993). The system has been proven as self-sustaining many times over, and is still working today. The results of this pioneering initiative greatly exceeded expectations: biomass production as high as 80 tons/h/y on a pilot-scale experiment, and as high as 35 tons/h/y on industrial-scale production; protein

loading up to 45 % of dcw, starch loading up to 70 % and carotenoid loading up to 1.25 mg/g of fresh biomass were reported (Table 3).

Thus, Duckweed biomass has a great potencial for an industrial scale production as a feedstock for biofuels and agricultural industries.

1.2.3.1 Properties of Duckweed

Duckweed (Lemnaceae) is an aquatic plant with the highest rate of growth of known vascular plants (Landolt and Kandeler, 1987), and has a very high protein and starch content (Xu et al, 2011, 2012). Duckweed species are small, floating aquatic plants naturally found in still, nutrient-rich fresh and brackish waters. The family consists of four genera - *Lemna, Spirodela, Wolffia*, and *Wolffiella* - among which about 40 species have been identified so far. The chemical and nutritional properties of Lemnaceae species are similar in natural environments, although some differences have been recognized (Table 3, 4). For any Duckweed species, fluctuations in the chemical composition are simply dependent on the chemical composition of the water and bioavailability of the aqueous compounds. Duckweed production systems do not require great investment, and consequently have gained interest for production of biofuels and bio-products. Until recently, Duckweed had been used only in commercial applications to treat wastewater in North America, and as additional feed for poultry in Asia.

1.2.3.2 Duckweed as a feedstock for biofuels and bioproducts

Compared with most plants, Duckweed fronds have low fiber content (Table 3), as low as 5 % in cultured plants while wild types have up to 15 % of fiber (Leng et al, 1995), because they do not need structural tissue to support leaves or stems. As a result, almost all tissue is metabolically active and easily digestible as an animal feed product. In a view of biofuel (bioethanol) production, this feature enables to consider Duckweed biomass as highly efficient for conversion processing not only due to its high starch loading (up to 70 % of dcw in optimized conditions, Bergmann et al., 2000), but also due to low percent of residual mass. This important characteristic contrasts favourably with many terrestrial crops such as

soybeans, rice, or corn used for the first generation of bioethanol production. Terrestrial crops have a large amount of residual biomass after the conversion and extraction processes. Importantly, Duckweed is capable of producing high levels of dextrose (10 % on average; Barett et al., 2012).

Table 3. Comparison of key-nutrients composition of two Duckweed species, % of total dry weight.

Plant species	**Lemna minor[1]**	**Lemna giba[2]**	**Rice[3]**
NFE	8-20	10-30	8
Ether extract	5-10	10-15	1.4
Fiber	5-20	5-15	2
Ash	20	20	1.1
TC*, mg/g fwt	1.25	0.4	0
TDM, %**	1.25	0.4	0
Starch	45-60 %	60-70 %	50-72 %
Ca	0.2 – 4.5		0.3 – 0.7
Mg	0.04 – 2.8		0.1 – 0.4
P	0.03 – 3.0		0.5 – 0.9
N	0.8 – 8.0		
K	0.3 – 7.0		3.5 – 4.6
Na	0.1 – 1.3		0.1 – 0.4
Fe	0.01 - 3.2		
Mn	$3x10^{-3}$ - 6.5		
Zn	$4x10^{-3}$ - 0.14		
Cu	$0.2x10^{-3}$ - 3.2		

Note: NFE- Nitrogen free extract (90 % as starch); * - TC is total carotenoids as concentration in fresh biomass weight (fwt); ** - TDM is total dry matter; 1, 2- data shown from Halver, 1972, Ogino, 1980, Leng et al, 1995, Landolt and Kandeler, 1987, Guimaraes et al., 2012; 3- data from Fageria, 1976; Wang and Wang (2001); Litskas et al, 2014 and Arns et al., 2015.

Duckweed biomass is used for fermentation to produce glucose and ethanol (Yu et al., 2014; Toyama et al., 2018). The target bio-products from Duckweed biomass are omega-3 fatty acids, pharmaceutical proteins consisting a vast variety of amino acids (Table 4), and carotenoids (Landesman, 2011; Cui and Cheng, 2014). There are some clear advantages of Duckweed proteins for pharmaceutical usage, including inexpensive technology, easy scale-up production, and the absence of potentially hazardous animal proteins, cells, and viruses. Thus, many of the challenges of producing biopharmaceuticals are vastly simplified and the cost of production is reduced accordingly. Synthon Inc. is the first pharmaceutical company to employ a novel technology developed by Anne-Marie Stomp (North Carolina State University, USA), to produce therapeutic proteins using genetically engineered Duckweed.

1.2.3.3 Duckweed for animal feed

The crude protein content, which closely resembles animal protein (Hillman and Culley, 1978) in terms of microelements, carotenoids (Table 3), and the amino acid composition (Table 4), suggests that Duckweed biomass could serve as a nourishing and highly digestible feed for poultry, livestock, and farmed fish (Leng et al., 1995; Fasakin et al, 1999). The main challenge of Duckweed as feed biomass is a relatively high (2-4 % dry weight) percent of calcium oxalate. Calcium oxalate is not a nutrient (nor a beneficial source of calcium), is indigestible, and can be toxic in large doses. However, the concentration of calcium oxalate in Duckweed can be controlled by adjusting the water chemistry (Mazen et al., 2003; Landesman, 2011).

Duckweed protein has higher concentrations of the essential amino acids lysine and methionine (Table 4) than most plant proteins, and more closely resembles animal protein in that respect (Hillman and Culley, 1978). Cultured Duckweed also has high concentrations of trace elements and pigments, particularly β-carotene and xanthophylls (mostly in form of zeaxanthin and canthaxanthin) that make Duckweed meal an especially valuable supplement for poultry, salmonid fish farming, and other animal

feeds. The total content of carotenoids in fresh Duckweed feed is roughly 10-times higher than that of terrestrial leaf-plants (Leng et al., 1995; Fasakin et al., 1999). Xanthophyll concentrations of over 1,000 ppm were documented in Duckweed grown agriculturally for poultry feed in Peru (Journey et al., 1991). This is economically important because of the relatively high cost of the pigment supplement, which improves quality and colorations of meat and eggs, in poultry feed (Haustein et al, 1988).

Table 4. Composition of amino acids (% of crude protein) of two aquatic plants grown on swine wastewater compared with soybean (Rusoff et al., 1980).

Species:	**Duckweed**	**Azolla**	**Soybean**
Crude protein, (mg/g frw)	25-45	31	44
Amino Acid (mg/g frw)			
Lysine	3.7/11*	3.4	6.6
Histidine	1.7	1.7	2.5
Agrinine	5.1	4.6	7.3
Threonine	4.2	3.5	3.9
Valine	5.8	5.1	4.6
Methionine	1.5	1.4	1.2
Isoleucine	4.3	3.8	4.5
Leucine	7.8	7.1	7.7
Tryptophane	4.2	3.5	3.6

Note: * average/max found in literature (max value from Yilmaz et al., 2004).

Freshly harvested Duckweed plants can be used without further processing as a complete feed for fish (Hillman and Culley, 1978; Gaigher et al., 1984). It also was reported that a monoculture of Nile tilapia and a poly-culture of Chinese and Indian carp species were observed to feed readily on fresh

Duckweed and dry Duckweed pellets (Robinette et al., 1980; Hassan and Edwards, 1992). The results showed that a diet consisting of up to 20 % content could be used as a complete replacement for commercial feed in diet formulation for common carp and tilapia (Wee, 1991; Yilmaz et al., 2004). Importantly, Duckweed grown as a fish-farm co-product can be used as an in-house source of feed for fish drastically decreasing the cost of fish production while increasing nutritional quality of fish.

1.2.4 Altering biosynthesis in microalgae: increasing yield vs. decreasing production costs

1.2.4.1 The goals of altering biosynthesis

The development of new technologies and novel approaches for scale-up production of microalgae and Duckweed biomass in cold climates is vital to Canadian economic sustainability and leadership in the industry. To achieve the cost-efficient production, the most suitable operating conditions for production, the co-products of interest, and the species/strains with optimal for production characteristics must be determined. Advanced technologies to improve bioreactors, harvesting, and conversion processes have to be designed in greatly contributing to cost-efficient operation. Application of genetic engineering is a powerful tool to achieve one of the prime goals of alterning biosynthesis patterns of high-value biomolecules in microalgae (and perhaps even Duckweed). Integration of biomass production based on maximum recycling of water and industrial wastes such as flue-gas CO_2, wastewaters, and heat is a relatively novel approach of bringing algae and Duckweed biomass production to the industrial scale reducing the investment for both a biomass production and a plant operation.

1.2.4.2 The tools of alteration

To date, valuable algae molecules production is based on induction of biosynthesis under extreme growth conditions. It is well known that lipid and carotenoid accumulate under the influence of various adverse conditions (stressors) and especially their combinations (Jin et al., 2003; Lemoine and Schefs, 2010). The most studied and therefore extensively employed stressors can be classified into two groups: extreme values of growth conditions and additional physico-chemical stressors. The first group is

represented by light intensity and quality, nutrients depletion, temperature, and pH. The second group includes, but is not restricted to trace metals, herbicides, salt-stress, additional organic compounds (such as hormones, organic carbon), ultrasound and artificial UV radiation (Alfalo et al., 1999; Boussiba, 2000). An outstanding tool of biosynthesis alteration is transgenics and this way of alteration will be discussed separately. Also separately will be conferred the powerful tool of hormones application, as the main subject of the presented project.

1.2.4.2.1 Growth conditions and carbon sources

Algae biosynthesis and synthesized molecules accumulation depend on growth conditions. Alteration of growth conditions can be used as a tool for induction of biosynthesis and/or accumulation of molecule of interest. It was recently hypothesized that the physiological role of TAG accumulation in oil bodies, is not limited to storing carbon and energy (Bigogno et al., 2002). A number of researchers showed that, in addition to traditional and most studied function of energy storage, microalgal TAG can also accomplish other important intracellular functions such as adaptation to environmental changes or membrane repair after the damaging effects of light or temperature (Alonso et al., 1998; Allakhverdiev and Murata, 2004; Mohanty et al., 2007; Khozin-Goldberg et al., 2006; Sforza et al., 2012).

TAG accumulation in microalgal cells occurs in two stages. First, when all elements required for culture growth are available in the medium, microalgal cells divide rapidly and synthesize mainly membrane lipids and chloroplast lipids in particular. Second, when any growth factor, such as nitrogen becomes limiting but photosynthetic CO_2 fixation continues, the lipogenic phase takes place as a reaction to an adverse condition. This phase can be characterized by the cessation of cell division and by accumulation of non-polar carotenoids and neutral lipids, TAG in particular (Leman, 1997; Guschina and Harwood, 2009) which are stored to support cell growth once normal growth conditions are restored (Harwood and Jones, 1989). Light regime, nutrient loading, the pH of growth medium, and temperature

are the key factors affecting lipid accumulation and their composition in microalgal cells (Harwood and Jones, 1989; Christi, 2007).

Carotenoid and protein accumulation also greatly depends on ambient conditions. Temperature, elevated to the upper biomass limited threshold level, benefits the accumulation of different carotenoids which has being proven for many algae species (García-González et al., 2005; Ip and Chen, 2005; Sánchez et al., 2008b; Fernández-Sevilla et al., 2010). The influence of flue-gas CO_2 application and the pH influence on algal carotenogenesis are more species specific and also depend on the mode of production (Fernández-Sevilla et al., 2010). High irradiation is another universal strategy for inducing carotenoid production (Ip and Chen, 2005; Imamoglu et al., 2009). However, a number of species-specific, reactor-specific and modes of growth-specific alterations of ambient conditions, including the combination of strategies, are in use at the industrial scale of algal carotenoids production (Kobayashi et al., 1990; Zhu et al., 2000; Kim et al., 2006; Fábregas et al., 2003; Sun et al., 2016).

1.2.4.2.1.1 Light intensity

Intensity, spectrum, and the photoperiod of light affect the metabolism of microalgae and Duckweed. Light induced changes in the lipid composition are related mainly to changes in chloroplast development (Harwood, 2004). Excess of light frequently results in fatty acid photo-oxidation (Richardson et al., 1983; Harwood, 1998) and suppresses TAG accumulation in algae (Solovchenko et al., 2014). Insufficient light induces the formation of polar lipids over neutral lipids (Brown et al., 1996), while already synthesized TAGs are used as an alternative source of energy and are metabolized (increased isocitratelyase activity) via the β-oxidation pathway. However, some microalgae species display the opposite relationship between light intensity and degree of TAG accumulation (e.g., microalgae *P. incisa* and *Porphyridium cruentum*) which highlights species-specific metabolism of neutral lipid in algae (Bigogno et al., 2002). Light intensity also affects the pigment composition of cells.

High light intensity usually increases the carotenoid content, especially that of β-carotene and lutein (Ip and Chen, 2005; Solovchenko et al., 2014), which serve to prevent photo-bleaching.

Several studies have demonstrated a positive correlation between light intensity and Lemnaceae biomass production (Landolt and Kandeler, 1987). It was demonstrated that the faster growth (evaluated as doubling-time) was obtained with Duckweed grown under red and blue wave-lengths of light, while the slowest growth was observed under green light (Tat et al., 2011). This observation is supported by the fact that photosynthetic rate has two peaks in red and blue spectrum (Moor et al., 1995).

1.2.4.2.1.2 Nutrition deficiency

Availability of mineral nutrients exerts a great influence on biomass production and lipid metabolism in microalgae (Harwood and Jones, 1989). In almost all cases, the decrease in the availability of nutrients results in the slowdown of cell division and the increase of TAG accumulation. Nutrient starvation is one of the most widely used lipid induction techniques for microalgal TAG production and has been reported for many species (Lynn et al., 2000; Rodolfi et al., 2009). However, recently it was demonstrated that negative effect of nutrients starvation on algae biomass production can be mitigated by applying organic carbon (Fan et al., 2012) or growth regulation substances such as 3-indoleacetic acid (IAA) and diethyl aminoethyl hexonate (Babu et al., 2017). Similar to microalgae, nutrient starvation stress triggers the starch accumulation in Duckweed plants with the starch content increased by 29.8 % after the Duckweed (*Landoltia punctata*) was grown in well-water for eight days (Xu and Shen, 2011). However, the protein content of Duckweed decreases in the water with low nutrients loading (Fasakin et al, 1999).

1.2.4.2.1.3 Effect of pH

Another factor that greatly affects microalgae growth and valuable molecules accumulation is pH of growth medium. Algae and Duckweed species are known to live in a very wide range of pH from 4 to 10 in nature (McLay, 1976; Lee, 2012). Optimal pH is highly species-specific and thus, the rule of pH-

dependence of lipids accumulation in microalgae and Duckweed cells can be defined as the following: species will accumulate neutral lipids or starch when the pH is shifted toward the opposite condition that is normal for the species – i.e. either alkaline or acidic (McLay, 1976; Wijanarko et al., 2008; Fernandez-Sevilla et al., 2010). Some algae species (e.g., *Scenedesmus sp*) can grow without significant change in production rate when pH shifts between 6 and 9.5 pH with no acclimatisation period needed (Martinez et al., 1999). It easies wastewater application for these species biomass production.

1.2.4.2.1.4 Dissolved carbon

In natural water ponds, the concentration of dissolved inorganic and organic carbon and their ratios vary greatly (Lower, 1999). When it comes to growth media used in industrial units, the combination of different dissolved carbon compounds (before CO_2 gas application) and its influence on growth and lipid yields in microalgae is often overlooked. The presence of organic sources of carbon stimulates TAG accumulation, shifting the C:N ratio toward carbon, which was found to be more pronounced under nitrogen starvation conditions (Cohen and Khozin-Goldberg, 2011). In *E. gracilis*, the addition of organic carbon to the medium under conditions of nitrogen deficiency enhanced the storage of TAGs enriched in C14:0 and C16:0 fatty acids (Regnault et al., 1995). Importantly, the recent research showed that under the excess carbon (acetates) condition and without nutrient deficiency, microalgae cells are kept growing while producing high percent of oil (Wang et al., 2011b; Fan et al., 2012). This finding rules out the previously held dogma that algae growth and increased oil production are mutually exclusive.

1.2.4.2.1.5 Effect of temperature

Growth rate of algae normally slows down as the temperature of medium lowers. However, some species important for industrial production are resist to wide-range of temperature changes. For example, *Scenedesmus sp*. can reach high biomass production with a little change in growth between 14 and 30 °C (Xu et al., 2012). Elevated neutral lipid synthesis (total TAG and omega-3 fatty acid) has been shown in several investigations when the temperature of growth medium is lowered (Tatsuzawa and Takizawa,

1995; Jiang and Gao, 2004; de Castro and Garcia, 2005; Ward and Singh, 2005). Hovewer, temperature affects TAG synthesis and accumulation in a species-dependent manner and the opposite effect of temperature also could be observrd (Converti et al., 2009).

For Duckweed, although lower water temperatures negatively affect biomass production (Leng et al., 1995; Skillicorn et al., 1993), it favours the accumulation of starch due to reduced respiration of Duckweed plants at night, when starch consumption is substantial. For instance, starch content can increase by approximately 59 % when growth temperature decreases from 25 ^{o}C to 5 ^{o}C (Cui et al., 2011).

1.2.4.2.2 Stress-induction strategies

1.2.4.2.2.1 Metals

Essential for living cells metals such as copper, iron, zinc stimulate microalgae growth rate (Fisher et al., 1984; Crist et al., 1994) and neutral lipids production. Importantly, metals can induced endogenous phytohormones biosynthesis (BL, IAA, abscisic acid (ABA) and zeatin (Zt)) with the result of further protein and monosaccharides biosynthesis and their accumulation enhancement (Bajguz, 2011).

However, the main problem in using metals as inductive factors is their high ability to intracellular accumulation. It brings up an issue of bio-products contamination.

1.2.4.2.2.2 Herbicides

Some evidence suggests that inhibiting glutamine synthase, and thereby obstructing nitrogen assimilation by herbicides (glufosinate), induced astaxanthin accumulation in *H. pluvialis* and provoked nitrogen starvation due to high efflux of intracellular ammonia (Alfalo et al., 1999). Similar to metals, the use of such toxic compounds for bio-products production is questionable. However, as well as for metals, assessment of herbicide influence on microalgae lipids and co-products yield is important when wastewater is applied for microalgae biomass production.

1.2.4.2.2.3 Combination of stresses

The combination of stress factors has different, sometimes controversial influence on algae biosynthesis and valued molecules accumulation. For example, extreme temperature in combination with high light intensity has synergistic effect on astaxanthin production while slowing down cell division of *H. pluvialis* (Collins et al., 2011). In the work conducted by Domiani et al. (2010) both single stress (high light) and combined stress (high light + low nitrogen) significantly and similarly increased TAG accumulation (Table 5). However, this work is an example in which the combined stress, enhancing TAG production compare to the control, greatly slows down biomass production resulting in overall loss in TAG production (Figure 2, Table 5). Interestingly, Li et al. (2008) observed significant increase in TAGs (3.5-fold), astaxanthin (7.8-fold) production and not greatly suppressed algae growth (Table 5).

Lemoine and Schefs (2010) explained such differences in outcome of combined astaxanthin-induction factors as the fact that under multiple stresses several defense pathways can be activated, each contributing to the overall protection or, at some degree, mediating each other.

Table 5. Combined data from two investigations on the influence of single versus multiple induction factors on astaxanthin and lipids production.

	Domiani et al., 2010			Li et al., 2008*		
Haematococcus pluvialis	Control	HL	HL+LN	Control	HL	HL+SA+FE
Doubling time (h)	21	30	246	25	46	36
TAGs (% dcw)	9.2 ± 0.67	19.8 ± 0.14	16.6 ± 0.41	8	15.5	28
Phospholipid (% dcw)	1.87 ± 0.05	9.5 ± 0.0	9.8 ± 1.84	n/a	n/a	n/a
Astaxanthin (% total carotenoid)	n/a	n/a	n/a	12	57	93

Note: FE, ferrous sulfate; HL, high light; SA, sodium acetate; LN, low nitrogen; dcw –dry cell weight; *, data is transferred from the graphs by Kozlova T.A.

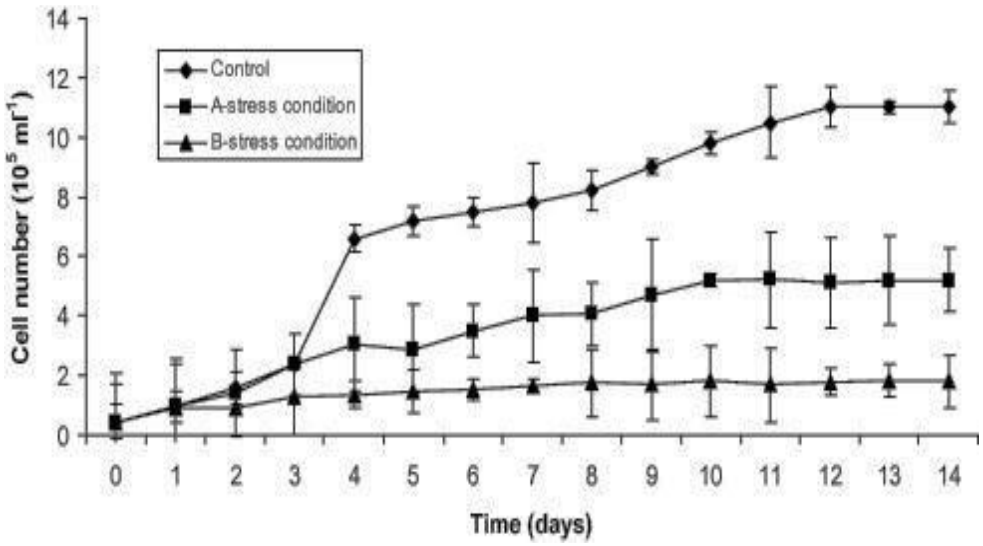


Figure 2. Growth of *Haematococcus pluvialis* in control cultures, in A-stress condition (continuous light with high intensity) and in B-stress-combined condition (continuous light with high intensity and without nitrogen) (after Domiani et al., 2010).

1.2.4.2.3 The application of hormones

Research on influence of hormones on microalgae physiology was initiated nearly 80 years ago, and focused primarily on the effect of growth regulator substances on cell growth and algae biomass production (Pratt, 1938; Conrad et al., 1959; Ahmad and Winter, 1968). More rigorous attention to the role of exogenous phyto- and animal hormones in algae physiology has been reported by numerous studies in recent decades, which have shown that hormones can significantly increase microalgal cell growth and biomass production (Zhou et al., 2012; Tate et al., 2013; Kozlova et al., 2017, 2018; Han et al., 2018), as well as accumulation of intracellular lipids (Liu et al., 2016; Babu et al., 2017; Park et al., 2013; Kozlova et al., 2017, 2018), protein (Hunt et al., 2010; Bajguz and Piotrowska, 2014; Salama et al., 2014; Talarek-Karwel et al., 2018), and pigments (Cowan and Rose, 1991; Bajguz and Piotrowska, 2014; Talarek-Karwel et al., 2018; Kozlova et al., 2017, 2018).

The most important achievement of hormone application for microalgae biomass production is that, similar to the effect of dissolved organic carbon from acetate (Wang et al., 2011b; Fan et al., 2012), phyto- and animal hormones clearly demonstrated an ability to simultaneously promote both microalgal cell growth and accumulation of secondary metabolites (Bajguz and Czerpak, 1996 a, b; Babu et al., 2017; Kozlova et al., 2017, 2018).

Hormone applications not only stimulate increases in total lipid accumulation, but can also alter fatty acids profiles of different algae species (Salama et al., 2014; Babu et al., 2017; Kozlova et al., 2017, 2018). Thus, hormones could be used as a tool for fatty acid profile manipulation in the biofuel and pharmaceutical industries. Another commercially valuable algal co-product, carotenoids, can accumulate to high concentrations in the presence of exogenous hormones. Significantly high levels of induction have been reported for total carotenoids (Czerpak and Bajguz, 1997; Kozlova et al., 2017, 2018), β-carotene (Cowan and Rose, 1991; Czerpak and Bajguz, 1997; Talarek-Karwel et al., 2018) and

astaxanthin/xanthophyll (Kobayashi et al., 1997; Czerpak and Bajguz, 1997; Gao and Mang, 2007) in the studies conducted on algae species of industrial interest.

Remarkably, plant and algae species can synthesize not only phytohormones (Tarakhovskaya et al., 2007; Kiseleva et al., 2012), but also trace concentrations of animal steroids (Zhang et al., 1991; Finsterbusch et al., 1999, Janeczko and Skoczowski, 2005). All three groups of animal sex steroids, androgens, estogens and progesterons, were detected in a number of plant species (Saden-Krehula et al., 1979; Stohs and El-Olemy, 1972; Simons and Grinwich, 1989; Maier et al., 1995; Lindemann and Luckner, 1997), and the enzymes responsible for biosynthesis and metabolism of these animal sex hormones have also been detected in plants (Brzostek et al., 1998; Rosati et al., 2003). Importantly, the distribution of sex hormones in plant tissues depends on location and gender of a plant (Zhang et al., 1991; Zhong-han et al., 1994). For instance, testosterone was found in pollen of some plants and the bulk hormone activity increases immediately before and during flowering (Zhong-han et al., 1994). In *Juglans regia* and a Taiwanese yam cultivar, progesterone- and progesterone-like hormones have been detected (Pauli et al., 2010; Yang et la., 2003). Thus, it was a logical finding that fishery wastewaters, which bear nano- to microconcentrations of fish hormones, can greatly induce agricultural plant growth and biomass production (Takeda et al., 1997; Rakocy et al., 2004, 2007; Bansal et al., 2005; Bailey and Ferrarezi, 2017).

Being an aquatic plant, Duckweed has been shown to be close to microalgae in its response to both phytohormones (McLaren and Smith, 1976; Czerpak et al., 2004) and fish steroids (Czerpak and Szamrej, 2003; Szamrel and Czerpak, 2004). In particular, the doubling-time of *Lemna minor* fronds was reduced by low concentration (10^{-6} M) of ABA, while relative growth rate of fronds was elevated (McLaren and Smith, 1976). Remarkably, and similar to algae, low ABA concentration enhanced biosynthesis and accumulation of all tested molecules, carbohydrates, and chlorophyll in Duckweed (McLaren and Smith, 1976). Auxin demonstrated a lower influence on Duckweed growth than that what has typically been

observed for vascular plants (Moon and Stomp, 1997; Czerpak et al., 2004). Animal sex steroids are capable of increasing soluble protein, sugars, photosynthetic pigments and carotenoids in Duckweed (Czerpak and Szamrej, 2003; Szamrel and Czerpak, 2004). However, animal sex hormones fall under the category of Endocrine Disruption Chemicals (EDC), which makes the applied concentrations and the algae growth units operating conditions are crucial in term of the synthesized bioproducts application for pharmaceutical, cosmetic industries andas animal feed. More study is needed for clarification of a possibility of animal steroid accumulation in algae cells and Duckweed tissue.

Industrial-scale hormone application is restricted by high cost of manufactured hormones into growth medium. Opportunely, it has been proven that hormones are effective to promote algae growth and biosynthesis in considerably low nanomolar concentrations (Bajguz and Czerpak, 1998; Nobel et al., 2014; Kozlova et al., 2017, 2018; Talarek-Karwel et al., 2018). Although naturally occurring hormones normally could be found in surface and well water in trace concentrations (Shore and Shemesh, 2003; Kashian and Dodson, 2004; Brennan et al., 2006), only a few studies have observed hormone loading in well waters at μM concentrations (Silva et al., 2012; Jobling & Owen, 2013). Agricultural wastewaters, such as those derived from hydroponic cultivation in greenhouses, agricultural run-off from crops treated with plant growth substances or hormone-herbicides (Cobb, 1992; Khripach et al., 2000; Crossmann, 2007), or effluences of fish and animal farms can bear significantly high concentrations of hormones, reaching hundreds of μM (Lopez et al., 2005; Silva et al., 2012; Jobling and Owen, 2013).

Thus, the sources of phytohormones in agricultural effluents are naturally produced plant hormones, as well as hormone contamination from agricultural fields. In vascular plants, phytohormones play crusial role in physiology as growth regulators, tissue and organs diferentiators and stress mitigators upon variety of environmental stressors. Most of the time phytohormones work synergistically or antagonistically on the same physiological functions creating signalling network (Verhage et al., 2010; Yang et al., 2013). In particular, brassinosteroids are growth-inducing agents also working with other hormones to modulate

plant disease resistance (De Vleesschauwer et al., 2012). Most common and well-studied auxin IAA coordinates cell division and elongation and plays important role in organs and tissue diffenciation in vascular plants (Zhao, 2010; Ludwig-Muller, 2011) often working synergistically with brassinolides on plant growth regulation (Katsumi, 1985; Sala and Sala, 1985; Vidhyasekaran, 2015). Envirinmental stresses affect the plant immune signalling system (Vidhyasekaran, 2015). While all the phytohormones play their role in protection against stresses and pathogens, ABA is a prime mitigator, which plays a multiple role in imunoregulation of of vascular plant. As a stress mitigator, ABA can work synergistically (with SA and ethylene) or antagonisticcaly (with JA and SA) with other phytohormones, which depends on a stress and ambient conditions (Pastori and Foyer, 2002; Chen et al., 2012; Sanchez-Vallet et al., 2012; Alazem et al., 2014). Oppositely, auxin works as a secondary factor affecting antagonistically or synergisticcaly powerful stress mediators such as SA or JA (Peer et al., 2013; Tatsuki et al., 2013). In addition to environmental stress mitigation, ABA plays an important role in carotenogenesis in vascular plants (Zeevaart and Creelman, 1989; Parry et al., 1990). More detales on phytohormones functions and their crosstalk in vascular plants and microalgae will be discussed in the following chapters.

Fishery wastewater bears sex and stress animal hormones released by fish first due to their reproductive activities. In fish physiology, 17,20β-P and its major metabolites have an internal role as the hormone that induces final oocyte maturation, and an external role as the pheromone that stimulates milt production, sperm motility, and behavioral spawning competence in males (Sorensen et al., 1995; Vermeirssen et al. 1996; Scott et al., 2010). In both genders E2 plays an important role for reproductive system development and motivation of sexual activity (Chaves-Pozo et al., 2007; Guiguen et al., 2010). E2 is mostly produced by females during final oocyte maturation and ovulation, although small amounts of E2 are synthesized by mature male fish (Sorensen and Scott, 1994; Van Weerd et al., 1991). From here, it could bee seen the importance of release of the steroids into surrounded water for motivation of sexual activity. Therefor, the sufficient amount of naturally occurring animal and plant hormones is expected in

the effluents from spawning tanks and hydroponics. Application of naturally occurring hormones from fishery and hydroponic wastewaters could be one of the solutions to reduce the cost of industrial scale microalgal production. The detailed assessment of the influence of phyto- and fish hormones on microalgae growth and biosynthesis will be discussed further in the following chapters.

1.2.4.2.4 Transgenics

One of the most powerful tools for modifying microalgal bio-production is genetic engineering. Currently, the efforts in the field are focused on increasing oil yield by overexpression of native genes, identification of transcriptional control factors or introduction of novel genes from exotic or native, robust algae species (Hannon et al., 2010).

Recently a novel genetic method of an active CO_2-concentrating mechanism (CCM) alteration was developed to increase biomass yield by 50 to 80 % (Fang et al., 2012). Another tendency in genetic engineering of microalgal strains is introduction of bacterial or even animal (Nematoda, protozoa) genes for modification of chemical structure of microalgal FAs to branched-chain fatty acids (BFAs) for biofuel production (Kniazeva et al., 2004; Machida et al., 2016, 2017). in Duckweed research the recent genome sequencing and the development of genetic engineering methods provide valuable technological resources for genetic modification of target molecules (Soltis et al., 2003; Mardanov et al., 2008; Wang and Messing, 2011).

1.2.4.2.5 Integration of biomass production

The novel and one of the most powerful ways to bring biofuel & bioproducts economy to the industrial-scale producing competitive to the current market bio-commodities is integration of biomass production on the basis of maximum nutrients and energy recycling, sharing space, and lighting. Thus, integration of biomass production could be categorized into two major types: 1) integration of two or more biomass production in one industrial unit; 2) and integration of different industrial companies on purpose of maximum waste recycling and end-products cross-utilisation. In particular, application of industrial

CO_2 emissions and wastewaters as nutrients sources and industrial waste-heat as an energy source coupled with integrated production of two or more types of biomass in one industrial unit can greatly improve the feasibility of production. These methods of integration could not only support alternative industrial production of biofuel and valuable co-products, but also provide concomitant reduction in atmospheric CO_2 and remediation of wastewaters.

1.3 Systems integration

1.3.1 Different types of integration

Industrial integration has proved to be an asset in a new world development (EC, SEC 1272, 1276, 2010; Navarri, 2015). In the field of biomass production the efforts of integration have been made in the last decade (McKendry, 2002; EC, 1997, 2004; DOE, 2010). The tactics of the integration can be organized into three sections: 1) Integrative production of biofuels and valuable co-products from one type of biomass; 2) Integrative production of biomass of different types for different applications; and 3) Integrative production of biomass with wastewater and other industrial wastes treatment;

The combination of the above methods accomplished with the involving of non-biomass production industrial companies. This classification is flexible. For example the first section can be combined with the following. The first method of integration is extensively used at most of the existing companies, which produce biofuels. Thus, the combination of biofuels and valuable molecules production by one company is vital for its persistence in the business. These new technologies allow widening the assortment of co-products from a biomass processing adding for example animal feed supplements and high quality fertilizers made from the residual mass (Mulbry et al., 2005; Brady and Weil, 2007).

1.3.2 Current progress of integrative production of biomass

One of the most established integrative production system is aquaponics: the combined production of fish and plants. This type of integration is based on the idea of sharing nutrients including dissolved in fish tanks CO_2 and to some degree sharing energy, to remediate and recycle water used in the system

(Figure 3). In particular, the wastewater from one type of biomass production (fish tanks) is used as a fertilizer for another type of biomass production (e.g., vegetables, Duckweed) while, in turn, the residues of the second biomass (e.g., Duckweed) is used as a feed supplement for fish or dry fertilizer for external application (agricultural crop) (Bansal et al., 2005; Diver and Rinehart, 2010).

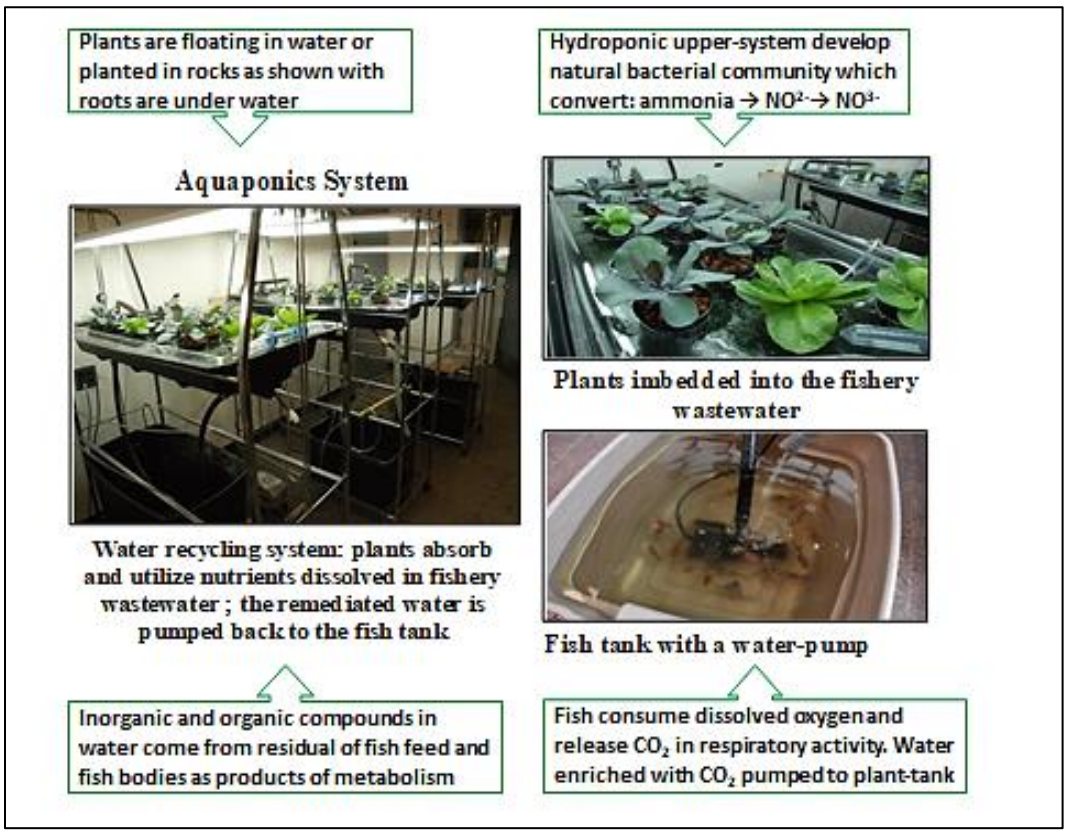


Figure 3. A schematic description of an aquaponics system designed by Kozlova T. A. at the Red River College, Winnipeg; photo by Kozlova T. A.

The combination of fish farming and hydroponics systems is a relatively new method however has been demonstrated good success in the combined production of both types of biomass (Rakocy et al., 2006; Bernstain, 2011). To date, a number of small farms and scientific pilot facilities have built to study the effects of such combination on fish and plant production and their biomass quality (Figure 4). Aquaponics have also been adapted to Duckweed biomass production (Figure 4). The main principles of aquaponics and other integrative biomass, such as microalgal, production can be summarized as: 1) Water

recycling: reduces water consumption and its effluences to environment; 2) Utilisation of dissolved in fish effluences nutrients as fertilisers for plants or algae; 3) Utilization of industrial CO_2 in form of flue-gases which, in addition to promoting biomass production, provides concomitant reduction in atmospheric CO_2; 4) Utilization of industrial waste heat; 5) Conversion of ammonia of fish wastes to the nitrogen compounds (nitrite and nitrate) bioavailable and beneficial for plant; 6) consumption of exsess of oxygen from algae production units by fish; 7) Combination of different biomass and a variety of the target molecules production; 8) In-house source of fish feed (residual of plant biomass or whole biomass in form of pellets).

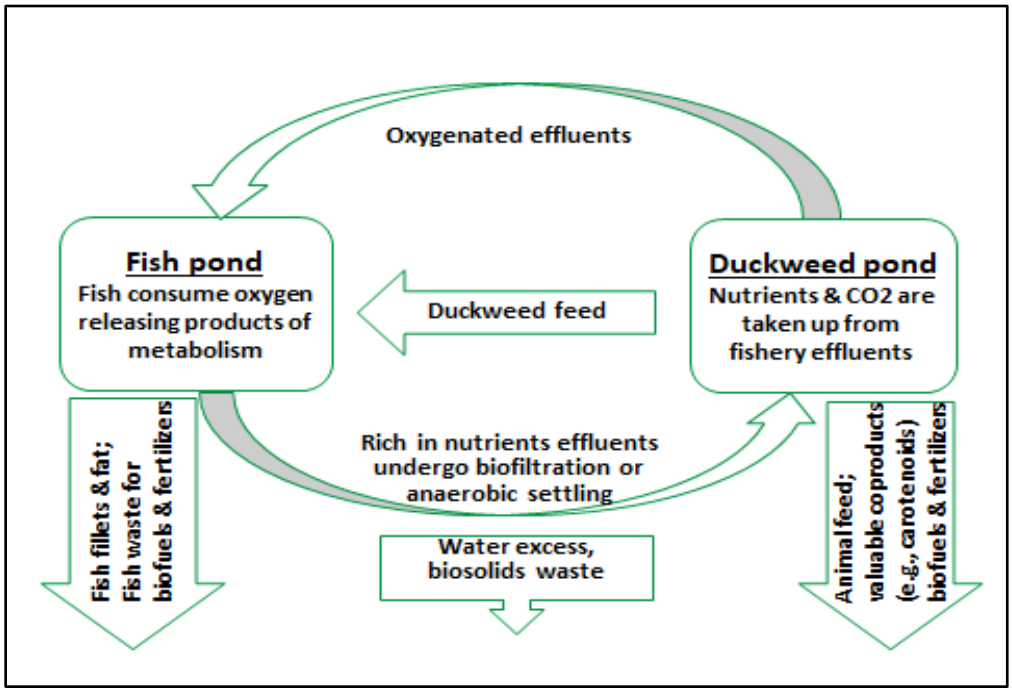


Figure 4. Profitability of an integrated system for duckweed and herbivore/mesocarnivore fish biomass production.

However, aquaponic systems face some challenges, which could be listed as following: a) water reuse may result in the accumulation and biomagnification of fish and/or plant metabolites (e.g., hormones); b) additional investments in bio-filtration may be necessary when plants are produced; c) fish and/or plants may not be treated for disease in the way as they are in single use (fish only or plant only)

production facilities. The second issue is not the case when microalgae production is combined with fish farming.

The use of a combined technology for microalgae biomass production is a completely novel approach. In recent years PhBRs have been used successfully for indoor, industrial scale hydroponic facilities, when reactors were housed within greenhouses, which allowed sharing artificial lighting and heat (Roquette, Germany). In 2011, Air Hydroponic Technology Ltd. of the United Kingdom established the technology of embedding medium-size raceways into greenhouses, passing the hydroponic wastewaters to the raceways for algae production. After the algae are harvested, water enriched with commercial fertilizers is pumped to the hydroponic cultures. For the industrial production of Duckweed, a successful attempt to combine plant and fish (common carp, *Cyprinus carpio*) cultivation was established in Bangladesh (Skillicorn et al., 1993). Comprehensive analyses of plant (Duckweed) and tilapia co-culture technology, and the benefits and bottlenecks of this co-culture system, have been published recently (Basco, 2012; Rosagast, 2012). Clearly, this approach offers significant reduction of an initial investment in the unit development as well as for its operating and maintenance.

The integration of biomass production, especially when water is recycled between the cultures of different origin (algae, plants, fish) brings the likelihood of co-influences of the culture's metabolites, competition for nutrient uptake and other possible competitive or symbiotic character of their interactions. The mechanism of such co-influences has to be carefully studied and taken in account for an accurate prediction of long-term cultures coexisting in an industrial unit.

1.3.3 Tools of integrated production of biomass

1.3.3.1 Wastewaters as a source of nutrients: pros and cons

In microalgae biomass-production research, attention is given to both the use of different types of wastewaters as a cheap source of nutrients, and to using microalgae as a highly efficient method for wastewater remediation. Most of the wastewater treatment plants have an open-pond concept, which

creates a problem of significant release of CO_2 and N_2 produced by bacterial communities (Benemann, 2013). Thus, the use of wastewaters of different origins for the microalgae biomass production not only supplies the algae population with a low-cost nutrient source, but also contributes to wastewater treatment and decreases the release of the gases produced by bacterial communities to the atmosphere. It was shown that microalgae were capable of easily cleaning wastewater with organic loadings of 100-150 kg BOD_5/ha/day while nutrient assimilation rates reached 600 kg CO_2/ha/day, 24 kg N/ha/day, and 3 kg P/ha/day giving biomass productivity up to 30 g/m^2/day (Cragg et al., 2011).

Despite the bright picture of wastewater applications and nutrient recycling for microalgae biomass production, this approach has some challenges. First, there is a high risk of algae culture contamination either by bacterial and planktonic wastewater communities, or by toxic compounds (metals, herbicides, detergents, etc.). Second, bacterial communities in wastewater will compete with the microalgae for nutrients, reducing algae biomass productivity (Benemann, 2013). To resolve these problems, additional wastewater treatments (filtration, sanitation) are necessary prior using the wastewater for algae culture. Additional treatments, however, increase the cost of wastewater application and may eliminate the positive effects of some organic compounds of the wastewaters, such as enzymes and hormones.

There still is a rather scattered knowledge of the influence of organic compounds of wastewaters on microalgae (or plant) physiology. The combined effect of dissolved organic compounds and their products of degradation is even less known. Thus, before to apply a particular wastewater to algae growth unit, scrupulous investigation of the water chemistry must be accomplished to avoid toxic or undesirable for algae biosynthesis effect. For example, high concentrations of animal steroids are toxic to algae and bring a risk of intracellular hormone accumulation. Thus, nanoconcentrations of sex hormones from fishery wastewater have been considered as a safer and cheaper source of hormones compared to those of commercial nature. The use of fishery wastewater for microalgae production has some advantages compared to other agricultural and municipal effluences. This type of wastewater normally has relatively

low chemical oxygen demand (COD), biological oxygen demand (BOD), and turbidity/total suspended solids (TSS) (Rakocy et al., 2005). Effluents from salmonid fish farming also have low HPC (heterotrophic plate counts) due to relatively low organics and low water temperature (4 to 15 ^{o}C). This eases the pretreatment of the wastewaters prior to pumping it to the algae production units.

Thus, the development of effective methods for the improvement of wastewater application to grow microalgae (and plant) biomass for biofuels and bioproducts will enable its use on industrial scale of production (McGinn et al., 2011). Another important point has to be considered for bringing wastewaters application to a feasible level is use of robust to wastewater algae species. For example, microalgae *S. quadricauda* can be characterized as a species which can be successfully grown on different types of wastewater first of all due to high tolerance to change in pH, temperature and nutrients fluctuation (Martinez et al., 1999; Xu et al., 2012) showing good adaptation ability to ambient conditions. Successful implementation of different types of wastewaters for *Scenedesmus sp* scale-up production was demonstrated in several studies (Kim et al., 2007; Hodaifa et al., 2008, 2009; Mata et al., 2013). A cost-effective method for the improved wastewater application to grow *Scenedesmus sp* has been shown to enable the scale-up algae production in the cold Canadian climate (McGinn et al., 2012). Growing on agricultural wastewaters *Scenedesmus* can reach biomass production of 1260 mg/L/day (Goswami, 2011) with specific growth rate up to 0.4/h (Makarevičienė et al., 2011).

1.3.3.2 Other wastes: industrial flue-gas CO_2 and heat

Microalgae and plants naturally sequester CO_2 from the atmosphere during photosynthetic reactions. Algae are capable of fixing several-fold more CO_2 per unit area than trees or crops and, hence, are considered as the greatest producers of oxygen on Earth (Steinman, 2000). However, to support the rapid growth and significant increase in biomass for industrial scale production, additional amounts of CO_2 are necessary (Wang et al., 2011b; Benemann, 2013). Thus, the use of industrial CO_2 emissions as an additive source of carbon can be considered as one of the most promising alterations of the microalgae

biomass production. In addition, this approach has the great potential of reduction of "greenhouse gas" in atmosphere due to negative emitted to consumed gas balance and prolonged period of decomposition of the pharmaceuticals and cosmetics products acquired from such biomass (de Morais and Costa, 2007; Vijayakumar and Menakha, 2015; Santhosh et al., 2016). Industrial flue-gas emissions contain 10-20 % of CO_2 on average (Ho et al., 2011). The tolerance of algae species to flue-gas CO_2 is wide and ranges from about 1-5 % (*Synechocystis sp*) to 80 % (*S. quadricauda*), and even 100 % (*Cyanidium caldarium*, *Chlorella* sp. T-1 – strain), without crucial compromising of their biomass production (Ono and Cuello, 2003; Salih, 2011). However, optimal biomass production rate normally occurs at lower percent of CO_2 application and flue-gas CO_2 influence also depends on the growth medium composition (Salih, 2011, Liu et al., 2013).

Not all the species that have high tolerance to flue-gas have sufficient ability to capture CO_2 and use it for biosynthesis of molecules. The best scenario is when a species keeping high production rate can demonstrate high rate of carbon fixation. For example, *Ch. vulgaris* was reported to achieve biomass production of 1060 mg/L/d and CO_2 fixation rate of 1992 mg/L/d (Zhao and Su, 2014). For Duckweed, elevated CO_2 concentrations have shown to increase their photosynthetic rates (Loats and others, 1981; Campbell and others, 1988; Baker and Allen, 1994) and reduce respiration rates (Baker and Allen, 1994). However, the study of CO_2 influents on Duckweed performance is usually conducted in short term experiments.

In North America the loss of heat per year is estimated at 20-50 % of industrial energy input (USA Department of Energy report, 2011). It is a significant waste of energy and money. This highlights the importance of industrial integration and supports companies focused on waste heat utilization. For algae production indoor facilities in a cold climate, utilization of waste heat is a great potential for cost reduction of biomass production. The modelling work and Life Cycle Assessment (LCA) are an important part of waste heat recycling in algae production (Lardon et al., 2009; Slade and Bauen, 2013).

1.3.3.3 Combined production of algae species

Two types of combined growth of microalgae could be distinguished: two or more species of algae grown in one production unit; a microalgae species with symbiotic bacteria. The purposes of co-culturing for both types are: retrenchment of production space; sharing nutrients and metabolites that could be beneficial to one or all the co-growing species; enhancement of harvesting biomass with combined synthesized by all co-cultured species molecules (Smith et al., 2010; Shurin et al., 2013). There is also opinion that mixed algae species accomplished with a symbiotic bacteria, alike in nature, develop more stable, balanced ecosystem in a production unit and as a result, more robust for contaminations (Novoveska et al., 2016).

For example, it was demonstrated that symbiotic bacteria *Azospirillum brasilense* grown with three strains of one genus *Chlorella* (*Ch. vulgaris* UTEX 2714; *Ch. vulgaris* UTEX 395; *Ch. sorokiniana* UTEX 1602) synergistically affected biomass production, chlorophyll, lutein, violaxanthin and lipid accumulation modifying the FAs profiles of all the tested strains (de-Bashan et al., 2002; Devi et al., 2012). Yet, notable differences in influence of co-culture on growth parameters were observed among the strains (de-Bashan et al., 2002). Importantly, significantly elevated number of valued for biofuel FAs could be harvested form mixed algae biomass (Mohan et al., 2011). The application of mixed algae-bacteria cultures for water treatment is well known technology; yet, recently, the technology has been improved with some novel features such as Microbial Fuel Cell (MFC) method which aimed for both, water treatment enhancement and production of algae-bacterial biomass for biofuels (Huarachi-Olivera et al., 2018).

1.3.3.4 The physiology of co-cultures

1.3.3.4.1 Competition for nutrients

In the case of water recycling between hydroponics and algae production units, the co-influence of vascular plants and algae metabolism has to be taken in account. Vascular plants and algae will compete

for bioavailable nutrients in media. However, the degree and the character of this competition depend on certain circumstances. Microalgae are less depend on dissolved inorganic compounds for photosynthesis than plant (Landolt and Kandeler, 1987). Sequentially, hydroponic (floating) plants are less dependent on the dissolved compounds than terrestrial plants (Ansari and Lanza, 2010). From here, the nutrient removal rate is different between these two groups of photosynthetic organisms when they would grow in the same media.

For algae grown on wastewater, the highest corresponding NH_4–N and PO_4–P removal rates are 2.4 g/m^2/day and 0.3 g/m^2/day (Cragg et al., 2011). Exceptionally high total P removal rate of 70 g/m^2/day was calculated by Dixner, 2013 when mixed phytoplankton of a eutrophic river in China was studied. For vascular plant grown in rich wastewater media the corresponding NH_4–N and PO_4–P removal rates can be as high as 1.3 g/m^2/day and 0.6 g/m^2/day , respectively (Cheng et al., 2002). This data shows difference in nitrogen to phosphorus demand between algae and vascular plant cultures. It gives 70-95 % of nutrients depletion in 3-4 days (Korner and Vermaat, 1998; Smith, 2007), where nitrogen and phosphorus removal ratio is usually 1:3 for algae and 2:1 for Duckweed. The ratio and removal rates can be altered by water physico-chemical parameters as such pH, light, temperature and initial N:P ratio in the water (Borowitzka, 1998; Adamsson, 2000; Larsdotter, 2004).

The preferences in ions of each macro- and micronutrients also differ among the algae, Duckweed and hydroponic plants. Ammonium ion is the preferred form of nitrogen for hydroponics plant (Skillicon et al., 1993). The main source of ammonium for wild colonies of Duckweed is from fermentation of organic material by anaerobic bacteria. Duckweed plants reportedly utilize all available ammonium before beginning to assimilate nitrate, and appear to grow more quickly in the presence of ammonium than with nitrate. In contrast to plant, unicellular algae prefer nitrate (Skillicon et al., 1993).

There are, however, differing opinions on microalgal preferences for nitrogen consumption. Bhaya et al. (2000) stated that ammonium is the preferred compound, and when this is available, no alternative

nitrogen sources are be assimilated. Other authors determined that nitrate uptake is greater by microalgae compared to other nitrogen sources (Halterman and Toetz, 1984). Dortch (1990) proposed the terms “ion preference” and “ion inhibition” and highlighted the strong dependence of both processes (in particular inhibition of nitrate uptake by ammonium) on the physico-chemical parameters of water. This study clearly indicated that although ammonium was the preferred nitrogen source for uptake, growth rates on nitrate usually equalled or exceeded those on ammonium, and emphasized the necessity of modeling work on this subject. Thus, modeling work on nutrient uptake is vital for co-culture production, as differences in ion preferences and the possibility of manipulation by their uptake coupled with the use of ammonium rich fishery wastewaters may provide a rational basis for co-culture technology with respect to ion competition and reduce the cost of production.

1.3.3.4.2 Oxygen

There are two main controversial processes in aqueous environments that can affect oxygen concentrations when plants and algae share the water. A dense crop cover on the surface of water reduces dissolved oxygen in the water column and suppresses nitrifying bacteria. An increase in anaerobic bacteria enhances the denitrification process and swings the nitrogen balance further in favor of ammonium over nitrate (Skillicon et al., 1993). This tends to lower pH as ammonium ions are assimilated by plant.

When algae present in the water column, they assimilate plant metabolites and supply water with oxygen which promotes growth of the bacterial community of the plants roots. The bacteria in turn, break-down organic compounds in the wastewater to bioavailable inorganic ions (Gallert, 2005; Svistoonoff et al., 2013). The first results concerning the interaction of aerobic bacteria with the root system of plant were presented in several reports from Japan. Yamaga et al. (2010) clearly demonstrated growth promotion and metabolic enhancement Duckweed in co-culture with specific species of bacteria. In one case, the signalling compound was identified to be a carbohydrate and was shown to stimulate growth of different Duckweed species as well as some other hydroponic plants.

1.3.3.4.3 Hormones and enzymes co-influence

Aqueous environments facilitate the interactions of metabolites released by co-cultured species. Combinations of phytohormones known to enhance growth and stress tolerance of algae species, but the effect of mixed hormones on microalgae biomass production and accumulation of synthesized molecules is not well studied. This subject will be discussed in details further. Another interesting challenge is the high probability that animal hormones can interact with phytohormones when hydroponics and fishery wastewaters are used as the sources of nutrients for algae and Duckweed production. There is deficiency of data on the subject of interactions of animal and plant hormones in aqueous solutions.

1.3.3.4.4 Co-culturing algae with vascular plants

In natural ponds Duckweed and microalgae interact with the aquatic biota. As in any ecosystem, such interactions can be symbiotic, antagonistic, or synergistic in nature. The production system at Air Hydroponic Technology Ltd. in the UK can be considered a co-culture system between microalgae and vascular plants, since wastewater from hydroponic vegetable tanks loaded with remained nutrients and enriched with plant hormones and enzymes is pumped to microalgae culture raceways. However, this unique system has no, or at best, a weak reverse link from algae to plant, since the water is filtered and then vigorously mixed with commercial fertilizers. This processing likely destroys plant hormones and enzymes, greatly changes physico-chemical parameters of water and also breaks a link of oxygen enrichment by algae metabolism. Usually microalgae are considered as unwelcome competitors in aqueous plant production systems (rice and lotus fields, hydroponics).

However, the possibility of beneficial relationship between plants grown in aqueous environment (hydroponics, rice) and algae has been discussed. For example, in rice agriculture blue-green algae has been considered as a symbiotic organism similar to the nitrification bacteria of soil (Roger and Kulasooriya, 1980; Mishra and Pabbi, 2004).

1.3.4 Modeling work for Integrated Systems

The majority of microalgae and Duckweed research for biofuel and other commodities production has been done at the laboratory scale and lacks validation at industrial scale. The 2-3 times difference in biomass production between the laboratory and industrial production (Skillicon et al., 1993) highlights the need for an accurate prediction of actual biomass productivity of Duckweed on industrial scale.

Because of the cost of implementing the laboratory results on even pilot scale systems, the scale-up of microalgae biofuel production has been estimated by computational modeling. An important part of these models is systems analyses to derive energy inputs and mass balances, in other words, Life Cycle Energy Assessment (LCEA). In recent decades the attention of investigators has been focused on the feasibility of the algae indoor biodiesel production in cold climates, based on LCEA and environmental impact parameters, a concept which allows for the quantification of mass and energy streams such as energy consumption, material usage, waste production, and generation of co-products (Baliga and Powers, 2010). This modeling work was considered as an approach of using greenhouse facilities, flue-gas CO_2 supplement and waste heat in attempt to the scale–up algae biomass production significantly lowering the cost of the indoor PhBR operation. The findings of this work demonstrated lower energy consumption and air emissions for the algae biodiesel production when compared to soy biodiesel under equal growth conditions.

Another trend of developing a model, which is flexible to be used for different types of facilities (open ponds, indoor and outdoor bioreactors), is presented by work of Fernandez et al. (2012). The flexibility of application is one of the advantages of their model. Other advantages are: incorporation into the model of comprehensive biological, ecological (e.g., seasonal changes or stress-induction strategies), chemical (e.g., water chemistry, additional compounds and their metabolism in medium) and physical parameters (e.g., fluid-dynamic, solar radiation) of a production unit ecosystem. A comprehensive model can be developed for a particular production unit with no option to adjust to other units. However, some

of these types of models incorporate a number of parameters for maximum accuracy of prediction and are also designed to predict the outcomes of downstream processes as well as the economics of production (Davis et al., 2016; Del Rio-Chanona et al., 2018).

More simple models, which incorporate a few parameters (Munoz-Tamayo et al., 2013) or developed for one particular facility (Malek et al., 2015), are designed to optimise the production process, usually are more accurate to predict the outcomes of alteration of the incorporated parameters. However, they are not flexible in adjusting to other production units and give a weak prediction of the unit ecosystem in time.

To date, there is a deficiency of data on modeling work for Duckweed biomass and target molecule production. Importantly, for the subject of this report, a model for Duckweed production with agricultural wastewater as a source of nutrients application was developed (Landesman et al., 2005). Another important for industrial production model was developed on the mat-density management (Monett et al., 2007). A significant gap in the microalgae biofuel production is that the majority of the studies have not been based on the detailed engineering designs and validated on the designed industrial units.

1.3.5 The probability of success

According to the Energy Efficiency & Renewable Energy (EERE) division of the United Sates Department of Energy, more than 1 billion gallons per year of cost-competitive algal biofuels can be achieved by 2022, if an algal biofuel intermediate yield of 2,500 gallons of crude algae oil (or equivalent dry weight basis) per acre per year can be harvested by 2019.

With wastewater or/and CO_2 application, the biomass production in open ponds typically lies in the range of 300-380 mg/L/d, but can be as high as to 8 g/L/d (see Tables 1 and 2), while lipids can be enhanced from 30-50 % to 70-80 % of biomass dry weight applying N-starvation (Borowitzka, 1988). In PhBRs and indoor raceways the biomass production is about ten to fifteen times higher than in open ponds due to more stable and easily manageable environment (Gardner et al., 2011). The most successful

companies in US (e.g., Cellana LLC, Sapphire Inc.) have achieved oil production of 1,500 gal/acre/year on average (see Table 2) with the goal to increase the oil production up to 5,000-6,500 gal/acre/year in two to three years. For 1 acre of 1 dm (100 cm) depth, a biomass production of 350 mg/L/d gives 51.7 tons/acre/year (density of crude oil for 20 ºC is $p \approx 900$ kg/m^3). If the oil content is 30 %, it gives 15.5 tons/acre/year, or 17.2 m^3 oil, or 4,544 gal/acre/year. The results of this calculation support the probability of the goal to achieve oil yield as 2,500 gal/acre/year in outdoor facilities (Table 2). The use of cold-adaptive species/strains and different species for different seasons coupled with the use of the cold-climate conditions as an advantage for the biofuel production bring the probability of sucsess in production of microalgae biomass in cold climat to high level (McGinn et al., 2011; Park et al., 2012).

1.4 General goals of the project and the research objectives

1.4.1 General goals

This PhD research project is connected with the goals of Myera Nu-Agrinomics Group Canada Inc and the Canadian Model Aquaculture Farm (CMAF). It is designed to assess the development of new technologies and approaches for scale-up production of microalgae and Duckweed in a cold climate. The general goal of this study was to determine the favorable conditions for enhanced microalgae production for accumulation of specific molecules of interest (neutral lipids and carotenoids). The project was aimed to elucidate an influence of wastewater organic compounds such as hormones on microalgae physiology.

1.4.2 Objectives

From the general goals, the following objectives were addressed in this research: 1) Clarification of the possible influence of aquaponics/hydroponics wastewater components such fish hormones, and phytohormones on the metabolic processes in the selected microalgae *Scenedesmus quadricauda* cultured under the optimal growth conditions; 2) Assessment of influence of waterborne phytohormones Abscisic acid (ABA), 3-Indoleacetic acid (IAA) and two types of brassinosteroids [brassinolide (BL) and 24-epibrassinolide (EBL)] singularly and in some combinations on growth parameters, biomass production,

pigment and lipid accumulation of microalgae *S. quadricauda*; 3) Comparison of influence of waterborne fish steroids 17β-estradiol (E2) and progesterone 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) on growth parameters, biomass production, pigment and lipid accumulation of microalgae *S. quadricauda*; 4) Assessment of influence of waterborne fish steroid estradiol (E2) on growth parameters, biomass production, pigment and protein accumulation of microalgae *S. quadricauda*; and Duckweed *Lemna minor*; and 5) Assessment of influence of the chemistry of a growth medium on a hormone ability to alter physiology of *S. quadricauda*; and *Lemna minor*.

1.4.3 Hypotheses

This PhD research is hypothesis driven, and the following hypotheses were tested:

Hypothesis 1: Phytohormones (ABA, IAA, BL and EBL) will stimulate microalgae growth and biomass production;

Hypothesis 2: Fish steroids (17β-estradiol and 17,20β-P) will stimulate microalgae growth and lipid accumulation;

Hypothesis 3: Two aquatic organisms, microalgae *S. quadricauda* and Duckweed *L. minor*, growth will be positively affected by nano-concentrations of 17β-estradiol;

Hypothesis 4: Microalgae *S. quadricauda* and Duckweed *L. minor*, will have significantly greater growth and valuable molecules accumulation in natural fish wastewater (FWW) with or without 17β-estradiol presence compared to synthetically reconstituted fish wastewater (RFWW) or standard Bold's Basal Medium (BMM).

Chapter 2

Methods and Experimental Approaches

2.0 General methodological approach

In this study, scrupulous attention was given to the relevancy of the analytical methods completing all appropriate quality assurance (QA) and quality control (QC) standards. After the live culture materials (algae and Duckweed species) were delivered to the laboratory, they underwent the acclimatisation period. The microalgae were grown on BBM medium for a minimum one month before they were used for all the tests under optimised growth conditions. After the acclimatisation period, microalgae and Duckweed species were assessed for health criteria identified by Environment Canada (Biological Test Method, EC, EPS1/RM/25, 2007) prior to the tests. All the analytical instruments that were used for this study were calibrated and internal/external standards and reference solutions were employed for quality control. Sufficient data with adequate amount of replicates (minimum four biological and minimum six technical) were collected in all the trials for comprehensive statistical analysis. Prior to the start the experimental work, key-protocols were created on the basis of appropriate Standard Methods to insure precision and consistency of the conducted assays.

2.1 Materials and Methods

2.1.1 Strain and culturing conditions

Scenedesmus quadricauda (CPCC-158) and *Lemna minor* (CPCC-140) were obtained from the Canadian Phycological Culture Centre (CPCC; Waterloo, Ontario). *S. quadricauda* was chosen because it is known to grow well on wastewaters, and exhibits high biomass, lipid, and carotenoid production (Kim et al., 2007; Dev Goswami, 2011; Mata et al., 2013). *L. minor* was selected as a model plant in this study due to its high growth rate, and its ability to accumulate high concentrations of protein, carotenoids, and lipids (Leng et al., 1995; Landolt and Kandeler, 1987; Guimaraes et al., 2012).

A stock culture of algae was grown aseptically in standard Bold's Basal Medium (BBM) (Stein, 1973) with some modifications (NH_4Cl was added to 4.5 ppm) for at least two months prior to the experiment at 23 ^oC, pH 6.9 ± 0.1, with a Plant Growing fluorescent Lamp at 5000 lux (100 μmol/m^2/s) measured with a CalRight CI-1010 digital lux-meter (CalRight Instrument Inc.). Stock cultures of *S. quadricauda* and *L. minor* were maintained in 500 mL flasks agitated at 95 rpm in a chamber shaker (New Brunswick, Innova-44R) (Figure 5).

Prior to the experiments with altered ambient conditions (e.g., lighting in walk-in growth chamber, aeration) all cultures were acclimated to the experimental conditions for minimum one month. Before starting the experimental work, the status of the cultures was checked using the health criteria identified by Environment Canada (Biological Test Methods, EC EPS1/RM/25, 2007). This ensures the quality and relevance of the obtained data. All experimental trials with phytohormones, and the 20 days steroid hormone experiments were conducted in 250 mL flasks with 150 mL of culture in each, using the same growth conditions and growth chamber as the stock cultures where no aeration of the medium was provided for either the stock cultures or experimental flasks (Figure 5).

However, in the 11 days 17β–estradiol (E2) experiment, aeration was applied aseptically to 250 mL beakers containing *S. quadricauda* and *L. minor* (Figure 6A & 6B). The air bubbled through the cultures was dispersed through the plastic discs with 2 mm pores situated at the bottom of the culture flasks (Figure 6A). This experiment was conducted in a walk-in growth chamber with controlled lighting (Plant Growing fluorescent Lamp at 4000 lux (80 μmol/m^2/s) and temperature (23 ^oC ± 1) (Figure 6A and 6B). The seeding density of *S. quadricauda* cells in all trials was 6.0 ± 1.0 x 10^4 cell/mL. The initial density of *L. minor* culture was ten plants with 3 fronds on each.

Figure 5. The experimental set-ups for algae trials in all phytohormones and 20 days steroids 17β-estradiol (E2) and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) experiments (photo by Kozlova T.A).

A **B**

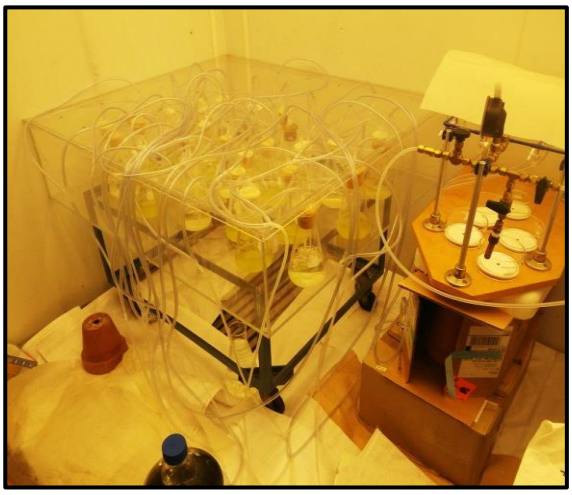


Figure 6. The experimental set-up for duckweed (A) and algae (B) grown in a walk-in chamber with aeration and flow control manifold (photo by Kozlova T.A).

2.1.2 Water chemistry

Monitoring of general water chemistry and ambient conditions was performed daily: temperature, pH, and conductivity were measured using an Orion pH-meter 420 (ThermoFisher Scientific Ltd). The composition of dissolved nutrients (phosphate, nitrate, nitrite, ammonium ions) and their depletion was

monitored using a HACH DR-900 colorimeter (HACH Co., Canada). Analysis of water ions (Ca, Mg, K, P, metals) was conducted by inductively coupled plasma mass spectrometry (ICP-MS) at the Manitoba Chemical Analysis Laboratory (MCAL) of the University of Manitoba. Water samples for ions analysis were acidified with 16N HNO_3 (1 % acidification).

2.1.3 The range of hormone concentrations and preparation of working solutions

The range of hormone concentrations used in each experiment was chosen based on the reported concentrations of each compound in surface and ground water in North America, as well as in aquaponics and hydroponic wastewaters of the same region (Hala et al., 2012; Beecher, 2013; Tate et al., 2013; Mota et al., 2014). All the tested hormones were purchased from Sigma Aldrich Inc. (Oakville, ON). A control-blank (no hormone) was assessed in each trial.

All stock solutions for the tested hormones were prepared in dimethyl sulfoxide (DMSO), except for E2, which is water-soluble. The working solutions for the tests were prepared in the modified BBM. The influence of the solvent, DMSO, on cell growth and other physiological parameters was evaluated using the following equation proposed in this study (see Table S1 in the Supplementary Matherials):

$$N = Xi – (Xs - Xc), \quad (1)$$

where N = final cell density, Xi = cell density in a hormone-solvent trial, Xs = cell density in a solvent only trial, and Xc = cell density in the control of the DMSO trial.

2.1.3.1 The ranges of single phytohormones concentrations

The ranges of hormone concentrations tested were: EBL - 0.5, 5, 10, 50, and 100 nM; BL - 0.5, 5, 50, 100, and 300 nM; IAA - 1, 5, 10, 50, 100, and 1000 nM; ABA - 5, 10, 50, 100, and 500 μM (Table 6).

Table 6. The ranges of the tested hormone concentrations.

Hormone	Concentrations tested
Single hormones trials	
EBL	0, 0.5, 5, 10, 50, 100 nM
BL	0, 0.5, 5, 50, 100, 300 nM
IAA	0, 1, 5, 10, 50, 100, 1000 nM
ABA	0, 5, 10, 50, 100, 500 nM
E2	0, 5, 15, 50, 100, 300 ng/L*
17,20β–P	0, 1, 5, 15, 50, 100, 500, 1000 ng/L*
Mixed trials	
0.5 EBL	+ 5, +100, 1000 IAA nM
2 EBL	+ 5, +100 IAA nM

Note: The unit ng/L was used in the steroids trials because this unit usually can be seen in the literature cites we used for comparison of the results.

2.1.3.2 The range of mixed phytohormone concentrations

Based on the theory of one-way communication between brassinosteroid hormones (BRs), like 24-epi-brassinolide (EBL) and Auxins, like Indole-3-acetic Acid (IAA) (Davies, 2013; Nakamura et al., 2003), EBL was used as a matrix hormone in this study. The two matrix concentrations of the EBL, 0.5 and 2 nM, were chosen accordingly to the reported optimal concentrations of this hormone in previous studies (Bajguz and Piotrowska-Niczyporuk, 2013; Nakamura et al., 2003). A range of IAA concentrations (5, 100, and 1000 nM) was mixed with 0.5 nM matrix EBL concentration, and 5 and 100 nM IAA were mixed with 2 nM matrix EBL (Table 6). The IAA concentrations were within the range of the hormone concentrations tested in single phytohormone experiments, which has been published (Kozlova et al., 2017). All the mixed EBL and IAA concentrations were also tested singularly as the

controls. The single phytohormone trials (reported in Chapter 3) were conducted simultaneously with the mixed hormone trials (reported in Chapter 4). The influence of the solvent (DMSO) on cell growth and other physiological parameters was taken in account as previously described (Kozlova et al., 2017).

2.1.3.3 The range of E2 and 17,20β-P concentrations

The range of E2 was chosen accordingly to the previously reported steroid loading in waste- and surface-waters (Beecher, 2013) and the reported impact of E2 on vascular plants and algae (Lai et al., 2002; Beecher, 2013). The range of 17,20β-P was wider, taking in account elevated 17,20β-P concentration in surface waters compared with the concentrations of E2 (Hala et al., 2012; Mota et al., 2014; Esteban et al., 2014). The range of E2 concentrations tested was 5, 15, 50, 100, and 300 ng/L. The range of 17,20β-P concentrations tested was 1, 5, 15, 50, 100, 500, and 1000 ng/L (Table 6). The working solutions for the test were prepared in natural fish wastewater (FWW). The same hormone concentrations were tested at the same time on synthetically reconstituted fish wastewater (RFWW), on FWW, and on BBM.

2.1.4 Sampling and Analytical methods

2.1.4.1 Algae cell counting

Cell density was determined for each culture by counting individual cells with a Bright-Line haemacytometer (Hausser Scientific) using a Nikon-Eclipse-Tι microscope (Nikon Inc.), taking 7 to 10 technical replicates. Cell size was assessed using the NIS-Elements-D3.1 software program of the microscope. Damaged cells were visualized by observation under Nikon-Eclipse-Tι microscope. Tose cells in which cytoplasma was shrinked or shifted to one side of a cell for about 50 % or more.

As the seeding density in each trial could not be absolutely identical, the proportional rate of increase between each time point was used to assess cell growth, using the following formula:

$$N_a = (N_t - N_o)/t, \quad (2)$$

where N_a = the calculated cell density, N_o = initial cell density, and N_t = cell density on day t.

2.1.4.2 Assessment of biomass production

To determine total dry cell weight (dcw), a 20 mL aliquot of well shaken culture was taken at the end of experiment from each flask, washed with 0.9 % NaCl and oven-dried (Zhu and Lee, 1997). The cells were pelleted using a Thermo Scientific^{TM} Sorvall^{TM} RC 6 Plus centrifuge at 4500 rpm (2,000 x g) for 15 minutes (min.).

2.1.4.3 Assessment of chlorophyll-a and total carotenoids concentrations

To determine concentrations of chlorophyll-a and carotenoids, samples were collected according to the sampling schedule, placed on ice, centrifuged, resuspended in 99.8 % methanol (Sigma Aldrich Inc.) and stored in the dark for a minimum of 24 hours. Aliquots of each extract were transferred into wells of a 96 well plate and measured for optical density using a microplate spectrophotometer (BioTek Synergy 4-Hybrid). Chlorophyll-a and total carotenoids were calculated using the method for methanol extraction as described by Eaton and Franson (2005). Chlorophyll and carotenoid concentrations per cell (ng/cell) were calculated using the following equation:

$$X = C/N*1000, \quad (3)$$

where X = pigment concentration per cell in ng/cell, C = pigment concentration in μg/mL of extract, and N = number of cells per mL.

2.1.4.4 Analyses of fatty acids

Analyses of fatty acids (FAs) synthesized by *S. quadricauda* were performed at the end of each trial. Algal biomass was washed with 0.9 NaCl and freeze-dried in a Labconco FreeZone-6 freeze-drier. Dried algal cells were first mechanically homogenized using a cell grinder, and then digested in chloroform spiked with internal standard and 15 % H_2SO_4 in methanol (1:1) solution (modified method from Folch et al., 1957) followed by the trans-esterification reaction. The total lipid fatty acid profiles where determined by analyzing Fatty Acid Methyl Esters (FAMEs) by Gas-Chromatography (Agilent-6890N-GC). The FAME peaks were identified against a Standard (Supelco 37-compounds FAMEs,

SigmaAldrich).

Calculation of FA concentrations in mg/g lipid was accomplished following the method described by Wrolstad et al. 2005:

$$C = (M^{IS} * PA^{FA} * RF / PA^{IS} * M^{DS}) * 1000, \quad (4)$$

where C = concentration of FA in mg/g lipid extracted; M^{IS} = mass of Internal Standard; PA^{FA} = peak area of a fatty acid; RF = response factor of a fatty acid; PA^{IS} = peak area of the appropriate Internal Standard; and M^{DS} = mass of digested algae sample.

Concentrations of FAs were classified as major FAs (> 3 % of total FAs), minor FAs (1-3 % total FAs), and Minimal (Min) FAs (< 1 % total FAs). Min FAs were calculated empirically from the percent and actual concentrations of major FAs, as:

$$\text{Min (mg/g lipid)} = \text{Major (mg/g lipid)} * \text{Min (\%)} / \text{Major (\%)} \quad (5)$$

2.1.4.5 Duckweed assessment

Growth parameters of *L. minor* were assessed according to the methods EPS 1/RM/37 (2014) and ISO/FDIS/20079 described by Environmental Canada. Minor adjustment was accomplished for roots growth evaluation taking in account the method described by Greenberg et al. (1992). Soluble proteins of dry Duckweed biomass were assessed with Bradford assay.

2.2 Statistical analyses

To determine the statistical relevance of the data, all trials were conducted using four to five independent biological replicates (n = 4 or 5) and 5 to 15 technical replicates depending on the analytical method to be used. The collected data was analyzed for significant differences ($P < 0.05$) against the control-blank (zero hormone concentration) using the Analysis of Variance test (ANOVA). The significance of measured differences in cell size and biomass was assessed with Mann-Whitney Rank Sum Test using SigmaStat software (version 3.5, Systat Software, Inc.). Isobolographic analysis (Chou, 2006; Tallarida, 2011) was used for the assessment of the mixed hormones interactions. This method takes

in account median dose-effect to determine the Combination Index (CI), which determines whether the relationship between two mixed hormones are synergistic (CI < 1), antagonistic (CI > 1) or additive (CI = 1) (Chou and Talalay, 1984). Unlike the original method proposed by Chou (2006), we took into account the statistical difference of the effect of mixed hormones from the effects of the single hormones mixed. The degree of increase for synergistic relationships was calculated as following:

$$N = M/\text{Average}(H1:H2), \quad (6)$$

where N = the degree of increase, M = mixed trial influence, H1 = influence of the first hormone mixed, and H2 = influence of the second hormone mixed. An explanation of how the calculations of the dose-effect relationships for single versus mixed compounds were used to determine the CI are provided in Tables S1, S2 and S3, and Figures S2 and S3, in the Supplementary Materials.

Chapter 3

Effect of Phytohormones on Growth and Accumulation of Pigments and Fatty Acids in the Microalgae *Scenedesmus quadricauda* (CPCC-158)

3.0 Abstract

The phytohormones abscisic acid (ABA), 24-epibrassinolide (EBL), brassinolide (BL), and 3-indoleacetic acid (IAA), at concentrations relevant to that in hydroponic wastewater, were investigated for their physiological effects on the microalga *Scenedesmus quadricauda*. All phytohormones tested had positive stimulatory effects on *S. quadricauda* cell growth, biomass production, as well as on intracellular concentrations of chlorophyll-a, carotenoid, and lipid biosynthesis. The most powerful inducers of chlorophyll-a and carotenoid biosynthesis were EBL and IAA, while the phytohormone effect on fatty acids biosynthesis followed the order: IAA ≥ ABA > EBL > BL. Both the quantities of fatty acids and their profiles depended on the phytohormone type and the specific concentrations tested. All tested phytohormones altered *S. quadricauda* cell size and positive or negative effects were dose-dependent. To our knowledge, this is the first report that systematically compares the influence of four phytohormones, including two types of brassinosteroids, on a broad-range of *S. quadricauda* physiological parameters.

3.1 Introduction

One of the most promising ways to improve the economic viability of the microalgae industry is to reduce the costs of production by maximizing energy and nutrient recycling. This can be accomplished via application of industrial flue-gas carbon dioxide and nutrient-rich wastewaters to support microalgal growth, and simultaneously offering a significant reduction of environmental emissions.

Growing interest in the use of wastewaters of different origins for industrial production of microalgal biomass, for biofuels and bioproducts, has stimulated studies on the influence of wastewater compounds on microalgal growth and physiology. Several studies have demonstrated that some wastewaters are capable of significantly increasing microalgal biomass production (Kim et al., 2007;

Kong et al., 2010; Dalrymple et al., 2013), as well as biosynthesis of intracellular lipids (Kim et al., 2007; McGinn et al., 2012; Han et al., 2016) and carotenoids (Kim et al., 2007; Kang et al., 2006; Safafar et al., 2015). Some inorganic compounds of wastewaters, such as iron and other microelements (Fabregas et al., 2003; Naito et al., 2005; Cai et al., 2009) and the carbon:nitrogen (C:N) ratio specific to the growth conditions (Kang et al., 2006; Geider and La Roche, 2002; Silaban et al., 2014) derived from both inorganic and organic molecules in water, have been found to enhance microalgal biomass production and synthesis of valuable bio-products. Numerous investigations have also highlighted the important role of organic compounds dissolved in wastewater or added to growth media for microalgal biomass production (Yang et al., 2000; Bumbak et al., 2011; Sun et al., 2016). Particularly, phyto- and animal hormones, which may be found in both surface water and wastewaters, have gained attention of investigators (Bajguz and Czerpak, 1998; Bajguz, 2003; Jobling and Owen, 2003; Park et al., 2013; Nobel et al., 2014).

Microalgae and aquatic plants produce phytohormones (Tsavkelova et al., 2006; Tarakhovskaya et al., 2007; Kiseleva et al., 2012; Stirk et al., 2013) that efflux in trace concentrations into aqueous environments (Nobel et al., 2014; Hirsch et al., 1989). Agricultural wastewaters, such as those derived from hydroponic cultivation in greenhouses and agricultural run-off from crops treated with plant growth substances or hormone-herbicides (Khripach et al., 2000; Cobb, 1992; Grossmann, 2007) can bear significantly high concentrations of phytohormones, reaching hundreds of μM (Dalrymple et al., 2013; Lopez et al., 2005; Tate et al., 2013).

Phytohormones regulate aspects of plant growth and development, as well as environmental stress responses (Davies, 2004; Vidhyasekaran, 2015). Importantly, all phytohormones are effective in the low nanomolar concentrations (Davies, 2004). It is well-established that the most important hormones in vascular plants are auxins [Indole-3-acetic acid (IAA) is the most abundant auxin], abscisic acid (ABA), brassinosteroids (BRs), gibberellin (GA), cytokinins, and ethylene. ABA, in particular, is an important two-way growth regulator, adjusting plant growth to environmental conditions such as cold or hot

weather, water deficit, the impact of pathogens, etc. (Davies, 2004; Schroeder et al., 2001; Pastori and Foyer, 2002; Tuteja, 2007). In microalgae, ABA has been found to increase biomass production, carotenogenesis, and lipid biosynthesis (Park et al., 2013; Nobel et al., 2014; Cowan and Rose, 1991) in *Chlamydomonas reinhardtii, Euglena gracilis,* and *Dunaliella salina*.

In vascular plants, auxins regulate cell growth and elongation, as well as tissue and organ differentiation (Davies, 2004; Ludwig-Muller, 2011). IAA was tested on different algae species including *Euglena gracilis Klebs* (Nobel et al., 2014), *Chlamydomonas reinhardtii* growth (Park et al., 2013), *Chlorella pyrenoidosa* (Czerpak et al., 1994, 1999; Liu et al., 2016), and *S. quadricauda* (Liu et al., 2016). The results of these studies agree on the positive effects of IAA on microalgae cell division, biomass production, and pigment biosynthesis. Remarkably, IAA was shown to increase algae cell size (Park et al., 2013; Nobel et al., 2014) and protein content (Park et al., 2013).

In contrast, there are few reports in the literature on the influence of brassinosteroids on microalgae productivity. Brassinolide and 24-epi-brassinolide, the two key brassinosteroids, were shown to work synergistically with IAA to elongate cells and promote tissue differentiation in vascular plants. Moreover, like ABA, brassinosteroids work to protect plant tissues from environmental stresses (Clouse and Sasse, 1998; Cano-Delgado et al., 2004) and pathogens (Vidhyasekaran, 2015). In *Chlorella vulgaris*, brassinosteroids have been shown to increase biomass production (Bajguz, 2003), cell growth, chlorophyll and monosaccharide production (Bajguz and Czerpak, 1998), and protein content (Bajguz, 2003).

The ability of phytohormones to promote microalgae growth and biosynthesis is dose dependent. The most effective concentrations of phytohormones vary somewhat in the literature. For example, IAA was reported to be most effective at concentrations between 10^{-9} and 10^{-4} M by several authors (Park et al., 2013; Czerpak et al., 1994, 1999; Piotrowska et al., 2008), including one of pioneering papers of Pratt (1938). However, Nobel et al. (2014) observed an inhibitory effect of IAA at concentrations of 10^{-7} M and higher. Possible explanation of such differences lies in morphological and physiological differences

between microalgae species and in dissimilarities in algae growth conditions.

Thus, despite the recent findings on the positive influence of phytohormones on microalgae biomass production and synthesis of valuable biomolecules, there are several gaps in our understanding of the impact of phytohormones on various features of microalgae physiology. Importantly for microalgae industry, there is little data available to date on the effect of phytohormones on the composition of fatty acids in microalgal cells. Moreover, the investigation of phytohormonal influence on microalgal physiology is relevant to the use of hydroponic wastewaters for microalgal biomass production.

S. quadricauda, a species of interest for biofuel (McGinn et al., 2012; Dev Goswami, 2011; Peng et al., 2012) and industrial carotenoid (lutein, β-carotene) production (Kim et al., 2007; Cordero et al., 2011), was chosen for this investigation. Influence of four phytohormones: 24-epi-brassinolide (EBL); brassinolide (BL); Indole-3-acetic acid (IAA) and abscisic acid (ABA) at concentrations relevant to those in hydroponic wastewater on growth parameters and physiology of microalgae was investigated. Influence of phytohormones on microalgae was evaluated comparing cell growth and biomass production, cell size, chlorophyll-a, total carotenoid, fatty acids of total lipid production, and the fatty acid profiles.

3.2 Results

3.2.1 Effect of phytohormones on *S. quadricauda* growth, biomass production and cell size

3.2.1.1 Algae growth and the solvent influence

Two sets of experiments to evaluate the influence of ABA on microalgal physiology were conducted simultaneously. The first set included a series of ABA concentrations where the hormone was first dissolved in DMSO and then diluted with BBM. The second set included a series of appropriate DMSO concentrations calculated from the first experiment. The results of the first series demonstrated some significant differences in *S. quadricauda* growth in 5 to 50 μM ABA dissolved in DMSO in comparison to the control (Figure S1A in the Supplimentary Materials). However, the assessment of DMSO influence on microalgal physiology also demonstrated some stimulation of *S. quadricauda* growth (Figure S1B).

After subtracting the solvent co-influence, a positive effect of ABA concentrations on *S. quadricauda* growth was confirmed at concentrations of 5 μM and 50 μM (Figure S1C). At the end of the experiment, *S. quadricauda* cell density was 1.6-fold greater in the 5 μM ABA trial compared with the control. No influence of the solvent was found in the EBL, BL, and IAA experiments, except at the highest solvent concentration of 0.1 % DMSO in the 1 μM IAA trial, where the influence of the solvent was also substracted (data before substraction is not shown).

Both EBL and BL demonstrated promotion of *S. quadricauda* growth (Figures 7A and 7B). However, brassinolides showed some dissimilarities in the influence. In the EBL experiment concentrations of 50 nM and lower demonstrated clear stimulation of the algal growth, while 100 nM showed a minor initial stimulation of growth, followed by significant effect after 15 days.

In the BL experiment, the lowest concentration tested (0.5 nM) had no effect on cell growth, and concentrations of 100 and 300 nM had either no effect or an inhibitory effect after 15 days. The order of efficacy in the EBL trial was $50 \geq 10 > 5 > 0.5$ nM while the order of eficacy in the BL trial was and $50 > 5$ nM. In the range of concentrations tested, auxin (IAA) was found to be the most effective stimulator of *S. quadricauda* growth (Figure 7C). Most of the tested auxin concentrations, excluding the lowest (1 nM) and the highest (1 μM), stimulated cell division. The greatest effect on cell growth was at 100 nM IAA, which increased cell density by 2.3-fold after 10 days. The order of efficacy for the effective IAA concentrations was $100 > 50 > 10 > 5$ nM. ABA demonstrated the weaker ability to increase *S. quadricauda* growth (Figure 7D).

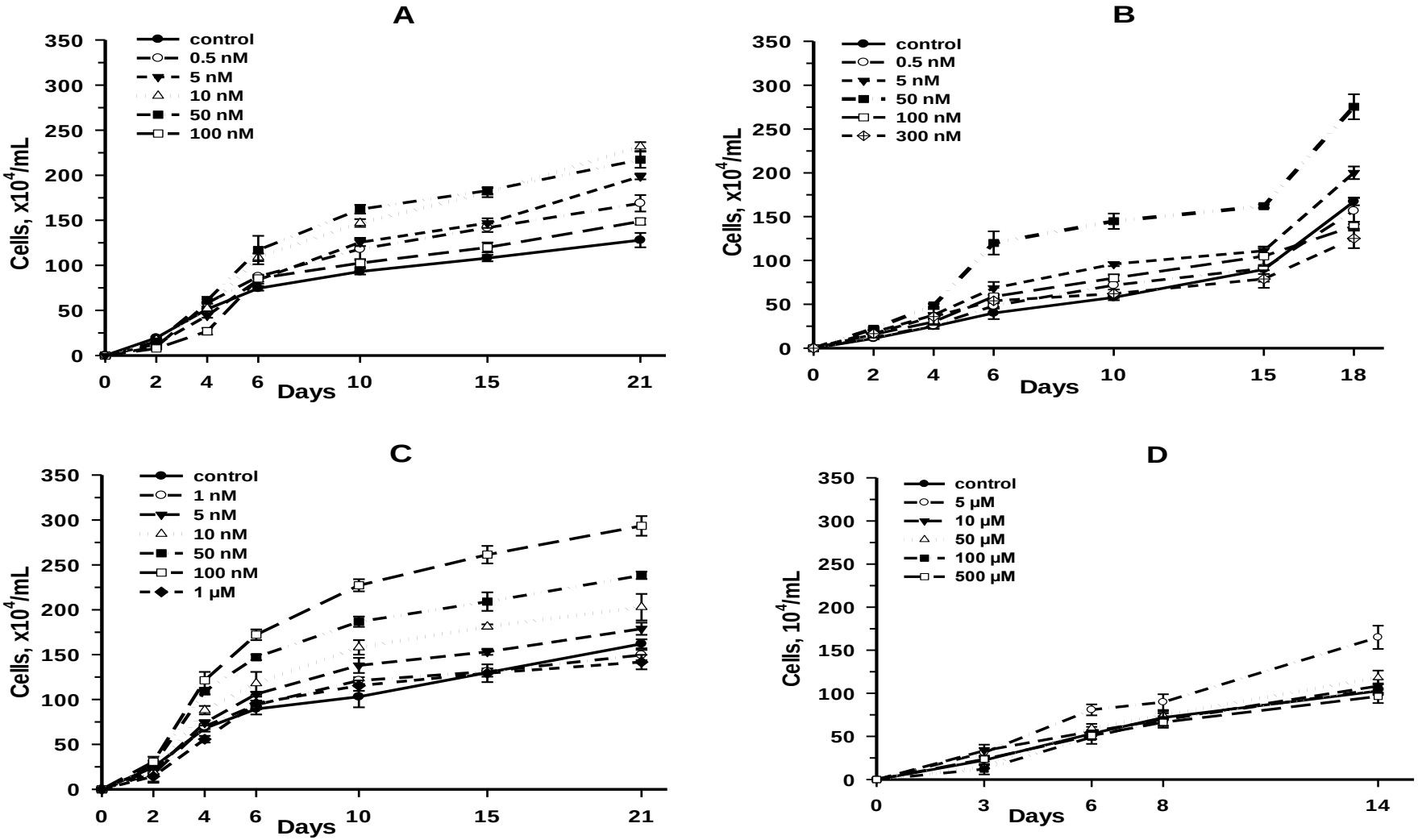


Figure 7. Growth of *S. quadricauda* in phytohormones: A) EBL; B) BL; C) IAA, and D) ABA trials. Data shown as the mean ± SD, n = 12.

3.2.1.2 Relationship between cell size, biomass and cell density

All the phytohormones tested demonstrated a significant effect on *S. quadricauda* cell size and biomass production (Table 7). However some differences in the extent of the influence were observed. The two brassinosteroids, EBL and BL, stimulated an increase in *S. quadricauda* cell size. The effect was observed only at 5 nM for BL, but was seen at most of the tested concentrations for EBL. At 50 nM and higher for BL and at 100 nM for EBL reduced cell size was observed.

Both brassinosteroids increased biomass production. BL stimulated an increase in *S. quadricauda* biomass production at 5, 50, and 100 nM, while EBL stimulated increased biomass production at all concentrations tested. IAA stimulated an increase in cell size at 5 and 10 nM only, while biomass production was increased at all six concentrations tested (Table 7). Unlike the other phytohormones tested, ABA negatively affected cell size at concentrations of 50, 100, and 500 μM with no-effect at 5 and 10 μM. Biomass production was stimulated by ABA at concentrations of 5, 10, and 50 μM resulting in 1.67-, 1.26-, and 1.27-fold greater production, respectively, compared with the control. An adverse effect on biomass production was observed at high (100 and 500 μM) ABA concentrations (Table 7). DMSO did not have any influence on *S. quadricauda* cell size.

Table 7. *S. quadricauda* cell size (µm), biomass (g dcw/L), and cell density (n x 10^4/mL) at the termination of each experiment.

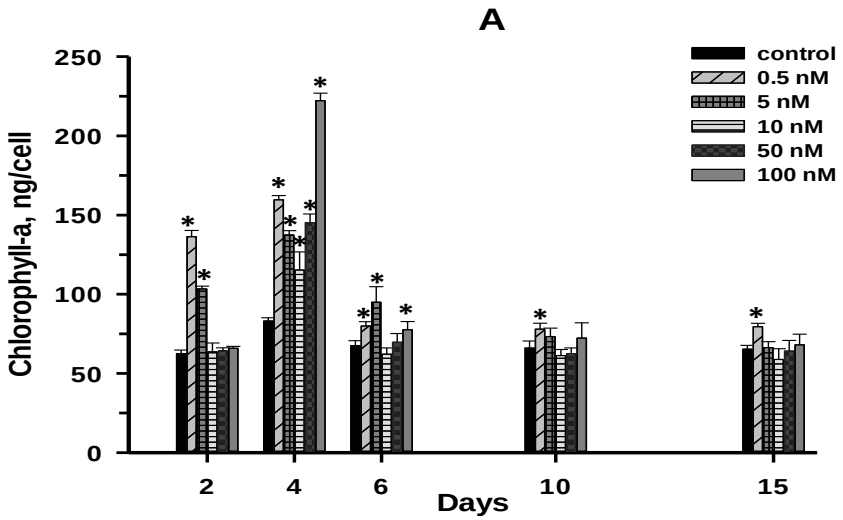
Hormone concentration	Cell size (µm)	Biomass (g dcw/L)	Cell density (x 10^4/mL)
EBL trial			
control	42.16 ± 0.81	0.47 ± 0.03	127.97 ± 8.03
0.5 nM	50.82 ± 2.31*	0.60 ± 0.06*	168.93 ± 5 .11*
5 nM	58.14 ± 0.35*	0.97 ± 0.11*	198.74 ± 3.28*
10 nM	45.05 ± 1.05*	1.17 ± 0.09*	231.86 ± 4.93*
50 nM	44.23 ± 0.85*	0.81 ± 0.13*	217.26 ± 8.93*
100 nM	38.82 ± 0.49$^+$	0.6 9 ± 0.05*	148.67 ± 3.47*
BL trial			
control	41.96 ± 1.75	0.44 ± 0.03	167.23 ± 4.15
0.5 nM	45.72 ± 1.07	0.48 ± 0.05	156.66 ± 15.22
5 nM	53.75 ± 1.02*	0.61 ± 0.06*	200.02 ± 7.22*
50 nM	38.44 ± 0.66$^+$	0.75 ± 0.08*	275.41 ± 1.25*
100 nM	38.82 ± 0.49$^+$	0.59 ± 0.05*	139.12 ± 5.11$^+$
300 nM	36.02 ± 0.75$^+$	0.50 ± 0.07	125.11 ± 11.05$^+$
IAA trial			
control	43.02 ± 2.25	0.47 ± 0.05	162.09 ± 4.90
1 nM	44.24 ± 1.33	0.59 ± 0.06	149.53 ± 5.92
5 nM	49.02 ± 1.43*	0.70 ± 0.07*	178.92 ± 6.78*
10 nM	49.85 ± 2.11*	0.80 ± 0.05*	202.91 ± 3.75*
50 nM	48.05 ± 2.31*	0.76 ± 0.05*	238.40 ± 4.16*
100 nM	45.44 ± 2.16	0.70 ± 0.04*	293.40 ± 10.93*
1 µM	44.12 ± 1.48	0.68 ± 0.05*	141.67 ± 8.02$^+$
ABA trial			
control	45.36 ± 6.14	0.46 ± 0.04	102.68 ± 3.24
5 µM	46.77 ± 5.26	0.77 ± 0.04*	165.03 ± 13.5*
10 µM	43.65 ± 4.73	0.58 ± 0.03*	108.45 ± 7.68
50 µM	35.12 ± 2.31$^+$	0.59 ± 0.05*	116.30 ± 8.05*
100 µM	32.45 ± 3.07$^+$	0.37 ± 0.02$^+$	104.77 ± 6.75
500 µM	28.34 ± 2.44$^+$	0.33 ± 0.04$^+$	96.39 ± 7.70

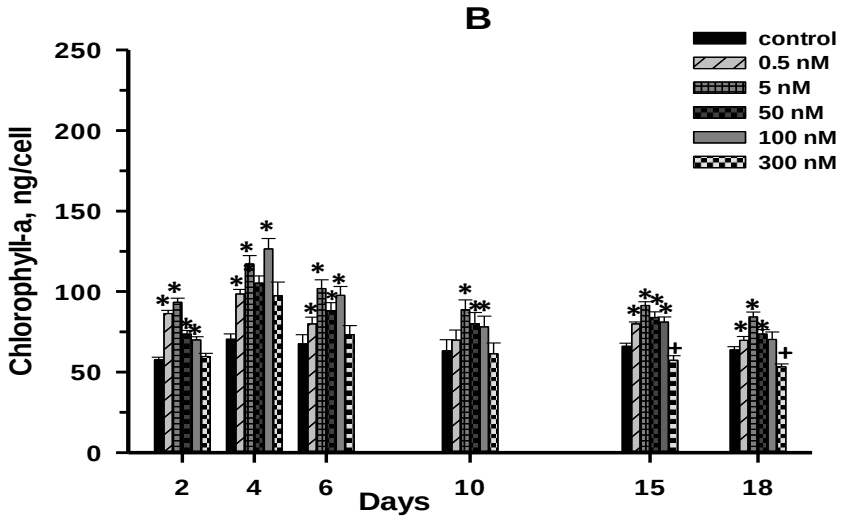
Note: * - significantly greater than control ($P < 0.05$); $^{+}$ - significantly lower than control ($P < 0.05$). Data shown as the mean ± SD (for cell size n = 15; for biomass n = 4; for cell density n = 12).

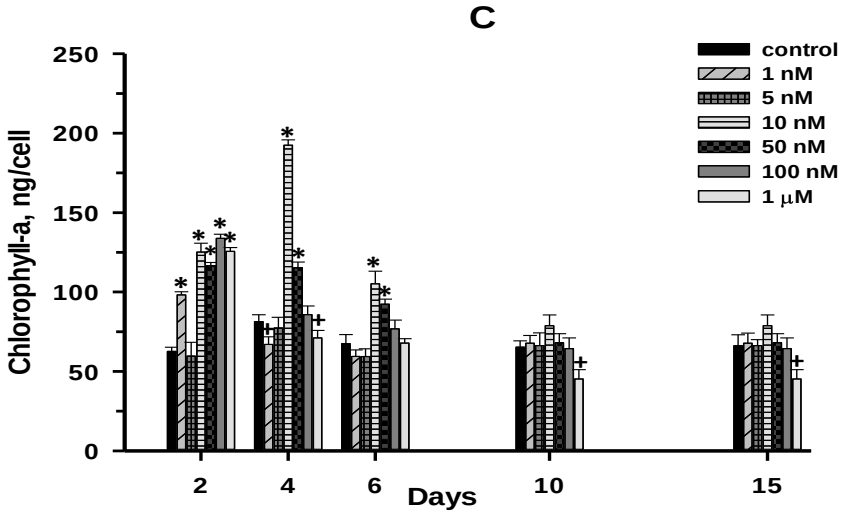
3.2.2 Effect of phytohormone type and concentrations on *S. quadricauda* pigments production

3.2.2.1 Chlorophyll-a accumulation

Chlorophyll-a concentrations per cell were significantly increased in *S. quadicauda* by all phytohormones tested. Chlorophyll-a concentrations increased within two to four days (Figure 8). EBL and IAA had the greatest effect on chlorophyll-a biosynthesis, with 2.5- and 2.3-fold increases, respectively, in chlorophyll-a concentrations compared with the control were observed (Figures 8A and 8C). However, by day six of the experiments, the stimulatory effect of all tested hormones was fully, or almost fully, mitigated in all the trials, except the BL trial, where most of the tested concentrations were effective until the end of the experiment (Figure 8B). The order of efficacy for stimulation of chlorophyll-a by each phytohormone was: 0.5 > 5 > 100 > 50 > 10 nM for EBL; 5 > 100 > 0.5 > 50 > 300 nM for BL; 10 > 50 > 100 nM ≈ 1 μM > 1 nM for IAA; and 10 > 5 > 50 μM for ABA (Figure 8). Adverse effects on chlorophyll-a production were observed at different concentration for different phytohormones: 300 nM for BL; 1 nM and 1 μM for IAA; and 100 and 500 μM for ABA (Figures 8B, 8C, and 8D). In the controls, chlorophyll-a production was slightly elevated between day four and day six in all the experiments (Figures 8A and 8C).


A
Chlorophyll-a, ng/cell
250
200
150
100
50
0
2
4
6
10
15
Days
control
0.5 nM
5 nM
10 nM
50 nM
100 nM


B
Chlorophyll-a, ng/cell
250
200
150
100
50
0
2
4
6
10
15
18
Days
control
0.5 nM
5 nM
50 nM
100 nM
300 nM


C
Chlorophyll-a, ng/cell
250
200
150
100
50
0
2
4
6
10
15
Days
control
1 nM
5 nM
10 nM
50 nM
100 nM
1 μM

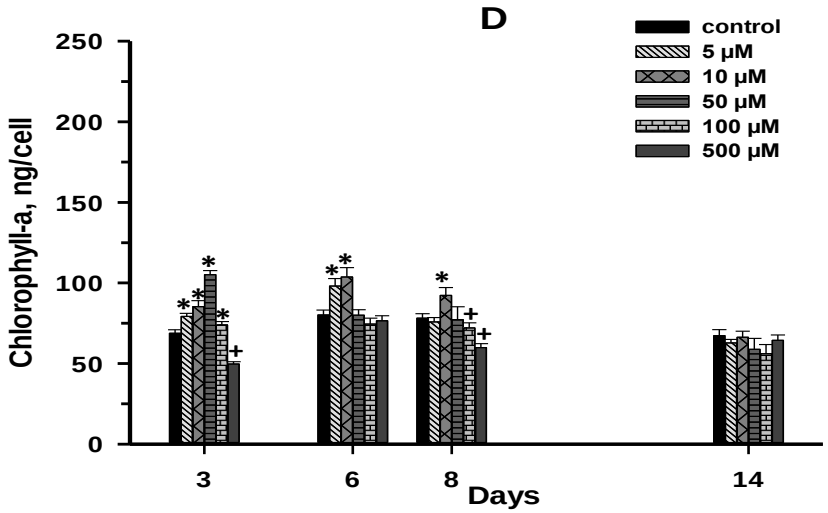

D
Chlorophyll-a, ng/cell
250
200
150
100
50
0
3
6
8
14
Days
control
5 μM
10 μM
50 μM
100 μM
500 μM

Figure 8. Chlorophyll-a concentrations (ng/cell) in *S. quadricuada* during growth in the presence of phytohormones: A) EBL; B) BL; C) IAA; and D) ABA. Statistically significant differences from the controls ($P < 0.05$) are indicated, where * - significantly greater than the control and $^{+}$ - significantly lower than the control. Data shown as the mean ± SD, n = 15.

3.2.2.2 Total carotenoids biosynthesis

Total carotenoid production per cell was stimulated by all phytohormones tested. As was observed for chlorophyll-a, the greatest effect occurred between day four and day six of the experiments in all the trials, except for IAA, where the greatest increase was observed from day two to day four of the experiment (Figure 9). EBL and IAA displayed the strongest enhancement, stimulating carotenoid production up to 4.0- and 2.8-fold, respectively (Figures 9A and 9C). The order of the efficacy for carotenoid stimulation was: 0.5 > 100 > 5 > 50 > 10 nM for EBL; 5 > 0.5 > 50 nM for BL; 10 > 50 > 100 nM ≈ 1 μM > 1 nM for IAA; and 10 > 5 > 50 μM for ABA (Figure 9). Adverse effects of the phytohormones on carotenoid production were observed for IAA at 1 μM (Figure 9C) and for ABA at 100 and 500 μM (Figure 9D). In the controls, similar to chlorophyll-a, total carotenoid production was slightly elevated between day four and day six (EBL and BL), day four and day ten (IAA), and day 6 and day 8 (ABA) compared with the first measurement in each trial, which was taken on the second day of each experiment (Figure 9).

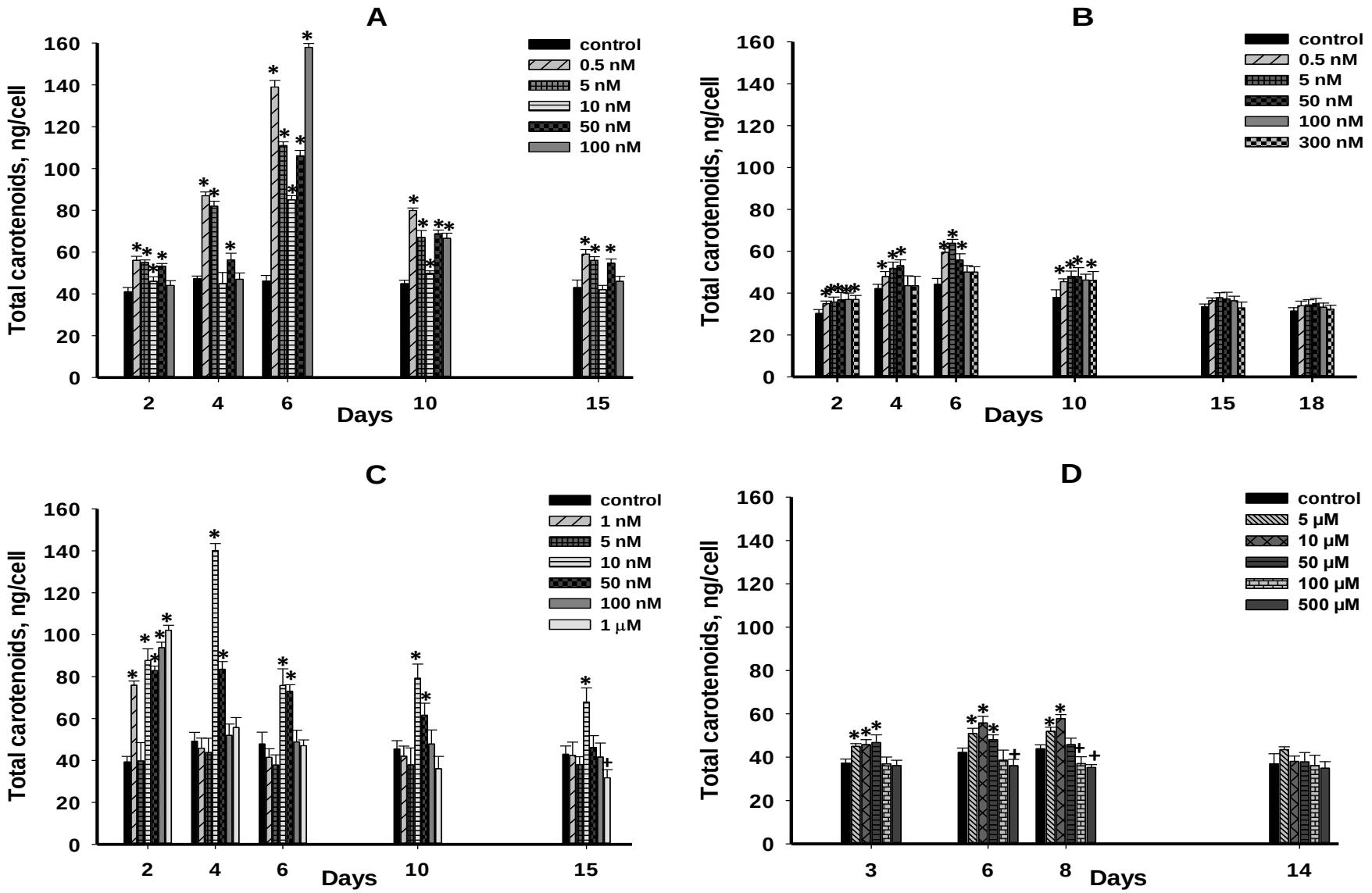

A
Total carotenoids, ng/cell
Days
control
0.5 nM
5 nM
10 nM
50 nM
100 nM
B
Total carotenoids, ng/cell
Days
control
0.5 nM
5 nM
50 nM
100 nM
300 nM
C
Total carotenoids, ng/cell
Days
control
1 nM
5 nM
10 nM
50 nM
100 nM
1 μM
D
Total carotenoids, ng/cell
Days
control
5 μM
10 μM
50 μM
100 μM
500 μM

Figure 9. Total carotenoid concentrations (ng/cell) in *S. quadricuada* during growth in the presence of phytohormones: A) EBL; B) BL; C) IAA; and D) ABA. Statistically significant differences from the controls ($P < 0.05$) are indicated, where * - significantly greater than the control and $^{+}$ - significantly lower than the control. Data shown as the mean ± SD, n = 15.

3.2.3 Effect of different phytohormones on lipid accumulation and their fatty acids profiles

All the phytohormones tested stimulated fatty acid accumulation in *S. quadricauda*. The fatty acid profiles were dependent on the type and concentration of phytohormone, and on time of harvesting (Figure 10; Table 8). The most pronounced increase in the accumulation of major fatty acids (each > 3 % of total FAs) was observed at 100 μM ABA, where palmitic (C16:0), palmitoleic (C16:1), and oleic acids (C18:1n9c) reached their maximum concentrations, which corresponded to 34.2, 12.1, and 42.4 mg/g lipid, respectively (Figure 10D). EBL also greatly stimulated accumulation of palmitic and oleic acids at 100 nM, and increased lauric acid (C12:0) accumulation to a maximum of 24.8 mg/g lipid at 10 nM (Figure 10A). Auxin stimulated the accumulation of three long chain FAs: arachidic (C20:0), γ-linolenic (C18:3n6), and heneicosanoic (C21:0), to their maximum values of 22.4, 19.1, and 7.8 mg/g lipids respectively while increasing lauric, palmitic, and oleic acids to concentrations just slightly lower than ABA or EBL (Figures 10A, 10C and 10D).

The order of phytohormone stimulation efficiency on major fatty acid synthesis was: EBL ≥ ABA > IAA > BL. However, total fatty acids were pronouncedly higher in the IAA trial, which reached the highest concentration (130.2 mg/g lipid at 10 nM) of all the tested hormones (Table 8). The most effective hormone concentrations in the trials were 100 nM for EBL, 5 nM for BL, 10 nM and 100 nM for IAA, and 100 μM for ABA.

However, adverse effects of phytohormones on selected FAs accumulation were also observed: with IAA at 50 nM, 100 nM, and 1μM; ABA at concentrations from 10 to 500 μM; and BL at 100 nM and 300 nM (Figures 10B, 10C and 10D).

The core fatty acid composition in the control cells (*S. quadricauda* cultured without phytohormone treatment) consisted of palmitic acid, palmitoleic acid, oleic acid, and γ-linolenic acid. The major fatty acid composition of lipids extracted from *S. quadricauda* cultured with BL and ABA (harvested on day 18 and day 14, respectively) were similar to the fatty acid profiles of the control cells (Figures 10B and 10D). In contrast, the major fatty acid composition of lipids from cells cultured with EBL and IAA (harvested on day 21 day), were very different from the core fatty acid profile of control cells (Figures 10A and 10C). Treatment with some phytohormones at different concentrations both enhanced production of some of these core FAs and stimulated the production of additional FAs, which were not found in the controls. The order of increased production of core FAs was: C18:1n9c > C16:0 > C18:3n6 > C16:1.

Increasing concentrations of EBL significantly induced production of core palmitic, oleic, and γ - linolenic acids, and 100 nM EBL resulted in a 2.8-, 2.6-, and 2.0-fold increase, respectively (Figure 10A). However, oleic acid was not detected in the fatty acid profile synthesized by *S. quadricauda* in the presence of 10 nM EBL. EBL also stimulated the synthesis of lauric acid and heneicosanois acid, which were not found in the core fatty acid profile of control cells.

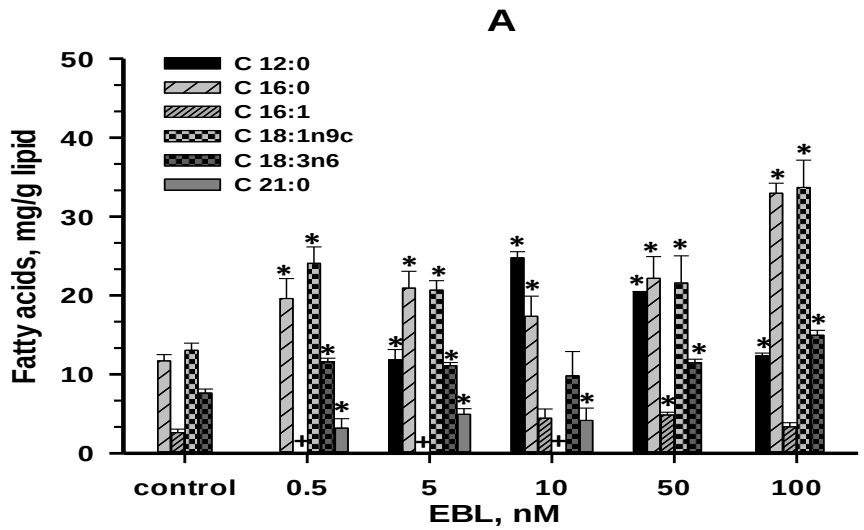
Minor FAs (each 1-3 % of total FAs) in the EBL trial displayed the highest accumulation (10.37 mg/g lipid) at 5 nM EBL, while all other tested concentrations did not increased the concentrations of minor FAs compared to the control (Table 8). The order of significance (% of total FAs) for minor FAs in this trial was: C11:0 (undecanoic acid) > C18:1n9t (9-trans-oleic acid) > C18:0 (octadecanoic acid) > C17:1 (cis-10-heptadecenoic acid). The percent of insufficient FAs (each < 1 % of total FAs) at each EBL concentration was greatly lower than in control (Table 8).

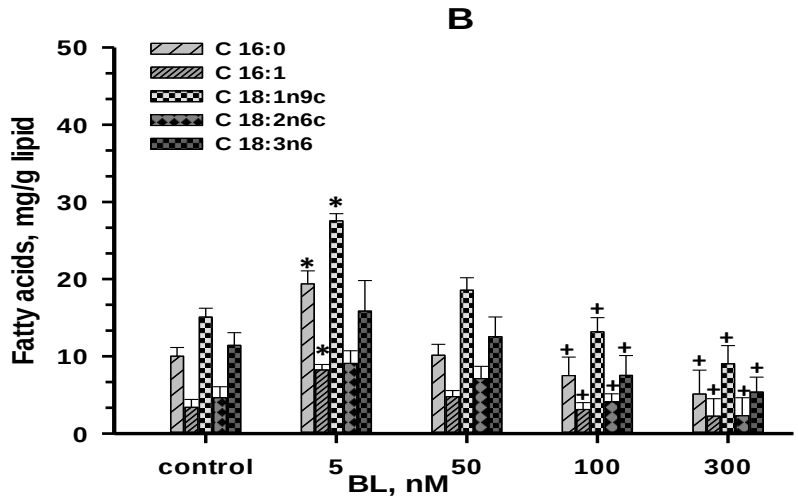
In the BL experiments, only one concentration (5 nM) was capable of increasing oleic, palmitic, and palmitoleic acids accumulation. In this trial, linoleic acid (C18:2n6c) was detected in the control. The fatty acid profiles were consistent across all the BL concentrations tested (Figure 10B). Although minor FAs accumulation was greater at 5 nM and 50 nM BL, the percent of minor FAs in this trial was

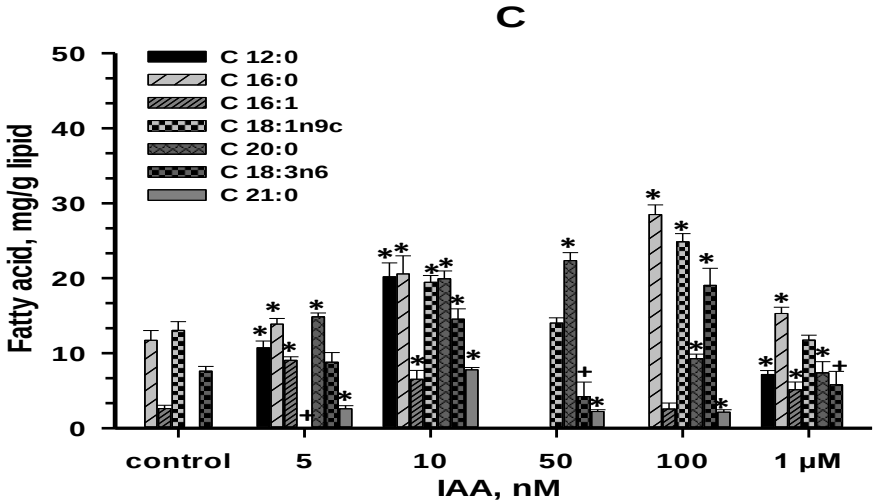
significantly higher than in the controls, similar to the accumulation observed in all tested hormone concentrations, and 2- to 3-times lower than the percent accumulation of Min FAs. The order of significance for the minor FAs was: C18:1n9t > C17:1 > C18:0 > C12:0 ≥ C20:1 (cis-11-eicosenoic) ≥ C24:1 (nervonic acid).

Increasing IAA concentrations stimulated the accumulation of all the FAs found in control *S. quadricauda* cells. Concentrations of palmitoleic, palmitic, γ-linolenic, and oleic acids were enhanced 3.4-, 2.4-, 2.5-, and 1.9-fold, respectively (Figure 10C). The concentrations of 10 nM and 100 nM IAA were most efficient in enhancing synthesis of all the core FAs. IAA also stimulated the synthesis of three additional major FAs compared to controls. Arachidic acid accumulated to a maximum concentration of 22.4 mg/g lipid in the presence of 50 nM IAA, while lauric acid and heneicosanoic acid accumulated to maximum concentrations of 20.2 mg/g lipid and 7.8 mg/g lipid, respectively, in the presence of 10 nM IAA. At 50 nM IAA, lauric, palmitic, and palmitoleic acids were not synthesized. Minor FAs accumulated to similar concentrations in this trial across all IAA concentrations tested, but achieved significantly higher concentrations in the IAA trails than in the controls (Table 8). The order of significance for the minor FAs was: C18:1n9t > C18:0 > C17:1 ≥ C18:2n6c.

The accumulation of Min FAs in this trial depended on the hormone concentration, and appeared to be inversely dependent to the accumulation of major FAs. In other words, Min FAs accumulated to their lowest concentrations in those IAA trials where the accumulation of major FAs was highest (Table 8).


A
C 12:0
C 16:0
C 16:1
C 18:1n9c
C 18:3n6
C 21:0
Fatty acids, mg/g lipid
0
10
20
30
40
50
control
0.5
5
10
50
100
EBL, nM


B
C 16:0
C 16:1
C 18:1n9c
C 18:2n6c
C 18:3n6
Fatty acids, mg/g lipid
0
10
20
30
40
50
control
5
50
100
300
BL, nM


C
C 12:0
C 16:0
C 16:1
C 18:1n9c
C 20:0
C 18:3n6
C 21:0
Fatty acid, mg/g lipid
0
10
20
30
40
50
control
5
10
50
100
1 μM
IAA, nM

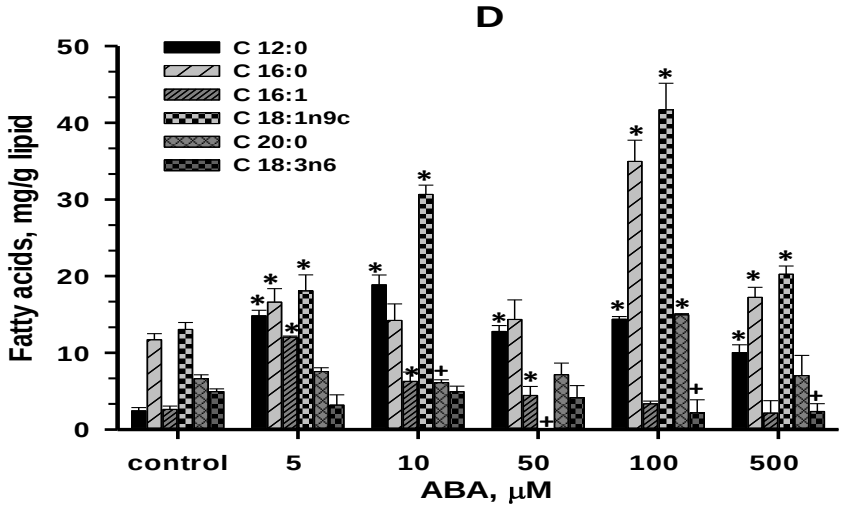

D
C 12:0
C 16:0
C 16:1
C 18:1n9c
C 20:0
C 18:3n6
Fatty acids, mg/g lipid
0
10
20
30
40
50
control
5
10
50
100
500
ABA, μM

Figure 10. The distribution of major fatty acids in *S. quadricuada* cells in the presence of phytohormones on the day of test termination: A) on day 21 with EBL; B), on day 18 with BL; C) on day 21 with IAA; and D) on day 14 with ABA. Statistically significant differences from the controls ($P < 0.05$) are indicated, where * - significantly greater than the control and $^{+}$ -significantly lower than the control. Data shown as the mean ± SD, n = 4.

Table 8. Fatty acid accumulation in *S. quadricauda* cells at the day of a test termination in the four phytohormone trials (Major FAs, each > 3 % of total FAs; Minor FAs, each 1-3 % of total FAs; Minimal (Min) FAs, each < 1 % of total FAs).

Hormone concentration	Major FAs, mg/g lipids	Major FAs, % of total FAs	Minor FAs, mg/g lipids	Minor FAs, % of total FAs	*Min FAs, mg/g lipids	^Min FAs, % of total FAs	*Total FAs, mg/g lipids
EBL trial							
control	35.00 ± 0.93^{a}	65.02 ± 0.78^{a}	7.75 ± 0.87^{a}	11.75 ± 0.65^{a}	**9.51**	23.27 ± 1.35^{a}	**52.24**
0.5 nM	58.42 ± 1.28^{b}	85.21 ± 1.14^{b}	8.48 ± 1.61^{ab}	$9.91 \pm 0.84^{a*}$	**3.36**	$5.02 \pm 0.53^{b*}$	**70.24**
5 nM	69.48 ± 0.78^{c}	$89.56 \pm 1.06^{c*}$	10.37 ± 1.28^{b}	7.19 ± 1.12^{b}	**2.52**	3.15 ± 1.16^{b}	**82.33**
10 nM	60.53 ± 2.26^{b}	85.92 ± 0.82^{b}	8.89 ± 1.05^{ab}	11.54 ± 0.73^{a}	**2.52**	$2.62 \pm 1.06^{b*c}$	**71.93**
50 nM	80.41 ± 1.73^{d}	90.14 ± 2.11^{c}	7.63 ± 2.21^{ab}	$7.27 \pm 1.62^{a*b}$	**2.32**	2.50 ± 0.82^{c}	**90.25**
100 nM	97.34 ± 0.63^{e}	$92.82 \pm 0.74^{c*}$	7.35 ± 0.37^{a}	5.98 ± 0.99^{b}	**2.72**	1.93 ± 0.65^{c}	**107.43**
BL trial							
control	43.71 ± 1.12^{a}	65.81 ± 0.28^{a}	5.71 ± 0.77^{a}	8.55 ± 0.85^{a}	**17.02**	25.43 ± 1.15^{a}	**69.30**
5 nM	63.68 ± 0.87^{b}	69.00 ± 1.53^{b}	11.14 ± 0.61^{b}	$11.97 \pm 1.64^{b*}$	**19.96**	21.06 ± 0.73^{b}	**94.99**
50 nM	48.63 ± 1.44^{c}	61.00 ± 0.76^{c}	11.29 ± 0.98^{b}	13.04 ± 1.52^{b}	**20.55**	24.85 ± 1.06^{a}	**80.52**
100 nM	34.71 ± 2.34^{d}	57.02 ± 1.46^{d}	6.74 ± 1.25^{a}	$11.04 \pm 0.63^{b*}$	**19.42**	31.9 ± 0.54^{c}	**60.85**

300 nM	23.78 ± 3.09^{e}	54.04 ± 2.80^{d}	5.69 ± 2.01^{a}	12.97 ± 0.82^{b}	**14.46**	32.87 ± 0.72^{c}	**43.95**
IAA trial							
control	35.02 ± 0.55^{a}	67.05 ± 1.21^{a}	6.97 ± 0.47^{a}	12.88 ± 1.25^{a}	**11.42**	21.77 ± 0.38^{a}	**53.39**
5 nM	59.88 ± 1.01^{b}	61.55 ± 0.58^{b}	16.33 ± 0.39^{b}	18.42 ± 0.48^{b}	**19.51**	19.88 ± 1.82^{a}	**95.71**
10 nM	108.99 ± 3.66^{c}	86.79 ± 0.33^{c}	17.18 ± 0.91^{b}	10.24 ± 2.04^{a}	**4.06**	3.11 ± 0.53^{b}	**130.18**
50 nM	42.80 ± 2.35^{d}	53.04 ± 1.16^{d}	17.66 ± 0.44^{b}	21.74 ± 0.62^{c}	**20.21**	25.12 ± 1.16^{c}	**80.63**
100 nM	86.38 ± 0.76^{e}	80.05 ± 0.77^{e}	16.99 ± 1.25^{b}	15.05 ± 1.13^{a}	**5.42**	4.9 ± 0.54^{b}	**108.78**
1 µM	52.49 ± 1.33^{f}	56.84 ± 2.81^{d}	16.73 ± 0.29^{b}	18.11 ± 0.82^{b}	**23.04**	25.07 ± 0.65^{c}	**92.22**
ABA trial							
control	41.38 ± 0.33^{a}	76.00 ± 0.59^{a}	3.15 ± 0.73^{a}	4.49 ± 1.34^{a}	**10.8**	19.51 ± 2.03^{a}	**55.33**
5 µM	72.27 ± 2.73^{b}	86.91 ± 1.68^{b}	2.61 ± 0.41^{a}	2.72 ± 0.85^{a}	**8.65**	12.64 ± 0.68^{b}	**83.53**
10 µM	81.05 ± 0.96^{c}	89.71 ± 0.83^{b*}	3.31 ± 0.88^{a}	3.71 ± 2.06^{a}	**5.9**	6.58 ± 0.79^{c}	**90.26**
50 µM	42.88 ± 0.51^{a}	79.69 ± 0.49^{c}	2.5 ± 0.45^{a}	4.42 ± 1.4^{a}	**8.55**	15.89 ± 1.64^{a}	**53.93**
100 µM	111.50 ± 2.85^{d}	93.47 ± 0.71^{d}	4.22 ± 0.27^{a}	3.14 ± 1.09^{a}	**4.5**	3.39 ± 0.95^{d}	**120.22**
500 µM	59.03 ± 0.76^{e}	84.75 ± 1.04^{b*}	3.45 ± 0.50^{a}	5.48 ± 1.07^{a}	**6.34**	9.08 ± 0.93^{c}	**68.82**

Note: Values in Bold font are empirically calculated numbers. Within a trial, and for each column, values labeled with the same letter are not significantly different from each other ($P > 0.05$), and values labeled with a letter plus an asterisk indicate significant difference from different letters, but no statistical difference from other equal letters. Data shown as the mean ± SD, n = 4.

In the ABA trial, while only small amounts of lauric acid and arachidic acid were detected in the controls, concentrations of lauric acid were significant elevated at all ABA concentrations tested, and the concentration of arachidic acid was significantly greater at 100 nM ABA (Figure 10D). The degree of increase for lauric, palmitoleic, oleic, palmitic, and arachidic acids in the ABA trial were 7.8-, 4.6-, 3.2-, 3.0-, and 2.3-fold, respectively (Figure 10D). Minor FAs accumulated to low concentrations at all ABA concentrations tested, and in the control (Table 8). The order of significance for the minor FAs was: C24:1 > C18:1n9t ≥ C17:1. As observed with the IAA trials, Min FAs accumulation depended on the ABA concentration tested. However, at most ABA concentrations tested, Min FAs were detected at concentrations that were lower than those detected in the controls.

3.3 Discussion

Wastewaters from aquaponics and/or hydroponics have been suggested as sources of nutrients and hormones to support industrial algae biomass production (Kim et al., 2007; Kong et al., 2010; McGinn et al., 2012; Dalrymple et al., 2013; Han et al., 2016). The actual loading of phytohormones in wastewaters, such as brassinolides and natural auxins, has been demonstrated to be in the range of 200-300 nM and lower (Khripach et al., 2000; Savidov, 2004), although ABA was found in hydroponic wastewater at concentrations as high as 500 μM (Lopez et al., 2005; Liu et al (2016) assessed the influence of four types of auxin on the physiology of *Chlorella pyrenoidosa* and *S. quadricauda* at concentrations between 28 and 340 μM. Such high concentrations can only be achieved in industrial production units by the addition of commercial hormones into growth medium, which is very costly and most likely not economically viable. Thus, application of naturally occurring hormones from fishery and hydroponic wastewaters could be one of the solutions to reduce the cost of industrial scale microalgal production.

Selection of a species is another important question for success in the industry. *S. quadricauda* is a species broadly used for commercial biomass production due to its high growth rate (Cordero et al., 2011), high lipids (McGinn et al., 2012; Dev Goswami, 2011), and carotenoid (lutein and β-carotene) content

(Kim et al., 2007; Cordero et al., 2011).

Our data support some recent reports that suggest that phytohormones can simultaneously promote both microalgal cell growth and end-product biosynthesis (Babu et al., 2017), though there are some dissimilarities between the assessed hormones influence on algal physiology, as well as between the results of previous investigations and our findings. Although the range of concentrations of all the phytohormones tested demonstrated a stimulatory effect on *S. quadricauda* growth, biomass production, and targeted molecules biosynthesis, the degree of influence and most effective concentrations were varied with the hormone type. In addition, we emphasize the importance of preparation of phytohormone solutions, where the solvent used may co-influence the results. This co-influence has been taken in account in our study and we did not observe any solvent (DMSO) influence when the solvent concentration was below 0.001 %, as was the case for the hormones tested in nM concentrations.

The two brassinolides hormones tested, EBL and BL, displayed similar stimulatory effects on *S. quadricauda* growth and biomass production enhanced both parameters by about 2-fold. However, the impacts of EBL and BL on chlorophyll-a, carotenoid, and lipid biosynthesis were dissimilar. EBL enhanced chlorophyll-a and carotenoid synthesis to a much greater extent than BL in the first six days of growth at concentrations from 0.5 nM to 100 nM. EBL also had a greater stimulatory effect on the fatty acids synthesis and on fatty acid profile alteration than BL.

Little data is available in the literature on brassinosteroids influence on microalgae. Our results are consistent with those of Bajguz and Czerpak (1998), who reported a clear stimulatory effect of two brassinosteroids, BL and castasterone, on cell growth, chlorophyll-a, and monosaccharide production in *Chlorella vulgaris* cells at low concentrations. However, in contrast to our study, Bajguz (2003) stated that BL demonstrated a greater impact on *Ch. vulgaris* cell growth, and biosynthetic activity than EBL. In induction of chlorophyll-a, carotenoids, and lipid synthesis in microalgae EBL is consistent with the role of the hormone in vascular plants (Davies, 2004; Sasse, 2003; Bishop and Yokota, 2001). Although

both EBL and BL play a role in cell elongation in plants, only EBL increased *S. quadricauda* cell size at all of the tested concentrations, except 100 nM, while BL stimulated an increase in cell size at 5 nM, but inhibited cell size at concentrations of 50 nM and higher.

Auxin IAA was found to be one of the most effective phytohormones for stimulation of *S. quadricauda* growth and biosynthesis. Our data are consistent with previous studies, which reported that IAA is capable of significant induction of microalgal growth and biomass yield (Park et al., 2013; Nobel et al., 2014; Liu et al., 2016; Babu et al., 2017; 61), chlorophyll (Park et al., 2013; Liu et al., 2016; Ozioko et al., 2015), and lipid (Park et al., 2013; 40, Babu et al., 2017) biosynthesis. However, some discrepancies in the results must be highlighted. For example, in contrast to the most effective concentrations for IAA in our study (between 5 and 100 nM), Nobel et al. (2014) observed stimulation of *Euglena gracilis* growth, biomass yield, and cell size at 1 nM and 10 nM IAA, and physiological inhibition at higher IAA concentrations. Liu et al. (2016) demonstrated induction of *S. quadricauda* growth and biomass production at very high IAA concentrations (228 and 342 μM), while in our study on *S. quadricauda*, 1 μM was not effective (on cell size), or had toxic effects (on growth, chlorophyll, and total carotenoid production).

Such significant differences are likely the result of different growth conditions and, most importantly, different growth medium composition. It is well known that the bioavailability of compounds, including hormones, dissolved in water depends on water chemistry, which affects competition among ions for uptake sites on algal cell membranes or binding capacity of dissolved molecules (Pollard and Rojas, 1988). Moreover, presence of waterborne auxin might alter algae membrane permeability, since auxin was shown to be capable of activating proton pump ATPase in the plasma membrane (Rober-Kleber at al., 2003; Perrot-Rechenmann, 2010) in vascular plants. As auxins regulate cell growth and elongation in vascular plants (Davies, 2004) it was no surprise that IAA at the concentrations 5 to 50 nM significantly increased *S. quadricauda* cell size in our experiments. The ability

of IAA to enlarge *S. quadricauda* cells has been previously reported for different algal species (Park et al., 2013; Nobel et al., 2014; Ozioko et al., 2015). However, contrary to our results, both growth stimulatory (1 nM) and growth inhibitory (0.01 mM) IAA concentrations in those experiments equally increased algae cell size.

Our results clearly demonstrate positive effect of IAA on *S. quadricauda* pigment biosynthesis, inducing chlorophyll-a and total carotenoid production. As was observed for all the phytohormones tested, the effect of IAA on pigment production was initially rapid, but decreased after day six, which likely occurs due to the degradation of the hormones in the aqueous environment.

A positive effect of IAA on lipid accumulation was also found to be pronounced in our research. Furthermore, IAA was the phytohormone that stimulated significant increases in the bioaccumulation of both major and minor FAs at all concentrations tested. The few reports in the literature on the effect of IAA on lipid synthesized by microalgae are highly variable. Recent studies of Liu et al. (2016), conducted on *S. quadricauda* and Babu et al. (2017), conducted on *Ch. sorokiniana*, demonstrated a clear induction of lipid production by IAA. However, Park et al. (2013) did not observe any effects when fatty acids were expressed as percent of *C. reinhardtii* cell biomass, neither Liu et al. (2016) could confirm the effect on *Ch. pyrenoidosa*. These discrepancies may be due to the fact that hormones often act in a species-specific manner, which has been demonstrated primarily with vascular plants (Davies, 2004; Bishop and Yokota, 2001; Xu et al., 2013), and may also be true for microalgae.

The effects of ABA on *S. quadricauda* cell physiology were more complex. Although ABA displayed the weakest effect on *S. quadricauda* growth and had an adverse effect on cell size, 5 to 50 μM ABA was capable of increasing biomass production and pigment biosynthesis. Moreover, in our study ABA and IAA had the greatest effects on the fatty acid biosynthesis by *S. quadricauda*, which was consistent with the results of Park et al. (2013). The weak ability of ABA to stimulate *S. quadricauda* growth could be explained by taking into account that the prime function of ABA in higher plants is as a

protective mediator against environmental stresses and pathogens (Davies, 2004; Schroeder et al., 2001; Pastori and Foyer, 2002; Tuteja, 2007), which cannot be demonstrated under laboratory conditions. Remarkably, this function of ABA was confirmed in microalgae (Hirsch et al., 1989; Cowan and Rose, 1991).

Induction of chlorophyll-a and total carotenoid production by ABA in our study was consistent with previous observations. Park et al. (2013) reported an approximate 2-fold increase in chlorophyll content in the presence of 40 μM ABA. Cowan and Rose (1991) demonstrated positive correlation between intracellular production of β-carotene and ABA in *Dunaliella salina*, suggesting that ABA is likely a regulator of carotenogenesis, particularly in response to an environmental stress. Moreover, ABA has been shown to be connected with carotenogenesis in vascular plants, where ABA is considered as a catabolite of neoxanthin and xanthoxin (Zeevaart and Creelman, 1989; Parry et al., 1990).

It is worth noting that alterations in the fatty acid profiles of lipids synthesized by *S. quadricauda* were observed for all phytohormones tested. However, differences in the manner in which the fatty acid profiles of major, minor and minimal FAs were altered were observed with different phytohormones. Our data are consistent with Park et al. (2013), who reported a significant increase in fatty acid yields (% of biomass) in the presence of ABA, despite the fact that cell growth was not affected by the hormone. Recent research by Babu et al. (2017) also supports our results. Their comprehensive study of microalgal FAs composition of *Ch. sorokiniana* cells, in the presence of IAA and reduced nitrogen, demonstrated high ability of the hormone to mitigate the adverse effects of nutrient depletion on lipid production. Salama et al. (2014) also demonstrated the ability of IAA to induce synthesis of certain FAs in *S. obliquus* cells. However the most affected FAs, the degree of induction, and the core FAs profile have some dissimilarity compared to our study, which is likely the result of the differences in growth conditions and time of harvesting (Salama et al., 2014). This comparison highlights the importance of investigating the influence of temperature and light intensity on phytohormone activity for algae production.

A possible explanation of the mechanism of fatty acid profile alteration by phytohormones may be that they can affect activity of acyl-ACP thioesterase (FAT), the enzyme that determines the final chain length of the fatty acids in algae (Borowitzka, 1988). Our results highlight the possibility that phytohormones could be used as a tool for fatty acids profile manipulation in the biofuel and pharmaceutical industries.

3.4 Conclusions

We have compared the influence of four phtyohormones - EBL, BL, IAA, and ABA - on several physiological parameters in *S. quadricauda*, with emphasis on the effect of these hormones on cell growth, cell biomass production, cell size, pigment biosynthesis (chlorophyll-a and total carotenoids), fatty acids biosynthesis and their profiles. All phytohormones tested had positive stimulatory effects on *S. quadricauda* cell growth, biomass production, chlorophyll-a, carotenoid, and lipids biosynthesis. To the extent of our knowledge, this is the first report on influence of EBL, BL, and ABA on the fatty acid composition of lipids in microalgae. Our research clearly demonstrated that EBL, IAA, and ABA, but not BL, are capable for both inducing production of fatty acids and altering the fatty acid profiles of lipids synthesized by *S. quadricauda*. Our data also suggest that even very small concentrations of the hormones tested were capable to enhance the growth, biomass production, and biosynthesis of valuable biomolecules, which raises the possibility of producing high-value fatty acids via industrial-scale microalgal production using hydroponic wastewaters containing phytohormones.

After obtaining the results on the single hormones influence on microalgae *S. quadricauda*, we conducted a set of the experimental work on mixed hormones influence, because in nature phytohormones normally crosstalk with eachother. In particular, the mixtures of EBL and IAA were investigated.

Chapter 4:

The Combined Influence of 24-epibrassinolide and 3-indoleacetic Acid on Growth and Accumulation of Pigments and Fatty Acids in Microalgae *Scenedesmus quadricauda* (CPCC-158)

4.0 Abstract

Interest in the use of wastewaters of different origins for the industrial production of microalgal biomass for biofuels and bioproducts has stimulated studies on the influence of a variety of compounds in wastewaters on algal physiology. Following the work on the impact of four single phytohormones on the physiology of the microalga *Scenedesmus quadricauda* (CPCC-158), described in Chapter 3, we investigated the combined effect of the 24-epibrassinolide (EBL) and 3-indoleacetic acid (IAA) on *S. quadricauda* biosynthesis and biomass production. Synergistic increases in *S. quadricauda* biomass production, chlorophyll-a accumulation, total carotenoid accumulation, as well as total and major neutral lipid (triacylglyceride) accumulation were observed for most of the mixed hormone trials. Mixed hormones had stimulatory effects on growth and biosynthesis of *S. quadricauda*, but the influence of mixed hormones on algae growth (maximum 1.6-times greater) was less than their influence on algal biosynthesized molecules content (maximum 1.7-, 2.7-, and 3.3-fold for chlorophyll-a, total carotenoids, and fatty acids, respectively) compared with single hormone treatments. The mechanism of EBL and IAA crosstalk is likely specific to targeted biosynthetic pathways. The type of relationship between two mixed phytohormones was assessed with Ting-Chao Chou Test. Our results highlight the need for further research on the combined influence of phytohormones on algae physiology.

4.1 Introduction

One of the groups of organic compounds dissolved in agricultural wastewaters are phytohormones, which have been demonstrated to induce simultaneously both microalgal growth and accumulation of valuable secondary metabolite molecules (Yang et al., 2000; Geider and La Roche, 2002; Bumbak et al., 2011; Silaban et al., 2014; Kozlova et al., 2017).

In vascular plants, some phytohormones support each other by working singularly, and/or synergistically, on the same physiological functions. For instance, abscisic acid (ABA) and auxin both regulate cell growth and elongation. However, ABA is also an important two-way growth regulator, that functions to adjust plant growth to environmental conditions and the impact of pathogens (Schroeder et al., 2001; Pastori and Foyer, 2002; Davies, 2004; Tuteja, 2007), while auxins also regulate tissue and organ differentiation (Davies, 2004; Ludvig-Muller, 2011). Brassinosteroids (BRs) can work synergistically with auxins to elongate cells and promote tissue differentiation, and with ABA as an environmental stress remediator (Clouse and Sasse, 1998; Cano-Delgado et al., 2004; Vidhyasekaran, 2015).

First discovered in different cells of vascular plants, all known phytohormones have also been found in microalgal cells (Tsavkelova et al., 2006; Tarakhovskaya et al., 2007; Kiseleva et al., 2012; Stirk et al., 2013). Importantly, in nature, phytohormones rarely, if ever, work as single agents. Inside living cells, phytohormones (synthesized by cells or absorbed from the environment) work by activating specific signaling pathways that normally crosstalk with each other (Vidhyasekaran, 2015; Sasaki et al., 2001; Verhage et al., 2010; Yang, 2013). This network is not constant in time, but depends on environmental conditions, such as temperature, soil and/or water chemistry, and the presence of pathogens (Vidhyasekaran, 2015; Robert-Seilaniantz et al., 2011; Thaler et al., 2012; Alazem and Lin, 2015). Moreover, crosstalk among phytohormones can have different characteristics, resulting in synergistic, additive, or antagonistic relationships.

A synergistic effect is defined as the outcome observed when the combined effect of two chemicals is greater than the sum of the effects (expressed as the proportional effect units) of each compound alone. In contrast, an antagonistic effect is defined as the outcome observed when the effect of mixed compounds is less than the effect of each compound alone. Over ten methods have been used in quantitative determination of these relationships. The most popular method used in recent decades is based on "median dose-effect relationship" where the combination effect (CI) is used to determine whether the combined compound works synergistically or antagonistically (Chou and Talalay, 1984; Chou, 2006). These outcomes are often dependent on the concentrations of the specific hormones in question, as well as on ambient conditions or on the specific plant or algae species under investigation (Thaler et al., 2012; Peleg and Blumwald, 2011; Van der Does et al., 2013; Hunt et al., 2010).

Synergistic interaction between BRs and auxins, such as Indole-3-acetic acid (IAA), was noted in several studies (Yopp et al., 1981; Katsumi, 1985; Sala and Sala, 1985) and has been widely applied to agricultural crop production (Mandava, 1988). Some authors (Nakamura et al., 2003; Vert et al., 2008) clearly demonstrated the molecular mechanism of BRs and IAA crosstalk, suggesting that the signaling pathways of both hormones are capable of triggering the transcriptional system of IAA, and BRs work to induce the early auxin-inducible genes. In contrast, De Vleesschauwer et al. (2012) demonstrated negative crosstalk of BRs with salicylic acid (SA) and gibberellin (GA) pathways, which in their study resulted in a dramatic change in BRs function from protection of plants from biotic stress to immune suppression in rice (*Oryza sativa*). Moreover, the most effective phytohormone combination is plant/algae species-specific. For rice production, the optimal combination of plant growth regulators treated to paddy water is GA and ABA (Moya et al., 1995). For commercially used algae species, the optimal combination of phytohormones is yet to be determined.

There is some evidence of a link between BRs and IAA metabolism in vascular plants. BRs synergistically affect ethylene production in inflorescences, leaves and stalks of *Arabidopsis thaliana* (L.)

when applied with IAA, but had no influence on ethylene production when the plant was exposed to BRs only (Arteca and Arteca, 2008). Synergistic interaction of BRs and auxin to elongate the stem tissue, hypocotyl of embryos and roots was reported for different species of vascular plants (Mandava, 1988; Clouse and Sasse, 1998; Kim et al., 2000; Khripach et al., 2000; Karnachuk et al., 2002). Davies (2004) proposed a one-way interaction between BRs and IAA where only BR can affect IAA biosynthesis and activity in plants, but not *vice versa*. This assumption was supported by Nakamura et al. (2003) who elucidated the molecular mechanism of the one-way communication of BL, the most active BRs, with IAA suggesting that exogenous BL gradually and, likely, independently from the IAA signaling pathway, induces IAA5 and IAA19 genes in seedlings of wild type (WT) *Arabidopsis* via activation of the IAA response element. Synergistic interaction between BRs and auxin in intensification of expression of transcription of auxin-induced genes (Vert et al., 2008) in vascular plants enhances sensitivity of plant tissues to auxin (Sasse, 1999). Remarkably, exogenous application of BL clearly triggers IAA genes expression (Nakamura et al., 2003) and, as a result, BL synergistically affects activity of IAA in plant tissue (Yopp et al., 1981; Nakamura et al., 2003; Bao et al., 2004; Davies, 2004).

Thus, although some data on synergistic, antagonistic, or additive effects of phytohormone combinations has been demonstrated in vascular plants in recent decades, our understanding of the role and effects of phytohormones on algal cell physiology, and the influence of exogenous combinations of phytohormones on microalgal physiology is limited. *S. quadricauda* is a species of interest for biofuel (Dev Goswami. 2011; Peng et al., 2012; Mata et al., 2013) and industrial carotenoid (lutein, β-carotene) production (Kim et al., 2007; Cordero et al., 2011). We report here, the physiological effects of combinations of the BR, 24-epibrassinolide (EBL), and IAA, on cell growth, cell size, and biomass production, as well as the intracellular concentrations (accumulation) of chlorophyll-a, total carotenoids, total fatty acids, and the fatty acid composition in *S. quadricauda.*

4.2 Results

4.2.1 Effect of mixed phytohormones on *S. quadricauda* growth, biomass production and cell size

4.2.1.1 Algal cell growth

Two sets of experiments were conducted to evaluate the co-influence of EBL and IAA on *S. quadricauda* physiology. The first set of experiments, where 0.5 nM EBL was mixed with 5, 100 and 1000 nM IAA, demonstrated a clear synergistic effect of the mixed hormones on algae growth at 5 and 1000 nM IAA from Day 4 to Day 15 of the experiment compared to cultures exposed to either one of those phytohormones at the same concentrations. The mixture of both EBL matrix concentrations with 5 nM IAA showed significant decrease in the lag-phase of algae growth (Figures 11A and 11B). The mixture of 0.5 nM EBL with 100 nM IAA had an additive effect in this trial, except on Day 4 when this mixture had an adverse effect on algae growth (Figure 11A). The synergistic increase in cell density was a maximum of 1.2- and 1.5-fold compared to the average of the effects of two mixed single hormones when 0.5 nM EBL was mixed with 5 nM or 1000 nM IAA respectively.

In the second set of experiments, where 2 nM EBL was mixed with 5 or 100 nM IAA, a significant synergistic effect of the mixture was observed with 5 nM IAA from Day 4 to Day 15 of the experiment. An average increased in cell density of 1.74-fold was observed, with the highest cell density reaching 248.1 cells x 10^4/mL (Figure 11B). The mixture of 2 nM EBL + 100 nM IAA demonstrated an additive effect only (Figure 11B). The order of efficacy of the tested mixtures on algal growth was: 2 nM EBL + 5 nM IAA > 0.5 nM EBL + 1000 nM IAA ≥ 2 nM EBL + 100 nM IAA > 0.5 nM EBL + 5 nM IAA > 0.5 nM EBL + 100 nM IAA.

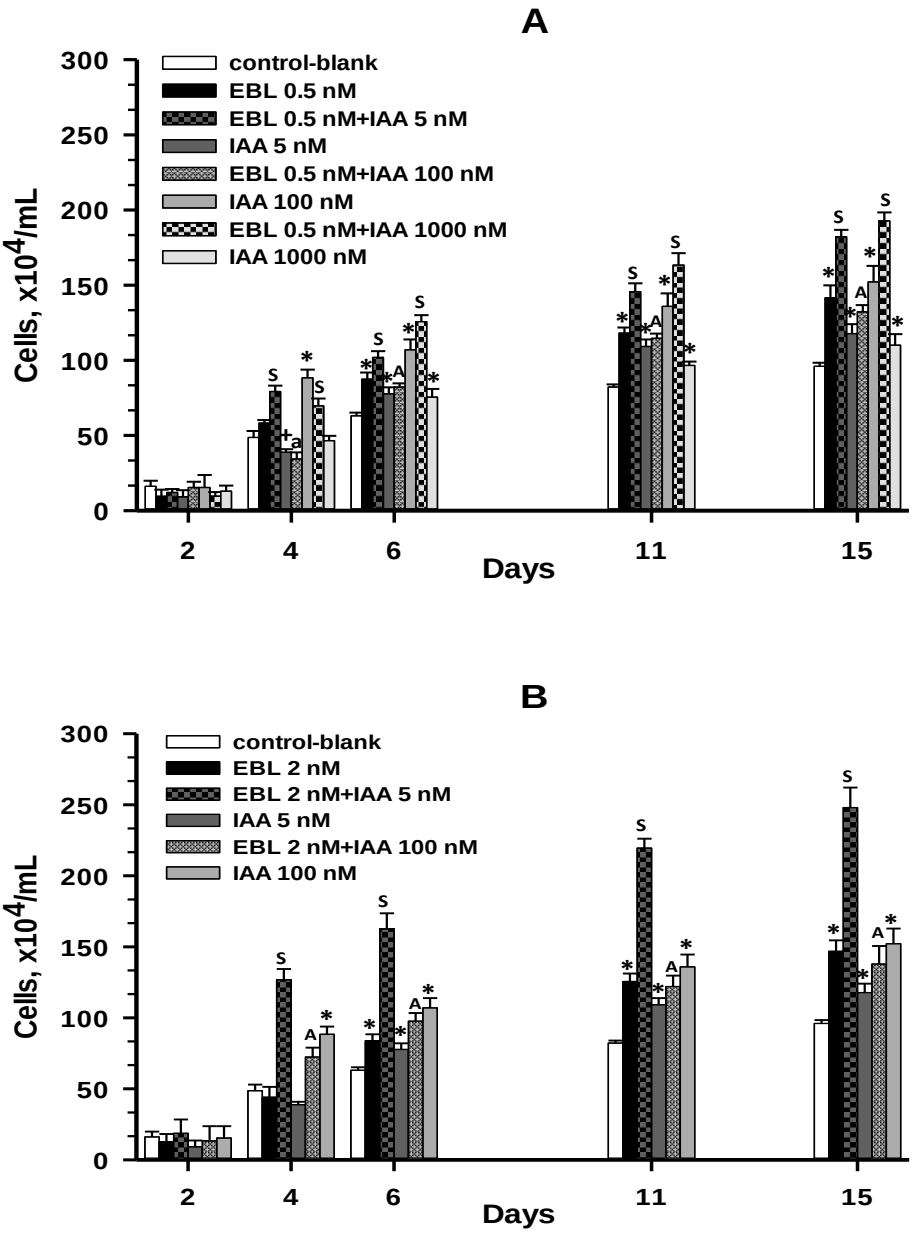


Figure 11. Growth of *Scenedesmus quadricauda* in the two mixed phytohormones experiments, where the effect of the mixtures (columns with patterns) were compared to the single hormones (columns with no patterns) used for the mixtures preparation. A) 0.5 nM 24-epibrassinolide (EBL) mixed with 5, 100, and 1000 nM 3-indoleacetic acid (IAA); B) 2 nM 24-epibrassinolide (EBL) mixed with 5 and 100 nM 3-indoleacetic acid (IAA). Statistically significant differences are indicated: *, for single hormones, significantly greater values compared with the control-blank ($P < 0.05$); for mixed hormones, S = synergistic effect; A = additive effect; a = antagonistic effect. Data shown as the mean ± SD, n = 12.

4.2.1.2 Relationship between cell size, biomass and cell density

All single hormone concentrations used in this study demonstrated clear induction of the growth parameters (cell size, biomass, and cell density) on the day of the test termination (Day 15) compared to the control-blank (Table 9). The mixtures of the EBL and IAA had different effects on *S. quadricauda* growth parameters demonstrating the dependence of the response on the concentrations of each phytohormone in the mixtures.

In the first set of experiments, where 0.5 nM EBL was used as a matrix concentration, the addition of 5 IAA significantly reduced cell size, but it did not prevent a great increase in cell density and biomass production (Table 9). In this trial, addition of 100 IAA had an additive effect on cell density, but cell size and biomass were greatly enhanced by this hormone combination, demonstrating a synergistic effect. The combination of 0.5 nM EBL with1000 nM IAA did not affect cell size having only an additive effect on this growth parameter, although cell density and biomass production were significantly increased (Table 9).

In the second set, the greater concentration (2 nM) of EBL demonstrated an antagonistic reduction in cell size when mixed with 5 nM IAA. However, in this trial, the highest cell density and the greatest value of biomass production were achieved (Table 9). When 2 nM EBL was mixed with 100 nM IAA, cell size and biomass production were synergistically affected, although cell density on Day 15 (when the experiment was terminated and the cells were harvested) showed an additive effect only.

Table 9. *Scenedesmus quadricauda* cell size (µm), biomass (g dcw/L) and cell density (n x 10^4/mL) at the day of a test termination (Day 15) in the single and mixed hormone trials, with indication of the type of interaction between the mixed hormones calculated using Isobolographic analysis.

Hormone concentration	Cell size, µm	Biomass, g dcw/L	Cell density, x 10^4/mL
Single hormones used for the combinations, nM			
control-blank	$34.71 \pm 0.62^{\alpha}$	$0.41 \pm 0.08^{\alpha}$	$95.96 \pm 2.42^{\alpha}$
0.5 EBL	$48.50 \pm 0.33^{\beta}$	$0.55 \pm 0.06^{\beta}$	$141.65 \pm 8.28^{\beta}$
2 EBL	$48.94 \pm 0.45^{\beta}$	$0.73 \pm 0.07^{\gamma}$	$146.86 \pm 7.71^{\beta}$
5 IAA	$45.25 \pm 0.32^{\gamma}$	$0.64 \pm 0.07^{\delta}$	$117.73 \pm 6.30^{\gamma}$
100 IAA	$43.11 \pm 0.37^{\delta}$	$0.69 \pm 0.12^{\gamma}$	$152.23 \pm 10.64^{\delta}$
1000 IAA	$36.38 \pm 0.41^{\varepsilon}$	$0.62 \pm 0.07^{\delta}$	$114.07 \pm 4.19^{\gamma}$
Mixed trials, nM			
0.5 + 5 IAA	36.81 ± 0.81^{a}	1.08 ± 0.11^{S}	182.14 ± 4.70^{S}
0.5 + 100 IAA	48.97 ± 0.41^{S}	1.21 ± 0.10^{S}	127.97 ± 4.53^{A}
0.5 + 1000 IAA	36.75 ± 0.35^{A}	1.11 ± 0.07^{S}	194.79 ± 5.61^{S}
2 + 5 IAA	35.44 ± 0.68^{a}	1.46 ± 0.15^{S}	248.06 ± 14.08^{S}
2 + 100 IAA	51.01 ± 0.39^{S}	1.26 ± 0.11^{S}	137.86 ± 12.72^{A}

Note: Within the single hormone trials, and for each column, values labeled with the same letter are not significantly different from each other ($P > 0.05$). Within the mixed hormone trials, and for each column: S = synergistic effect; A = additive effect; a = antagonistic effect. EBL - 24-epibrassinolide; IAA - 3-indoleacetic acid. Data shown as the mean ± SD (for cell size n = 15; for biomass n = 4; for cell density n = 12).

4.2.2 Effect of mixed phytohormones on *S. quadricauda* pigment production

4.2.2.1 Chlorophyll-a accumulation

Chlorophyll-a accumulation per cell was significantly increased in *S. quadicauda*, compared to the control-blank, by all single hormone concentrations used in this study (Figures 12A and12B). The mixture of 0.5 nM EBL with 100 nM or 1000 nM IAA demonstrated synergistic relationships on Day 2 of the experiment, but synergistic effect was kept at 0.5 nM EBL + 100 nM IAA only and was antagonistic at 0.5 nM EBL + 1000 nM IAA by Day 4. Both 100 nM and 1000 nM IAA demonstrated antagonistic relationship with 0.5 nM EBL by Day 6. By the end of the experiment (Day 15), the mixture of 0.5 nM EBL + 100 nM IAA recovered to an additive effect (Figure 12A). The addition of 5 nM IAA to 0.5 nM EBL had an additive effect on chlorophyll-a accumulation, which was reduced to an antagonistic effect by the last day of the experiment (Day 15). The mixture of 0.5 nM EBL + 1000 nM IAA exhibited an additive effect only on Day 2 of the experiment, but displayed a slightly antagonistic effect on chlorophyll-a accumulation by Day 6 until the end of the experiment (Figure 12A).

In the second set of experiments, with 2 nM EBL as a matrix concentration, both tested combinations of phytohormones demonstrated synergistic effect on pigment accumulation by Day 2 (Figure 12B). On Day 4 only the mixture of 2 nM EBL + 5 nM IAA kept synergistic effect while the mixture with 100 nM IAA demonstrated only an additive effect on the pigment accumulation. From Day 6 to the end of experiment (Day 15), both combinations showed antagonistic effects on chlorophyll-a accumulation (Figure 12B). The greatest increase in chlorophyll-a concentration was observed at 0.5 nM EBL + 100 nM IAA on Day 2 of the experiment when chlorophyll-a accumulation reached its highest value in this study at 210.6 ng/cell (Figure 12A). Generally, the stimulatory effect of all tested single and mixed hormones was most pronounced from Day 2 to Day 6 of the experiment (Figure 12A and 12B). The order of efficacy for stimulation of chlorophyll-a accumulation by the tested mixtures of phytohormones was: 0.5 nM EBL + 100 nM IAA > 2 nM EBL + 100 nM IAA ≥ 0.5 nM EBL + 1000 nM IAA > 2 nM EBL +

5 nM IAA ≥ 0.5 nM EBL + 5 nM IAA (Figures 12A and 12B). In the blank-control, chlorophyll-a accumulation was slightly elevated on day 4 of the experiment (Figures 12A and 12B).

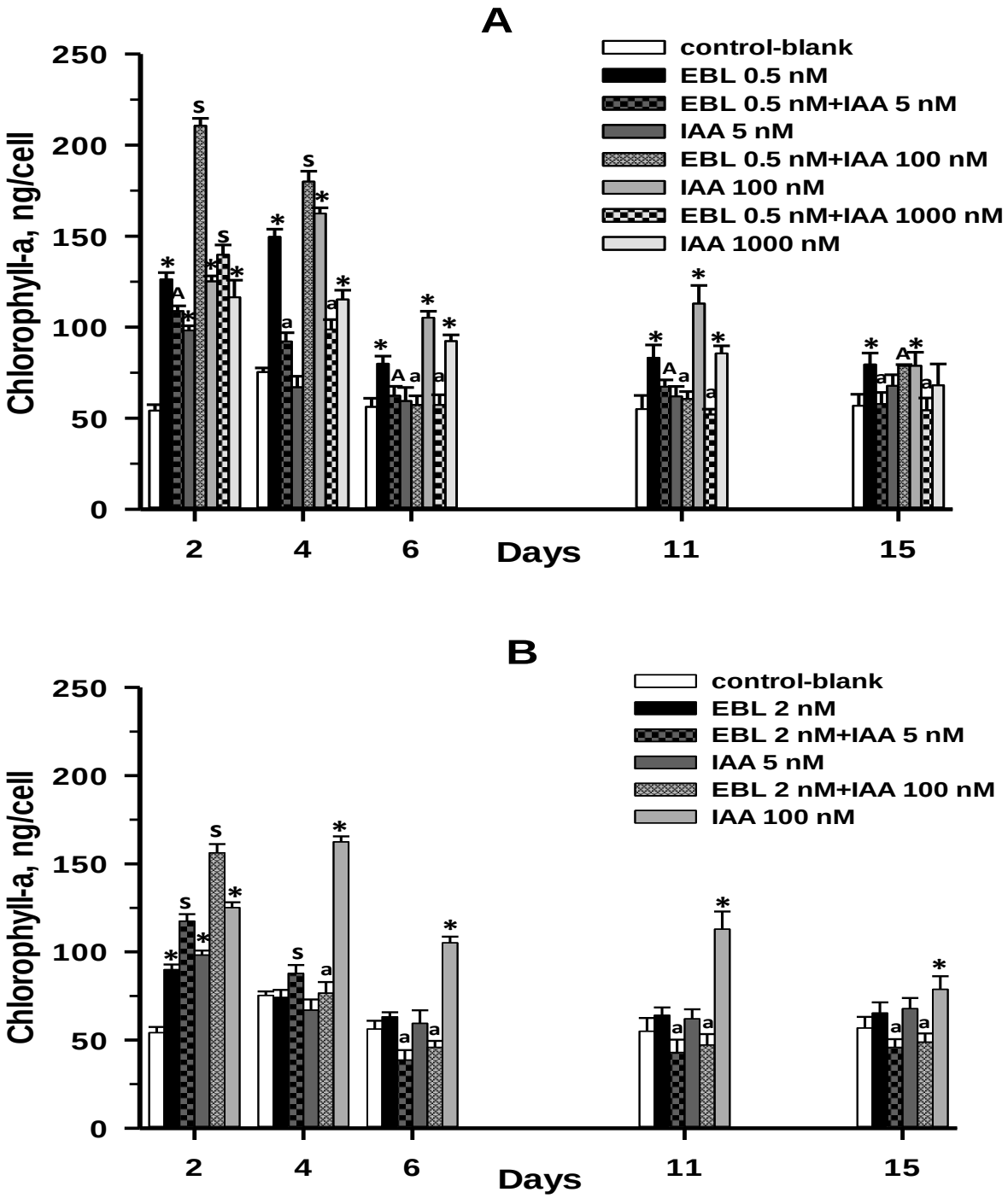


Figure 12. Chlorophyll-a concentrations (ng/cell) in *Scenedesmus quadricuada* during growth in the presence of mixed (columns with patterns) and singular (columns with no patterns) phytohormones used

for the mixtures preparation. A) 0.5 nM 24-epibrassinolide (EBL) + 5, 100 or 1000 nM 3-indoleacetic acid (IAA); B) 2 nM 24-epibrassinolide (EBL) + 5 or 100 nM 3-indoleacetic acid (IAA). Statistically significant difference is indicated: *, for single hormones treatments with significantly greater values from the blank-control ($P < 0.05$); for mixed hormones: S = synergistic effect; A = additive effect; a = antagonistic effect. Data shown as the mean ± SD, n = 15.

4.2.2.2 Total carotenoid accumulation

Similar to chlorophyll-a, accumulation of total carotenoids per cell was stimulated by all the single hormones used for mixed hormone combinations in this study compared to the control-blank. However, unlike chlorophyll-a, total carotenoid biosynthesis was synergistically stimulated by all the hormones combinations tested and the effect was stable to Day 4 of the experiment (Figures 13A and 13B). After Day 4, the effect became additive (combination 0.5 EBL + 100 nM IAA) or antagonistic (all other combinations). From Day 6 to the end of the experiment (Day 15) all the hormone combinations in this trial demonstrated an antagonistic effect on total carotenoids accumulation (Figure 13A).

In the second set of experiments, where 2 nM EBL mixed with 5 nM or 100 nM IAA, the effect on total carotenoids accumulation was similar to that of observed for chlorophyll-a production, although the antagonistic effect of mixed hormones in this trial was more pronounced (Figures 13A and13B).

The order of the efficacy of the tested hormones combinations for carotenoid stimulation was: 0.5 nM EBL + 100 nM IAA > 2 nM EBL + 100 nM IAA > 0.5 nM EBL + 5 nM IAA ≥ 2 nM EBL + 5 nM IAA > 0.5 nM EBL + 1000 nM IAA (Figures 13A and 13B). In the control-blank, total carotenoid production did not show a significant difference during the experiment (Figures 13A and 13B).

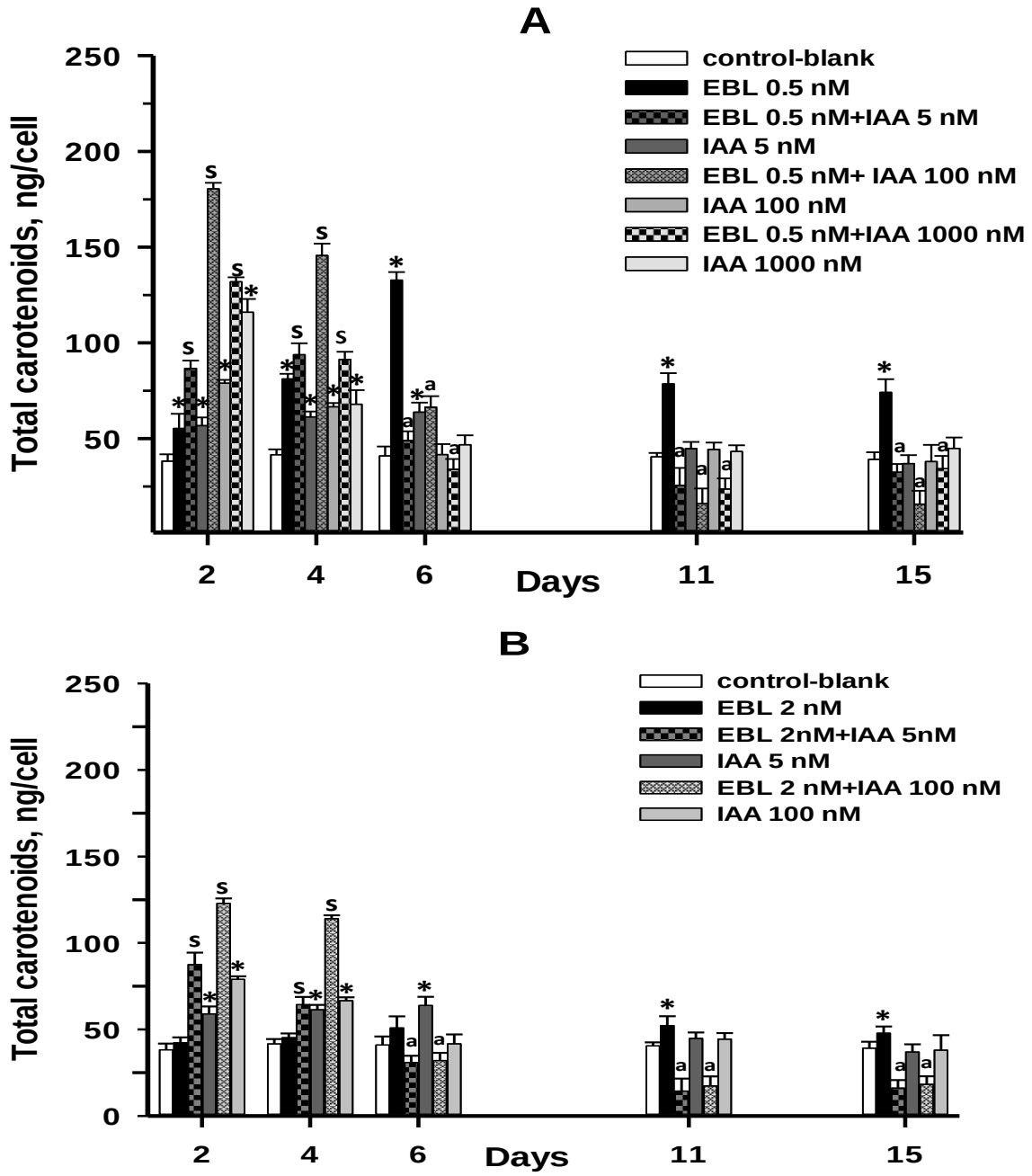


Figure 13. Total carotenoids concentrations (ng/cell) in *Scenedesmus quadricuada* during growth in the presence of mixed (columns with patterns) and singular (columns with no patterns) phytohormones used for the mixtures preparation. A) 0.5 nM 24-epibrassinolide (EBL) + 5, 100 or 1000 nM Indole-3-acetic acid (IAA); B) 2 nM 24-epi-brassinolide (EBL) + 5 or 100 nM 3-indoleacetic acid (IAA). Statistically significant difference is indicated: *, for single hormones treatments with significantly greater values from the blank-control ($P < 0.05$); for mixed hormones: S = synergistic effect; A = additive effect; a = antagonistic effect. Data shown as the mean ± SD, n = 15.

4.2.3 Effect of mixed phytohormones on lipid accumulation and their fatty acids profiles

All the single phytohormones used for mixed hormone combinations in this study demonstrated stimulatory effect on lipid biosynthesis in *S. quadricauda* (Figure 14A and 14B; Table 10). Both EBL concentrations of 0.5 and 2 nM, used as the matrix solutions in the mixed hormone trials were effective in increasing accumulation of major fatty acids (each > 3 % of total FAs) and minor fatty acids (each 1-3 % of total FAs) expressed as mg/g lipid, resulting in significant increase of total FAs accumulation (1.7- and 1.8-fold respectively) compared to the control-blank (Table 10). Single auxin concentrations of 5, 100 and 1000 nM used for the mixed hormone trials also elevated major and minor fatty acids biosynthesis resulting in greater total FAs accumulation (1.82-, 2.26- and 2.0-fold respectively). Total FAs concentration reached the highest number of 106.24 mg/g lipid at 100 nM IAA (Table 10). Single hormones greatly affected fatty acids profiles at all tested concentrations (Figures 14A and 14B). In these trials, the core fatty acids found in the control-blank reached their maximum values of: 31.57 mg/g lipid of oleic acid (C18:1n9c); 28.21 mg/g lipid of palmitic acid (C16:0); 11.53 mg/g lipid of γ-linolenic acid (C18:3n6) at 100 nM IAA; and 7.95 mg/g lipid of palmitoleic acid (C16:1) at 5 nM IAA (Figures 14A and 14B). Moreover, all the single hormone concentrations tested promoted synthesis of additional fatty acids and the FAs reached their maximum values of: 16.9 mg/g lipid of lauric acid (C12:0) at 2 nM EBL; 10.76 mg/g lipid of arachidic acid (C20:0) at 1000 nM IAA and 3.54 mg/g lipid of heneicosanoic acid (C21:0) at 2 nM EBL.

When the influence of the hormone combinations on FAs accumulation and their profile was assessed, all three types of relationships between the hormones were detected: synergism, antagonism and additivity. Importantly, some fatty acids biosynthesis was synergistically affected in any of the tested mixtures (Figures 14A and 14B). Moreover, major FAs (expressed either as mg/g lipid or % of total FAs) and total FAs accumulation were synergistically affected in any of the tested mixtures despite the fact that minor and Min FAs accumulation was mostly antagonistically affected when expressed as % of total FAs

(Table 10). The order of the mixture efficiency on both the total FAs and major FAs synthesis (mg/g lipid) was: 0.5 nM EBL + 100 nM IAA > 2 nM EBL + 100 nM IAA > 0.5 nM EBL + 1000 nM IAA > 2 nM EBL + 5 nM IAA ≥ 0.5 nM EBL + 5 nM IAA. Total FAs accumulation in these trials was 3.3-, 2.6-, 2.2-, 1.9- and 1.9-fold respectively when compared to the control-blank (Table 10).

Throughout all the mixed hormone trials total FAs concentration reached the highest number of 154.07 mg/g lipid when 0.5 nM EBL was mixed with 100 nM IAA. The order of the mixture efficiency on the minor FAs (mg/g lipid) was similar to that on the major FAs: 0.5 nM EBL + 100 nM IAA > 2 nM EBL + 100 nM IAA > 2 nM EBL + 5 nM IAA ≥ 0.5 nM EBL + 1000 nM IAA > 0.5 nM EBL + 5 nM IAA. The order of significance (mg/g lipid) for the minor FAs was: C18:3n3 > C20:2 ≥ C14:0 > C17:0 > C18:2n6c (data is not shown).

In the mixed hormone trials, the core fatty acids found in the control-blank reached their maximum values of 51.74 mg/g lipid of oleic acid and 48.51 mg/g lipid of palmitic acid in the trial containing 0.5 nM EBL + 100 nM IAA, while the trial containing 2 nM EBL + 100 nM IAA produced 15.11 mg/g lipid of γ-linoleic acid and 3.77 mg/g lipid of palmitoleic acid (Figures 14A and 14B).

FAs profiles in the mixed trials were similar to those in single hormone trials with two exceptions: arachidic acid was not detected in the trials containing 2 nM EBL + IAA (Figure 14B). Also, in both EBL single trials, comparable amounts of heneicosanoic acid were detected, but not arachidic acid (Figures 14A and 14B). Additional fatty acids reached their maximum values of: 19.54 mg/g lipid of lauric acid and 9.67 mg/g lipid of arachidic acid at 0.5 nM EBL+ 5 nM IAA; 5.23 mg/g lipid of heneicosanoic acid at 2 nM EBL + 100 nM IAA (Figures 14A and 14B). In trials with either 0.5 nM or 2 nM EBL, the addition of 100 nM IAA resulted in the greatest values of total, major, and minor FAs and all produced similar FA profiles (Figures 14A and 14B, Table 10).

Table 10. Fatty acid accumulation in *Scenedesmus quadricauda* cells at the day of a test termination (on Day 15th) in the single and mixed hormone trials with indication of the type of interaction between the mixed hormones calculated using Isobolographic analysis (Major FAs, each > 3 % of total FAs; Minor FAs, each 1-3 % of total FAs; Minimal (Min) FAs, each < 1 % of total FAs).

Hormone concentration	Major FAs, mg/g lipid	Major FAs, % of total FAs	Minor FAs, mg/g lipid	Minor FAs, % of total FAs	Min FAs, mg/g lipid	Min FAs, % of total FAs	Total FAs, mg/g lipid
Single hormones used for the combinations, nM							
control-blank	$31.48 \pm 0.43^{\alpha}$	$67.23 \pm 0.85^{\alpha*}$	$4.46 \pm 0.74^{\alpha}$	$9.15 \pm 1.35^{\alpha}$	**11.06**	$23.62 \pm 2.15^{\alpha}$	**47.00**
0.5 EBL	$61.37 \pm 1.08^{\beta}$	$77.25 \pm 1.16^{\beta}$	$8.46 \pm 1.52^{\beta}$	$10.57 \pm 0.61^{\alpha}$	**9.68**	$12.18 \pm 0.74^{\beta}$	**79.51**
2 EBL	$72.08 \pm 1.12^{\gamma}$	$85.45 \pm 0.28^{\gamma}$	$7.54 \pm 0.77^{\beta}$	$8.90 \pm 0.85^{\alpha}$	**4.76**	$5.65 \pm 1.15^{\gamma}$	**84.38**
5 IAA	$59.29 \pm 1.14^{\beta}$	$69.60 \pm 0.76^{\alpha}$	$13.59 \pm 0.58^{\gamma}$	$15.44 \pm 1.32^{\beta}$	**12.74**	$14.96 \pm 1.06^{\delta}$	**85.62**
100 IAA	$88.56 \pm 1.59^{\delta}$	$83.96 \pm 1.87^{\gamma}$	$14.02 \pm 2.31^{\gamma}$	$12.57 \pm 0.74^{\gamma}$	**3.66**	$3.46 \pm 0.62^{\gamma}$	**106.24**
1000 IAA	$67.45 \pm 0.98^{\varepsilon}$	$71.04 \pm 1.74^{\alpha*}$	$11.65 \pm 0.68^{\delta}$	$12.21 \pm 0.96^{\alpha\gamma}$	**16.01**	$16.75 \pm 2.25^{\delta}$	**95.11**
Mixed trials, nM							
0.5 EBL + 5 IAA	80.98 ± 1.32^{S}	90.22 ± 1.16^{S}	4.38 ± 1.34^{a}	4.69 ± 1.22^{a}	**4.57[a]**	5.09 ± 0.58^{a}	**89.93[S]**
0.5 EBL + 100 IAA	137.48 ± 2.44^{S}	89.62 ± 0.62^{S}	12.19 ± 0.55^{A}	7.51 ± 0.75^{a}	**4.41[A]**	2.87 ± 0.86^{a}	**154.07[S]**
0.5 EBL + 1000 IAA	93.85 ± 1.39^{S}	92.82 ± 0.82^{S}	7.41 ± 0.45^{a}	5.98 ± 0.76^{a}	**2.75[a]**	1.93 ± 0.33^{a}	**104.02[S]**
2 EBL + 5 IAA	81.13 ± 0.87^{S}	89.73 ± 1.53^{S}	7.74 ± 0.61^{A}	8.51 ± 1.64^{a}	**1.25[a]**	1.36 ± 0.73^{a}	**90.12[S]**
2 EBL + 100 IAA	109.18 ± 2.34^{S}	90.02 ± 1.46^{S}	9.74 ± 1.15^{A}	6.34 ± 0.63^{a}	**4.48[A]**	3.74 ± 0.54^{A}	**121.45[S]**

Note: Values in Bold font are empirically calculated numbers. Within single hormone trials, and for each column, values labeled with the same letter are not significantly different from each other (P > 0.05), and values labeled with a letter plus an asterisk indicate significant difference from different letters, but no statistical difference from other equal letters. Within a mixed hormone trial, and for each column: S = synergistic effect; A = additive effect; a = antagonistic effect. Data shown as the mean ± SD, n = 4.

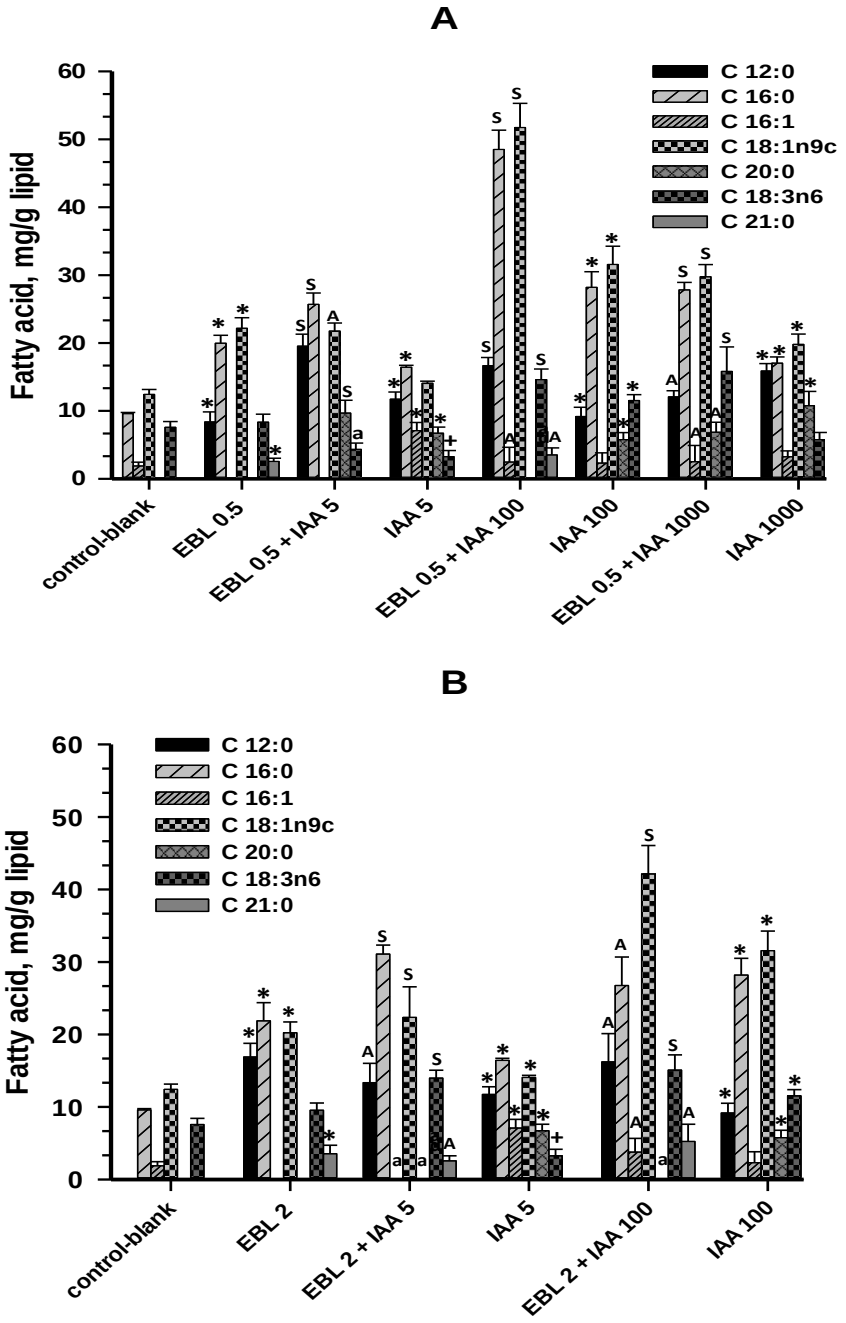


Figure 14. Major fatty acid distribution in *Scenedesmus quadricuada* cells on the day of test termination (Day 15) in the presence of mixed and single phytohormones used for the mixtures preparation: A) 0.5 nM 24-epibrassinolide (EBL) + 5, 100, or 1000 nM 3-indoleacetic acid (IAA); B) 2 nM 24-epibrassinolide (EBL) + 5 or 100 nM 3-indoleacetic acid (IAA). Statistically significant differences are indicated: *, for single hormones with significantly greater values from the control-blank ($P < 0.05$); $^{+}$, for single hormones with significantly lower values from the control-blank ($P > 0.05$); for mixed hormones: S = synergistic effect; A = additive effect; a = antagonistic effect. Data shown as the mean ± SD, n = 4.

4.3 Discussion

The primary purpose of this study was to investigate synergistic, additive, and antagonistic influences of a combination of two phytohormones, EBL and IAA, on microlagal physiology. The green microalga, *S. quadricauda*, is known to grow well in wastewaters of different origins (McGinn et al., 2012; Safafar et al., 2015). This species is used for industrial biomass production (Kim et al., 2007; Dev Goswami, 2011; Cordero et al., 2011; McGinn et al., 2012), and we have previously investigated the influence of a number of individual hormones on *S. quadricauda* physiology (Kozlova et al., 2017). In this study, the influence of mixtures of EBL and IAA, at different concentrations on *S. quadricauda* growth, cell size, and biomass production, as well as the accumulation of pigments (chlorophyll-a and total carotenoids) and fatty acids, was assessed against the performance of each individual hormone and against the control-blank. Nano-concentrations of EBL and IAA were chosen, since low concentrations are expected in hydroponic effluences (Moya et al., 1995; Hartung, 2010; Dalrymple et al., 2013; Nobel et al., 2014), which have been proposed for use as a growth medium for microalgae industrial production. Successful performance of phytohormones in nano-concentrations for microalgae biomass and valuable molecules production was confirmed in several studies (Nobel et al., 2014; Salama et al., 2014; Kozlova et al., 2017; Talarek-Karwel et al., 2018).

When applied to microalgae production units, the influence of mixed dissolved phytohormones on

microalgae physiology is expected. However, the combined influence of two or more phytohormones on microalgae is less studied. Interrelationships between phytohormones occur via transduction cascades of their metabolic pathways forming a signaling network (Harrison, 2012; Vidhyasekaran, 2015). The excessive activity of auxins in the signaling network is likely due to the fact that auxin homeostasis has multiple pathways in vascular plants. The activation of the auxin pathway depends on particular environmental factors, which trigger certain combinations of genes and enzymes (Ligung et al., 2002; Davis, 2004). Moreover, auxin has high binding capacity and a tendency to form conjugated conformations, which facilitate its involvement in phytohormones crosstalk network (Rock and Sun, 2005; Robert-Seilaniantz et al., 2011; Tatsuki et al., 2013; Vidhyasekaran, 2015), although formation of conjugates is reported to be different in microalgae (Stirk et al., 2013).

Signaling networks of phytohormones and their metabolic pathways are not well understood in microalgae. A few phytohormones crosstalk experiments conducted on different algae species gained some insights on the influence of different plant hormone combinations on microalgae physiology. Hunt et al. (2010) reported significant increase in biomass and chlorophyll accumulation, but not in lipid or protein production, when *Chlorella sorokiniana* was exposed to combined phytohormones from different taxa (auxin, gibberellin and cytokinin), while no effect was confirmed with a mix of different auxins (Vance, 1987; Hunt et al., 2010). Ozioko et al. (2015) also observed clear synergistic effects of mixed auxin with gibberellin on biomass and chlorophyll-a production in *Ch. sorokiniana* cells. Our results confirmed the ability of auxin IAA to synergistically increase cell division, biomass production, chlorophyll, total carotenoids and lipid accumulation, although our study, unlike the research of Hunt et al. (2010) and Ozioko et al. (2015), was conducted on *S. quadricauda*, using nearly 100 times lower hormone concentrations, and, more importantly, on the combinations of IAA + EBL.

Two assumptions previously proposed by some researchers led us to use two EBL concentrations, 0.5 and 2 nM, as the matrix solutions for the mixed hormones trials. First, the theory of one-way

relationship between BRs and auxins metabolism proposed by Davies (2004) and supported by other authors (Nakamura et al., 2003; Vert et al., 2008). Second, previous results highlighted the ability of exogenous BRs to enhance endogenous IAA accumulation and/or to elevate plant sensitivity to auxin (Sasse, 1999; Nakamura et al., 2003). This finding was supported by the only study, to our knowledge, conducted on co-influence of BRs and IAA on microalgae *Chlorella vulgaris* (Bajguz and Piotrowska-Niczyporuk, 2013).

When either 0.5 or 2 nM EBL was combined with 5 nM IAA, a similar impact on *S. quadricauda* growth, biomass production, cell size and all the tested metabolites accumulation was noted. However, when 2 nM EBL was a matrix solution, the lag phase of algae growth was clearly shorter resulting in the highest cell density of 248.06 cells $x10^4$/ml achieved in this study. The ability of phytohormones to reduce the lag phase of algae growth was previously reported for auxin (Hunt et al., 2010) and EBL (Talarek-Karwel et al., 2018). Thus, certain phytohormone combinations synergistically promote both greater biomass yield and its rate of production. When 100 nM IAA was combined with two EBL concentrations the results were similar only for the growth parameters and lipid accumulation, but not for the pigment accumulation where the pigments reached their maximum values of 219.3 ng/cell (chlorophyll-a) and 176.5 ng/cell (total carotenoids) at 0.5 nM EBL + 100 nM IAA mixture.

Our results on pigments accumulation suggest that in the mixed trials low IAA concentration of 5 nM is responsible for the gained effect, not the brassinosteroid. This supports the theory that BRs contribute to the synergistic effect with auxin independently from its concentration and likely independently from IAA signaling pathway, but via induction of the early auxin-inducible genes (Nakamura et al., 2003; Davies, 2004), which results in enhancement of IAA activity. However, the complex outcome of auxin (100 nM IAA) mixed with either of the two matrix concentrations of EBL suggests that the mechanism of EBL and IAA crosstalk was specific to targeted biosynthetic pathways. This assumption is supported by the work of Bajguz and Piotrowska-Niczyporuk (2013), who

demonstrated that auxin can also increase the activity and effectiveness of BRs in a synergistic manner. Nevertheless, the mechanism of formation of a signaling network in aqueous environment between exogenously applied phytohormones and their endogenous accumulation and the influence of this network on different algae species physiology is not yet well understood.

Our work was conducted as a batch-type experiment. Thus, the degradation of applied hormones by the end of the experiment was expected. Indeed, mitigation of the phytohormone effect on chlorophyll-a and total carotenoinds accumulation was noted. In most of the mixed trials, we observed a reduction in the influence of the hormone on pigment accumulation toward the end of the experiment (Day 15) that was more pronounced than in single hormone trials, suggesting that the phytohormones were unstable and were less effective over time.

An alternative explanation may be that a combination of the products of hormone degradation negatively affected pigment biosynthesis or resulted in an increase in intracellular pigment degradation. In previous studies, the attenuation of the positive effect of phytohormones on chlorophyll and carotenoid accumulation toward the end of the experiments was also observed (Tatkowska and. Buczek, 1980; Kozlova et al., 2017; Talarek-Karwel et al., 2018). However, some authors reported a stable increase in chlorophyll and carotenoid accumulation after 6-7 days of exposure (Tatkowska and. Buczek, 1980; Hunt et al., 2010; Nobel et al., 2014; Liu et al., 2016). A possible explanation of this discrepancy lies, as it was mentioned earlier, in differences in growth conditions. Different factors can singly, or in combination, alter hormone degradation, algae cell membrane permeability (Rober-Kleber et al., 2003; Perrot-Rechenmann, 2010), or directly affect pigment biosynthesis in algae species-specific manner (Pei et al., 2017).

Cell growth, biomass production, and total FA concentrations on the day of the test termination (Day 15) were significantly higher in all the single and combined hormone trials compared to the control-blank. This gives the impression that despite the possibility of hormone degradation, the initial exposure

of the cells to the hormones stimulated the enhancement of growth parameters and lipid accumulation (and possibly pigment production under certain conditions) in microalgae cells. This assumption is supported by recent work conducted on *S. quadricauda* and *Chlorella ellipsoidea* (Pei et al., 2017). A wider-range of algae species and physiological responses has to be further investigated for better prediction of the influence of particular hormone combination.

4.4 Conclusions

We have compared the co-influence of two phtyohormones, EBL and IAA, on several physiological parameters in *S. quadricauda*, with emphasis on the effect of these hormones on cell growth, cell biomass production, cell size, pigment biosynthesis (chlorophyll-a and total carotenoids), fatty acids biosynthesis and their profiles. We confirmed the synergistic relationship between a brassinosteroid (EBL) and an auxin (IAA) when *S. quadricauda* cells were exposed to the range of two hormones mixtures. Our results are generally consistent with previously reported observations on synergistic effects of combined BRs and auxins on the physiology of vascular plants and algae, and support the suggestion that phytohormones can simultaneously promote both microalgal cell growth and end-product biosynthesis. Even very small concentrations of the hormones tested were capable of enhancing the growth, biomass production, and biosynthesis of valuable biomolecules, which raises the possibility of producing high-value fatty acids via industrial-scale microalgal production using hydroponic wastewaters containing phytohormones.

In this project, wastewaters from hydroponics and fishery farms are considered as growth mediums for microalgae production. Thus, our next step of investigation was focussed on possible influence of fish steroids on *S. quadricauda* growth and valuable molecules accumulation.

Chapter 5

Effect of Fish Steroids 17β-estradiol and 17,20β-dihydroxy-4-pregnen-3-one on Growth, Accumulation of Pigments, and Fatty Acid Profiles in the Microalgae *Scenedesmus quadricauda* (CPCC-158)

5.0 Abstract

The influence of fish steroid hormones on algal (*Scenedesmus quadricauda*) physiology was investigated, as it is relevant to the use of aquaponics wastewaters for microalgae biomass production. *S. quadricauda* was exposed to two steroids, 17β-estradiol (E2) and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P), commonly found in fish farm effluents. The tested ranges of E2 and 17,20β-P were 15, 50, 100 and 300 ng/L and 1, 5, 15, 50, 100, 500, 1000 ng/L, respectively, reflecting the expected concentrations of the steroids in the effluents of fish spawning tanks. Both steroids were tested using modified Bold Basal Medium (BBM). The tested steroids demonstrated stimulatory effects on *S. quadricauda* cell growth, biomass production, as well as on intracellular accumulation of chlorophyll-a, total carotenoid, and lipids. However, the influence on the kinetics of the population growth and most effective concentrations were different for the two hormones. Both sex steroids promote total carotenoid accumulation to higher degree than they promote chlorophyll accumulation. E2 was a more powerful inducer of the tested pigments and lipid accumulation compared with 17,20β–P in this study. The work was conducted to demonstrate the possibility of integration of cold-water aquaculture with algae production companies to maximize dissolved in wastewater nutrients recycling and induction of algae biomass and valuable molecules yield using nano-concentrations of naturally occurred fish steroids.

5.1 Introduction

The composition and concentrations of dissolved nutrients, the possibility of chemical interference and/or physical interactions among the compounds, and the possibility of their accumulation and/or bio-magnification in algal cells are important characteristics for accurate prediction of their influence on the physiology of a particular microalgal species. Clarification of these characteristics is highly important when a wastewater is designed to be used as a growth medium for microalgae biomass production. Generally, the closer physico-chemical characteristics of the chosen wastewater are to the optimal conditions for microalgae maximum production and its targeted molecule(s) biosynthesis, the more efficient and less costly the use of the wastewater will be.

One of the major groups of organic compounds of agricultural and domestic wastewaters is animal hormones. Numerous reports provide the range of residual E2 in surface waters as 1 to 200 ng/L E2 in North America and Europe (Pojana et al., 2007; Zhou et al., 2011; Zhang et al., 2011; Esteban et al., 2014; Liu et al., 2018). Concentrations of 17,20β-P in surface waters are approximately 5-10-fold higher (Hala et al., 2012; Mota et al., 2014).

Several studies were conducted to evaluate fish steroids concentration in effluents from fish farms. Different fish species can release up to 40-50 ng/g/h of 17,20β-P, as was shown in studies conducted on *Carassius auratus* (Sorensen et al., 2005) and rainbow trout (Barry et al., 1997), where hundreds of μg/L of this hormone were detected at maximum fish density in a spawning tank. Ratios of 17,20β-P and E2 in a reproduction tank varied from about 4:1 to 20:1, depending on fish species and their age (Hala et al., 2012).

Fishery wastewaters have gained a reputation as a great inducer of agricultural plant growth and biomass production (McMurtry et al., 1997; Takeda et al., 1997; Adler et al., 2000; Rakocy et al ., 2004, 2007; Bansal et al., 2005; Bailey and Ferrarezi, 2017), because they contain all macro- and micronutrients required for successful growth of higher plants (Xu and Shen, 2011). Fishery wastewaters have been

utilized successfully as a source of nutrients in integrated aquaponic-systems and have had positive effects on hydroponic crop quality and production (Zweig, 1986; Rakocy et al., 2004).

Beside the micro- and macronutrients dissolved in fishery wastewater, fish hormones and other organics may play important role in promotion of plants growth and biosynthesis of valuable molecules. Upadhyay and Maier (2016) reported that E2 induced shoot and root growth and enhanced the photosynthetic rate, carbohydrate and protein accumulation in *Arabidopsis sp.* treated with low hormone concentrations of 10 and 100 nM (2.72 and 27.2 μg/L). In the studies conducted on many high plants, including Lemnaceae, E2 demonstrated great ability in promoting flowering and ovary formation (Czygan, 1962; Ylstra et al., 1995; Bhardwaj and Thukral, 2000; Janeczko et al., 2003). However, for most vasular plants, fish effluents that are rich in ammonium ions have to be biologically treated to convert the ammonium to nitrate and nitrite. Thus, additional investment for bio-filtration has to be taken in account. There is no need in such a treatment for microalgal biomass production since they can consume ammonium ions as a source of nitrogen.

Growing evidence of the positive effects of fishery farms effluents on microalgal physiology suggests that: a) the macro- and micronutrients dissolved in these wastewaters are in sufficient amount for elevated algal biomass production; b) unlike hydroponic plants, microalgae can consume ammonium released by fish without preliminary bioconversion by nitrification bacteria; c) fish metabolites released into the water may play an important role in overall induction of microalgal growth and biosynthesis by fishery wastewater.

Mammal hormones have been reported to induce microalgae growth (Czerpak and Szamrej, 2000; Bajguz and Czerpak, 1996a, b) and production of valuable molecules, such as carotenoids (Bajguz and Czerpak, 1996a; Czerpak et al., 2001b), proteins (Bajguz and Czerpak, 1996b; Czerpak and Szamrej, 2000) and soluble sugars (Bajguz and Czerpak, 1996b; Czerpak and Szamrej, 2000). The mechanism of the influence is complex. When applied to the growth medium, E2 and 17,20β-P are taken-up rapidly by

algal cells (absorption > adsorption), with about 90 % uptake in 48 hours (Liu et al., 2018). Further biodegradation occurs during 4-6-days, resulting in intracellular accumulation of steroid metabolites, of both known (e.g., E2 transfer to E1) and unknown nature (Larsson et al. 1999; Shi et al., 2010; Liu et al., 2018). The influence of these metabolites on microalgae growth and biosynthesis needs to be studied further.

High concentrations of steroids are toxic to algae and bring a risk of intracellular hormone accumulation. Toxicity of E2 to microalgae is relatively low, normally more than 3.2 mg/L EC50 (Liu et al., 2018), depending on the species and water chemistry. Thus, a reduced range of steroid concentrations, compared to previous studies, was chosen for investigation, considering the following key points: a) expected loading of the hormones in fishery wastewater effluents; b) toxicity of the hormones to plants and algae; and c) an attempt to avoid of the possibility of hormone accumulation in the algal cells.

Scenedesmus quadricauda was chosen for this study because of its tolerance to environmental changes which would be expected in an aquaponics system: little change in growth was observed between 14 and 30 °C (Xu et al., 2012) and between pH 6 and 9.5 (Martinez et al, 1999). Being an industrial scale production species, *S. quadricauda* can demonstrate high growth rate (0.8 - 1.2 d^{-1}), high accumulation of pigments (22 mg/L of lutein, 3 mg/L of β-carotene), and high lipid production (55-80 % drw) including 37 % of total lipid as omega-3-FA (Makarevičienė et al., 2011; Li et al., 2011; Cordero et al., 2011).

Taking into account that in Canada the aquaculture industry has grown more than 5-fold in the past 20 years (CAIA, 2015-2016; DFO, 2016) and that the primary species of fish cultured in the Prairies are cold-water fish (Rainbow trout (*Oncorhynchus mykiss*) and Arctic charr (*Salvelinus alpinus*) (DFO, 2012), integration of cold-water aquaculture and algae production companies is highly beneficial for the development of industry in Manitoba.

5.2 Results

5.2.1 Effect of steroids on *S. quadricauda* growth, biomass production, and cell size

5.2.1.1 Algal cell growth

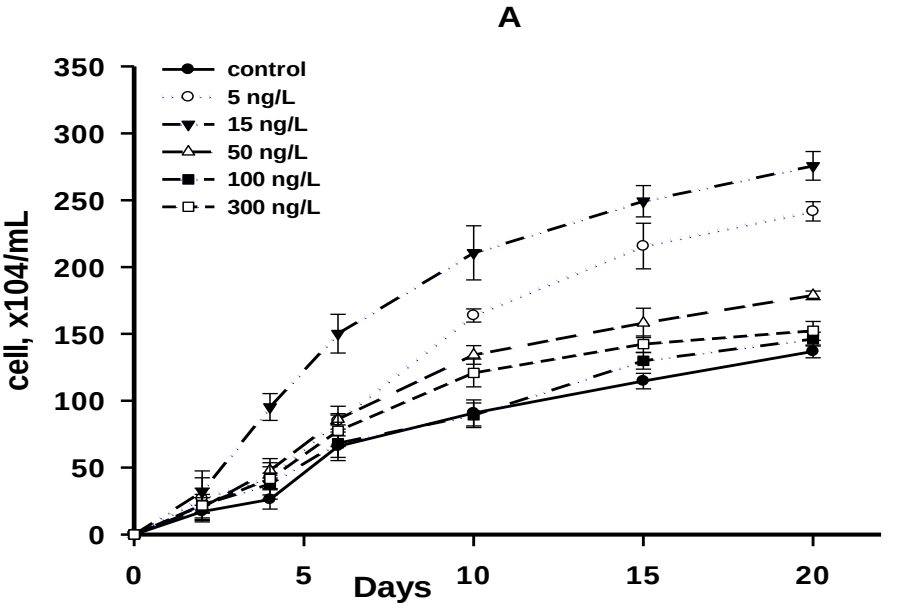
Both investigated fish steroids, E2 and 17,20β-P, were capable of significantly stimulating *S. quadricauda* growth. However, at the most effetive concentrations, the degree of increase and the kinetics of cell growth were dissimilar over the 20 days of the experiment (Figures 15A and 15B). The most effective concentration was 15 ng/L for both hormones. However, 17,20β-P was effective at a concentration of 500 ng/L, while no significant increase was observed at two of the lowest concentrations tested: 1 and 5 ng/L (Figure 15B). E2 was effective from 5 to 50 ng/L (Figure 15A). The maximum increase in E2 trial was 2-fold compared with the control cultures, reaching 275.74 cells x 10^4/mL at 15 ng/L (Figure 15A, Table 11). The greatest increase in cell density was observed in the 17,20β-P trial at 15 ng/L, reaching 303.5 cells x 10^4/mL, which was 2.6-fold greater than in the control cultures (Figure 15B, Table 11). However, in the E2 trial, a significant difference from the control was observed earlier (from day 4 at 15 ng/L and from day 10 at 5 ng/L) compared to the 17,20β-P trial, where 5, 15 and 100 ng/L were effective from day 10 and 500 ng/L from day 15 of the experiment.

The order of efficacy of the steroids tested was (ng/L): $15 \geq 5 > 50 > 300 \geq 100 \approx$ control for E2; and $15 > 50 > 100 > 500 > 5 \geq 1 \approx$ control ≤ 1000, for 17,20β-P. Despite the fact that *S. quadricauda* cells exposed to a concentration of 1000 ng/L, cells in the 17,20β-P trial grew to a slightly lower density than cells in the untreated (control cultures), no statistically significant difference was detected. Thus, the highest concentration of 17,20β-P tested was not considered to be toxic to *S. quadricauda* cells.

5.2.1.2 Relationship between cell size, biomass and cell density

Most of the E2 concentrations tested (except the highest concentration of 300 ng/L) resulted in increased *S. quadricauda* cell size and biomass production (Table 11). Cell density at the day of test termination (day 20) was significantly increased at all the E2 concentrations tested, except 100 ng/L.

Exposure of *S. quadricauda* cells to 17,20β-P increased cell size at 100 and 500 ng/L only (Table 11). Biomass production was elevated at 17,20β-P concentrations of 15, 50, and 100 ng/L. An influence of 17,20β-P at the highest concentrations tested could not be confirmed, due to the loss of the samples. Cell density at the day of test termination (day 20) was significantly increased at 15, 50, and 100 ng/L of 17,20β-P. Maximum biomass production was at 15 ng/L, resulting in 2.6-fold increase compared with the control in both trials.



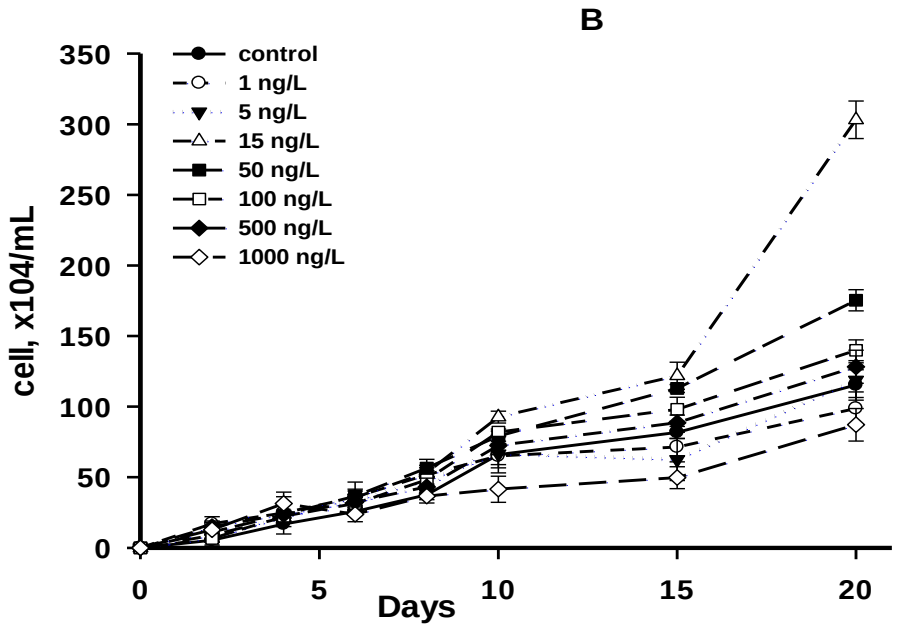


Figure 15. Growth of *S. quadricauda* in BBM containing different concentrations of steroid hormones: A) E2; and B) 17,20β-P. Data shown as the mean ± SD, n = 12.

Table 11. *S. quadricauda* cell size (μm), biomass (g dcw/L), and cell density (n x 10^4/mL) at the termination of each experiment (day 20).

Hormone concentration, ng/L	Cell size (μm)	Biomass (g dcw/L)	Cell density (x 10^4/mL)
E2 trial			
control	45.06 ± 1.22	0.49 ± 0.12	136.87 ± 4.75
5	54.96 ± 2.00*	0.77 ± 0.07*	241.68 ± 7.24*
15	57.64 ± 1.19*	1.29 ± 0.06*	275.74 ± 10.69*
50	48.05 ± 1.07*	0.85 ± 0.10*	178.93 ± 8.73*
100	48.85 ± 1.15*	0.81 ± 0.14*	146.12 ± 5.75
300	44.82 ± 0.64	0.73± 0.08	152.34 ± 4.75*
17,20-P trial			
control	43.42 ± 1.61	0.46 ± 0.07	112.34 ± 8.75
1	41.44 ± 2.03	0.48 ± 0.07	98.67 ± 11.74
5	41.52 ± 3.23	0.50 ± 0.10	118.72 ± 12.27
15	42.85 ± 2.41	1.19 ± 0.04*	303.17 ± 13.32*
50	46.85 ± 1.91	0.96 ± 0.08*	175.39 ± 8.46*
100	48.54 ± 1.76*	0.88 ± 0.04*	139.87 ± 7.33*
500	48.52 ± 3.02*	***	128.22 ± 3.16
1000	43.02 ± 2.16	***	97.17 ± 8.59

Note: * - significantly greater than control (P < 0.05); ***** -** samples were lost;

Data shown as the mean ± SD (for cell size n = 15; for biomass n = 4; for cell density n = 12).

5.2.2 Effect of fish steroids on the accumulation of pigments in *S. quadricauda* cells

5.2.2.1 Chlorophyll-a accumulation

Chlorophyll-a concentrations per cell were significantly increased in *S. quadicauda* by both steroids tested, with similar maximum values (Figure 16A and 16B). However, the most effective concentrations and the kinetics of chlorophyll-a accumulation in cells were dissimilar between two steroids over the 20 days of the experiment. E2 stimulated increases in chlorophyll-a accumulation were in the range of 1.6- to 2.2-fold compared with chlorophyll-a accumulation in untreated control cells at concentrations of 5 to 100 ng/L, while the highest tested concertation of 300 ng/L was slightly less effective, with maximum increase in 1.6-fold on day 15 (Figure 16A). Importantly, all the E2 concentrations tested exhibited significant stimulation of chlorophyll-a accumulation between day 2 and day 20 of the experiment. The effect was maximum and relatively stable between day 6 and day 20, with the maximum value of 162.8 ng/cell of chlorophyll-a in cells exposed to 5 ng/L on day 10 of the experiment (Figure 16A).

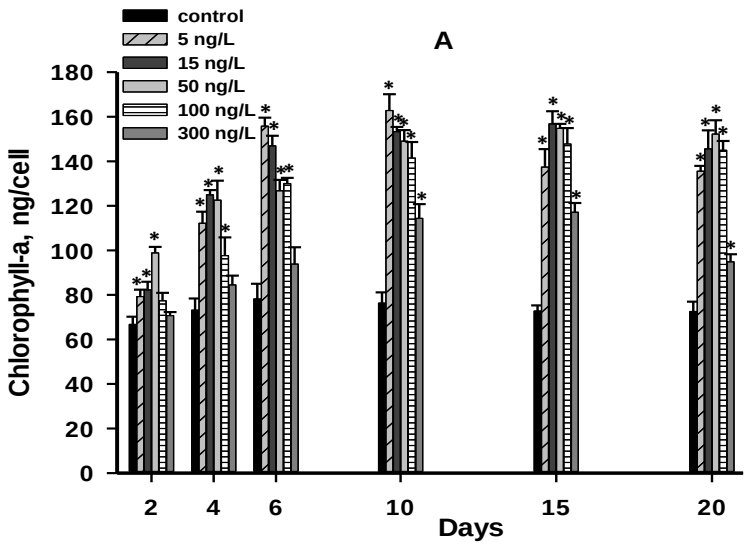
In the 17,20β-P trial, the most effective stimulation of chlorophyll-a accumulation was observed in cells exposed to 15 to 500 ng/L of the hormone, with a maximum of 1.5- to 2.8-fold increase compared with untreated (control) cells on day 2 of the experiment (Figure 16B). Unlike in the E2 trial, the stimulatory effect of 17,20β-P was most pronounced between day 2 and day 6, after which the effect diminished to the end of the experiment (day 20). The maximum values of chlorophyll-a accumulation in this trial was 165.6 ng/cell, observed in cells exposed to 100 ng/L 17,20β-P on day 4 of the experiment (Figure 16B). The order of efficacy for stimulation of chlorophyll-a by the two steroids was: $5 \geq 15 \geq 50 > 100 > 300$ ng/L for E2; and $100 > 50 > 500 > 15 > 5 \geq 1 \geq 1000$ ng/L for 17,20β-P. No toxic effects on chlorophyll-a accumulation were observed for either hormone at any concentration tested.

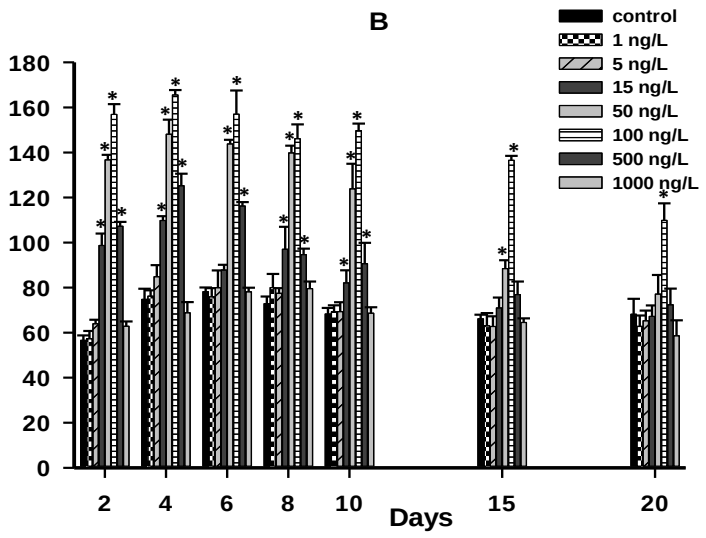
5.2.2.2 Total carotenoid accumulation

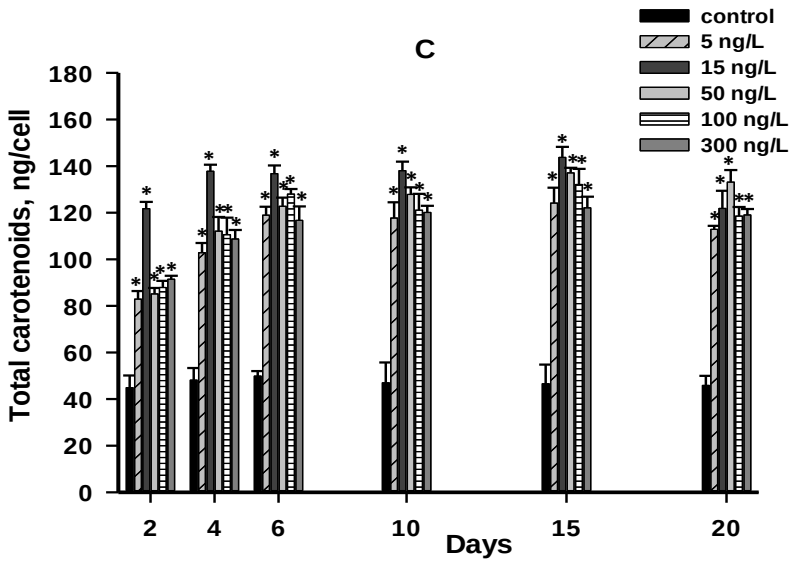
The steroids tested also stimulated total carotenoid production and intracellular accumulation in *S. quadricauda* cells (Figure 16C and 16D). A more pronounced effect was observed in the E2 trials, where

all concentrations tested stimulated accumulation of carotenoids 2.1- to 2.9-fold, compared with the untreated control cells from day 4. The greatest total carotenoid accumulation, 143.75 ng/cell, was stimulated by E2, at 15 ng/L on day 15 of the experiment (Figure 16C). The effect remained relatively stable until the end of the experiment.

In the 17,20β-P trial, the maximum increase in total carotenoids was observed on day 4 at 100 ng/L, resulting in 103 ng/cell (Figure 16D). Unlike the effect on chlorophyll-a accumulation, all concentrations of 17,20β-P tested significantly stimulated total carotenoids production in *S. quadricauda* cells. However, similar to chlorophyll-a, the effect of 17,20β-P was most pronounced between day 2 and day 6, with further mitigation of its impact by the end of the experiment (day 20). The order of the efficacy for carotenoid stimulation was: $15 > 50 \geq 100 > 5 \geq 300$ ng/L for E2 and $100 > 50 > 500 > 15 \geq 5 \geq 1000 \approx$ 1ng/L for 17,20β-P (Figure 16D). No toxic effects on total carotenoid accumulation were observed for either hormone at any concentration tested.


A
control
5 ng/L
15 ng/L
50 ng/L
100 ng/L
300 ng/L
Chlorophyll-a, ng/cell
Days


B
control
1 ng/L
5 ng/L
15 ng/L
50 ng/L
100 ng/L
500 ng/L
1000 ng/L
Days


C
control
5 ng/L
15 ng/L
50 ng/L
100 ng/L
300 ng/L
Total carotenoids, ng/cell
Days

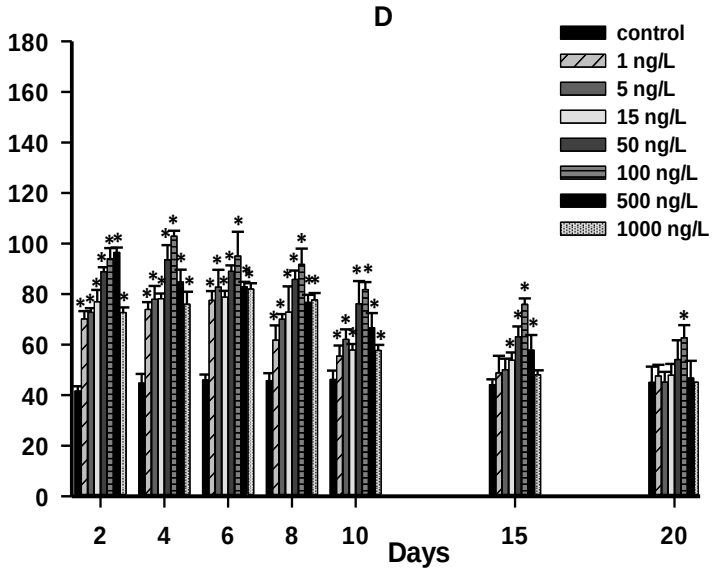

D
control
1 ng/L
5 ng/L
15 ng/L
50 ng/L
100 ng/L
500 ng/L
1000 ng/L
Days

Figure 16. Chlorophyll-a accumulation in *S. quadricuada* cells exposed to different concentrations (ng/L) of A) E2 and B) 17,20β–P; and total carotenoid accumulation in *S. quadricuada* cells exposed to different concentrations of C) E2; and D) 17,20β-P. *, Statistically significant differences of treated versus controls ($P < 0.05$) are indicated, Data shown as the mean ± SD, n = 15.

5.2.3 Effect of fish steroids on lipid accumulation and their fatty acids profiles

Two steroids tested significantly increased fatty acids (FA) accumulation in *S. quadricauda* cells (Table 12, Figures 17A and 17B). All the E2 concentrations tested stimulated major FAs (each > 3 % of total FAs), minor FAs (each 1-3 % of total FAs), and total FA accumulation (Table 12). In this trial, the most pronounced increase in the accumulation of major and total fatty acids was observed at 50 and 100 ng/L, with maximum values of 165.11 mg/g lipids for major FAs at 50 ng/L E2 (3.1-fold increase) and 190.59 mg/g lipids for total FAs at 100 ng/L E2 (2.5-fold increase). Other tested concentrations were slightly less effective in the induction of major and total FAs synthesis (2.6- to 2.7-fold compared with untreated control cells).

Accumulation of minor FAs was also greatly stimulated by all E2 concentrations tested, resulting in 2.4- to 3.9-fold increase compared to untreated control cells (Table 12). In the 17,20β-P trial, all concentrations tested stimulated major FAs biosynthesis. However, the lowest concentrations of 1 and 5 ng/L did not affect total FAs accumulation, and minor FAs accumulation was negatively affected by 17,20β-P concentrations from 1 to 100 ng/L. Interestingly, no effect on FA biosynthesis was observed in cells exposed to 17,20β-P concentrations of 500 and 1000 ng/L (Table 12).

The most pronounced increase in the accumulation of major and total fatty acids was observed at 50, 100, and 500 ng/L with maximum values of 159.94 mg/g lipids for major FAs (3.4-fold increase) and 174.5 mg/g lipids for total FAs at 100ng/L 17,20β-P (2.4-fold increase). The core FAs in both trials were palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1n9c), linoleic (C18:2n6c), and γ-linolenic (C18:3n6) acids (Figure 17A and 17B).

Table 12. Fatty acid accumulation in *S. quadricauda* cells at the day of a test termination (day 20) (Major FAs, each > 3 % of total FAs; Minor FAs, each 1-3 % of total FAs; Minimal (Min) FAs, each < 1 % of total FAs)

Hormone concentration	Major FAs, mg/g lipids	Major FAs, % of total FAs	Minor FAs, mg/g lipids	Minor FAs, % of total FAs	*Min FAs, mg/g lipids	^Min FAs, % of total FAs	*Total FAs, mg/g lipids
E2 trial							
Control	52.82 ± 0.97^{a}	61.12 ± 1.18^{a}	6.99 ± 1.09^{a}	9.95 ± 1.05^{a}	**17.98**	24.75 ± 0.77^{a}	**77.02**
5	139.43 ± 1.37^{b}	81.35 ± 0.64^{b}	24.18 ± 2.11^{b}	13.73 ± 1.27^{a}	**8.82**	$4.97 \pm 1.33^{b*}$	**172.43**
15	144.83 ± 3.25^{c}	88.16 ± 0.98^{c}	16.57 ± 1.54^{c}	9.91 ± 2.15^{a}	**3.02**	1.78 ± 2.42^{b}	**164.42**
50	165.11 ± 1.66^{d}	88.72 ± 1.84^{c}	18.80 ± 0.85^{c}	$10.11 \pm 1.82^{a*}$	**2.12**	$1.17 \pm 1.55^{b*c}$	**186.03**
100	161.84 ± 2.43^{d}	84.74 ± 1.72^{d}	26.65 ± 2.00^{b}	$14.07 \pm 0.97^{a*b}$	**2.10**	1.06 ± 0.49^{c}	**190.59**
300	144.81 ± 1.41^{c}	80.82 ± 2.04^{b}	$27.15 \pm 0.58^{b*}$	15.28 ± 1.05^{b}	**6.14**	3.73 ± 0.88^{b}	**178.10**
17,20β-P trial							
Control	46.63 ± 1.62^{a}	62.37 ± 0.49^{a}	8.71 ± 0.53^{a}	11.86 ± 0.45^{a}	**18.83**	25.72 ± 2.05^{a}	**74.17**
1	50.18 ± 0.78^{b}	82.55 ± 0.56^{b}	4.17 ± 1.03^{b}	6.43 ± 0.53^{b}	**7.35**	11.12 ± 1.66^{b}	**61.70**
5	63.09 ± 1.07^{c}	85.30 ± 1.57^{c}	5.54 ± 0.84^{b}	7.17 ± 1.44^{b}	**5.76**	7.49 ± 0.82^{c}	**74.39**
15	100.86 ± 2.22^{d}	87.95 ± 0.53^{d}	$6.89 \pm 0.75^{b*}$	5.71 ± 0.53^{b}	**7.55**	6.33 ± 1.00^{c}	**115.30**
50	157.24 ± 1.52^{e}	94.06 ± 1.61^{e}	5.71 ± 0.88^{b}	3.04 ± 0.57^{c}	**2.35**	2.79 ± 0.76^{d}	**165.30**
100	159.94 ± 0.98^{e}	91.58 ± 0.98^{e}	6.76 ± 1.67^{ab}	3.86 ± 0.78^{c}	**7.79**	4.52 ± 1.24^{cd}	**174.49**
500	142.51 ± 3.26^{f}	88.42 ± 1.14^{cd}	9.44 ± 1.14^{a}	5.62 ± 0.69^{b}	**10.07**	$5.93 \pm 0.65^{c*d}$	**162.02**
1000	93.68 ± 1.05^{g}	86.02 ± 1.57^{cd}	8.72 ± 2.31^{a}	7.88 ± 0.82^{b}	**6.78**	$5.99 \pm 1.22^{cd*}$	**109.18**

Note: Values in Bold font are empirically calculated numbers. Within a trial, and for each column, values labeled with the same letter are not significantly different from each other ($P > 0.05$), and values labeled with a letter and an asterisk indicate significant difference from each other, but no statistical difference from other equal letters. Data shown as the mean ± SD, $n = 4$.

In the E2 trial, the core FAs reached maximum concentrations of 23.33 mg/g lipid for palmitic acid (1.5-fold increase), 10.53 mg/g lipid for palmitoleic acid (1.7-fold increase), 36.79 mg/g lipid for oleic acids (2.1-fold increase), 39.82 mg/g lipid for linoleic acids (7.9-fold increase), and 11.19 mg/g lipid for γ-linolenic (1.4-fold increase), at 50, 300, 50, 15 and 100 ng/L E2, respectively (Figure 17A). Four additional FAs were detected when E2 was added to the growth medium. Lauric (C12:0), myristic (C14:0) and 9-trans-oleic acid (C18:1n9t) acids were found at all E2 concentrations tested, while heneicosanoic acid (C21:0) was detected at 50 and 100 ng/L E2 (Figure 17A). Lauric and myristic acids accumulated to a maximum of 12.2 and 8.9 mg/g lipid respectively at 15 ng/L E2. Elaidic and heneicosanoic acids had their maximum values of 24.3 and 9.2 mg/g lipid at 100 ng/L E2 (Figure 17A).

In the 17,20β-P trial, the core FAs reached maximum concentrations of 31.67 mg/g lipid for palmitic acid (2.2-fold increase at 50 ng/L), 8.83 mg/g lipid for palmitoleic acid (1.9-fold increase at 500 ng/L), 45.68 mg/g lipid for oleic acid (3.0-fold increase at 100 ng/L), 29.44 mg/g lipid for linoleic acid (7.8-fold increase at 50 ng/L), and 10.83 mg/g lipid for γ-linolenic acid (1.2-fold increase at 50 ng/L) (Figure 17B). Three additional FAs were detected when 17,20β-P was added to the growth medium: lauric, margaric, and 9-trans-oleic acid acids were found at 15 to 1000 ng/L 17,20β-P (Figure 17B). Low amounts of margaric acid also was detected at 1 ng/L of 17,20β–P. Lauric acid accumulated to a maximum of 21.6 mg/g lipid at 50 ng/L 17,20β-P. Margeric and elaidic acids had their maximum values of 16.8 and 12.0 mg/g lipid at 500 ng/L 17,20β-P (Figure 17B).

The order of significant increase in concentrations of core FAs in the E2 trial was: C18:2n6 > C18:1n9c > C16:0 > C16:1 ≥ C18:3n6, while for the minor FAs this was: C18:3n3 (α-linolenic acid) > C18:0 (octadecanoic acid) > C17:0 > C20:1 (cis-11-eicosenoic acid) > C17:1 (cis-10-heptadecenoic acid) ≥ C24:1 (nervonic acid). The order of significance of core FAs in the 17,20β-P trial was: C18:1n9c > C16:0 > C18:2n6 > C18:3n6 > C16:1, and for the minor FAs, this was: C18:3n3 > C17:1 > C14:0 > C24:1.

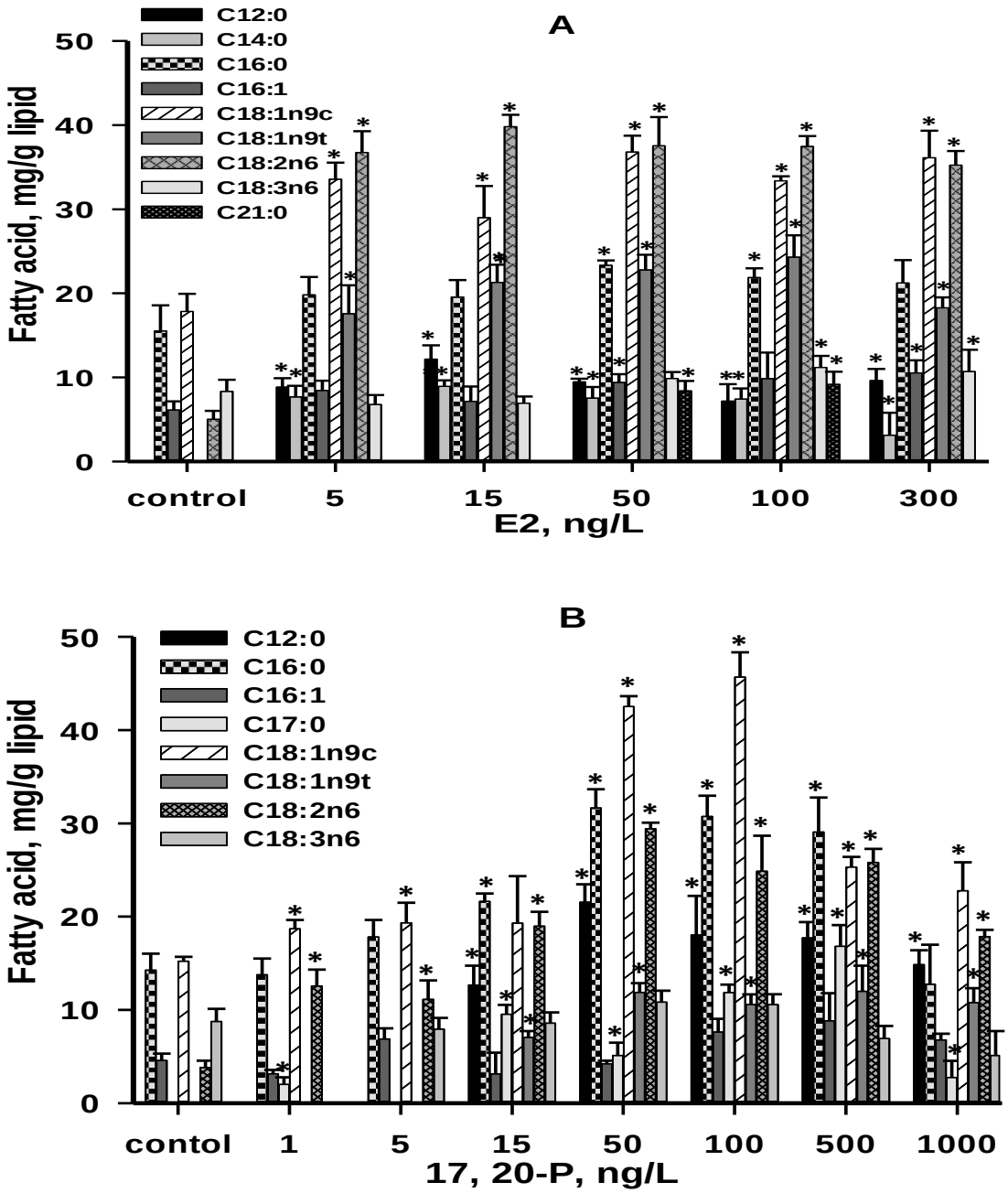


Figure 17. The distribution of major fatty acids in *S. quadricuada* cells on the day of test termination (on day 20). A) Fatty acid profiles in cells exposed to different concentrations of E2; B) Fatty acid profiles in cells exposed to different concentrations of 17,20β-P. *, Statistically significant differences of treated versus controls ($P < 0.05$). Data shown as the mean ± SD, n = 4.

5.3 Discussion

Integration of microalgae and fish biomass production in one recirculating system such as aquaponics brings up the question of the influence of algal and fish metabolites dissolved in the water on the physiology of co-grown biomass, and consequentially on the quality of commercial products extracted from both biomasses (e.g., quality of algal oils for biofuels, carotenoids for pharmaceuticals, and quality of fish flesh). In this project, wastewater from a Rainbow Trout and Arctic Char hatchery farm were specified to be applied as a growth medium for algal biomass production. Thus, it is highly important to understand if and how fish metabolites, in this case two fish steroids commonly found in water, E2 and 17,20β-P, can affect algae physiology. Therefore, the primary goal of this research was to investigate whether low concentrations of two selected steroids are able to affect growth performance and valuable molecules (FAs and pigment) biosynthesis and their intracellular accumulation in *S. quadricauda* cells.

Low concentrations of the two steroids were selected for the following reasons. First, the concentrations must be within the range of the expected E2 and 17,20β-P loadings in hatchery farms effluents. Second, the range has to be lower than the Lowest Effective Concentration (LOEC) for different plants and algae species to avoid not only toxic effects on algal physiology, but also the possibility of hormones accumulation in algal cells. The toxicity of E2 is well studied and the EC50 48 hour value lies between 1 and 5 mg/L for aquatic (Lemnaceae) and terrestrial plants (Heftmann, 1975; Mohsen et al., 1987; Bhardwaj and Thukral, 2000; Oost et al., 2016), which gives an LOEC value lower than 1 mg/L. However, in the study conducted on fungus *Pythium periplocum*, a significant decrease in sexual reproduction was observed at much lower E2 concentrations of 272 µg/L, while 900 µg/L E2 was lethal for this species (Hendrix & Guttman, 1968).

Quite a wide-range of toxicities may be found in the literature for microalgae. E2 was reported to have the lowest observed adverse effect on growth of *Chlorella vulgaris* cells, as well as on carotenoid

and chlorophyll accumulation, at a concentration of 272 μg/L, between 10 and 20 days of exposure (Bagjuz and Cserpak, 1996a, b). However, Beecher (2013) reported a toxic effect of E2 on *P. subcapitata* growth at concentrations of 500 ng/L or greater. To our best knowledge, it is the highest LOEC for E2 toxicity reported for an algal species.

Data on 17,20β-P toxicity is very limited, although Sorensen et al. (1995) suggested that olfactory system of male goldfish can react to waterborne 17,20β-P at concentrations as low as 3 ng/L, but no diverse effect of such small concentrations of the steroid was reported..

Nevertheless, the application of fishery wastewaters with the steroids loading for microalgae biomass production raises the concern of possible bioaccumulation of hormones in the biomass generated by algal cultures exposed to these hormones, and, hence, the suitability of the biomass for agriculture (animal feed) and pharmaceutical industry. There are some reports that microalgae can bioconcentrate in their cells and subsequently biomagnify steroids into higher trophic levels at high (> 1 mg/L) waterborne exposures (Pflugmacher et al., 1999; Sijm et al., 1998). This assumption is supported by the fact that estrogens can easily cross membranes via a simple diffusion pathway (Williams and Stancel, 1996), although the mechanism for membrane crossing by 17,20β-P in algal cells is not well studied.

Despite the probability of bioaccumulation of sex steroids in algae cells, there is some evidence that suggests this risk is low in fish hatchery farm effluents. First, it was reported that the concentration factors of natural estrogens in algae are significantly lower than those reported for synthetic estrogens (e.g., ethinyloestradiol EE2) and other endocrine-disrupting substances (Larsson et al., 2000). Moreover, Lai et al. (2002) suggested that biotransformation and biodegradation of intracellular estrogens would work well up to the saturation point, which depends on the waterborne exposure concentration, which corresponds to 400 g/L/g wwt or greater.

Numerous studies conducted on algae species elucidated the mechanism of steroid removal (including passive adsorption and active absorption) and their biodegradation. The corresponded percent of biodegradation (BDP) and the speed of action vary among the studies. For natural estradiols it takes from 3 hours to 4-5 days to be removed and biotransformed, which depends on algal species, water chemistry, lighting, the hormone concentration to algae density ratio, and the presence of bacteria (Shi et al., 2010; Li et al., 2010; Norvill et al., 2017; Liu et al., 2018). Importantly, it was reported that E2 likely cannot be transferred via the food chain (Beecher, 2013).

Thus, the results of these studies coupled with the fact that estrogenic activity of natural steroids, such as E2, is about 100 to 7,000 times lower (Nendza and Wenzel, 2006) than their synthetic analogues, suggests that no intracellular accumulation of the tested steroids is expected. However, more research is needed for different algal species and growth conditions.

Previously we investigated the influence of four phytohormones (EBL, BL, IAA, and ABA), individually and in some combinations, on physiology of *S. quadricauda* (Kozlova et al., 2017, 2018). In this study, we were interested to investigate whether or not two selected fish steroids are also capable of stimulation of both *S. quadricauda* biomass production and valuable molecules biosynthesis. Our results demonstrate that both E2 and 17,20β-P promoted algal cell growth and biomass production, although the kinetics of the population growth and most effective concentrations were different for the two hormones.

Moreover, the lag phase of algal growth was clearly shorter in the E2 trial at the most effective concentrations of 15 and 5 ng/L. This may be due to the specific influence of E2 on the early growth stages of aquatic organisms such as fish juveniles (Liu et al., 2012), plant shoots, buds and young roots (Janeczko and Skoczowski, 2005; Lino et al., 2007) and may also be true for microalgae.

Unlike the 17,20β-P concentrations tested in this study, and the phytohormones investigated previously (Kozlova et al., 2017, 2018), the influence of E2 on pigment production and accumulation

was relatively stable until the end of the experiment (day 20). A possible explanation of this fact may be related to the mechanism of E2 degradationBiological degradation is rapid where E2 is taken-up by algal cells within 3-4 hours for 80-90 % and then converted to estrone (E1), which can be further metabolized to estriol and hydroxyestrone (Hom-Diaz et al., 2015; Liu et al., 2018), with assistance of 17β-hydroxysteroid dehydrogenase (Itagaki and Iwaya, 1988) and, importantly, with a little portion of inverse reaction (Larsson et al. 1999; Shi et al., 2010; Ghasemi et al., 2011). Thus, it is possible that the products of E2 degradation are mainly responsible for the observed effects on algal physiology.

Our results support earlier research conducted by Bajguz and Czerpak (1996a), suggesting that sex steroids promote total carotenoid accumulation more than they promote chlorophyll accumulation

In vascular plants, E2 was shown to induce pigments accumulation, simultaneously increasing intracellular content of GA and auxin (Kopcewicz and Rogozinska, 1974). According to the Janeczko, 2012 flower buds contain 2-3 times higher estrogen and progesterone concentration compared to developed flowers. The same pattern was found to be true in ripening tomatoes: immature green tomatoes contain 40 times higher progesterone loading compared to red tomatoes (Lino et al., 2007). It gives a clue about involving phytosteroids in carotenogenesis in plant. From here, it is possible that E2 and 17,20β–P may also directly and indirectly, via activation of phytohormones synthesis, affect photosynthesis and carotenogenesis in algae cells. The fact that both tested hormones were detected in seeds (wheat, rice, corn), vegetable oils and plant tubers (potatoes) in concentrations as high as 5.1 ng/g wwt (Hartmann et al., 1998; Lino et al., 2007) suggests that the steroids involved in regulation of lipogenesis and/or in lipid deposition in high plant. Indeed, a pronounced positive effect of both steroids on lipid accumulation was found in our research with a stronger effect of E2 compared to 17,20β-P. 1

Similarly to the influence of some phytohormones (Salama et al., 2014; Kozlova et al., 2017; Babu et al., 2017), the two animal hormones tested in this study altered the fatty acid profiles of lipids synthesized by *S. quadricauda*. Yet, while all the tested concentrations of E2 were effective. the lowest

concentration of 17,20β-P tested did not alter the core FA profiles. Both E2 and 17,20β-P additionally stimulated synthesis of other FAs.

Thus, we can report that, similar to the effect of four phytohormones on fatty acid profiles in *S. quadricauda* (Kozlova et al., 2017), the two animal steroids tested could be used as a tool for fatty acids profile manipulation in the biofuel and pharmaceutical industries. A possible explanation of the mechanism of fatty acid profile alteration by animal steroids may be that steroids undergo chemical transformation during an experiment resulting in their hydroxylation, oxidation hydroxidation, etc. It leads to form conjugates (e.g., glycosides) (Salama 1980; Pollio et al., 1994). This transformation is different in time and depends on hormone concentrations and surrounded chemistry (water or cell plasma) and likely intefear with FAs formation, since both steroids and FAs have ketone and hydroxyl groups in their structure (Zhang et al., 1991; Zhong-han et al., 1994; Finsterbusch et al., 1999, Janeczko and Skoczowski, 2005). Moreover, appropriate to metabolic pathways of steroid enzymes and estrogen receptors were detected in many species of higher plants (Janik and Adler, 1984 These receptors and enzymes may have similar roles in algal cells: possibly they involved in a process of cell division and maturation

Dissimilarities between the reactions of different algal species to steroid hormone exposure are expected, as it was evidently demonstrated by Perron and Juneau (2011) when two green algae (*Chlamydomonas reinhardtii*, CC125 and *Pseudokirchneriella subcapitata*, CPCC37) and two strains of cyanobacteria *Microcystis aeruginosa* (CPCC299 and CPCC632) were subjected to high E2 concentration (4 mg/L). They found that photosynthetic activity of green algae, have much weaker reaction to E2 exposure, compared to cyanobacteria, even at high hormone concentrations. Their finding was supported by Nendza and Wenzel (2006) and at least partially explains why no adverse effect of E2 in nano-concentrations was observed.

The results of the toxicological research on natural estrogen E2 and intensive studies of steroid biodegradation by algal cells, coupled with the fact that estrogenic activity of natural steroids such as E2 is low, suggest that intracellular accumulation of the tested steroids applied in low concentrations and their transfer via food chain is unlikely. Although, further research on this subject is needed.

5.4 Conclusions

We have investigated the influence of two animal steroids, E2 and 17,20β-P, on several physiological parameters in *S. quadricauda*, with emphasis on the effect of these hormones on cell growth, cell biomass production, cell size, pigment biosynthesis (chlorophyll-a and total carotenoids), fatty acids biosynthesis and their profiles. We have found that both steroids demonstrated positive stimulatory effects on *S. quadricauda* cell growth, biomass production, chlorophyll-a, carotenoid, and lipid biosynthesis E2 was a more effective promoter of all the tested parameters compared to 17,20β-P with one exception: chlorophyll-a biosynthesis was greater affected by 17,20β-P steroid. To the extent of our knowledge, this is the first report on influence of 17,20β-P on growth, biomass production, chlorophyll-a and carotenoid accumulation, and the fatty acid composition of lipids in microalgae. The results of this study support to the statement that no intracellular accumulation of the tested steroids applied in nanoconcentrations and/or their transfer via food chain is expected. This conclusion is important in term of application of fishery wastewaters with animal steroids loading for industrial microalgal biomass production. Yet, more research is needed on animal hormones for different algae species and growth conditions.

Since not microalgae only, but also Duckweed was planned to be used for biomass production by the industrial collaborator, Myera Nu-Agrinomics Group Canada Inc., the next experiment we conducted to compare an influence of 17β-estradiol on growth and biosynthesized molecules accumulation of both microalgae *S. quadricauda* and Duckweed *Lemna minor*. Also the effect of water chemistry on the hormone influence was one of the focus of this study.

Chapter 6

Effect of 17β-estradiol on Growth and Biosynthesized Molecules accumulation of Microalgae *Scenedesmus quadricauda* (CPCC-158) and Duckweed *Lemna minor* (CPCC-490) Grown in Three Different Media.

6.0 Abstract

Fish farm wastewaters have detectable levels of fish hormones such as 17β-estradiol (E2). An understanding of the influence of fish steroids on algal (*Scenedesmus quadricauda*) and Duckweed (*Lemna minor*) physiology is relevant to the planned use of fishery wastewaters for microalgae and plant biomass production. The study was conducted using three types of media: Bold Basal Medium (BBM), natural fishery wastewater from a hatchery fish farm (FWW), and reconstituted fishery wastewater (RFWW) with the nutrient composition adjusted to mimic FWW. We investigated the influence of the steroid hormone (E2), which was previously shown to have a drastic effect on *S. quadricauda* growth and biosynthesis in a 20 days experiment (Chapter 5). During the experiment, the media were aerated and scrupulous attention was paid to changes in the pH and conductivity of the water. The results of this study are generally in accordance to the previous experiment on E2 (Chapter 5), showing great promotion of growth by *S. quadricauda* and *L. minor* with significant accumulation of high valuable molecules at very low steroid concentrations. However, clear differences were observed in both *S. quadricauda* and *L. minor* performance grown in different media, and the most effective hormone concentrations were also evidently different for the algae and the plant.

6.1 Introduction

There is great difference in the ability of different organic compounds to bind to, and cross cell membranes under the growth conditions. Most of organic molecules strongly bind to ions dissolved in water, which normally reduces membrane permeability, and this, bioavailability of both molecules (Smith et al., 2002; Richards et al., 2001). Consequently, the concentrations of dissolved ions (hardness, conductivity), the pH, light quality and quantity, and temperature are among the major factors that influence of organic compounds on the growth and biosynthesis of microalgae and aquatic plants (Shi et al., 2010; Li et al., 2010; Liu et al., 2018).

With respect to the fate of estradiol in aqueous environments, three major processes must be considered. The first process is the "reverse uptake" of the steroid into fish, although the percentage of E2 reabsorbed by fish is small (Scott and Ellice, 2007), and in case of water flowing out of fish tanks to the microalgae or plant production units, this possibility can be ignored. The second consideration is abiotic degradation (e.g., photodegradation) of E2. It has been established that between 2 % and 10 % of spiked E2 concentration may be photodegraded in 48 hours (Norvill et al., 2017). The third process is biotic degradation of the steroid by the bacterial community and/or algae. Most exogenous estrogens undergo biotic degradation, which can be simply described as an oxidative process beginning with hydroxylation that increases the solubility of estrogen, followed by glycosylation and methylation. During this process, E2 is converted to estrone (E1), then to estriol (E3) plus low concentrations of an unknown metabolite (Lai et al., 2002; Shi et al., 2010; Liu et al., 2018). A recent paper by Liu et al. (2018) reported the ratio of extracellular (adsorbed) to intracellular (absorbed) E2, as well as residual E2, in water over four days of exposure. The ratio appeared to be dependent on the initial concentration of E2, time, and water chemistry.

Different algae species have been shown to remove and degrade estradiols from aquatic environments via biodegradation or biotransformation processes, rather than simple sorption and

accumulation in the cells (Wang et al., 2017; Liu et al., 2018). The ratio of adsorbed to biodegraded estradiols varied among the studies, but is often reported more or less as half and half (Hom-Diaz et al., 2015). However, some studies suggest that most of the adsorbed estradiol becomes absorbed and biodegraded (Beecher, 2013; Liu et al., 2018) and, importantly, does not accumulate intracellularly, at least not when the concentrations of estradiol were below 100 ng/L (Beecher, 2013).

It has been reported that mammalian sex steroids can be synthesized by plants. About 70-80 % of plant species tested were found to synthesize progesterones (including 17,20β-P) and androgens. Estrogens were found to be synthesized by about 50 % of the plant species tested (Zhang et al., 1991; Janeczko and Skoczowski, 2005). The metabolic pathways of sex steroids in plants are suggested to be very similar to those in animals and humans (Itagaki and Iwaya, 1988; Rosati et al., 2003; Milanesi et al., 2001), and the same enzymes that carry-out these reactions in animal cells (for example, 17β-hydroxysteroid dehydrogenase). Likely, it is also true for microalgae species, but research on mammal steroid synthesis by algae cells is not as well advanced.

The hormone, E2, has been shown to promote plant and microalgae growth and biosynthesis. Increases in growth, crop yield, and flowering stimulation has been observed in maize plants at 3-300 μg/L estrogen (Bhardwaj and Thukral, 2000). Other studies confirmed the positive effect of E2 on flowering in *Cichorium intybus L*. and *Arabidopsis thaliana L.* (Janeczko and Filek, 2002; Kopcewicz, 1970), as well as an improvement in the development of reproductive organs in *Salvia splendens* (Kopcewicz and Porazinski, 1974). Thus, the processes targeted by estrogens in plants are primarily early stages of plant development (shoots and young roots) and formation of the sexual reproduction organs (Geuns, 1977; Zhang et al., 1991; Zhong-han et al., 1994). In aquatic plants, and particularly in the members of the family Lemnaceae, E2 was reported to increase chlorophyll and total carotenoid content (Czerpak and Szamrej, 2003), nucleic acids (DNA and RNA), soluble protein content, and reducing sugars content (Szamrej and Czerpak, 2004). Stimulation of *L. minor* flowering was also

observed (Czygan, 1962).

Growing interest in microalgae biomass production for biofuels, pharmaceuticals, and the agriculture industry has motivated investigations of the influence of steroids on algal growth and biosynthesis, although the initial studies on this subject were conducted more than 45 years ago (Sayegh and Greppin, 1973). It was demonstrated that E2 increases growth and biomass production, as well as biosynthesis of chlorophyll, carotenoids, proteins, and sugars in *Ch. vulgaris* cells (Bajguz and Czerpak, 1996a, b; Czerpak and Szamrej, 2000; Czerpak et al., 2001a), when concentrations between 0.3 to 3000 ng/L exogenous E2 were applied.

In our previous study on the influence of the estrogen E2, and the progesterone 17,20β-P, on *S. quadricauda* growth and biosynthesis, we observed positive effect of both steroids on cell division and biomass production, and biosynthesis of chlorophyll-a, total carotenoids, and lipids (Chapter 5; Kozlova et al., 2018). In the present study, the general goal was to compare the performance of low concentrations of E2 in three different media - Bolt Basal Medium (BBM), fishery wastewater from a hatchery farm (FWW) and reconstituted fishery wastewater (RFWW) in 11-day experiment conducted with *S. quadricauda* and *L. minor*. In this experiment the microalgae and the Duckweed growth paramenters, *S. quadricauda* pigments accumulation, *L. minor* soluble protein accumulation and changes in water chemistry (pH and conductivity) were assessed. Major macro- and micronutrients of FWW were also analysed.

6.2 Results

6.2.1 Water chemistry

The FWW from a hatchery fish farm was screened for major inorganic and organic compounds, and it was found that concentrations of key macro- and micronutrients in the wastewater were sufficient for algae and duckweed biomass production (Tables 13 and 14). Concentrations of copper (Cu) were

found to be above LOEC, which are about 1 to 3 μg/L for Cu (Franklin et al., 2000; Wilde et al., 2006).

The RFWW was designed taking in account both BBM and natural fish wastewater physico-chemical paramenters. RFWW was used to examination of influence of macro- and micro-nutrients in FWW, eliminating possible co-influence of unknown dissolved inorganic and/or organic compounds in the water derived form an actual fish hatchery farm.

To eliminate possible influence of pH on *S. quadricauda* and *L. minor* physiology and on the steroid performance, pH of all the media tested was adjusted to 7 with 1N HCl or 1N NaOH solutions. All pH-adjusted media were equilibrated for 1 day prior to being used in the tests. During the 11 days of experiments with *S. quadricauda,* pH of the growth media was shifted to alkaline conditions, but differently for each medium tested (Figures S4A, S4B, and S4C in the Supplimentary Materials). In both artificial media, pH increases occurred to lesser degree compared with FWW. It appears that E2 presence did not influence pH changes in the media over the 11 days of the experiment at the concentrations of steroid tested. However, a change in pH was observed in the FWW medium at E2 concentrations of 5 to 50 ng/L. The pH increase was significantly less than that observed in the control cultures (with no steroid present) or in cultures with 100 and 300 ng/L E2 (Figure S4A). Interestedly, the pH of the FWW medium was more alkaline at all concentrations of E2 tested, compared with the equivalent E2 concentrations in the two artificial mediums (Figure S4A, S4B, and S4C).

The conductivity of the media did not change significantly over the 11 days of the experiment (data is not shown). However, the conductivity of RFWW was about 2.2-fold and 1.3-fold higher than the conductivity of FWW and BBM respectively.

Table 13. Macronutrients and buffering capacity of standard synthetic Bold's Basal Medium (BBM), 30 % Hoagland's medium (30 % HG), natural fishery wastewater (FWW), and reconstituted fishery wastewater (RFWW) used in the experiment, ppm.

	DOC	TC	TDS	TN	NO_3	NO_2	NH_4-N	PO_4-P	TDP	pH	Alkalinity (as $CaCO_3$)	Conductivity (mS/cm)
BBM	<0.5	<1	635	41	182	0	1.5	50	50	6.8	354	720
30%HG	20	60	590	20.5	200	0	1.5	15	40	7	350	1000
FWW	19.4	60.4	585.5	20.5	175	0.2	0.7	0.9	30	7.75	363.7	555
RFWW	<0.5	50	600	40	180	0	1	50	50	7	350	750

Note: DOC, Dissolve Organic Carbon; TC, Total Carbon ($KHCO_3$; $NaHCO_3$); TDS, Total Dissolved Solids; TN, Total Nitrogen; TDP, Total Dissolved Phosphorus.

Table 14. Micronutrients in standard synthetic Bold's Basal Medium (BBM), 30 % Hoagland's medium (30 % HG), natural fishery wastewater (FWW), and reconstituted fishery wastewater (RFWW) used in the experiment, ppm.

Ions	Ca	Mg	K	Na	B	Fe	Cu	Zn	Al	Ni	Pb	Cd
BBM	68	25	62	68	0.5	1.5	0.005	0.005	0	0	0	0
30%HG	70	60	25	74	0.25	1.5	<0.005	<0.005	0	0	0	0
FWW	73.5	60	16	75.6	0.3	1.06	0.04*	0.007	0.04	0	0.001	0.005
RFWW	70	40	52	68	0.5	1.5	0.005	0.005	0	0	0	0

Note: * - above LOEC for algae species (Fujiwara et al., 2008; Morris et al., 2013).

6.2.2. Effect of E2 on *S. quadricauda* and Duckweed (*Lemna minor*) growth and biomass production

6.2.2.1 *S. quadricauda* growth and biomass production under different E2 concentrations in three mediums

The growth of *S. quadricauda* in the presence of E2 varied with the different types of media (Figures 18A, 18B, and 18C). All E2 concentrations tested showed significant stimulation of *S. quadricauda* growth in natural FWW and BBM. The greatest (3.5 fold) effect was observed in FFW medium at 15 ng/L E2 (Figure 18A). Ranking the growth stimulating concentrations revealed that 15 ng/L > 5 ng/L > 50 ng/L ≈ 100 ng/L in both FWW and BBM mediums.

In RFWW, E2 inhibited growth of *S. quadricauda* at all concentrations tested, but inhibition was particuary strong between 5 and 50 ng/L. In addition, a high percent of unhealthy cells were observed in both all cultures containing E2 (Figure 18B). E2 concentrations of 100 and 300 ng/L increased total cell density, although the percent of healthy cells was lower at these concentrations compared to the control. *S. quadricauda* grown in control (no E2 added) increased cell density to a similar degree in FWW and BBM (Figure 18A and 18C). However, in RFWW trial the amount of healthy cells and total cell density were significantly greater in control (2-2.4 times) compared to that in other mediums (Figure 18A, 18B, and 18C).

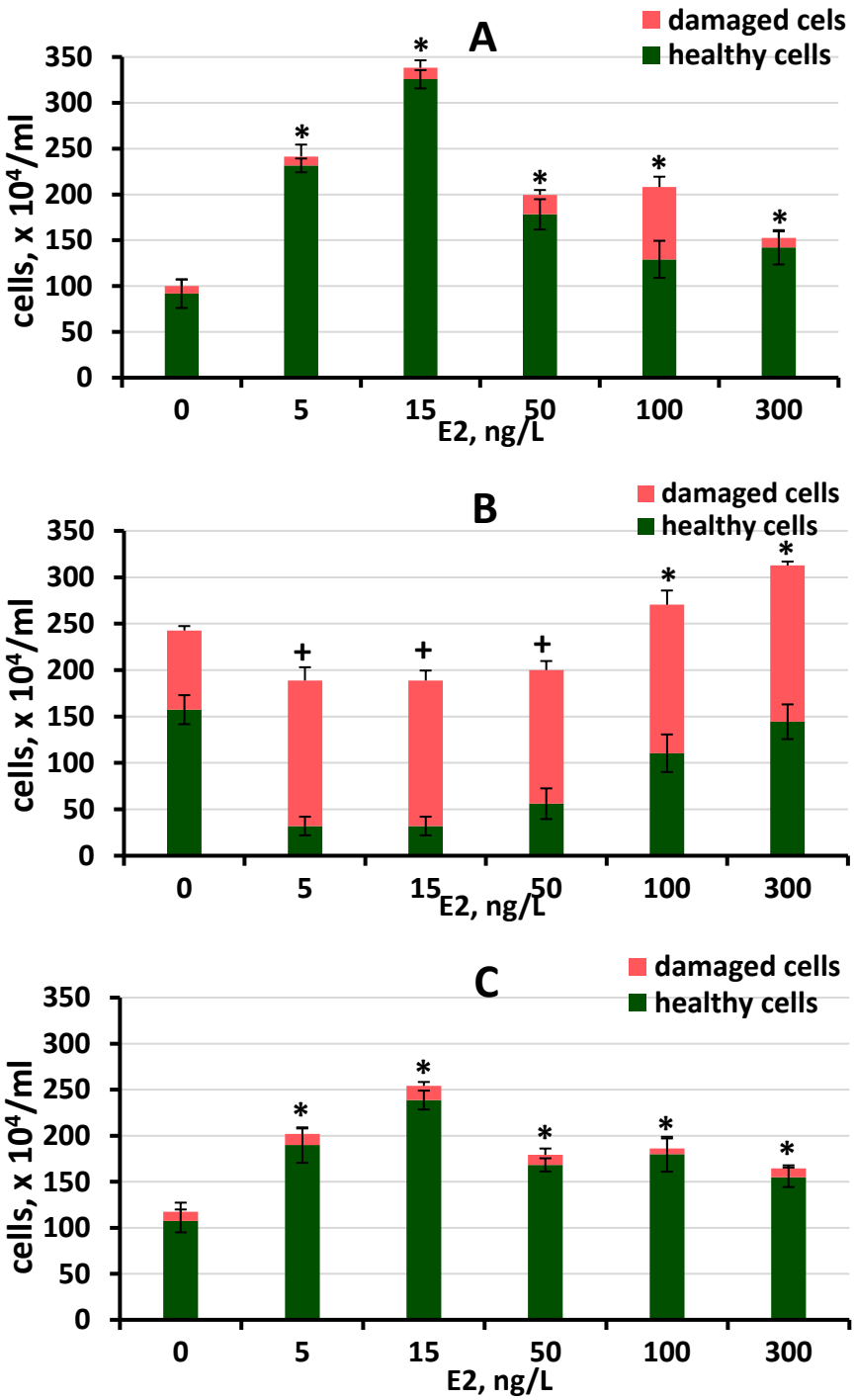


Figure 18. Cell density of *S. quadricauda* cultured in the three media containing E2 concentrations of 5 ng/L to 300 ng/L on day 11 of the experiment: A) FFW; B) RFWW; C) BBM. Statistically significant differences from the controls ($P < 0.05$) are shown for the total cells density, where * - significantly greater than the control; + - significantly lower than the control. Data shown as the mean ± SD, n = 15. Control cultures did not contain E2, indicated by "0".

Table 15. Changes in *S. quadricauda* cell size, total biomass, production, and cell density when cultured in the three growth medias on day 11 of the experiment (the day on which the experiment was terminated).

E2 concentration, ng/L	Cell size (µm)	Biomass (g drw/L)	Cell density (x 10^4/mL)
FWW trial			
control	44.77 ± 3.26	0.40 ± 0.07	100.07 ± 5.23
5	46.65 ± 4.73	1.15 ± 0.11*	241.43 ±3 .87*
15	46.87 ± 2.31	1.79 ± 0.05*	338.34 ± 3.18*
50	42.05 ± 1.45	0.88 ± 0.10*	199.61 ± 4.93*
100	37.23 ± 1.85	0.86 ± 0.14*	208.12 ± 8.89*
300	31.65 ± 3.22$^+$	0.78 ± 0.08*	152.63 ± 5.47*
RFWW trial			
control	43.77 ± 4.25	0.69 ± 0.09	242.67 ± 4.45
5	47.14 ± 3.43	0.44 ± 0.05$^+$	188.81 ± 3.22$^+$
15	48.25 ± 2.62	0.46 ± 0.04$^+$	191.52 ± 3.42$^+$
50	39.84 ± 5.66	0.41 ± 0.07$^+$	200.21 ± 5.25$^+$
100	35.92 ± 4.32	0.72 ± 0.11	270.42 ± 8.61*
300	26.52 ± 3.65$^+$	0.76 ± 0.06	312.71 ± 6.05*
BBM trial			
control	44.76 ± 0.92	0.44 ± 0.11	128.51 ± 10.66
5	52.67 ± 1.01*	0.85 ± 0.09*	202.12 ± 12.54*
15	56.72 ± 1.25*	1.24 ± 0.04*	254.24 ± 8.42*
50	46.74 ± 0.63*	0.79 ± 0.08*	179.13 ± 5.57*
100	46.84 ± 1.05	0.81 ± 0.08*	186.02 ± 14.05*
300	42.95 ± 1.48	0.75 ± 0.06*	164.64 ± 7.33*

Note: * - significantly greater than control ($P < 0.05$); $^+$ - significantly lower than control ($P < 0.05$). Data shown as the mean ± SD (for cell size n = 15; for biomass n = 4; for cell density n = 12).

Biomass yields were significantly higher in FFW and BMM at all E2 concentrations tested compared with the control cultures (Table 15). The greatest increase in biomass production (4.5-fold) was observed in FWW medium at 15 ng/L E2. In RFWW no increase of biomass production was found. Moreover, concentrations from 5 to 50 ng/L E2 demonstrated adverse effects on biomass yield on day 11

of the experiment. When comparing biomass production in the controls, the highest value was obtained in RFWW.

S. quadricauda cell size was differently affected by E2 in all three media. In FWW and RFWW, cell size was not affected, except by the highest tested E2 concentration of 300 ng/L, at which an adverse effect was observed (Table 15). In BBM medium, concentrations of 5 to 50 ng/L E2 resulted significantly increased cell size, with no effect being observed at 100 and 300 ng/L. Higher diversity in cell size was noted in RFWW medium at all E2 concentrations tested (Table 15). No change in cell size was observed in the control cultures of all three media.

6.2.2.2 *L. minor* growth under different E2 concentrations in three mediums

The influence of E2 on *L. minor* growth was assessed by measuring the development of the roots and fronds. All E2 concentrations tested significantly stimulated the growth of *L. minor* fronds, in all three media, except in BBM at 5 and 300 ng/L E2 (Figure 19A), with the greatest effect observed in RFWW at 5 and 15 ng/L E2. The influence of E2 on frond development was comparable in FWW and BBM media, while stimulation of *L. minor* fronds was greatest in RFWW medium (Figure 19A). Stimulation of *L. minor* root development was observed in FWW at 5 and 300 ng/L E2 and in RFWW at 15 and 100 ng/L E2. However, no significant influence on root development was observed at any E2 concentration in BBM trial (Figure 19B). The variation in root length was significant in all three media, although the SD was lower in FFW compared to the other media.

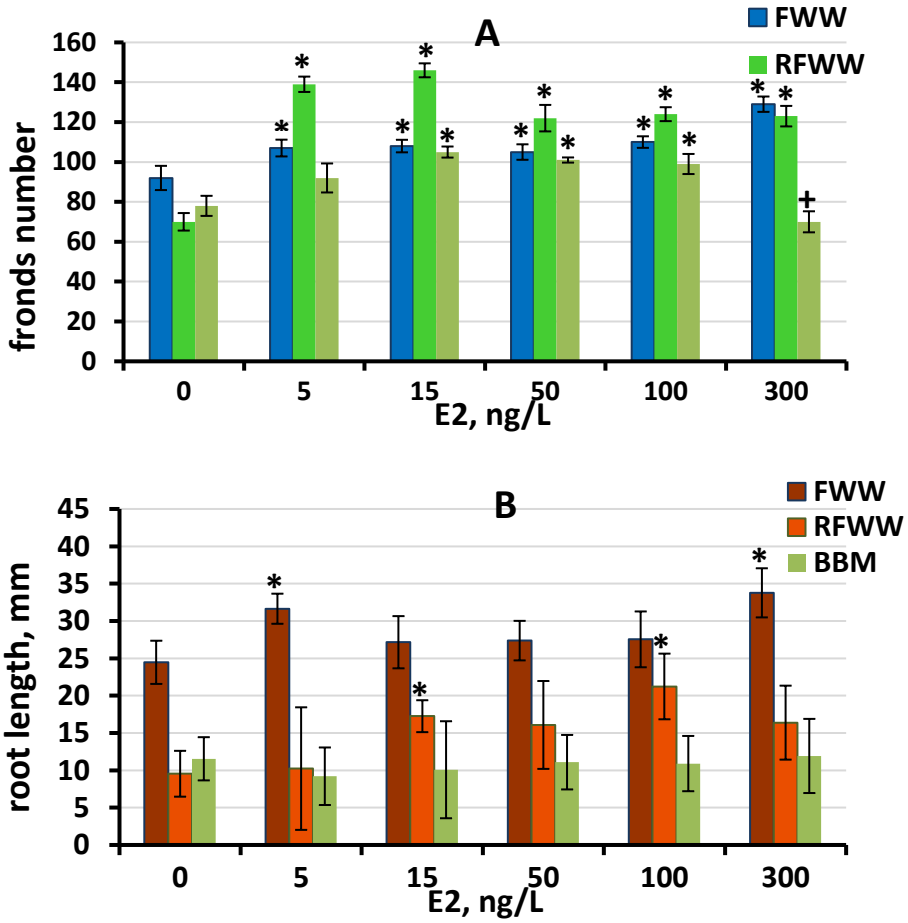


Figure 19. Growth of *L. minor* cultured in the three media containing E2 concentrations of 5 ng/L to 300 ng/L on day 11 of the experiment: A) foliage development in FFW, RFWW, and BBM.media; B) roots length in FFW, RFWW, and BBM media. Statistically significant differences from the controls ($P < 0.05$) are indicated, where * - significantly greater than the control; + - significantly lower than the control. Data shown as the mean ± SD, n = 15. Control cultures did not contain E2, indicated by "0".

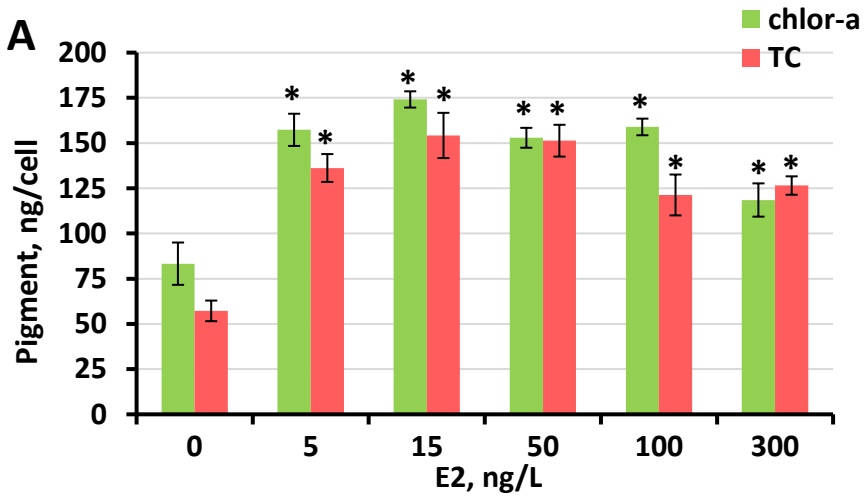
6.2.3 Effect of E2 on chlorophyll-a and total carotenoid production by *S. quadricauda* cells

Significant increases in chlorophyll-a and total carotenoids were observed at all E2 concentrations tested in FWW and BBM (Figure 20A and 20C). In contrast, no increase in either pigment was observed in RFWW (Figure 20B). Concentrations of both chlorophyll-a and total carotenoids increased in *S. quadricauda* cells to higher levels than in untreated control cells in FWW medium. A maximum chlorophyll-a concentration of 174.1 ng/cell was obtained in FWW at 15 ng/L E2, although at E2

concentrations of 5 to 100 ng/L, chlorophyll-a concentrations increased by 1.8- and 2.1-fold compared to the untreated control cultures.

In this trial, the maximum concentration of total carotenoids was nearly equal at 15 and 50 ng/L E2 (154.2 and 151.7 respectively), with an increase of 2.7-fold over the concentrations of total carotenoids observed in the untreated control cultures (Figure 20A). Ranking the efficacy of stimulation of chlorophyll-a by E2 in FWW, we determined that: 15 ng/L > 5ng/L ≥ 100ng/L ≥ 50 ng/L > 300 ng/L ng/L. Ranking the efficacy of stimulation of total carotenoids by E2 in FWW, we determined that: 15 ng/L ≈ 50 ng/L > 5 ng/L ≥ 100 ng/L ≥ 300 ng/L.

In BBM medium, maximum values of 153.4 and 151.2 ng/cell of chlorophyll-a were observed at 50 and 15 ng/L E2, respectively (Figure 20C). The accumulation of chlorophyll-a was stimulated to a lesser extent at 5, 100, and 300 ng/L E2. Total carotenoid accumulation in *S. quadricauda* cells was stimulated 2.4- to 2.5-fold at all E2 concentrations tested in BBM medium (Figure 20C). Ranking the efficacy of stimulation of chlorophyll-a in BBM medium, we found that 50 ng/L ≈ 15 ng/L > 100 ng/L ≥ 50 ng/L ≥ 300 ng/L. Ranking the efficacy of stimulation of total carotenoids in BBM medium, we found that 15 ng/L ≥ 50 g/L ≈ 100 ng/L ≈ 300 ng/L ≥ 5 ng/L.



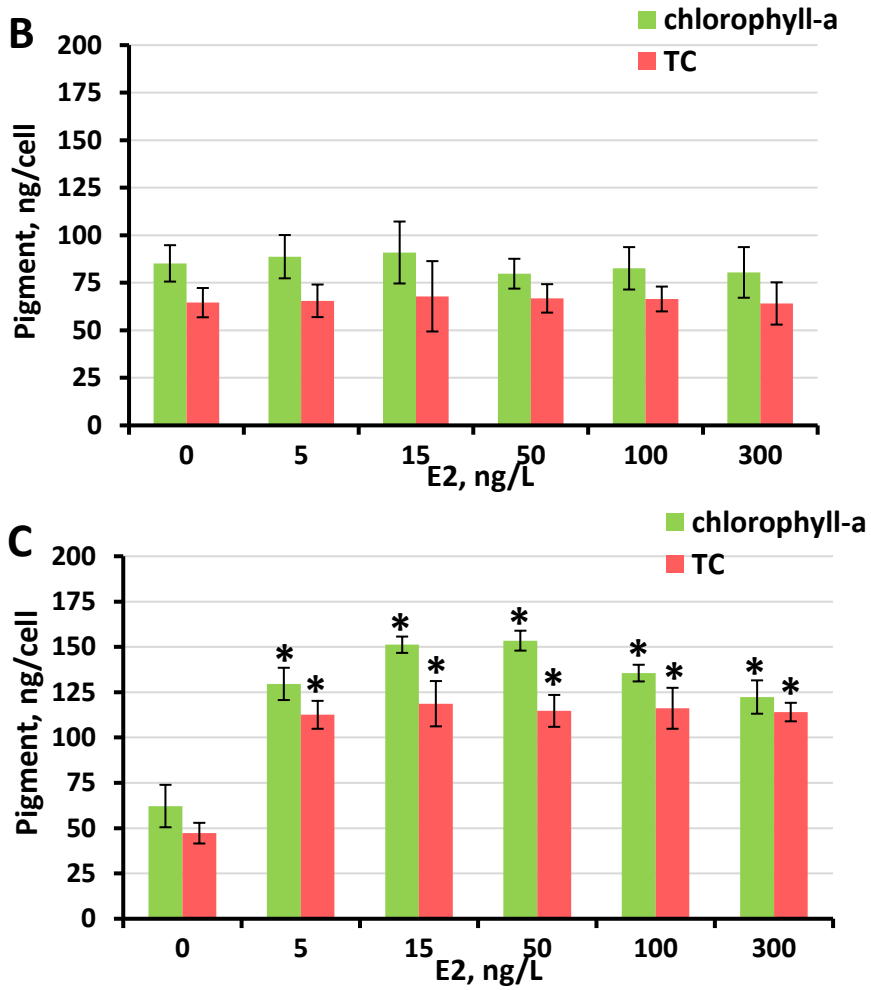


Figure 20. Chlorophyll-a and total carotenoid content in *S. quadricauda* cells cultured in the three media containing E2 concentrations of 5 ng/L to 300 ng/L on day 11 of the experiment: A) FWW; B) RFWW; and C) BBM. Statistically significant differences from the controls ($P < 0.05$) are shown for total cells density, where * - significantly greater than the control. Data shown as the mean ± SD, n = 12. Control cultures did not contain E2, indicated by "0".

6.2.4 Soluble protein production by *L. minor*

Accumulation of water-soluble protein in *L. minor* biomass was different when the plant was grown in three media containing different concentrations of E2, compared to untreated control cultures (Table 16). In the untreated control cultures, *L. minor* cultured in FWW medium produced the concentration of soluble proteins (36.12 mg/g frw), compared with the untreated controls cultured in RFWW and BBM. In the presence of E2, elevated protein concentrations (compared with the untreated

control cultures) were observed in *L. minor* cultured in FWW at concentrations of E2 from 5 to 300 ng/L, although the greatest stimulation of protein accumulation in the cells was at 50 ng/L. In RFWW, elevated protein concentrations were observed in cultures containing 5, 15, and 50 ng/L E2 (compared with the untreated control cultures), while in BBM medium, elevated concentrations of protein compared with the untreated control cultures were only detected in cultures containing 300 ng/L.

Table 16. Protein accumulation in *L. minor* grown in three different mediums on the day of the test termination (11 day).

E2, ng/L	control	5	15	50	100	300
FWW	36.12 ± 1.06	34.77 ± 0.76	38.95 ± 0.48*	40.41 ± 0.57*	38.92 ± 0.91*	39.68 ± 0.66*
RFWW	31.73 ± 0.72	40.74 ± 1.21*	41.12 ± 0.43*	36.02 ± 0.79*	32.82 ± 1.04	32.51 ± 0.72
BBM	24.75 ± 0.66	25.34 ± 0.26	25.28 ± 1.01	26.25 ± 0.76	25.78 ± 0.86	27.78 ± 0.58*

Note: * - significantly greater than control ($P < 0.05$). Data shown as the mean ± SD, n = 12.

6.3 Discussion

The range of E2 concentrations examined in this experiment was consistent with the concentrations typically found in Canadian surface and ground waters, and agricultural and fish-farming wastewaters (Nichols et al., 1998; Peterson et al., 2001; Ying et al., 2002). E2 is one of the frequently detected hormones in fishery wastewaters, which is why it may be used as a beneficial component of growth media in combined fish-algae-duckweed integrated production systems. In our previous study (Chapter 5), E2 demonstrated a powerful ability to simultaneously induce growth and biosynthesis of valuable bio-molecules in *S. quadricauda* cells. The current study was conducted to clarify whether the influence of E2 on the algae could be affected by physicochemical differences in three different growth media, including RFWW, which mimics the concentrations of micro- and macro-nutrients in real fish wastewaters (FWW).

Moreover, this study compares the effect of E2 on *S. quadricauda* physiology with the steroid influence on *L. minor*, when these two aquatic organisms were grown under the same conditions.

Despite the fact that ambient conditions in our previous study had some dissimilarities with this experimental work (the light intensity was 20 % higher and constant aeration of the growth mediums was not provided during the first experiment), the results of E2 influence on *S. quadricauda* growth parameters and biosynthesis of pigments were similar, comparing the values on days 10 and 11 of the experiments conducted in BBM medium.

However, clear dissimilarities in the algae population growth and biosynthetic activity were observed in three media tested. Remarkably, the dissimilarities in algae performance were found between the controls of three trials as well as between the test-cultures containing equal concentrations of E2. Furthermore, the pattern of dissimilarity between the controls varied with the type of medium used. For instance, cell density was about two times higher in untreated control cultures in RFWW media compared to the control cultures with BBM and FWW media. However, when E2 was present in the cultures, the effect was opposite: in RFWW, cell density and biomass yield of *S. quadricauda* were negatively affected by the three lowest E2 concentrations (5, 15, and 50 ng/L), while in FWW and BBM, these concentrations stimulated increase in cell density and biomass yield.

Why E2 negatively affected cell growth in the RFWW and why RFWW compromised cell health needs further investigation. Possible reason may be related to the buffering capacity of the medium, where sodium and potassium carbonites were used to stabilize pH of RFWW. This buffering strategy decreased the shifting of the pH to alkaline conditions, and increased the dissolved carbon value, which could directly affect the binding capacity of E2, and/or membrane permeability (Shi et al., 2010). It is also possible that the period of time for cells acclimatization to the RFWW media was insufficient for the algae to stabilize its physiology prior the test. When considering the effect of water chemistry on chlorophyll-a and carotenoids, it may be that stimulatory effect of E2 observed in the FWW and BBM media was abolished

in the RFWW medium.

In contrast to RFWW, the positive effect of E2 on *S. quadricauda* growth and biosynthesis in natural FWW was the most pronounced. The effect of water chemistry on the promoting efficiency of an induction factor (such as E2) has been highlighted previously by research conducted on aquatic plants (Agami and Reddy, 1989; Davis, 2004; Borker et al., 2013) and microalgal species (Wang et al., 2004; Li et al,. 2008; Domiani et al., 2010). It was demonstrated that a change in water chemistry could activate more than one defense pathways at the same time in algae cells. The combination of these pathways and dominance of one of them depend on the factor altered, whether it is a media chemistry parameter or an additional stressor such as a hormone or both (Wang et al., 2004; Lemoine and Schefs, 2010).

Remarkably, the influence of RFWW on *L. minor* growth was opposite to its influence on *S. quadricauda* growth. The growth of foliage, roots development, and protein accumulation in the duckweed was stimulated by E2 in RFWW medium, compared with the effect of E2 in the other two media, although FWW was a significantly better medium for *L. minor* growth when E2 was not present. Our results on the influence of E2 on the soluble protein accumulation by *L. minor* are consistent with research conducted on another duckweed species, *Wolffia arrhizal* (Szamrej and Czerpak, 2004). However, we observed the maximum effect at much lower E2 concentrations in both RFWW (15 and 5 ng/L E2) and FWW (between 15 and 300 ng/L E2) media, compared to the *W. arrhizal* study, where the concentrations of 272, 2720, and 27.2 μg/L E2 (in than order) were most stimulatory. In another study (Szamrej and Czerpak, 2004), soluble proteins were induced to a greater degree (1.8-fold increase) compared to maximum induction in our experiment (1.3-fold increase). The species-specific differences (e.g., *W. arrhizal* does not have roots) and growth media chemistry are likely responsible for the difference in results.

Lemnaceae species are broadly used for wastewater treatment, including domestic and pharmaceutical effluences, which are the prime resources of animal steroids in surface water. Some previous research indicates that duckweed species are one of the best agents for removing hormones from

water (Joss et al., 2004; Shi et al., 2010; Muradov et al., 2014). The low E2 waterborne concentrations were likely inactivated and biotransformed by *L. minor* shortly after the exposure. E2 and other animal steroids have also been shown to induce biosynthesis of valuable molecules in Lemnaceae plants, when applied in relatively low concentrations in the range of 10^{-5} to 10^{-7} M. For example, corticosteroids significantly stimulated the accumulation of pigments (chlorophylls and carotenoids), nucleic acids, soluble proteins, and sugar biosynthesis in *W. arrhizal* tissues, although the extent of stimulation was less than the effect of E2 (Czerpak and Szamrej, 2003; Szamrej and Czerpak, 2004). To our best knowledge, no study has measured duckweed growth performance under the impact of waterborne steroids. The effect of *L. minor* on buffering and stabilizing the pH of the growth media has also not previously been reported. However, efforts were made for development of a mathematical model for better understanding the influence of growth medium alteration on Lemnaceae performance *in vitro* and *in situ* (Landesman et al., 2005; Frederic et al., 2006; Khvatkov et al., 2018).

Our results on the influence of E2 on *S. quadricauda* growth and biomass production (cell size and density), and the accumulation of pigments (chlorophyll-a and total carotenoids) in BBM medium over 11 days was similar to that obtained in the previous 20 day experiment (Chapter 5). However, the stimulatory effects in this study were more pronounced than the effects observed at days 10 to 15 in the previous experiment. Likely, the better results are due to constant aeration condition applied in this (11 day) experiment.

6.4 Conclusions

In this Chapter, we have compared the influence of an animal steroid, E2 on the growth and physiology of two primary producers: the microalga *S. quadricauda*, and the aquatic plant *L. minor*. The results of this study may be organized in two groups. First, it was confirmed that E2 in nano-concentrations has strong positive effect on cell growth (cell size and final cell density) and biosynthesis of valuable molecules of *S. quadricauda*, as was previously demonstrated in a 20 days experiment (Chapter 5).

Moreover, we have demonstrated that E2 is capable of increasing foliage growth, root growth, and protein biosynthesis in *L. minor*, although root development was stimulated by the presence of E2 to a lesser extent. Second, we have compared the influence of three growth media (FWW, RFWW and BBM) on the two test organisms, both in the absence and the presence of E2. The results of this comparison show clearly the importance of water chemistry on the physicochemical parameters of the growth medium on the physiology and metabolism of aquatic organisms, as well as on the extent to which E2 affected the physiology of *S. quadricauda* and *L. minor*. Our results highlight the need for further investigations of the relationships between physicochemical parameters of growth media, the particular organism to be cultured, and any additional stressor, or chemical inducer that may be applied to stimulate biomass production and biosynthesis of the molecules of interest.

Chapter 7

General Conclusions and Further Work

7.1 Objectives and Hypotheses

This work was designed and implemented with consideration of industrial integration of microalgae and Duckweed biomass production for biofuels and bioproducts with fish farming and hydroponics, with the goal of maximizing nutrient, energy, water, and flue-gas CO_2 recycling. The main subject of this research was examination of the influence of organic compounds (plant and animal hormones) of hydroponic and fishery effluents on *Scenedesmus quadricauda* and *Lemna minor*, with emphasis on the effect of these hormones on cell growth, cell biomass production, cell size, pigment biosynthesis (chlorophyll-a and total carotenoids), and fatty acid biosynthesis and profiles.

Addressing the results of this study to the objectives, which were selected for investigation, we can confirm that:

1) All the tested hormones (four phyto-hormones and two animal steroids) in nano-concentrations were able to simultaneously induce *S. quadricauda* growth parameters, biomass production, pigment and lipid biosynthesis. The order of stimulation efficiency of the hormones tested on: a) growth parameters was: E2 > EBL ≈ 17,20β-P ≥ IAA > BL > ABA; b) chlorophyll-a synthesis was: 17,20β-P > EBL > IAA ≥ E2 > BL > ABA; c) total carotenoids synthesis was: EBL > E2 ≥ IAA >17,20β-P > BL ≥ ABA; and d) major and total fatty acid synthesis was: E2 > 17,20β-P > ABA ≈ IAA > EBL > BL. Therefore, both tested animal steroids and phytohormones EBL and IAA were the most powerful inducers of growth and pigment production/accumulation in *S. quadricauda* cells, while FAs biosynthesis was greater affected by presence of E2,17,20β-P, ABA and IAA.

Importantly, all the hormones were most effective in low concentrations within the tested ranges of nano-concentrations. This finding gives an impression that the expected concentrations of phytohormones

in the effluences of hydroponics facilities likely are sufficient for induction of both, microalgae biomass production and valuable molecules biosynthesis/accumulation.

2) When comparing the influence of four phytohormones on *S. quadricauda* physiology, it was found that within the concentrations tested, under the optimized growth conditions in BBM media, EBL and IAA demonstrated the greatest induction of growth parameters and the pigments biosynthesis. The most pronounced effect on FA production and alteration of the FA profile was confirmed in IAA and ABA trials. BL was less effective for induction of any of the parameters tested, compared with the other phytohormomes. It also was noted that the manner in which the fatty acid profiles were altered was different for each phytohormone tested. Moreover, all the phytohormones could stimulate additional FAs synthesis compared with the controls. Thus, our results highlight the possibility that phytohormones could be used as a tool for fatty acids profile manipulation in the biofuel and pharmaceutical industries.

In the mixed EBL and IAA trials, the investigated physiological parameters (growth, chlorophyll-a and total carotenoids biosynthesis, fatty acids biosynthesis and their profiles) demonstrated the synergistic relationship between a brassinosteroid (EBL) and an auxin (IAA) when *S. quadricauda* cells were exposed to the range of two hormones mixtures. Our results also demonstrated that certain phytohormone combinations synergistically promote both greater biomass yield and its rate of production where the lag phase of algae growth in mixed hormone trials was clearly shortened. The results on pigment accumulation supports the previously stated theory that BRs contribute to the synergistic effect with auxin, independent of its concentration, and likely independently from the IAA signaling pathway. However, the complex outcome of some combinations also suggests that the mechanism of EBL and IAA crosstalk was specific to targeted biosynthetic pathways and, thus, auxin can increase the activity and effectiveness of BRs in a synergistic manner at certain circumstances. An adverse effect at highest tested concentrations was observed for most of the tested parameters in all the trials, when negative effect of BL and ABA was more pronounced.

3) Both tested animal steroids, E2 and 17,20β-P, exhibited strong stimulatory effects on the physiology of *S. quadricauda*. E2 was a more effective promoter of all the tested parameters compared to 17, 20β-P with one exception: chlorophyll-a biosynthesis was affected to a greater extend by the 17,20β-P steroid. Similarly to the influence of the phytohormones tested, the two animal hormones altered the fatty acid profiles of lipids synthesized by *S. quadricauda*. Both E2 and 17,20β-P stimulated synthesis of additional FAs. Thus, we can report that, as with the four phytohormones tested, the two animal steroids could be used as a tool for fatty acids profile manipulation in the biofuel and pharmaceutical industries.

The results of this study support to the statement that no intracellular accumulation of the tested steroids applied in nano-concentrations is expected. This conclusion is important in term of application of fishery wastewaters with animal steroids loading for industrial microalgal biomass production.

4) Two aquatic organisms, the microalga *S. quadricauda*, and the aquatic plant *L. minor*, exhibited positive reaction on presence of exogenous E2 in nano-concentrations. Regardless of the significant changes in ambient conditions (light intensity and the media aeration) the results were similar to the previous test, where growth parameters and the pigments biosynthesis increased in *S. quadricauda*, grown on BBM media. E2 was capable of increasing foliage growth, root growth, and protein biosynthesis in *L. minor*, although root development was stimulated by the presence of E2 to a lesser extent.

5) Physico-chemical parameters of a growth media demonstrated drastic effect on E2 influence on both, *S. quadricauda* and *L. minor* physiology. The designed RFWW negatively affected E2 ability to induce growth, chlorophyll-a, total carotenoid in *S. quadricauda*. Remarkably, RFWW did not prevent E2 ability to persuade growth and protein production in *L. minor*. For both tested aquatic organisms, the positive effect of E2 on growth and biosynthesis in natural FWW was the most pronounced with the only one exception of foliage development in *L. minor* where E2 in RFWW was more effective. The most effective E2 concentrations were different between three tested mediums for both aquatic organisms and all the tested parameters.

As anticipated, physico-chemical parameters of the growth media had strong influence on *S. quadricauda* and *L. minor* physiology at equal ambient conditions in the absence of steroid in the culture media. In controls, the best algae growth, biomass production, and pigment accumulation was observed in RFWW, while *L. minor* growth (roots, fronds) and protein accumulation were significantly greater in FWW media.

Therefore, we can conclude that all of the hypotheses tested in this thesis have been supported by the experimental data. The first hypothesis tested was supported by the data: all the examined phytohormones (ABA, IAA, BL, and EBL) stimulated *S. quadricauda* growth. The second hypothesis was supported by the data: two fish steroids (E2 and 17,20β-P) stimulated microalgae growth and lipid biosynthesis. The third hypothesis was supported by the data: in both the microalgae *S. quadricauda* and the Duckweed species, *L. minor*, growth was positively affected by nano-concentrations of E2. The fourth hypothesis was supported by the data: *S. quadricauda* and *L. minor* demonstrated significantly greater growth and activity of biosynthesis in natural FWW compared to RFWW or BMM when E2 was present, with the only one exception of foliage development in *L. minor* where E2 in RFWW was more effective. In the absence of steroid in FWW, hypothesis four was true for *L. minor*, but not for *S. quadricauda*, since the best algae growth, biomass production, and the pigment accumulation was observed in RFWW, not in FWW. Remarkably, for *S. quadricauda* the results in BBM were significantly greater, while all the tested parameters of *L. minor* were inhibited in BBM compared to RFWW.

7.2 Future work

The results of our investigation clearly demonstrated significant positive effects of the tested phytohormones and animal steroids on the physiology of *Scenedesmus quadricauda* and *Lemna minor*, emphasizing the possibility of simultaneously enhanced biomass produciotn and synthesis of valuable biomolecules.

Despite of our in-depth investigation of the microalgae physiology in the presence of phytohormones and steroids in concentrations relevant to hydroponics and fishery wastewater (in the nano–concentration range), there are number of questions on the relationship of hormones to microalgal- and Duckweed physiology that remain to be elucidated. These questions may be organized into two major groups: 1) subjects derived from our study that need clarification or further investigation to confirm our findings; and 2) subjects related to our study that currently have rather scattered knowledge, and require further investigation to improve industrial-scale microalgal and Duckweed biomass production and/or reduce the costs of the production processes.

7.2.1 Future work derived from our study

The following subjects should be considered as a high-priority list for further investigations:

1) Investigation and monitoring of the physico-chemical parameters and hormone-loading of fishery and/or hydroponic effluents, and the kinetics of hormones degradation;
2) Clarification of the influence of the products of hormone degradation on microalgae and Duckweed physiology:
3) Influence of aeration of the medium on hormones ability to induce microalgae and Duckweed growth and biosynthesis;

4) Ability of some hormone mixtures to decrease the lag-phase of algal growth needs extensive investigation, as hormones are a powerful tool for enhancement of both biomass yield and the rate of biomass production;
5) Animal hormone accumulation in algae/duckweed cells, as well as bio-transfer via food chain when hormones applied in nano-concentrations;
6) Carotenoid profiles in the presence of hormones: □-carotene, lutein, zeaxanthin, astaxanthin, and canthaxanthin accumulation in selected microalgae and Duckweed species;
7) Protein profiles: Key proteins, such as RuBisCO and radial spoke proteins, are important high-value commodities for pharmaceutical and agricultural (animal feed) industries;
8) For Duckweed: dextrose, starch, calcium oxalate monitoring/adjusting;
9) Comparison of all the above subjects in continuous mode of biomass growth vs a batch experiment (in particular comparison of pigments accumulation);
10) Validation of the obtained results on the designed pilot and then industrial units;
11) Multivariate Statistics on the obtained results: clarification whether or not population growth, biomass production and cell size statistically correlate to each other.

7.2.2 Future work on additional subjects closely related to our study

When considering important subjects, which are closely related to our research, the following gaps in knowledge need to be filled:

1) Influence of temperature, CO_2 applications and light intensity/spectrum on hormones ability to alter microalgae and Duckweed growth and biosynthesis;
2) In-depth consideration of the role of Photosystem II (PSII) in a view of hormone applications: the link between photosynthetic rate and pigment of interest biosynthesis; the link between TAGs accumulation and photosynthetic apparatus activity.

3) Investigation of phyto– and animal hormones, commonly found in hydroponics and fishery effluents, influence on other commercial species of microalgae and Duckweed;
4) Investigation of interactions between hormones in aqueous solutions: relationship between different mixtures of hormones. For commercially used algae species, the optimal combination of the types of phytohormones is yet to be determined;
5) A wider-range of algae species and physiological responses has to be further investigated for better prediction of the influence of a particular hormone combination;
6) Investigation of the mechanism of formation of a signaling network in aqueous environment between exogenously applied phytohormones, the intracellularly synthesized hormones and endogenous accumulation of the hormones;
7) Investigation of other than hormones organics of the effluents, first of all dissolved organic matter (DOM) as the potential ligands for inorganic ions and organic molecules;
8) Investigation of the genetic basis of the influence of hormones on microalgae/Duckweed biosynthesis.

Chapter 8

Industrial Relevance and Innovation of the Work

8.1 Industrial relevance

Two of the major challenges for industrial-scale biomass production of microalgae and Duckweed are the cost of production and yields of the high-value biomolecules synthesized by these organisms. The entirely novel technology that integrates hydroponics, fish farming, and microalgae biomass productions, where algae, plants, and fish share space, nutrients, and light in a combined industrial unit offers substantial cost reduction and extends the variety of bioproducts that may be produced, but raises the issue of the influence of metabolites of various co-culture species on each other, especially with the recycling of water between the production units. These influences could be negative or positive for a biomass growth and/or biosynthesis of valuable molecules. Therefore, investigation of the influence of plant and fish metabolites dissolved in recycling water on the biomass grown is crucial for success of the industry.

It is also vital for the industrial sector to achieve enhanced yields of high-value biomolecules from the same biomass such as TAGs for biofuels, as well as carotenoids and protein for broader applications in the production of food, animal feeds, dietary supplements, and pharmaceuticals. There is a great need to reduce the costs of biofuel production from microalgal biomass. It has been estimated that a 4-5 fold increase in productivity [with microalgae biomass containing 60% (w/w) of oil] could make biofuel production from microalgae economically viable (without the cost of co-products). When considering the co-products from microalgae biomass, only lutein demand in United States is estimated to be nearly $150,000,000 per year. The scientific community should find alternative ways for enhanced biomass production with improved yield of other high-value biomolecules like leutin.

The results of our study on the influence of selected phytohormones and fish steroids in nano-concentrations, commonly found in hydroponics and fishery effluents, revealed the great potential of these

organic compounds simultaneously increase both *S. quadricauda* and *L. minor* growth and biosynthesis. This finding supports the use of effluents as growth medium for industrial microalgae biomass production and offers the way for significant decrease in the cost of biomass production. Importantly and in support to the previous studies, some phytohormones and their combinations, as well as fish steroid E2 were capable of reducing the lag phase of algae growth, resulting in promotion of both greater biomass yield and its rate of production.

Consequently, our study supports the goals of industrial integration in at least three different ways: confirming that sufficient for industrial-scale production of biofuels and valuable co-products from one type of biomass is possible; demonstrating that cost reduction of microalgae and/or Duckweed biomass production applying naturally occurring hormones in nano-concentrations commonly found in hydroponics and fishery effluents is achievable; and offering significant reduction in environmental emissions due to the improved energy- and nutrient-recycling system, and due to the use of industrial wastewater as the sources of nutrients for grown biomass.

Thus, industrial integration creates more opportunities for flexible combination of biofuels, valuable co-products, and food and/or animal feed production according to the market demand, addressing unique niche markets (off-grid communities), and maximizing revenues.

8.2 Innovation of the research

This study was part of a collaborative effort between industry and academia to establish a completely novel technology that integrates different types of biomass production (fish farming, microalgae, Duckweed, and potentially, commercial vegetable production) in one industrial unit that maximizes recycling of nutrients and energy. The most important findings of our work were:

1) We demonstrated that integration of aquaculture and hydroponics with microalgae and/or Duckweed biomass production is possible in term of sufficiency of nano-concentrations of naturally

occurring fish steroids and phytohormones for significant induction of algal and/or Duckweed biomass production and valuable molecules yield in not mutually exclusive way;

2) We found that even very small concentrations of the tested hormones were capable of enhancing FAs yield and, importantly, alter the FAs profile of accumulated TAGs. Our results highlight the possibility that hormones could be used as a tool for fatty acids profile manipulation in the biofuel and pharmaceutical industries. Thus, common method of FAs cracking which used for adjusting the suitability of molecules to green gasoline could be exchanged with a new technology of algae biosynthesis manipulation applying a single or a blend of hormones;

3) Importantly, we found that time of harvesting has a great influence on phytohormones ability to alter FAs profile, although this results have to be confirmed in future investigation;

4) We found that majority of the mixtures of EBL with IAA demonstrated synergistic effect on *S. quadricauda* population growth, biomass production, the tested pigments and FAs accumulation, although our study was examined several times lesser phytohormones concentrations than any previously reported;

5) To the extent of our knowledge, this is the first report on influence of EBL, BL, and ABA on the fatty acid composition of lipids in microalgae and on influence of 17, 20β -P on growth, biomass production, chlorophyll-a, carotenoid accumulation, and the fatty acid composition of lipids in microalgae. Moreover, as far as we know, no study previously has measured Duckweed growth performance (fronds and roots development) under the impact of waterborne animal steroids E2;

6) The results of this investigation could be considered as a solid framework for future modeling work on the hormones to *S. quadricauda* and *L. minor* influence.

Supplementary Materials

For Chapter 3: Effect of Phytohormones on Growth and Accumulation of Pigments and Fatty Acids in the Microalgae *Scenedesmus quadricauda* (CPCC-158)

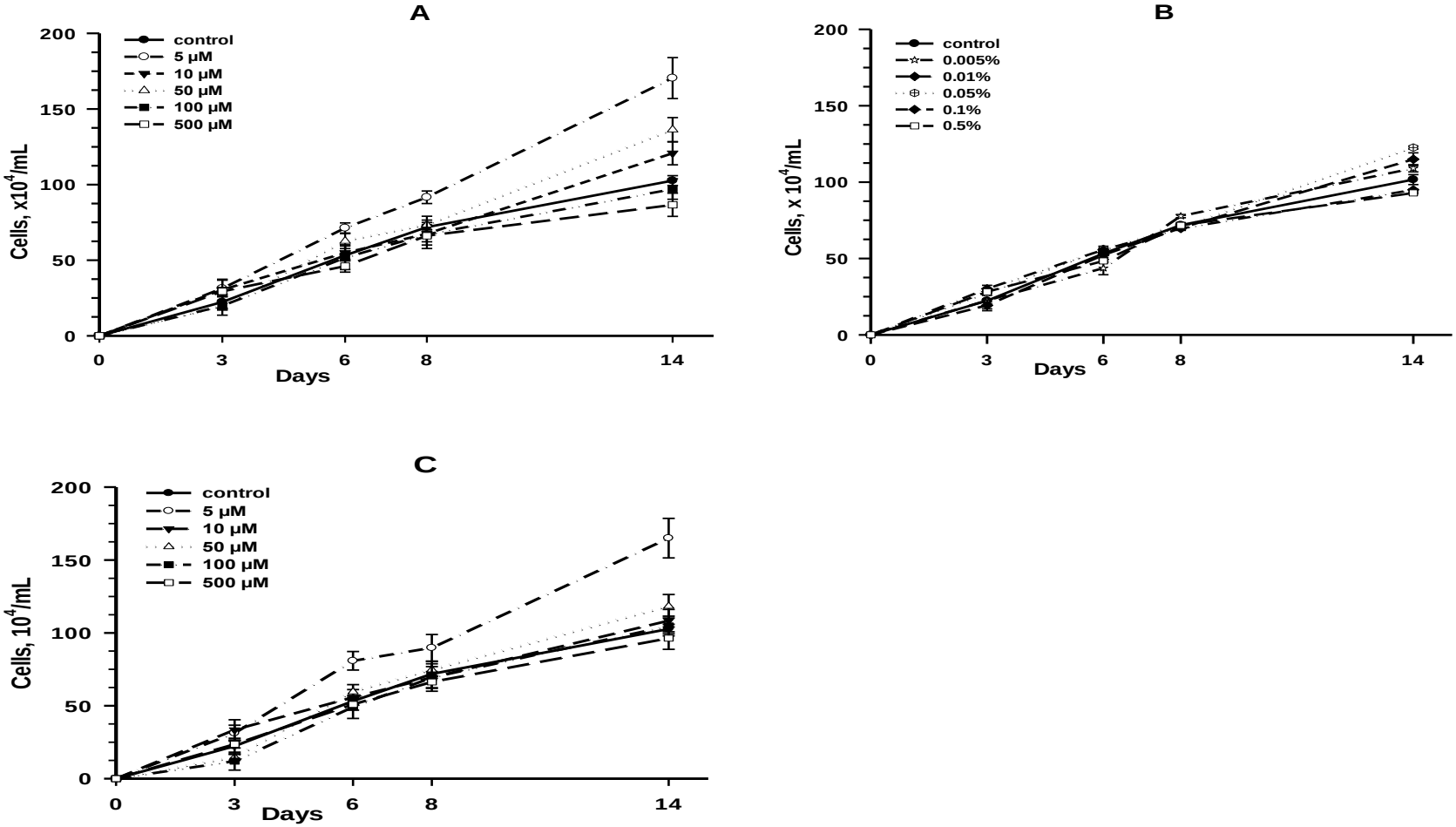


Figure S1. Growth of S. quadricauda in the presence of the phytohormone ABA with and without DMSO: A) ABA+DMSO; B) DMSO-only trial, as persent of the solvent in working solutions; C) the calculated influence of ABA on algae cell growth.

For Chapter 4: Combined Hormones Effect Calculation Based on Chou and Talalay Method

Here we present an explanation of how the calculations of the dose-effect relationship for individual and mixed hormone treatments were used to calculate the Combination Index (CI).

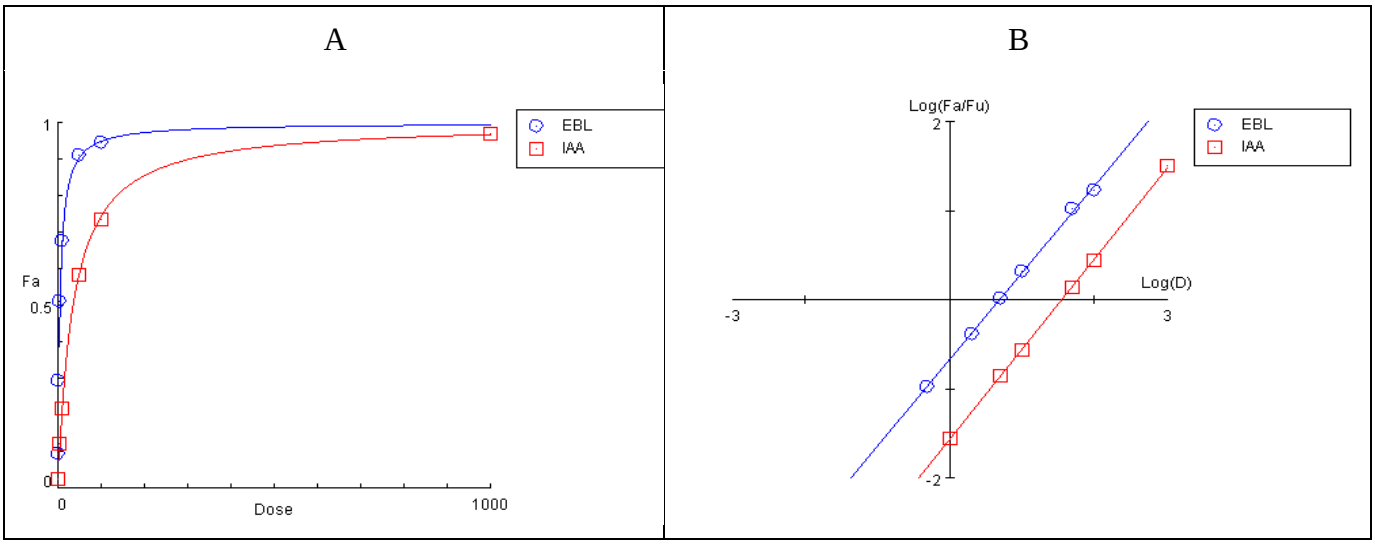


Figure S2. Determination of the parameters for Table 1. A) Dose-Effect Curve; B) Median-effect Plots for single hormone treatments (CompuSyn software).

The Median-effect equation (Chou and Talalay, 1984):

$fa/fu = (D/EC50)^m$; from here $fa=1/[1+(D_m/D)^m]$ or $D_x = D_m[fa/(1-fa)]^{1/m}$, (1S)

where fa is the fraction of dose affected (as a proportion of a medium-effect dose); fu is the fraction of dose unaffected (fu=1-fa); $EC50=D_m$; m, slope of the kinetic order; D, the dose of the hormone applied. In this study, the dose-effect relationship for cell growth had a hyperbolic shape until further increases in a hormone concentration resulted in an adverse effect (Table S1). At this point, m = 1 (Chou and Talalay, 1984).

Example 1

Table S1. Dose-effect relationship for mixed hormones with the input values used to calculate the CI.

EBL, 11d				**IAA, 11d**				**Mix[1: n]**				
D, nM	Growth, cells x 10^4/mL	fa	Input parameters	D, nM	Growth, cells x 10^4/mL	fa	Input parameters	D, nM	Growth, cells x 10^4/mL	fa-mix	Input parameters	
											0.5 nM EBL	2 nM EBL
0.5	118.06	0.095	Dm= 4.856	1.0	93.09	0.027	Dm= 34.695	0.5+5	145.75	0.019	Dm=277.3	Dm=54.5
2.0	125.52	0.296	X=0.686	5.0	109.95	0.123	X=1.54	0.5+100	114.58	0.266	X=1.82	X=1.44
5.0	136.14	0.513	Y= -0.668 ± 0.02	10.0	128.09	0.219	Y= -1.57 ± 0.013	0.5+1000	163.39	0.783	Y=-0.625	Y=-0.27
10.0	146.99	0.678	m=0.974 ± 0.016	50.0	151.72	0.583	m=1.0 ± 0.006	2+5	219.55	0.114	m=1.0	m=-1.0
50.0	162.38	0.913	R2 = 0.999	100.0	165.73	0.737	R2 = 0.999	2+100	121.99	0.652	R2=1.0	R2=1.0
100.0	102.45	0.955		1000.0	105.42	0.966						

Note: D, dose of a hormone applied; Dm, median effect dose; m, slope of the kinetic order derived from a plot log(D) vs log[(fa)/(1-fa)]: m < 1, m = 1, m > 1, indicating negative sigmoidal, hyperbolic, and sigmoidal, respectively; fa, fraction of dose affected (as a proportion of a medium-effect dose); X and Y, the coordinates of Dm on the plot.

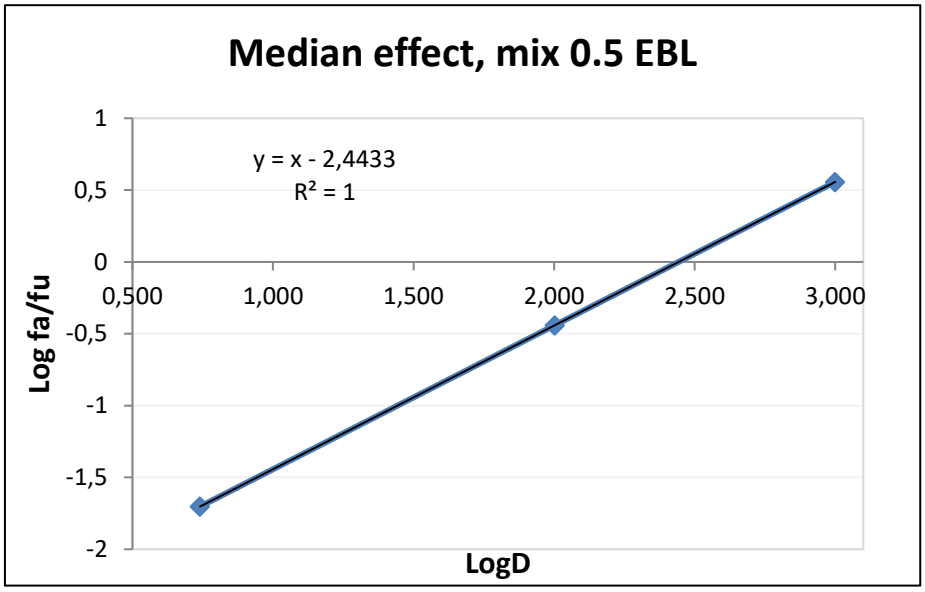


Figure S3. Example of a Median-effect plot for a mixed hormones treatment: 0.5 nM EBL + "n", nM IAA.

The Combination Index (CI) equations, also known as the Isobologram equation (Chou, 2006):

$$(CI)_x = \sum_{j=1}^{n} (D_x)1 - n\{[D]j /\Sigma^{n}_{1} [D]\}/ (D_x)j \{(fa_x) j/1-(fa_x) j\}^{1/mj}, \qquad (2S)$$

where $([D]j/\Sigma^{n}_{1} [D]\}$ is the proportionality of the concentration of each of n compounds causing x% effect; $(Dx)\, j\{(fax)\, j/1-(fax)\, j\}^{1/mj}$ is the concentration of each compound alone that exerts x% effect.

The Combination Index (coefficient) may also be expressed as:

$CI=(D_{com})_1/(D_{singl})_1+(D_{com})_2/(D_{singl})_2=\{(D_{com})_1/(D_m)_1\,[fa/(1-fa)]^{1/m1}\} + \{(D_{com})_2/(Dm)2[fa/(1-fa)]^{1/m2}\};$

if $(D)_1/(D)_2=P/Q$ then $= ((D)_{1,2}[P/(P+Q)])/((D_m)_1[fa/(1-fa)]^{1/m1}+ ((D)_{1,2}[Q/(P+Q)])/((D_m)_2[fa/(1-fa)]^{1/m2}$

(3S)

Non-constant hormone ratios for each pair were applied.

Table S2. Calculations from Table S1.

CI values for actual experimental points		
fa mix	CI	**Total dose, nM**
	0.5 nM EBL trial	
0.019	0.69	5.5 (0.5 EBL+5 IAA)
0.266	0.89	100.5 (0.5 EBL+100 IAA)
0.783	0.62	1000.5 (0.5 EBL+1000 IAA)
	2 nM EBL trial	
0.114	0.29	7 (2 EBL+5 IAA)
0.652	0.86	102 (2 EBL+100 IAA)

Note: fa, fraction of dose affected (as a proportion of a medium-effect dose); CI, Combination Index (isobologram equation) outcomes: CI < 1 = Synergistic effect; CI = 1 = Additive effect; CI > 1 = Antagonistic effect (Chou and Talalay, 1984).

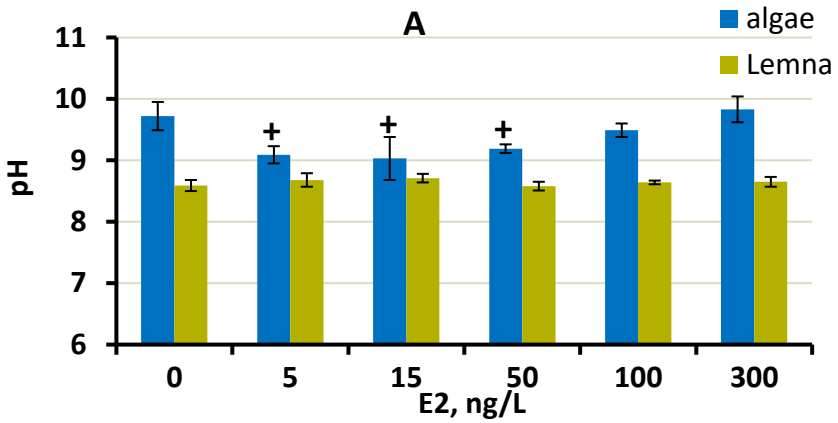
As may be seen from Table S2, all the calculated CI values were < 1. Thus, it may be concluded that all hormone relationships corresponding to these values in the combined hormone treatments had Synergistic effects. However, taking in the account statistical differences (SD) between single and mixed hormone treatments, we postulated Synergistic effects only for hormone combinations that resulted in statistically higher responses than each single hormone treatment. The same criteria was done to evaluate Antagonistic effects. Economically, it is not viable to apply two hormones for biomass production if treatment with mixed hormones does not demonstrate statistically greater productivity than each single hormone treatment.

There are more gradations for relationship between the mixed compounds often in use in pharmaceutical or toxicological research as it could be seen from Table S3.

Table S3. CI-value descriptions and symbols used in pharmaceutical or toxicological research (Chou, 2006).

Range of combination index	Description	Graded symbols	Graphic symbols
<0.1	very strong synergism	+++++	
0.1-0.3	strong synergism	++++	
0.3-0.7	synergism	+++	
0.7-0.85	moderate synergism	++	
0.85-0.90	slight synergism	+	
0.90-1.10	nearly additive	±	
1.10-1.20	slighht antagonism	−	
1.20-1.45	moderate antagonism	−−	
1.45-3.3	antagonism	−−−	
3.3-10	strong antagonism	−−−−	
>10	very strong antagonism	−−−−−	

For Chapter 6: Effect of 17β-estradiol on Growth and Biosynthesis of Microalgae *Scenedesmus quadricauda* (CPCC-158) and Duckweed *Lemna minor* (CPCC-490) Grown in Three Different Media.



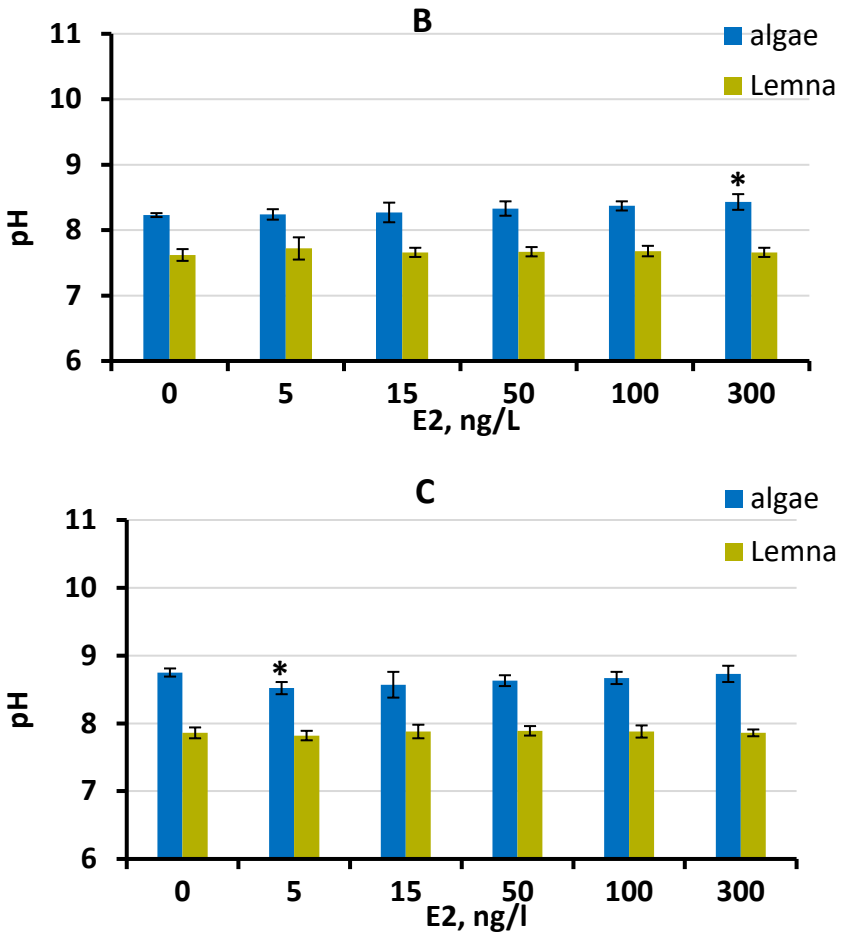


Figure S4. pH of the three culture media on day 11 of the experiment. A) FFW; B) RFWW; C) BBM. Statistically significant difference from the controls ($P < 0.05$) are indicated, where * - significantly greater than the control and + - significantly lower than the control. Data shown as the mean ± SD, n = 4. Control cultures did not contain E2, indicated by "0".

REFERENCES

Adamsson M., 2000. Potential use of human urine by greenhouse culturing of microalgae (*Scenedesmus acuminatus*), zooplankton (*Daphnia magna*) and tomatoes (*Lycopersicon*), Ecol. Eng. 16(2): 243-254.

Adarme-Vega T.C., Lim D.K.Y., Timmins M., Vernen F., Li Y., and P.M. Schenk, 2012. Microalgal biofactories: a promising approach towards sustainable ω-3 fatty acid production, Microb. Cell Factories 11: 96-109.

Agami M., and R. Reddy, 1989. Inter-relationships between *Salvinia rotundifolia* and *Spirodela polyrhiza* at various interaction stages, 27: 96-102.

Ahmad M.R., and A. Winter, 1968. Studies on the hormonal relationships of algae in pure culture: I. The effect of indole-3-acetic acid on the growth of blue-green and green algae, Planta, 78(3): 277-86. doi: 10.1007/BF00386428

Allakhverdiev S.I., and N. Murata, 2004. Environmental stress inhibits the synthesis de novo of proteins involved in the photodamage-repair cycle of Photosystem II in Synechocystis sp. PCC 6803, Biochimica et Biophysica Acta 1657(1): 23-32. DOI: 10.1016/j.bbabio.2004.03.003

Alazem M., Lin K.Y. and N.S. Lin, 2014. The abscisic acid pathway has multifaceted effects on the accumulation of *Bamboo mosaic virus*, Mol. Plant Microbe Interact., 27:177-189.

Alazem M., and N.S. Lin, 2015. Roles of plant hormones in the regulation of host–virus interactions, Molec. Pl. Pathol. 16(5): 529–540.

Aflalo C., Bing W., Zarka A., and S. Boussiba, 1999. The effect of the herbicide glufosinate (Basta) on astaxanthin accumulation in the green alga *Haematococcus pluvialis*, Z. Naturforsch., C: BioSci., 54: 49–54.

Alonso D.L., Belarbi E.H., Rodríguez-Ruiz J., Segura C.I., and A. Giménez, 1998. Acyl lipids of three microalgae, Phytochem. 47: 1473–1481.

An J.Y., Sim S.J., Lee J.S., and B.W. Kim, 2003. Hydrocarbon production from secondarily treated piggery wastewater by the green alga *Botryococcus braunii*, J. of Appl. Phycol. 15: 185–191.

Ansari A.A., KhanFareed F.A., KhanSarvajeet A., Gill S., Gill S.S, and J. Varshney, 2010. Aquatic plant diversity in eutrophic ecosystems, 12: 122-141. DOI: 10.1007/978-90-481-9625-8_12.

Arns B., Bartz J., Radünz M., Evangelho J.A., Pinto V.Z., Zavareze E.daR., and Á.R. Guerra Dias, 2015. Impact of heat-moisture treatment on rice starch, applied directly in grain paddy rice or in isolated starch, Food Sci. and Technol. 60(2): 257-289. DOI: 10.1016/j.lwt.2014.10.059

Arteca R.N., and J.M. Arteca, 2008. Effects of brassinosteroid, auxin, and cytokinin on ethylene production in *Arabidopsis thaliana* plants, J. Exp. Bot. 59 (11): 3019–3026, http://dx.doi.org/10.1093/jxb/ern159.

Avagyan A., 2008. A contribution to global sustainable development: inclusion of microalgae and their biomass in production and bio cycles, Clean Technol. and Environ. Policy 10(4): 313–317.

Babu A.G., Wu X., Kabra A.N., and D.P. Kim, 2017. Cultivation of an indigenous *Chlorella sorokiniana* with phytohormones for biomass and lipid production under N-limitation, Algal Res. 23: 178–185.

Bailey D.S and R.S. Ferrarezi, 2017. Valuation of vegetable crops produced in the UVI Commercial Aquaponic System. Aquaculture Reports 7: 77–82.

Bajguz A., and R. Czerpak, 1996a. Metabolic activity of estradiol in *Chlorella vulgaris* Beijerinck (*Chlorophytaceae*) Part 1. Content of photosynthetic pigments. Pol Arch Hydrobiol 43: 421-426.

Bajguz A., and R. Czerpak, 1996b. Metabolic activity of estradiol in Chlorella vulgaris Beijerinck (Chlorophyceae) Part II. Content of the cellular sugar and protein accumulation, Pol. Arch. Hydrobiol. 43: 427-430.

Bajguz A., and R. Czerpak, 1998. Physiological and biochemical role of brassinosteroids and their structure-activity relationship in the green alga *Chlorella vulgaris* Beijerinck (Chlorophyceae), J. Plant Growth Regul. 17: 131–139.

Bajguz A., 2000. Effect of brassinosteroids on nucleic acids and protein content in cultured cells of *Chlorella vulgaris*, Plant Physiol. Biochem. 38: 209–215.

Bajguz A., 2003. Brassinosteroids and lead as stimulators of phytochelatins synthesis in *Chlorella vulgaris*, J. Plant Physiol. 159: 321–324.

Bajguz A., 2011. Suppression of *Chlorella vulgaris* growth by cadmium, lead, and copper stress and its restoration by endogenous brassinolide, Arch. Environ. Contam. Toxicol. 60: 406. doi:10.1007/s00244-010-9551-0

Bajguz A., and A. Piotrowska-Niczyporuk, 2013. Synergistic effect of auxins and brassinosteroids on the growth and regulation of metabolite content in the green alga *Chlorella vulgaris* (Trebouxiophyceae), Plant Physiol. Biochem. 71: 290-297, http://dx.doi.org/ 10.1016/j.plaphy.2013.08.003.

Bajguz A., and A. Piotrowska-Niczyporuk, 2014. Interactive effect of brassinosteroids and cytokinins on growth, chlorophyll, monosaccharide and protein content in the green alga *Chlorella vulgaris* (Trebouxiophyceae). Plant Physiol. Biochem. 80:176–83.

Baker J.T., and Jr. L.H. Allen, 1994. Assessment of the impact of rising carbon dioxide and other potential climate changes on vegetation. Environ. Pollut. 83: 223–235.

Baliga R., and S.E. Powers, 2010. Sustainable algae biodiesel production in cold climates international, J. of Chem. Enginer. Volume 2010: 13 pages, Article ID 102179, doi:10.1155/2010/102179

Bansal V., Savidov N. and J. Rakocy, 2005. The effects of aquaponics and hydroponics nutrient solutions on shoot and root growth of six greenhouse crops, Austral. J. of Crop Sci. AJCS 4(2): 85-90. ISSN:1835-2707

Bao F., Shen J., Brady S.R., Muday G.K., Asami T., and Z. Yang, 2004. Brassinosteroids interact with auxin to promote lateral root development in *Arabidopsis*, Plant Physiol. 134: 1624-1631.

Barrett C.B., Bachke M.E., Bellemare M.F., Michelson H.C., Narayanan S., and T.F. Walker, 2012. Smallholder participation in contract farming: comparative evidence from five countries, World Development, 40 (4): 715-730.

Barry T.P., Riebe J.D., Parrish J.J., and J.A. Malison, 1997. Effects of 17α, 20β-Dihydroxy-4-pregnen-3-one on cortisol production by Rainbow Trout interrenal tissue *in vitro*, Gener.and Comparat. Endocrin. 107(2): 172-81.

Basco M., 2012. Duckweed profitable feed for tilapia farming: A Practical Manual to Tilapia Feeding (Tilapia Fish Farming) (Volume 2), by Create Space Independent Publishi ISBN 10: 1481123971

de-Bashan L.E., Bashan Y., Moreno M., Lebsky V.K., and J.J. Bustillos, 2002. Increased pigment and lipid content, lipid variety, and cell and population size of the microalgae *Chlorella spp*. when co-immobilized in alginate beads with the microalgae-growth-promoting bacterium *Azospirillum brasilense*, Can. J. Microbiol. 48(6): 514-21.

Becker E.W., 2004. Micro algae in human and animal nutrition. In Hand book of microalgal culture, ed. A. Richmond: 312–351 Oxford: Blackwell.

Becker E.W., 2006. Micro-algae as a source of protein, Biotech. Advan. 25: 207-10. http://dx.doi.org/10.1016/j.biotechadv.2006.11.002

Beecher L. 2013. Assessment of 17β-estradiol removal from wastewater via abiotic and biotic routes and potential effects on food chain pathways, A Dissertation Presented to the Graduate School of Clemson University: 152pp.

Ben-Amotz A., Shaish A., and M. Avron, 1989. Mode of action of the massively accumulated β-carotene of *Dunaliella bardawil* in protecting the alga against damage by excess irradiation, Plant Physiol. (86): 1286–1291.

Benemann J., 2010. Photobioreactors vs. ponds for microalgae biomass production, Aquacult. Soc. Annual Meeting, World Aquaculture, San Diego, CA, March 3.

https://www.was.org/documents/MeetingPresentations?AQ2010/AQ2010_0027

Benemann J., 2013. Microalgae for biofuels and animal feeds, Energies. 6: 5869-5886.

Bergmann B. A., Cheng J., Classen J., and A.M. Stomp, 2000. Nutrient removal from swine lagoon effluent by Duckweed. Transactions of the ASABE, 43: 263-269.

Bernstein S., 2011. Aquaponic gardening: A Step-by-Step Guide to Raising Vegetables and Fish Together, New Society Publishers.

Bibeau E., 2009. Microalgae technologies and processes for biofuels/bioenergy production in British Columbia: Current technology, sustability and barriers to implementation. Final Report Submitted to The British Collumbia Innovation Council, pp.74.

Bigogno C., Khozin-Goldberg I., Boussi S., Vonshak A., and Z. Cohen, 2002. Lipid and fatty acid composition of the green oleaginous alga *Parietochloris incisa*, the richest plant source of arachidonic acid, Phytochem. 60: 497–503.

Bishop G.J., and T. Yokota, 2001. Plants steroid hormones, brassinosteroids: current highlights of molecular aspects on their synthesis/metabolism, transport, perception and response, Plant Cell Physiol. 42: 114–120.

Bjerkeng B., Hamre K., Hatlen B., and E. Wathne, 1999. Astaxanthin deposition in fillets of Atlantic salmon (*Salmo salar L.*) fed two dietary levels of astaxanthin in combination with three levels of á-tocopheryl acetate, Aquacult. Res. 30: 637-646.

Bhardwaj R., and A.K. Thukral, 2000. Effects of steroids on growth and biochemical parameters of maize plants. Conference materials of the 12th Congress of the Federation of European Societies of Plant Physiology. 21-25 August 2000, Budapest, Hungary, p 82.

Bhaya D., Bianco N.R., Bryant D., and A. Grossman, 2000. Type IV pilus biogenesis and motility in the cyanobacterium *Synechocystis* sp. PCC6803, Molec. Microbiol. 37: 941–951.

Borker A.R., Mane A.V., Saratale G.D., and G.R. Pathade, 2013. Phytoremediation potential of Eichhornia crassipes for the treatment of cadmium in relation with biochemical and water parameters, Emir. J. Food Agric. 25(6): 443-456. doi: 10.9755/ejfa.v25i6.13970

Borowitzka M.A., 1988. Fats, oils and hydrocarbons. In, Micro-algal biotechnology, edited by M.A. Borowitzka and L. J. Borowitzka, Cambridge University Press, Cambridge: 257-287.

Borowitzka M.A., 1998. Limits to growth. In: Y. Wong and N.F.Y. Tam (Eds), Wastewater treatment with algae, Springer Verlag, 203-226.

Boussiba S., and A. Vonshak, 1991. Astaxanthin accumulation in the green alga *Haematococcus pluvialis*, Plant Cell Physiol. 32: 1077–1082.

Boussiba S., 2000. Carotenogenesis in the green alga *Haematococcus pluvialis*: Cellular Physiology and Stress Responce, Physiol. Plantar. 108(2): 111–117.

Brady N.C., and R.R. Weil, 2007. The nature and properties of soils, 14th edition. Pearson Education, Inc., Upper Saddle River, NJ.

Brennan S.J., Brougham C.A., Roche J.J., and A.M. Fogarty, 2006. Multi-generational effects of four selected environmental oestrogens on Daphnia magna, Chemosph. 64(1): 49–55.

Briens C., Piskorz J., et al., 2008. Biomass valorization for fuel and chemicals production. A Review. Internat. J. of Chemical Reactor Engineer. 6(6): 2-9.

Brown M.R., Dunstan G.A., Norwood S.J., and K.A. Miller, 1996. Effects of harvest stage and light on the biochemical composition of the diatom *Thalassiosira pseudonana*, J. Phycol. 32: 64–73.

Brzostek A., Popławski T., Murooka Y., and J. Dziadek, 1998. Genes and enzymes involved in conversions of steroids (in Polish). Biotechnologia 2: 65-85.

Bumbak F., Cook S., Zachleder V., Hauser S., and K. Kovar, 2011. Best practices in heterotrophic high-cell-density microalgal processes: achievements, potential and possible limitations, Appl. Microbiol. Biotechnol. 91: 31–46.

CAIA: Canadian Aquaculture Industry Alliance. 2012. www.aquaculture.caCAIA

Campbell, P. and T.H. Hutchinson, 1998. Annual review wildlife and endocrine disrupters: Requirements for hazard identification. Environ. Toxicol. and Chem.17(1): 127–135.

Caño-Delgado A., Yin Y., Yu C., Vafeados D., Mora-Garcia S., Cheng J.C., Nam K.H., Li J., and J. Chory, 2004. BRL1 and BRL3 are novel brassinosteroid receptors that function in vascular differentiation in *Arabidopsis*, Development (Cambridge, England) 131(21): 5341–5351, doi:10.1242/dev.01403

de Castro A.S., and V.M.T. Garcia, 2005. Growth and biochemical composition of the diatom *Chaetoceros cf. wighamii brightwell* under different temperature, salinity and carbon dioxide levels. I. Protein, carbohydrates and lipids, Aquacult. 246(4): 405–412.

Cai M., Li Z., and A. Qi, 2009. Effects of iron electrovalence and species on growth and astaxanthin production of *Haematococcus pluvialis*, Chin. J. Oceanol. Limnol. 27: 370–375.

Challouf R., Trabelsi, L., Dhieb, R.B., Abed, O.E., Yahia, A., Ghozzi, K., Ammer, J.B., Omram, H. and Ouada, H.B., (2011). Evaluation of cytotoxicity and biological activities in extracellular polysaccharides released by cyanobacterium Arthospira platensis. Brazilian Archives Biol and Technol., 54(4): 831-838.

Chaves-Pozo E., Liarte S., Vargas-Chacoff L., García-López A., Mulero V., Meseguer J., Mancera J.M., and A. García-Ayala, 2007. 17β-Estradiol triggers postspawning in spermatogenically active gilthead seabream (*Sparus aurata* L.) males, Biol. of Reproduc. 76(1): 142–148. https://doi.org/10.1095/biolreprod.106.056036

Chen R., Jiang H., Li L., Zhai Q., Qi L., Zhou W., Liu X., Li H., Zheng W., Sun J., and C. Li, 2012. The Arabidopsis mediator subunit MED25 differentially regulates jasmonate and abscisic acid signaling through interacting with the MYC2 and ABI5 transcription factors, Plant Cell., 24(7): 2898-916. doi: 10.1105/tpc.112.098277

Chekanov K., Lobakova E., Selyakh I., Semenova L., Sidorov R., and A. Solovchenko, 2014. Accumulation of astaxanthin by a new *Haematococcus pluvialis* strain BM1 from the White Sea coastal rocks (Russia), Mar. Drugs 12: 4504–4520. 10.3390/md12084504

Cheng J., Landesman L., Bergmann B.A., Classen J.J., Howard J.W., Yamamoto Y.T. et al., 2002. Nutrient removal from swine lagoon liquid by *Lemna minor*-8627, Trans ASAE 45(4): 1003–1010.

Chin M., 2009. Malaysian Palm Oil Association. MOPA annual report. Global Agricultural Information Network (GAIN).

Chisti Y., 2008. Biodiesel from microalgae beats bioethanol, Trends Biotechnol. 26: 126–131.

Chisti Y., 2007. Biodiesel from microalgae, Biotechnol. Adv. 25: 294–306.

Choi Y.E., Yun Y.S. and J.M. Park, 2002. Evaluation of factors promoting astaxanthin production by a unicellular green alga, *Haematococcus pluvialis*, with fractional factorial design, Biotechnol. Prog. 18(6): 1170-1175.

Christenson L., and R. Sims, 2011. Production and harvesting of microalgae for wastewater treatment, biofuels,and bioproducts, Biotechnol. Adv. 29: 686–702.

Chou T.C., 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug-combination studies, Pharmacol. Rev. 58: 621–681.

Chou T.C., and P. Talalay, 1984. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors, Adv. Enzym. Regul. 22: 27-55.

Chumpolkulwong N., Kakizono T., Ishii H., and N. Nishio, 1997. Enzymatic conversion of b-carotene to astaxanthin by cell-extracts of a green alga *Haematococcus pluvialis*, Biotechnol. Letters, 19(5): 443–446.

Clouse S.D., and J.M. Sasse, 1998. Brassinosteroids: Essential regulators of plant growth and development, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49: 427–51, doi:10.1146/annurev.arplant.49.1.427

Cobb A., 1992. Herbicides and plant physiology, Auxin-type herbicides, Chapman Hall: 82–106.

Cohen Z. and I. Khozin-Goldberg, 2011. Searching for PUFA-rich Microalgae, Single Cell Oils, Cohen, Z. and Ratledge, C. Eds., Am. Oil Chem. Soc.: Champaign I.L.: 201–224.

Colla L.M., Reinehr C.O., Reichert C. and J.A. Vieira Costa, 2007. Production of biomass and nutraceutical compounds by *Spirulina platensis* under different temperature and nitrogen regimes, Biores. Technol. 98(7): 1489-93.

Collins A.M., Jones H.D.T., Han D., Hu Q., Beechem T.E., et al.. 2011. Carotenoid distribution in living cells of *Haematococcus pluvialis* (Chlorophyceae), PLoS ONE 6(9): e24302 doi:10.1371/journal.pone.0024302

Collins M.L., Lynch B., Barfield W., Bull A., Ryan A.S., and J.D. Astwood, 2014. Genetic and acute toxicological evaluation of an algal oil containing eicosapentaenoic acid (EPA) and palmitoleic acid, Food and Chem. Toxicol. 72: 162–68. doi:10.1016/j.fct.2014.07.021

Conrad H., Saltman P., and R. Eppley, 1959. Effects of auxin and gibberellic acid on growth of *Ulothrix*. Nature 184: 556-557.

Converti A., Casazza A.A., Ortiz E.Y., Perego P., and M.D. Borghi, 2009. Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella*

vulgaris for biodiesel production, Chem. Eng. Prog. Process Intensification 48: 1146–1151. 10.1016/j.cep.2009.03.006

Cordero B.F., Obraztsova I., Couso I., Leon R., Vargas M. A., and H. Rodriguez, 2011. Enhancement of lutein production in *Chlorella sorokiniana* (Chorophyta) by improvement of culture conditions and random mutagenesis, Mar. Drugs, 9: 1607-1624. doi:10.3390/md9091607.

Cowan A.K., and P.D. Rose, 1991. Abscisic acid metabolism in salt-stressed cells of *Dunaliella salina*, Plant Physiol. 97: 798-803.

Craggs R.J., Heubeck S., Lundquist T.J., and J.R. Benemann, 2011. Algal biofuels from wastewater treatment high rate algal ponds, Water Sci. Technol. 63: 660–665.

Crist R.H., Robert M.J., Carr D., Watson J.R., and H.J. Clarke, 1994. Interactions of metals Ni^{2+}, Cd^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+}) and protons with algae. Ion exchange vs. adsorption models and a reassessment of Scatchard plots; ion exchange rates and equilibrium compared with calcium alginate, Environ. Sci. Technol. 28(11): 1859– 1866.

Crossmann K., 2007. Auxin herbicide action: lifting the veil step by Step, Plant signal. & Behave. 2(5): 421-3. DOI: 10.4161/psb.2.5.4417

Cui W., Xu J., Cheng J.J., A.M. Stomp, 2011. Starch accumulation in duckweed for bioethanol production. Biolog. Engin. 3: 187–197.

Cui Y., and J.J. Cheng, 2014. Growing Duckweed for biofuel production: a review, Plant Biol. 17(1): 16-23. doi:10.1111/plb.12216

Czerpak R., Bajguz A., Bialecka B., Wierzcholowska L.E., and M.M. Wolanska, 1994. Effect of auxin precursors and chemical analogues on the growth and chemical composition in *Chlorella pyrenoidosa*, Chick. Acta Soc. Bot. Pol. 63: 279-285.

Czerpak R., and A. Bajguz, 1997. Stimulatory effect of auxins and cytokinins on carotenes, with differential effects on xanthophylls in the green alga *Chlorella pyrenoidosa*, Chick. Acta Soc. Bot. Pol. 66: 41–46.

Czerpak R., Krotke A., and A. Mical, 1999. Comparison of stimulatory effect of auxins and cytokinins on protein, saccharides and chlorophylls content of *Chlorella pyrenoidosa*, Poloniae Arch. Hygrobiol. 46: 71-82.

Czerpak R., and I.K.Szamrej, 2000. Metabolitic activity of 11-deoxycorticosterone and prednisolone in the alga *Chlorella vulgaris* Beijerinck, Acta Soc. Bot. Pol. 69: 25.

Czerpak R., Bajguz A., and D. Iwaniuk, 2001a. Comparison of the influence of hydrocortisone and progesterone on the content of protein and sugar in the green alga *Chlorella vulgaris* Beijerinck, Ecohydrol. Hydrobiol. 1: 473.

Czerpak R., Bajguz A., and J. Kalinowska. 2001b. The stimulative effect of hydrocortisone on photosynthetic pigments in the green alga *Chlorella vulgaris* Beijerinck, Ecohydrol. Hydrobiol. 1: 465.

Czerpak R., and I.K. Szamrej, 2003. The Effect of β-estradiol and corticosteroids on chlorophylls and carotenoids content in *Wolffia arrhiza* (L.) Wimm. (Lemnaceae) Growing in Municipal Bialystok Tap Water, Polish J. of Environ. Studies 12(6): 677-684.

Czerpak R., Piotrowska A., and A. Krotke, 2004. Biochemical activity of auxins in dependence of their structures in *Wolffia arrhizal* (L.) Wimm. 73(4): 269-275.

Czygan J.C., 1962. Blütenbildung bei *Lemna minor* nach Zusatz von Oestrogen. Naturwissenschaften 49: 285-286.

Dalrymple O.K., Halfhide T., Udom I., Gilles B., Wolan J., Zhang Q., and S. Ergas, 2013. Wastewater use in algae production for generation of renewable resources: a review and preliminary result, Aqua. Biosyst. 9(1): 1-11.

Davis D.A., and C.R. Arnold, 2000. Replacement of fish meal in practical diets for the Pacific white shrimp *Litopenaeus vannamei*. Aquacult., 185: 291-298.

Davies P.J., 2004. Plant Hormones, Kluwer Academic Publishers, 716pp. doi:10.1186/2046-9063-9-2.

Davis R., Markham J., Kinchin Ch., Grundl N., and E.C.D. Tan, 2016. Process design and economics for the production of algal biomass: Algal biomass production in open pond systems and processing

through dewatering for downstream conversion, National Renewable Energy Laboratory, Process Consulting Prepared under Task No. BB14.1110; Technical Report NREL/TP-5100-64772; Contract No. DE-AC36-08GO28308

Department for Environment, Food and Rural Affair (DEFTA), UK 2008 The Impact of Biofuels on Commodity Prices., Report.

Del Rio-Chanona E.A., Liu J., Wagner J.L., Zhang D., Meng Y., Xue S., Shah Net al., 2018, Dynamic modeling of green algae cultivation in a photobioreactor for sustainable biodiesel production, Biotechol. and Bioengin. 115: 359-370. ISSN: 0006-3592

Department of Energy of the United States (DOE), 2010. Energy Efficiency and Renewable Energy, Golden Field Office, US

De Vleesschauwer D., Van Buyten E., Satoh K., Balidion J., Mauleon R., Choi Il-R., Vera-Cruz C., Kikuchi S., and M. Höfte, 2012. Brassinosteroids antagonize gibberellin and salicylate mediated root immunity in rice, Plant Physiol. 158 (4): 1833–1846. http://dx.doi.org/10.1104/pp.112.193672.

Dev Goswami R.Ch., 2011. *Scenedesmus dimorphus* and *Scenedesmus quadricauda*: two potent indigenous microalgae strains for biomass production and CO_2 mitigation - A study on their growth behavior and lipid productivity under different concentration of urea as nitrogen source, J. Algal Biomass Utln. 2(4): 42– 49.

Devi M.P., Subhash G.V., and S.V. Mohan. 2012. Heterotrophic cultivation of mixed microalgae for lipid accumulation and wastewater treatment during sequential growth and starvation phases: effect of nutrient supplementation, Renew Energy 43: 276–83. https://doi.org/10.1016/j.renene.2011.11.021.

DFO (2012): Department of Fisheries and Oceans, Canada. 2012. Communities and Employment/ Revitalizing coastal, rural and Aboriginal communities. Aquaculture Association of Canada.

DFO (2016): Department of Fisheries and Oceans, Canada. 2016. Canadian Aquaculture R&D Review. Aquaculture Association of Canada, Special Publication 16.

Di Mascio P., Murphy M.E., and H. Sies, 1991. Antioxidant defense systems: the role of carotenoids, tocopherols, and thiols, Am. J. C. in Nuir. 53: 194S-200S.

Diver, S., & Rinehart, L. (2010). Aquaponics — Integration of hydroponics and aquaculture (Apropriate technology transfer for rural areas: Horticulture systems guide), NCAT, 28pp.

Dolezel Y., Greilhuber J., and J. Suda, 2007. Estimation of nuclear˘DNA content in plants using flow cytometry, Nature Protocols 2(9): 2233–2244.

Damiani M.C., Popovich C.A., Constenla D., and P.I. Leonardi, 2010. Lipid analysis in *Haematococcus pluvialis* to assess its potential use as a biodiesel feedstock. Bioresour. Technol. 101: 3801–3807.

Dortch Q., 1990. The interaction between ammonium and nitrate uptake in phytoplankton, Mar. Ecol. Prog. Ser. 61: 183-201.

Dong W., Bian Y., Liang L., and B. Gu, 2011. Binding constants of mercury and Dissolved Organic Matter determined by a modified ion exchange technique, Environ. Sci. Technol. 45(8): 3576-3583. DOI: 10.1021/es104207g

Eaton A.D., and M.A.H. Franson, 2005. American Public Health, American Water Works, and Water Environment: Standard methods for the examination of water & wastewater. Washington DC: American Public Health Association.

EC, (2007) Biological Test Method: Growth Inhibition Test Using a Freshwater Alga, EPS1/RM/25.

EC, (2014) Biological Test Method: Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte, Lemna minor, EPS1/RM/37.

EC, SEC 1272, 1276, 2010: Planning for a sustainable future: a Federal Sustainable Development Strategy for Canada, 2010. Environment Canada, ISBN: 978-1-100-16796-1.

EIA IEO, (2009) Energy Information Administration. International Energy Outlook (EIAIEO). 284. EIA; DC, USA.

El- Baz F.K., Aboul-Enein M.A, El-Baroty G.S., Youssef A.M. and H.H.A. El-Baky, 2002. Accumulation of antioxidant vitamins in *Dunaliella salina*. Online J. Biol. Sci. 2: 220– 3.

El-Sayed, 2010. Carotenoids accumulation in the green alga *Scenedesmus sp*. Incubated with industrial citrate waste and different induction stresses, Nature and Science 8(10): 34-39.

Esteban S., Gorga M., Petrovic M., Gonzalez-Alonso S., Barcelo D., and Y. Valcarcel, 2014. Analysis

and occurrence of endocrine-disrupting compounds and estrogenic activity in the surface waters of central Spain, Sci. Total Environ. 466–467, 939–951. Doi:10.1016/j.scitotenv.2013.07.101.

Fábregas J., Dominguez A., Maseda A., and A. Otero, 2003. Interactions between irradiance and nutrient availability during astaxanthin accumulation and degradation in *Haematococcus pluvialis*, Appl. Microbiol. Biotechnol. 10: 253–261.

Fageria N.K., 1976. Critical P, K, Ca, and Mg contents in the tops of rice and peanut plants, Plant and Soil, 45(2): 421-431.

Fan J., Yan C., Andre C., Shanklin J., Schwender J., and C. Xu, 2012. Oil accumulation is controlled by carbon precursor supply for fatty acid synthesis in *Chlamydomonas reinhardtii*, Plant Cell Physiol. 53(8): 1380-1390.

Fang W., Si Y., Douglass S., Casero D., Merchant S., Pellegrini M., Ladunga I., Liu P., and M.H. Spalding, 2012. Transcriptome-wide changes in *Chlamydomonas* gene expression regulated by carbon dioxide and the CO_2 concentrating mechanism regulator CIA5/CCM, Plant Cell 24: 1876–1893.

Fasakin E.A., Balogun A.M., and E. Fasuru, 1999. Use of duckweed *Spirodella polyrrhiza* L. Schleiden, as a protein feedstuff in practical diets for Tilapia *Oreochromis niloticus* L. Aquacult. Res. 30: 313-318.

Fernández F.G.A., González-López C.V., Fernández Sevilla J.M., and E. Molina Grima, 2012. Conversion of CO_2 into biomass by microalgae: how realistic a contribution may it be to significant CO_2 removal? Appl. Microbiol. Biotechnol. 96: 577–586. DOI 10.1007/s00253-012-4362-z

Fernández-Sevilla J.M., Acién F.G., and E. Molina, 2010. Biotechnological production of lutein and its applications, Appl. Microbiol. Biotechnol. 86: 27–40.

Fiedor J., and K. Burda, 2014. Potential role of carotenoids as antioxidants in human health and disease, Nutrients, 6(2): 466–488. doi: 10.3390/nu6020466,

Finsterbusch A., Lindemann P., Grimm R., Eckerskorn C., and M. Luckner, 1999. δ5-3β-hydroxysteroid dehydrogenase from *Digitalis lanata* Ehrh. - a multifunctional enzyme in steroid metabolism, Planta 209: 478-486.

Fisher N.S., Bohe M., and J.L. Teyssie, 1984. Accumulation and toxicity of Cd, Zn, Ag, and Hg in four marine phytoplankters, Mar. Ecol. Prog. Ser. 18: 201-213.

Folch J., Lees M., and G.H.S. Stanley, 1957. A simple method for the isolation and purification of total lipids from animal tissues, J. of Biol. Chem. 226: 497.

Franklin N.M., Stauber J.L., Markich S.J., and R.P. Lim, 2000. pH- dependent toxicity of copper and uranium to a tropical freshwater alga (*Chlorella* sp.), Aquat. Toxicol. 48: 275–289.

Frédéric M., Samir L., Louise M., and A. Abdelkrim, 2006. Comprehensive modeling of mat density effect on duckweed (*Lemna minor*) growth under controlled eutrophication, Water Res. 40(15): 2901-2910. https://doi.org/10.1016/j.watres.2006.05.026

Fujiwara K., Matsumoto Y., Kawakami H., Aoki M., and M. Tuzuki, 2008. Evaluation of metal toxicity in *Chlorella kessleri* from the perspective of the Periodic Table, Bulletin of the Chemical Society of Japan DOI: 10.1246/bcsj.81.478

Gaigher I.G., Porath D., G. Granoth, 1984. Evaluation of duckweed (*Lemna gibba*) as feed for tilapia (*Oreochromis niloticuscross* & *Oreochromis aureus*) in a recirculating unit. Aquacul. 41(3): 235-244.

Gallert C., and J. Winter, 2005. Bacterial metabolism in wastewater treatment systems, Environ. Biotechnol. Concepts and Applications. Edited by H.-J. Jördening and J. Winter Weinheim ISBN: 3-527-30585-8

Gao Z.Q., and C.X. Meng, 2007. Impact of extraneous ethylene concentrations to astaxanthin accumulation of *Haematoccus pluvialis*, Food Sci. 10: 90-93.

García-González M., Moreno J., Manzano C., Florencio F.J., and M.G. Guerrero, 2005. Production of *Dunaliella salina* biomass rich in 9-cis β-carotene and lutein in a closed tubular photobioreactor, J. Biotechnol. 115: 81–90.

Gardner J., Nigel C., Quinn W.T., Van Gerpen J., and J. Simonpietri, 2011. Oilseed and algal oils as biofuel feedstocks, In: Sustainable Alternative Fuel Feedstock Opportunities, Challenges and Roadmaps for Six U.S. Regions. Braun R., Karlen D., and D. Johnson (Eds.), Soil and Water Conservation Society, Ankeny, IA.

Geider R.J., and J. La Roche, 2002. Redfield revisited: variability of C:N:P in marine microalgae and its biochemical basis, Eur. J. Phycol. 37: 1–17, doi:10.1017.S09670262010 03456

Geuns J.M.C., 1978. Steroid hormones and plant growth and development. Phytochem. 17: 1-14.

Ghasemi Y., Rasoul-Amini S., and E. Fotooh-Abadi, 2011. The biotransformation, biodegradation and bioremediation of organic compounds by microalgae. J. of Phycol. 47(5): 969–980.

Gojkovic Z., Vílchez C., Torronteras R., Vigara J., Gómez-Jacinto V., Janzer N, Gómez-Ariza J,L., Márová I., and I. Garbayo, 2014. Effect of selenate on viability and selenomethionine accumulation of *Chlorella sorokiniana* grown in batch culture, The Sci. World J. Article ID 401265, 13 pages, http://dx.doi.org/10.1155/2014/401265

Greenberg B.M., Huang X.D., and D.G. Dixon, 1992. Applications of the aquatic higher plant *Lemna gibba* for Ecotoxicological Assessment, J. of Aqua. Ecosys. Health 1: 147–155.

Grönlund E., Klang A., P-Å. Vikman, 2004. Emergy and socio-ecological principles in sustainability assessment: methodological considerations from a wastewater treatment case study, Conference: Emergy Synthesis 3. Proceedings from the 3rd Biennial Emergy Research Conference, Gainesville, Florida, January, 2004.Grossmann K., 2007. Auxin herbicide action, Plant Signal Behav. 2(5): 421–423.

Grunewald K., Hirschberg J., and C. Hagen, 2001. Ketocarotenoid biosynthesis outside of plastids in the unicellular green alga *Haematococcus pluvialis,* J. of Biol. Chem. 276: 6023–6029.

Guedes A.C., Amaro H.M., and F.X. Malcata, 2011. Microalgae as sources of carotenoids, Mar. Drugs 9: 625-644. doi:10.3390/md9040625

Guerin M., Huntley M.E., and M. Olaizola, 2003. *Haematococcus astaxanthin*: applications for human health and nutrition, Trends in Biotech. 21(5): 23-29.

Guimaraes F.P, Aguiar R., Oliveira J.A., Silva J.A.A., and D. Karam, 2012. Potential of macrophyte for removing arsenic from aqueous solution, *Planta daninha* 30(4): 112-118. dx.doi.org/10.1590/S0100-83582012000400001.

Guiguen Y., Fostier A., Piferrer F., and Ch.F. Chang, 2010. Ovarian aromatase and estrogens: A pivotal role for gonadal sex differentiation and sex change in fish, General and Compar. Endocrin. 165(3): 352-366.

Guschina I., and J. Harwood, 2009. Algal lipids and effect of the environment on their biochemistry, In book: Lipids in Aquatic Ecosystems. DOI: 10.1007/978-0-387-89366-2_1

Hala D., Petersen L.H., Martinovic D., and D.B. Huggett, 2012. Constraints-based stoichiometric analysis of hypoxic stress on steroidogenesis in fathead minnows, *Pimephales promelas*, The J. of Experim. Biol. 215: 1753-1765.

Halterman S.G., and D.W. Toetz, 1984. Kinetics of nitrate uptake by freshwater algae, Hydrobiol. 1149(3): 209-214.

Halver J.E., 1972. Fish nutrition. Academic Press, London, pp 713.

Han S., Jin W., Chen Y., et al., 2016. Enhancement of lipid production of *Chlorella Pyrenoidosa* cultivated in municipal wastewater by magnetic treatment, Appl. Biochem. Biotechnol. 180(6): 1043-1055, doi:10.1007/s12010-016-2151-3.

Han X., Zeng H., Bartocci P., Fantozzi F., and Y. Yan, 2008. Phytohormones and effects on erowth and metabolites of microalgae: A Review. Fermentation, 4(2): 25.

Hanexakowski P., Szymczyk B. and M. Wawrzynski, 1995. Composition and nutritive value of sewagegrown duckweed (*Lemna minor* L.) for rats, Anim. Feed Sci. Technol. 52: 339-343.

Hannon M., Gimpel J., Tran M., Rasala B. and Mayfield S., 2010. Biofuels from algae: challenges and potential, Biofuels 1(5): 763–784.

Hagen C., Braune W., and L. Björn, 1994. Functional aspects of secondary carotenoids in *Haematococcus lacustris* (Volvocales), Action as a Sunshade, J. Phycol. 30: 241–248.

Harrison M.A., 2012. Cross-talk between phytohormone signaling pathways under both optimal and stressful environmental conditions, in N.A. Khan et al. (eds.), Phytohormes and Abiotic Stress Tolerance in Plants, http://dx.doi.org/10.1007/978-3-642-25829-9_2.

Hartmann S., Lacorn M., and H. Steinhart, 1998. Natural occurrence of steroid hormones in food, Food Chem., 62(1): 7-20

Hartung W, 2010. The evolution of abscisic acid (ABA) and ABA function in lower plants, fungi and lichen, Funct. Plant Biol. 37: 806–812.

Harwood J.L. 1998. Environmental effects on plant lipid bio-chemistry, In: Harwood JL, ed. Plant lipid biosynthesis. Cam-bridge, UK: Cambridge University Press, 305–347.

Harwood J., 2004. Involvement of chloroplast lipids in the reaction of plants submitted to stress, lipids in photosynthesis: structure, function and genetics, Sigenthaller P.A., and Murata, N., Eds, Berlin: Springer Verlag: 287–302.

Harwood J.L., and A.L. Jones, 1989. Lipid metabolism in algae. In Advances in Botanical Research; Callow, J.A., Ed.; Academic Press: Waltham, MA, USA, Volume 16: 1–53.

Hassan S.M. and P. Edwards, 1992. Evaluation of Duckweed (*Lemna perpusilla* and *Spirodela polyrrhiza*) as feed for Nile Tilapia (*Oreochromis niloticus*). Aquacul. 104(3): 315-326.

Haustein A.T., Gilman R.H., Skillicorn P.W., Hannan H., Díaz F., Guevara V., Vergara V., Gastañaduy A., and J.B. Gilman, 1988. Performance of broiler chickens fed diets containing duckweed (Lemna gibba). J Agric Sci, 122(2): 285-289.

Haustein A.T., Gilman R.H., Skillicorn P.W., Vergara V., Guevara V., and A. Gastañaduy, 1990. Duckweed, a useful strategy for feeding chickens: performance of layers fed with sewage-grown Lemnaceae species, J. Poultry Sci. 69 (11): 1835-1844.

Heftmann E., 1975. Function of steroids in plants, Phytochem. 14: 891.

Hendrix J.W., and S.M. Guttman, 1968. Annulment of sterol-induced sexual reproduction by estradiol in *Pythium periplocum*, Sci. 161: 1252.

Hillman W.S., and D.D. Culley, 1978. The uses of duckweed, Am. Sci. 66(4): 442–451.

Hirsch R., Hartung W., and H. Gimmler, 1989. Abscisic acid content of algae under stress, Botan. Acta 102(4): 326–334. doi:10.1111/j.1438-8677.1989.tb00113.x

Ho S., Chen C., Lee D., and J. Chang, 2011. Perspectives on microalgal CO_2 -emission mitigation systems, A review. Biotechnol. Adv. 29: 189-198.

Hodaifa G., Martínez M.E., and S. Sánchez, 2008. Use of industrial wastewater from olive-oil extraction for biomass production of *Scenedesmus obliquus*, Bioresour. Technol. 99: 1111-1117.

Hodaifa G., Martínez M.E., and S. Sánchez, 2009. Daily doses of light in relation to the growth of *Scenedesmus obliquus* in diluted three-phase olive mill wastewater, J. Chem. Technol. Biotechnol. 84: 1550-1558.

Hom-Diaz A., Llorca M., Rodríguez-Mozaz S., Vicent T., Barceló D., and P. Blánquez, 2015. Microalgae cultivation on wastewater digestate: β-estradiol and 17α-ethynylestradiol degradation and transformation products identification, J. Environ. Manag. 155: 106–113.

Huarachi-Olivera R., Dueñas-Gonza A., Yapo-Pari U., Vega P., Romero-Ugarte M., Tapia J, Molina L, Lazarte-Rivera A., Pacheco-Salazar D.G. and M. Esparza, 2018. Bioelectrogenesis with microbial fuel cells (MFCs) using the microalga *Chlorella vulgaris* and bacterial communities, Electronic J. of Biotechnol. 31: 34–43.

Hunt R.W., Chinnasamy S., Bhatnagar A., and K.C. Das, 2010. Effect of biochemical stimulants on biomass productivity and metabolite content of the microalga, *Chlorella sorokiniana*, Appl. Biochem. Biotechnol. 162 (8): 2400–2414.

Imamoglu E., Dalay M.C., and F.V. Sukan, 2009. Influence of different stress media and high light intensities on accumulation of astaxanthin in green alga *Haematococcus pluvialis*. New Biotechnol. 26: 199–204. doi: 10.1016/j.nbt.2009.08.007

Inborr J., 1998. *Haematococcus*, the poultry pigmentor. Feed Mix, 6: 31-34.

Ip P.-F., and F. Chen, 2005. Production of astaxanthin by the green microalga *Chlorella zofingiensis* in the dark, Process Biochem 40: 733–738.

Iskandarov U., Khozin-Goldberg I., and Z. Cohen, 2011. Selection of a DGLA-producing mutant of the microalga *Parietochloris incisa*: I. Identification of mutation site and expression of VLC-PUFA biosynthesis gene, Appl. Microbiol. and Biotech. 90(1): 249-56. DOI: 10.1007/s00253-010-3005-5

Itagaki E., and T. Iwaya, 1988. Purification and characterization of 17β-hydroxysteroid dehydrogenase from *Cylindrocarpon radicicola*, J. Biochem. 103: 1039–1044.

James G.O., Hocart Ch.H., Hillier W., Chen H., Kordbacheh F., Price D., and M.A. Djordjevic, 2011. Fatty acid profiling of *Chlamydomonas reinhardtii* under nitrogen deprivation Bioresour. Technol. 102(3): 3343-51.

Janeczko A., and W. Filek, 2002. Stimulation of generative development in partly vernalized winter wheat by animal sex hormones, Acta Physiol. Plant 24: 291-295.

Janeczko A., Filek W., Biesaga-Koscielniak J., Marcinska I., and Z. Janeczko, 2003. The influence of animal sex hormones on the induction of flowering in *Arabidopsis thaliana*: comparison with the effect of 24-epibrassinolide, Plant Cell Tissue Organ Cult. 72: 147-151

Janeczko A., and A. Skoczowski, 2005. Mammalian sex hormones in plants, Folia Histochemica et cytobiologica 43(2): 71-79.

Janik J.R., and J.H. Adler, 1984. Estrogen receptors in *Gladiolus ovules*, Plant Physiol. 75: 135.

Jarvis E., Davis R., and C. Frear, 2013. Efficient use of algal biomass residues for biopower production with nutrient recycle: Final Project Report, National Renewable Energy Laboratory. US

Jiang H., and K. Gao, 2004. Effects of lowering temperature during culture on the production of polyunsaturated fatty acids in the marine diatom *Phaeodactylum tricornutum* (bacillariophyceae), J. Phycol. 40(4): 651–654.

Jin E., Lee C.G., and J.E.W. Polle. 2006. Secondary carotenoid accumulation in *Haematococcus* (Chlorophyceae): biosynthesis, regulation, and biotechnology, J.of Microbiol. and Biotechnol. 16: 821-831.

Lino M., Nomura T., Tamaki Y., Yamada Y., Yoneyama K., Takeuchi Y., Mori M., Asami T., Nakano T., and T. Yokota, 2007. Progesterone: its occurrence in plants and involvement in plant growth, Phytochem. 68(12):1664-73

Jobling S., and R. Owen, 2003. Ethinyl oestradiol in the aquatic environment, Emerg. Lessons Ecosyst. 13: 279-307.

Joss A., Andersen H., Ternes T., Richle P.R., and H. Siegrist, 2004. Removal of estrogens in municipal wastewater treatment under aerobic and anaerobic conditions: Consequences for Plant Optimization, Environ. Sci. Technol. 38(11): 3047–3055. DOI: 10.1021/es0351488

Journey W.K., Skillicorn P., and W. Spira, 1991. A new aquatic farming system for developing countries. The world bank Emena technical Department Agriculture DMSION.

Kang C.D., An J.Y., Park T.H., and S.J. Sim, 2006. Astaxanthin biosynthesis from simultaneous N and P uptake by the green alga *Haematococcus pluvialis* in primary-treated wastewater, Biochem. Eng. J. 31: 234–238.

Karnachuk R.A., Golovatskaya I.F., Efimova M.V., and V.A. Khripach, 2002. The effect of epibrassinolide on *Arabidopsis* seedling morphogenesis and hormonal balance under green light, Russ. J. Plant Physiol. 49: 530–533.

Katsumi M., 1985. Interaction of a brassinosteroid with IAA and GA3 in the elongation of Cucumber hypocotyl sections, Plant Cell Physiol. 26: 615–625.

Khozin-Goldberg I., Shrestha P., and Z. Cohen, 2005. Mobilization of arachidonyl moieties from triacyl glycerols into chloroplastic lipids following recovery from nitrogen starvation of the microalga *Parietochloris incisa*, Biochim. Biophys. Acta. Mol. Cell Biol. Lipids, 1738: 63–71.

Khozin-Goldberg I., and Z. Cohen, 2006. The effect of phosphate starvation on the lipid and fatty acid composition of the fresh water eustigmatophyte *Monodus subterraneus*. Phytochem.67: 696–701.

Khripach V., Zhabinski V., and A. De Groot, 2000. Twenty years of brassinosteroids: steroidal plant hormones warrant better crops for the XXI century, Annals of Botany 86: 441-447. doi:10.1006/anbo.2000.1227.

Khvatkov P., Chernobrovkina M., Okuneva A., and S. Dolgov, 2018. Creation of culture media for efficient Duckweeds micropropagation (*Wolffia arrhiza* and *Lemna minor*) using artificial mathematical optimization models. Plant Cell, Tissue and Organ Culture (PCTOC). https://doi.org/10.1007/s11240-018-1494-6

Kim S.K., Chang S.C., Lee E.J., Chung W.S., Kim Y.S., Hwang S., and J.S. Lee, 2000. Involvement of brassinosteroids in the gravitropic response of the primary root of maize, Plant Physiol. 123: 997-1004.

Kim Z.H., Kim S.H., Lee H.S., and C.G. Lee, 2006. Enhanced production of astaxanthin by flashing light using *Haematococcus pluvialis*. Enzyme Microb. Technol. 39: 414–419.

Kim M.K., Park J.W., Park C.S., Kim S.J., Jeune K.H., Chang M.U., and J. Acreman, 2007. Enhanced production of *Scenedesmus spp*. (green microalgae) using a new medium containing fermented swine wastewater, Bioresour. Technol. 98: 2220–2228.

Kim J.H., Nam S.W., Kim B.W., Kim W.J., and Y.H. Choi, 2010. Astaxanthin improves the proliferative capacity as well as the osteogenic and adipogenic differentiation potential in neural stem cells, Food Chem. Toxicol. 48(6): 1741-5.

Kiseleva A.A., Tarachovskaya E.R., and M.F. Shishova, 2012. Biosynthesis of phytohormones in algae, Russ. J. Plant Physiol. 59: 595–610.

Kniazeva M., Crawford Q.T., Seiber M., Wang C.T., and M. Han, 2004. Monomethyl branched-chain fatty acids play an essential role in *Caenorhabditis elegans* development. PLOS Biol. 2: 1446–1459.

Kobayashi M., Kakizono T., and S. Nagai, 1991. Astaxanthin production by a green alga, *Haematococcus pluvialis* accompanied with morphological changes in acetate media, J. Ferment. Bioeng. 71: 335–339.

Kobayashi M., Kurimura Y., Sakamoto Y., and Y. Tsuji, 1997. Selective extraction of astaxanthin and chlorophyll from the green alga *Haematococcus pluvialis*, Biotechnol. Techniq. 11(9): 657–660.

Kobayashi M., Kakizono T., and S. Nagai, 1993. Enhanced carotenoid biosynthesis by oxidative stress in acetate-induced cyst cells of a green unicellular alga, *Haematococcus pluvialis*, Appl. Environ. Microbiol. 59: 867-873.

Kobayashi M., Kurimura Y., Kakizono T., Nishio N., and Y. Tsuji, 1997. Morphological changes in the life cycle of the green alga *Haematococcus pluvialis*, J. Ferment. Bioeng. 84: 94–97.

Kobayashi M., and Y. Sakamoto, 1999. Singlet oxygen quenching ability of astaxantin esters from the green algae *Haematococcus pluvialis*, Biotech. Let. 21: 265-269.

Kong Q., Li L., Martinez B., Chen P., and R. Ruan, 2010. Culture of microalgae *Chlamydomonas reinhardtii* in wastewater for biomass feedstock production, Appl. Biochem. Biotechnol. 160(1): 9-18, doi:10.1007/s12010-009-8670-4.

Kopcewicz J., 1970. Influence of estrogens on the auxins content in plants. Naturwissenschaffen, 57: 48.

Kopcewicz J., and Z. Porazinski Z, 1974. Effect of growth regulators, steroids and estrogen fraction from sage plants on flowering of a long-day plant, *Salvia splendens*, grown under non-inductive light conditions, Biol. Plant (Praha) 16: 132-135.

Körner S., and J.E. Vermaat, 1998. The relative importance of *Lemna gibba* L., bacteria and micro-algae for the treatment of domestic sewage in duckweed-covered systems, Water Res. 32: 3651-3661.

Körner S., Vermaat J.E., and S. Veenstra, 2003. The capacity of duckweed to treat wastewater: ecological considerations for a sound design, J. Environ. Qual. 32(5): 1583-90.

Kozlova T.A., Hardy B.P., Krishna P., and D.B. Levin, 2017. Effect of phytohormones on growth and accumulation of pigments and fatty acids in the microalgae *Scenedesmus quadricauda*, Algal Res. 27: 325-334.

Kozlova T.A., Hardy B.P., and D.B. Levin, 2018. The combined influence of 24-epibrassinolide and 3-indoleacetic acid on growth and accumulation of pigments and fatty acids in the microalgae *Scenedesmus quadricauda* (CPCC-158), Algal Resear. 35: 22-32.

Krittaphol W., McDowell A., Thomson C.D., Mikov M., and J.P. Fawcett, 2011. Biotransformation of L-selenomethionine and selenite in rat gut contents, Biol. Trace Elem. Res. 139(2): 188-96. doi: 10.1007/s12011-010-8653-x

Lai K.M., Scrimshaw M.D., and J.N. Lester. 2002. Prediction of the bioaccumulation factors and body burden of natural and synthetic estrogens in aquatic organisms in the river systems. Sci. Total Environ. 289(1-3): 159-68.

Landesman L., Parker N.C., Fedler C.B., and M. Konikoff, 2005. Modeling duckweed growth in wastewater treatment systems, Livestock Res. for Rural Develop. 17(6), 8 pages.

Landesman L., Fedler C., and R. Duan, 2011. Plant nutrient phytoremediation using Duckweed (*Lemna minor*), 54(1): 35-45.

Landesman L., Fedler C., and D. Runbin, 2017. Plant nutrient phytoremediation using Duckweed, A.A. Ansari et al. (eds.), Eutrophication: Causes, Consequences and Control, 341-354. DOI 10.1007/978-90-481-9625-8_17

Landolt E., and R. Kandeler, 1987. The family of Lemnaceae - a monographic study, Phytochemistry, physiology, application and bibliography, In Biosystematic investigations in the family of Duckweeds (Lemnaceae), Geobotanischen Instutites der ETH, Stiftung Rubel, Zurich, 4: 638 pp.

Lardon L., Elias A.H., Sialve B., Steyer J.P., and O. Bernard, 2009. Life-Cycle Assessment of biodiesel production from microalgae, Environ. Scien. Technol. 43(17): 6475-81.

Larsdotter K., Söderbäck E., and G. Dalhammar, 2004. Phosphorus removal from wastewater by microalgae in a greenhouse in Sweden, Water Environ. Manag. Ser.3: 183-188.

Larsdotter K., 2006. Wastewater treatment with microalgae-a literature review. VATTEN, 62, 31-38.

Larsson D.G.J., Adolfsson E.M., Parkkonen J., Pettersson M., Berg A.H., Olsson P.E., and L. Forlin, 1999. Ethinyloestradiol - an undesired fish contraceptive? Aquat. Toxicol. 45(2–3): 91–97.

Larsson, P., A. Andersson, D. Broman, J. Nordback, and E. Lundberg. 2000. Persistent organic pollutants (POPs) in pelagic systems. Ambio 29: 202–209.

Laurens L.M.L., Nagle N., Davis R., Sweeney N., Van Wychen S., Lowell A., and P.T. Pienkos, 2015. Acid-catalyzed algal biomass pretreatment for integrated lipid and carbohydrate-based biofuels production, Green Chem. 17: 1145–1158.

Lee R.E., 2012. Effects of medium components and light on callus induction, growth, and frond regeneration *Lemna gibba* (Duckweed), Phycology. Cambridge University Press, 4th edition, 546pp.

Leema J.T.M., Kirubagaran R., Vinithkumar N.V., Dheenan P.S., and S. Karthikayulu, 2010. High value pigment production from *Arthrospira* (Spirulina) platensis cultured in seawater, Bioresour.Technol. 101: 9221–9227.

Leman, 1997. Oleaginous microorganisms: An Assessment of the Potential, Advances in applied microbiology 43: 195-243. DOI: 10.1016/S0065-2164(08)70226-0

Lemoine Y., and B. Schoefs, 2010. Secondary ketocarotenoid astaxanthin biosynthesis in algae: A multifunctional response to stress, Photosynth. Res. 106: 155–177.

Leng R.A., Stambolie J.H., and R. Bell, 1995. Duckweed –a potential high-protein feed resource for domestic animals and fish, Livestock Resear. for Rural Develop. 7(1).

Levin D.B., Verbeke T.J., Munir R., Islam R., Ramachandran U., Lal S., Schellenberg J., and R. Sparling, 2014. Omics Approaches for Designing Biofuel Producing Co-cultures for Enhanced Microbial Conversion of Lignocellulosic Substrates. In Direct Microbial Conversion of Biomass to Advanced Biofuels, M. Himmel ed., Elsevier Publishing. ISBN 9780444595928

Li J., Biswas M.G., Chao A., Russell D.W., and J. Chory, 1997. Conservation of function between mammalian and plant steroid 5α-reductases, Proc. Natl. Acad. Sci. USA 94: 3554-3559.

Li Q., Du W., and D. Liu D, 2008. Perspectives of microbial oils for biodiesel production. Appl. Microbiol. and Biotechnol. 80: 749–756.

Li Y., Qin J.G., Ball A.S., and R.BM oore, 2009. Perspectives of marine phytoplankton as a source of nutrition and bioenergy. In Kersey, William T., and Munger, Samuel P., (eds.) Marine Phytoplank. Oceanography and Ocean Engineering Series. Nova Science, Hauppage, New York, USA, pp. 187-203.

Li X., Hu H.Y., Gan K., and Y.X. Sun, 2010. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus sp.*, Bioresour. Technol. 101: 5494.

Li P., Mao X., Li R., and J. Zhong, 2011. In situ biodiesel production from fast-growing and high oil content *Chlorella pyrenoidosa* in rice straw hydrolysate, J. of Biomed. and Biotech. Vol. 2011, 8 pages doi:10.1155/2011/141207.

Lignell A., and J. Inborr, 2000. Agent for increasing the production of/in breeding and production mammals. Patent US6054491.

Lindemann P., and M. Luckner, 1997. Biosynthesis of pregnane derivatives in somatic embryos of *Digitalis lanata*. Phytochem. 46: 507-513.

Litskas V.D., Aschonitis V.G., Lekakis E.H., and V.Z. Antonopoulos, 2014. Effects of land use and irrigation practices on Ca, Mg, K, Na loads in rice-based agricultural systems, Agricult. Water Manag. 132(31): 30–36.

Liu S., Ying G.G., Zhao J.L., Zhou L.J., Yang B., Chen Z.F., and H.J. Lai, 2012. Occurrence and fate of androgens, estrogens, glucocorticoids and progestagens in two different types of municipal wastewater treatment plants, J. Environ. Monit. 14: 482–491.

Liu Z., Zhang F., and F. Chen, 2013. High throughput screening of CO_2-tolerating microalgae using GasPak bags, Aquat Biosyst. 9: 23. doi: 10.1186/2046-9063-9-23

Liu J., Qiu W., and Y. Song, 2016. Stimulatory effect of auxins on the growth and lipid productivity of *Chlorella pyrenoidosa* and *Scenedesmus quadricauda*, Algal Res. 18: 273–280.

Liu W., Chen Q., He N., Sun K., Sun D., Wu X., and S. Duan, 2018. Removal and biodegradation of 17β-Estradiol and diethylstilbestrol by the freshwater microalgae *Raphidocelis subcapitata*, Int. J. Environ. Res. Public Health 15: 452. doi:10.3390/ijerph15030452

Ljung K., Hull A.K., Kowalczyk M., Marchant A., Celenza J., Cohen J.D., and G. Sandberg, 2002. Biosynthesis, conjugation, catabolism and homeostasis of indole-3-acetic acid in *Arabidopsis thaliana*, Plant Mol. Biol. 50: 309–332.

Loats K.V., Noble R., and B. Takemoto, 1981. Photosynthesis under low level SO, and CO, enhancement conditions in three duckweed species, Bot. Gaz. 142: 305-314.

Lopez M.L., Peralta-Videa J.R., Benitez T., and J.L. Gardea-Torresdey, 2005. Enhancement of lead uptake by alfalfa (*Medicago sativa*) using EDTA and a plant growth promoter, Chemosph. 61: 595–598.

Lower S.K., 1999. Carbonate equilibria in natural waters, A Chem1, Simon Fraser University.

Ludwig-Müller J., 2011. Auxin conjugates: their role for plant development and in the evolution of land plants, J. Exp. Bot. 62 (6): 1757-1773. http://dx.doi.org/10.1093/jxb/erq41.

Lynn S.G., Kilham S.S., Kreeger D.A., and S.J. Interlandi, 2000. Effect of nutrient availability on the biochemical and elemental stoichiometry in the freshwater diatom *Stephanodiscus minutulus* (bacillariophyceae), J. Phycol. 36: 510–522.

Machida S., Shiraiwa Y., and I. Suzuki, 2016. Construction of a cyanobacterium synthesizing cyclopropane fatty acids, Biochim. Biophys. Acta 1861: 980–987.

Machida S., Bakku R.K., and I. Suzuki, 2017. Expression of genes for a flavin adenine dinucleotide-binding oxidoreductase and a methyltransferase from *Mycobacterium chlorophenolicum* is necessary for biosynthesis of 10-methyl stearic acid from oleic acid in *Escherichia coli*, Front Microbiol. 8: 2061. doi: 10.3389/fmicb.2017.02061.

Maiani G., Caston M.J., Catasta G., Toti E., Cambrodon I.G., Bysted A., Granado-Lorencio F., et al., 2009. Carotenoids: Actual knowledge on food sources, intakes, stability and bioavailability and their protective role in humans, Mol. Nutr. Food Res. 53: S194–S218.

Maier C.G.A., Chapman K.D., and D.W. Smith, 1995. Differential estrogenic activities of male and female plant extracts from two dioecious species, Plant Sci. 109: 31-43.

Makarevičienė V., Andrulevičiūtė V., Skorupskaitė V., and J. Kasperovičienė, 2011. Cultivation of microalgae *Chlorella sp*. and *Scenedesmus sp*. as a potentional biofuel feedstock, Environ. Resear. Engineer. and Managem. 3(57): 21–27.

Malca J., and F. Freire, 2006. Renewability and life-cycle energy efficiency of bioethanol and bio-ethyl tertiary butyl ether (bioETBE): Assessing the implications of allocation, Energy 31: 3362–3380.

Malek A., Zullo L.C., and P. Daoutidis, 2016. Modeling and dynamic optimization of microalgae cultivation in outdoor open ponds, Eng. Chem. Res. 55: 3327-3337. DOI: 10.1021/acsiecr.5b03209

Mandava N.B., 1988. Plant growth-promoting brassinosteroids, Ann. Rev. Plant Physiol. Plant Mol. Biol. 39: 23-52. http://dx.doi.org/10.1146/annurev.pp.39.060188.000323.

Mardanov, A.V. et al., 2008. Complete Sequence of the Duckweed (*Lemna minor*) chloroplast genome: structural organization and phylogenetic relationships to other angiosperms, J. Molec. Evol. 66(6): 555-564. http://www.springerlink.com/content/8042060xp665646x/

Martín J.F., Gudiña E., and J.L. Barredo, 2008. Conversion of β-carotene into astaxanthin: Two separate enzymes or a bifunctional hydroxylase-ketolase protein? Micro.Cell Factories 7: 3-13. doi:10.1186/1475-2859-7-3

Martinez M.E, Jimenez J.M., and F. El-Yousfi, 1999. Influence of phosphorous concentration and temperature on growth and phosphorous uptake by the microalga *Scenedesmus obliquus*. Bioresor. Biotech. 67: 233-240.

Mata T., Meloa A.C., Meireles S., Mendes A.M., Martins A.A., and N.C. Caetano, 2013. Potential of microalgae *Scenedesmus obliquus* grown in brewery wastewater for biodiesel production, Chem. Engi. Transact. 32: 901-907.

Mazen A.M.A, Zhang D., and V.R. Franceschi, 2003. Blackwell Publishing Ltd. Calcium oxalate formation in *Lemna minor*: physiological and ultrastructural aspects of high capacity calcium sequestration, New Phytologist 161: 435–448. doi: 10.1046/j.1469-8137.2003.00923.x

McGinn P.J, Dickinson K.E, Bhatti S., Frigon J.C, Guiot S.R, and S.J.B. O'Leary, 2011. Integration of microalgae cultivation with industrial waste remediation for biofuel and bioenergy production: opportunities and limitations, Photosynth. Resear. 109: 231-247.

McGinn P.J., Dickinson K.E., Park K.C., Whitney C.G., MacQuarrie S.P., Black F.J., Frigon G.S., Guiot S.R., and S.G.B. O'Leary, 2012. Assessment of the bioenergy and bioremediation potentials of the microalga *Scenedesmus sp*. AMDD cultivated in municipal wastewater effluent in batch and continuous mode, Algal Res. 1(2): 155-165. doi:10.1016/j.algal.2012.05.00.

McKendry P., 2002. Energy production from biomass (part 2): conversion technologies. Biores. Technol. 83: 47–54.

McLay, 1976. The effect of pH on the population growth of three species of duckweed: *Spirodela oligoruhiza*, *Lemna minor* and *Woffia arrhizal*, Freshwater Biol. 6: 125-136.

McLaren J.S., and H. Smith H, 1976. The effect of abscisic acid on growth, photosynthetic rate and carbohydrate metabolism in *Lemna minor L*, New Phytolog. 76: 11–20.

McMurtry M.R., Snaders D.C., Cure J.D., and R.G. Hodson, 1997. Effects of biofilter/culture tank volume ratio on productivity of s recirculating fish/vegetable co-culture system, J. Appl. Aquacult. 7 (4): 33-51.

Metting J. F., 1996. Biodiversity and application of microalgae. J. of Industr. Microbiol. 17(5-6): 477-489.

Milanesi L., Monje P., and R. Boland, 2001. Presence of estrogens and estrogen receptor-like proteins in *Solanum glaucophyllum*, Biochem. Biophys. Res. Commun. 289: 1175-1179.

Mishra U., and Pabbi, 2004 Biodiversity and biological degradation of soil, Resonance 9(1): 26-33. DOI: 10.1007/BF02902526

Mohan N., Rao P.H., Kumar R.R. and V. Sivasubramanian, 2010. Mass cultivation of *Chroococcus turgidusand* Oscillatoria sp. and effective harvesting of biomass by low-cost methods, Nature Precedings, http://dx.doi.org/10.1038/npre. 2010.4331.1

Mohanty P., Allakhverdiev S.I., and N. Murata, 2007. Application of low temperatures during photoinhibition allows characterization of individual steps in photodamage and the repair of photosystem II, Photosynth. Res. 94: 217–224. DOI 10.1007/s11120-007-9184-y

Mohsen A.A., El–Shourbagy M.N., Naguib M.I., and B.A. Abdel-Ghaffar, 1987. Effect of some endocrine hormones on yield quality of flax plants, Egypt J. Bot., 29/30, 89, 1986/87.

Monette F., Lasfar S., Millette L., and A. Abdelkrim, 2007. Comprehensive modeling of mat density effect on Duckweed (*Lemna minor*) growth under controlled eutrophication, Water Resear. 40(15): 2901-10. DOI:10.1016/j.watres.2006.05.026

Moon H.K., and A.M. Stomp, 1997. Effects of medium components and light on callus induction, growth and frond regeneration in *Lemna giba* (Duckweed), In Vitro Cell. Dev. Biol. Plant 33: 20-25.

Moon H.S., Abercrombie J.M., Kausch A.P., and C.N. Stewart, 2010. Sustainable use of biotechnology for bioenergy feedstocks, Environ. Managem. 46: 531–538.

Moor R., Stern K.R., Vodopich D., and W.D. Clark, 1995. Botany. William C Brown Pub.

de Morais M.G., and J.A.V. Costa 2007. Biofixation of carbon dioxide by Spirulina sp. and Scenedesmus obliquus cultivated in a three-stage serial tubular photobioreactor, J. of Biotechnol. 129(3): 439-45 DOI: 10.1016/j.jbiotec.2007.01.009

Morris J.M., Lipton J., and S. Brinkman, 2013. Copper toxicity to Rainbow Trout and Fathead Minnows in low-hardness waters: Comparisons of BLM predictions of toxicity to bioassay results using laboratory water and site-collected water from upper Talarik Creek in Bristol Bay, Southwest Alaska Salmon Science Workshop, Anchorage, AK, December 4–5.

Mota V.C., Martins C.I.M., Eding E.H., Canário A.V.M., and J.A.J. Verreth, 2014. Steroids accumulate in the rearing water of commercial recirculating aquaculture systems, Aquacult. Engineer. 62: 9–16.

Moya J.L., Ros R., and I. Picazo, 1995. Heavy metal-hormone interactions in rice plants: Effects on growth, net photosynthesis, and carbohydrate distribution, J. Plant Growth Regul. 14: 61-67.

Mulbry W.W., E.K. Westhead, C. Pizarro, and L. Sikora, 2005. Recycling of manure nutrients: Use of algal biomass from dairy manure treatment as a slow release fertilizer, Biores. Technol. 96: 451-458.

Mu˜noz-Tamayo R., Martinon P., Mairet G.elB.F.., and O. Bernard, 2013. Design of optimal experiments for parameter estimation of microalgae Growth Models, Preprints of the 12th IFAC Symposium on Computer Applications in Biotechnology, The International Federation of Automatic Control: 16-18.

Muradov N., Fidalgo B., Gujara A.C., and A. T-Raissi, 2014. Pyrolysis of fast-growing aquatic biomass – *Lemna minor* (Duckweed): Characterization of pyrolysis products, Biores.Technol. 101(21): 8424-8428.

Naik S.N., Goud V.V., Rout P.K., and A.K. Dalai, 2010. Production of first and second generation biofuels: A comprehensive review, Renewable and Sustainable Energy Reviews 14(2): 578–597. http://dx.doi.org/10.1016/j.rser.2009.10.003

Naito K., Matsui M., and I. Imai, 2005. Ability of marine eukaryotic red tide microalgae to utilize insoluble iron, Harmful Algae 4: 1021–1032.

Nakamura K., Higuchi K., Goda H., Fujiwara M.T., Sawa S., Koshiba T., et al., 2003. Brassinolide

induces IAA5, IAA19, and DR5, a synthetic auxin response element in *Arabidopsis*, implying a cross talk point of brassinosteroid and auxin signaling, Plant Physiol, 133: 1843-1853.

Navarri P., 2015. Integration Software – Optimizing heat recovery and reducing waste heat generation in industrial processes, peer-review report CETC number: 2015-036, Natural Resources Canada.

Nendza M., and A. Wenzel, 2006. Discriminating toxicant classes by mode of action, Environ. Sci. Pollut. Res. 13(3): 192-203.

Nichols D.J., Daniel T.C., Edwards D.R., Moore Jr. P.A., and D.H. Pote, 1998. Use of grass filter strips to reduce 17 beta-estradiol in runoff from fescue-applied poultry litter, J. Soil Water Conserv. 53: 74 – 77.

Nishino H., Murakosh M., Ii T., Takemura M., Kuchide M., Kanazawa M., Mou X.Y., Wada S., Masuda M., Ohsaka Y., Yogosawa S., Satomi Y., and K. Jinno, 2002. Carotenoids in cancer chemoprevention, Cancer Metastasis Rev. 21(3-4): 257-64.

Nishino H., Murakoshi M., Tokuda H., and Y. Satomi, 2009. Cancer Prevention by Carotenoids, Arch. Biochem. Biophys. 483: 165–168.

Nigam S., Prakash Rai M., and R. Sharma, 2011. Effect of nitrogen on growth and lipid content of *Chlorella pyrenoidosa*, Amer. J.of Biochem. and Biotech. 7(3): 124-129.

Nobel A., Kisiala A., Galer D., and R.J.N. Clysdale, 2014. Emery, *Euglena gracilis* (Euglenophyceae) produces abscisic acid and cytokinins and responds to their exogenous application singly and in combination with other growth regulators, Eur. J. Phycol. 49(2): 244–254.

Norvill Z.N., Toledocervantes A., Blanco S., Shilton A., Guieysse B., and R. Muñoz, 2017. Photodegradation and sorption govern tetracycline removal during wastewater treatment in algal ponds, Bioresour. Technol. 232: 35–43.

Novoveská L., Dylan T., Franks T.A., Wulfers W., and J. Henley, 2016. Stabilizing continuous mixed cultures of microalgae, Algal Resear. 13: 126-133.

Ogbonda K.H., Aminigo R.E., and G.O. Abu, 2007. Influence of temperature and pH on biomass production and protein biosynthesis in a putative *Spirulina* sp., Bioresour. Technol.. 98: 2207-2211.

Ogino C., 1980. Requirement of carp and rainbow trout for essential amino acids, Bull. Jpn. Soc. Sci. Fish. 46: 171-174.

Olaizola M., 2000. Commercial production of astaxanthin from *Haematococcus pluvialis* using 25,000-liter outdoor photobioreactors, J. of Appl. Phycol. 12 (3-5): 499-506.

Ono E., and J.L. Cuello, 2003. Selection of optimal microalgae species for CO_2 sequestration, Proceedings 2nd Annual Conference on Carbon Sequestration, Alexandria, pp. 1-7.

van der Oost R., Sileno G., and M.S. Munoz, 2016. Ecological key factor toxicity, University of Amsterdam, STOWA, 156 pp. ISBN 978.90.5773.727.5

Órpez R., Martínez M.E., Hodaifa G., Yousfi F., Jbari N. and Sánchez S., 2009. Growth of the microalga *Botryococcus braunii* in secondarily treated sewage, Desalination 246: 625-630.

Osborne D.J., and M.T. McManus, 2005. Hormones, signals and target cells in plant development, Cambridge University Press. p. 158. ISBN 978-0-521-33076-3.

Ozioko F.U., Chiejina N.V., and J.C. Ogbonna, 2015. Effect of some phytohormones on growth characteristics of *Chlorella sorokiniana* IAM-C212 under photoautotrophic conditions, Afric. J. Biotech. 14(30): 2367-2376. doi:10.5897/AJB2015. 14641

Panis G., and R. Carreon, 2016. Commercial astaxanthin production derived by green alga *Haematococcus pluvialis*: A microalgae process model and a techno-economic assessment all through production line, Algal Res. 18: 175-190. https://doi.org/10.1016/j.algal.2016.06.007

Park K.C., Whitney C., McNichol J.C., Dickinson K.E., MacQuarrie S., Skrupski P., Zou J., Wilson K.E., O'Leary S.J.B., and P.J. McGinn, 2012. Mixotrophic and photoautotrophic cultivation of 14 microalgae isolated from Saskatchewan, Canada: potential applications for wastewater remediation, Appl. Phycol. 24: 339-348.

Park W., Yoo G., Moon M., Kim C.W., Choi Y.E., and J.W. Yang, 2013. Phytohormone supplementation significantly increases growth of *Chlamydomonas reinhardtii* cultivated for biodiesel broduction, Appl. Biochem. Biotechnol. 171: 1128–1142. doi:10.1007/s12010-013-0386-9.

Parry A.D., Babiano M.J., and R. Horgan, 1990. The role of ciscarotenoids in abscisic acid biosynthesis. Planta. 182: 118-128.

Parry M.L., 2007. Intergovernmental panel on climate change, working group II, world meteorological organization, United Nations Environment Programme. Summary for Policymakers. In: Parry, ML.; Canziani, OF.; Palutikof, JP.; van der Linden, PJ.; Hanson, CE., editors. Climate Change 2007: Impacts, Adaptation and Vulnerability Contribution of Working Group II to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press; Cambridge, UK.

Pastori G.M., and C.H. Foyer, 2002. Common components, networks, and pathways of cross-tolerance to stress. The central role of "redox" and abscisic acid-mediated controls, Plant Physiol. 129(2): 460-468.

Pauli G.F., Friesen J.B., Gödecke T., Farnsworth N.R., and B. Glodny, 2010. Occurrence of progesterone and related animal steroids in two higher plants, J. of Natur. Prod. 73(3): 338–45. doi:10.1021/np9007415

Peer W.A., Cheng Y., and A.S. Murphy, 2013. Evidence of oxidative attenuation of auxin signalling, J Exp Bot., 64(9): 2629-39. doi: 10.1093/jxb/ert152.

Pei H., Jiang L., Hou Q., and Z. Yu, 2017. Toward facilitating microalgae cope with effluent from anaerobic digestion of kitchen waste: the art of agricultural phytohormones, Biotechnol. Biofuels 10: 76. http://dx.doi.org/10.1186/s13068-017-0759-3.

Peleg Z., and E. Blumwald, 2011. Hormone balance and abiotic stress tolerance in crop plants, Curr. Opin. Plant Biol. 14: 290–295. http://dx.doi.org/10.1016/j.pbi.2011.02.001.

Peng J., Yin K., Yuan J.P., Cao G.X., Xue M., Wu C.F., et al., 2012. Characterization of a newly isolated green microalga *Scenedesmus sp*. as a potential source of biodiesel, Afr. J. Biotechnol. 11: 16083–16094, doi:10.5897/AJB12.1319

Perez-Garcia O., Bashan Y. and M.E. Puente, 2011. Organic carbon supplementation of sterilized municipal wastewater is essential for heterotrophic growth and removing ammonium by the microalga *Chlorella vulgaris*, J. Phycol. 47: 190–199.

Perrot-Rechenmann C., 2010. Cellular responses to auxin: division versus expansion, Cold Spring, Harb. Perspect. Biol. 2(5): 1446. doi: 10.1101/cshperspect.a001446

Perron M.C., and P. Juneau, 2011. Effect of endocrine disruptors on Photosystem II energy fluxes of green algae and cyanobacteria, Environ. Res. 111(4): 520-529.

Peterson E.W., Davis R.K., and H.A. Orndorff, 2001. 17h-Estradiol as an indicator of animal waste contamination in mantled karst aquifers, J. Environ. Qual. 29: 826–34.

Petrusa L., and A.M. Noordermeer, 2006. Biomass to biofuels, a chemical perspective, Green Chem. 8(10): 861-67. doi 10.1039/B605036K

Pflugmacher S., Wiencke C., and H. Sandermann, 1999. Activity of phase I and phase II detoxication enzymes in Antarctic and Arctic macroalgae, Marine Environ. Res. 48:23-36.

Piotrowska A., Czerpak R., Adamowicz J., Biedrzycka A., and M. Potocka, 2005. Comparison of stimulatory effect of cytokinins adenine and urea derivatives on the level of some components in *Wolffia arrhiza* (L.) Wimm. (Lemnaceae), Acta Societatis Botanicorum Poloniae, 74(2): 111-118.

Piotrowska A., Czerpak R., Pietryczuk A., Olesiewicz A., and M. Wędołowska, 2008. The effect of indomethacin on the growth and metabolism of green alga *Chlorella vulgaris* Beijerinck, Plant Grow. Regul. 55: 125–36.

Pojana G., Gomiero A., Jonkers N. and A. Marcomini, 2007. Natural and synthetic endocrine disrupting compounds (EDCs) in water, sediment, and biota of a coastal lagoon, Environ. Internat. 33(7): 929-936.

Pollard H.B., and E. Rojas, 1988. Ca^{2+}-activated synexin forms highly selective, voltage-gated Ca^{2+} channels in phosphatidylserine bilayer membranes, Proc. Natl. Acad. Sci. USA 85: 2974-2978.

Pollio A, Pinto G, Della Greca M, Di Maio A, Fiorentino A,Previtera L., 1994. Progesterone bioconversion by microalgal cultures, Phytochem., 37:1269–1272

Pratt R., 1938. Influence of auxins on the growth of *Chlorella vulgaris*, Amer. J. Botany. 25(7): 498-501.

Pratt R., and E. Johnson, 1977. Production of thiamine, riboflavin, folic acid, pyridoxine and biotin by *Chlorella vulgaris* and *Chlorella pyrenoidosa*, 54(6): 871-874.

Priyadarshani I., and B. Rath, 2012. Commercial and industrial applications of micro algae. Phycospectrum Inc. Commercial and industrial applications of micro algae, A review, J. Algal Biomass Utln. 3(4): 89–100. ISSN: 2229- 6905 89.

Rakocy J.E., Shultz R.C., Bailey D.S. and E.S. Thoman, 2004. Aquaponics production of tilapia and basil: comparing a batch and staggered cropping system, Acta Hortic. 648: 63-69. DOI: 10.17660/ActaHortic.2004.648.8

Rakocy J.E., Losordo T.M., and M.P. Masser, 2006. Recirculating aquaculture tankmproduction systems: integrating fish and plant culture, southern region, Aquacul. Center Pub. 454: 1–16.

Rakocy J.E., Masser M.P., and M.T. Losordo, 2011. Recirculating aquaculture tank production systems: Aquaponics—Integrating Fish and Plant Culture, Oklahoma Cooperative Extension Fact Sheets, http://osufacts.okstate.edu

Ramos T.R.P. et al., 2013. Planning waste cooking oil collection systems. Waste Manag. 33: 1691-1703.

Regnault A., Chervin D., Chammai A., Piton F., Calvayrac R., and P. Mazliak, 1995. Lipid composition of *Euglena gracilis* in relation to carbon–nitrogen balance, Phytochem. 40: 725–733.

Reid W.S.Jr., 2004. Exploring Duckweed (*Lemna gibba*) as a protein supplement for ruminants using the boer goat (*Capra hircus*) as a model. (North Carolina State University, Master of Science Thesis.

Richards J.C., Curtis P.J., Bumison B.K., and R. Playle, 2001. Effect of natural organic matter sources on reducing metal toxicity to rainbow trout (*Oncorhynchus mykiss*) and on metal binding to their gills, Envir. Toxic. Chemis. 20: 1159-1166.

Richardson K., Beardall J., and J.A. Raven, 1983. Adaptation of unicellular algae to irradiance: An analysis of strategies, New Phytol. 93: 157–191.

Robert-Seilaniantz A., Grant M.R., and J.D.G. Jones, 2011. Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism, Annu. Rev. Phytopathol. 49: 2621-2627.

Rober-Kleber N., Albrechtova J.T., Fleig S., Huck N., Michalke W., Wagner E., Speth V., Neuhaus G., and C. Fischer-Iglesias, 2003. Plasma membrane H^+-ATPase is involved in auxin-mediated cell elongation during wheat embryo development, Plant Physiol. 131: 1302–1312.

Robinette H.R., Brunson M.W., and E.J. Day, 1980. Use of Duckweed in diets of channel catfish, Proceedings. 13th Annual.Conference. SE Association. Fish Wildlife Age: 108-114.

Rock C.D., and X. Sun, 2005. Crosstalk between ABA and auxin signaling pathways in roots of *Arabidopsis thaliana* (L.), Heynh. Planta 222: 98-106.

Rodolfi L., Zittelli G.C., Bassi N., Padovani G., Biondi N., Bonini G., and M.R. Tredici, 2009. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor, Biotech. and Bioengin. 102(1): 100-112.

Roger P.A., 1985. Methods for utilizing free living blue-green algae in rice cultivation. The Technology Transfer Workshop, IRRI, Los Banos.

Roger P.A., and S.A. Kulasooriya, 1980. Blue-green algae and rice. The Internat. Rice Res. Institute, Los Banos, Philippines. 112 pp.

Romanenko E.A., Kosakovskaya I.V., and P.A. Romanenko, 2015. Phytohormones of microalgae: Biological Role and Involvement in the Regulation of Physiological Processes. Pt I. Auxins, Abscisic Acid, Ethylene. Int. J. Algae 17: 275–289.

Rosagast M.R., 2012. Tilapia Fish Farming: Practical Manual. Createspace Independent Publishing Platform, ISBN-10 1481040901

Rosati F., Danza G., Guarna A., Cini N., Racchi M. L., and M. Serio, 2003. New evidence of similarity between human and plant steroid metabolism: 5α-reductase activity in *Solanum malacoxylon*, Endocrinol. 144: 220-229.

Ruiz J., Olivieri G., Barbosa M.J. et al., 2016. Towards industrial products from microalgae, Energy & Environ. Sci. 2016(10): 1145-1169.

Rusoff L.L., Blakeney W.W., and D.D. Culley, 1980. Duckweed (Lemnaceae): A potencial source of protein and amino acids, J. Agric. Food Chem. (28): 848-850.

Russo P., and A. Cesario, 2012. New anticancer drugs from marine cyanobacteria, Curr. Drug. Targets. 13(8): 1048–1053.

Šaden-Krehula M., Tajic M., and D. Kolbah, 1979. Sex hormones and corticosteroids in pollen of Pinus nigra. Phytochem. 18: 345-346.

Safafar H., van Wagenen J., Møller P., and Ch. Jacobsen, 2015. Carotenoids, phenolic compounds and tocopherols contribute to the antioxidative properties of some microalgae species grown on industrial wastewater, Mar. Drugs 13(12): 7339-7356. doi:10.3390/md13127069.

Sajilata M.G., Singhal R.S. and M.Y. Kamat, 2008. Fractionation of lipids and purification of γ-linolenic acid (GLA) from *Spirulina platensis*. Food Chemis. 109(3): 580-586.

Sakuradani E., Nojiri M., Suzuki H. and S. Shimizu, 2009. Identification of a novel fatty acid elongase with a wide substrate specificity from arachidonic acid-producing fungus *Mortierella alpina* 1S-4. 84(4): 709-716.

Sala C., and F. Sala, 1985. Effect of brassinosteroid on cell division and enlargement in cultured carrot (*Daucus carota* L.) cells, Plant Cell Rep. 4: 144–147.

Salama el.-S., Kim H. C., Abou-Shanab R. I., Ji M. K., Oh Y. K., Kim S. H., et al., 2013. Biomass, lipid content, and fatty acid composition of freshwater *Chlamydomonas mexicana* and *Scenedesmus obliquus* grown under salt stress, Bioprocess Biosyst. Eng. 36: 827–833. 10.1007/s00449-013-0919-1

Salama El-S., Kabra A.N., Ji M.K., Kim J. R., Min B., and B.H. Jeon, 2014. Enhancement of microalgae growth and fatty acid content under the influence of phytohormones, Bioresour. Technol. 172: 97–103.

Salih F.M., 2011. Microalgae tolerance to high concentrations of carbon dioxide: A Review. J. of Environ. Protec. 2: 648-654.

Sa´nchez J.F., Ferna´ndez J.M., Acie´n F.G., Rueda A., Pe´rez-Parra J., and E. Molina, 2008a. Influence of culture conditions on the productivity and lutein content of the new strain *Scenedesmus almeriensis*, Process Biochem. 43: 398–405.

Sa´nchez J.F., Ferna´ndez J.M., Acién F.G., Cerón M.C., Pérez-Parra J., and E. Molina-Grima , 2008b. Biomass and lutein productivity of *Scenedesmus almeriensis*: influence of irradiance, dilution rate and temperature, Appl. Microbiol. Biotechnol. 79: 719–729. DOI 10.1007/s00253-008-1494-2

Sánchez-Vallet A., López G., Ramos B., Delgado-Cerezo M., Riviere M.-P., and five other, Molina A. (2012). Disruption of abscisic acid signaling constitutively activates arabidopsis resistance to the necrotrophic fungus *Plectosphaerella cucumerina*, Plant Physiol., 160: 2109–2124.

Santhosh S., Dhandapani R., and R. Hemalatha, 2016. Bioactive compounds from microalgae and its different applications—a review. Adv. Appl. Sci. Res. 7(4): 153–158.

Sasaki Y., Asamizu E., Shibata D., Nakamura Y., Kaneko T., Awai K., Amagai M., Kuwata C., Tsugane T., Masuda T., Shimada H., Takamiya K., Ohta H., and S. Tabata, 2001. Monitoring of methyl jasmonate-responsive genes in arabidopsis by cDNA macroarray: self-activation of jasmonic acid biosynthesis and crosstalk with other phytohormone signaling pathways, DNA Res. 8: 153–161.

Sasse J., 1999. Physiological actions of brassinosteroids. In A. Sakurai, T. Yokota, S.D. Clouse, eds, Brassinosteroids: Steroidal Plant Hormones. Springer-Verlag, Tokyo, 137-161.

Sasse J.M., 2003. Physiological actions of brassinosteroids: An Update, J. Plant Growth Regul. 22: 276–288. doi:10.1007/s00344-003-0062-3

Savidov N., 2004. Evaluation and development of aquaponics production and product market capabilities in Alberta ids initiatives fund final report, Project #679056201, Crop Diversification Centre South Brooks, AB.

Sayegh and Greppin, 1973. Chlorella rubescens essai de synchronisation et mise en evidence de rythmes endogens, Arch. Sci. Geneve 8: 6-18.

Schindler J., and W. Zittel, 2008. Crude oil – The supply outlook, 102. Energy Watch Group; Ottobrunn, Germany.

Scott A.P., and T. Ellis, 2007. Measurement of fish steroids in water—a review. Minireview, General and Compar. Endocrinol. 153: 392–400.

Scott A.P., Sumpter J.P., and N. Stacey, 2010. The role of the maturation-inducing steroid, 17,20beta-dihydroxypregn-4-en-3-one, in male fishes: a review, J. Fish Biol. 76(1): 183-224. doi: 10.1111/j.1095-8649.2009.02483.x.

Schroeder J.I., Kwak J.M., and G.J. Allen, 2001. Guard cell abscisic acid signaling and engineering drought hardiness in plants, Nature 410: 327-330.

Shi W., Wang L., Rousseau D.P.L., and P.N.L. Lens, 2010. Removal of estrone, 17α-ethinylestradiol, and 17ß-estradiol in algae and Duckweed-based wastewater treatment systems, Environ. Sci. Pollut. Res. 17: 824–833. DOI 10.1007/s11356-010-0301-7

Shore L.C and M. Shemesh, 2003. Naturally produced steroid hormones and their release into the environment, Pure Appl. Chem., 75(11–12): 1859–1871.

Shurin J.B., Abbott R.L., Deal M.S., Kwan G.T., Litchman E., McBride R., Mandal S., and V.H. Smith, 2013. Industrial-strength ecology: trade-offs and opportunities in algal biofuel production, Ecol. Lett. 16: 1393–1404.

Sijm D.T., Broersen K.W., de Roode D.F., and P. Mayer, 1998. Bioconcentration kinetics of hydrophobic chemicals in different densities of *Chlorella pyrenodosa*, Environ. Toxicol. Chem. 17: 1695–1704.

Silaban A., Bai R., Gutierrez-Wing M.T., Negulescu I.I., and K.A. Rusch, 2014. Effect of organic carbon, C:N ratio and light on the growth and lipid productivity of microalgae/cyanobacteria co-culture, Eng. Life Sci. 14(1): 47–56.

Silva C.P., Otero M., and V. Esteves, 2012. Processes for the elimination of estrogenic steroid hormones from water: a review, Environ. Pollut. 165: 38-58. doi:10.1016/j.envpol.2012.02.002.

Simons R.G., and D.L. Grinwich DL, 1989. Immunoreactive detection of four mammalian steroids in plants, Can. J. Bot. 67: 288-296.

Skillicorn, P., W. K. Journey, and W. Spira, 1993. Duckweed Aquaculture. A new aquatic farming system for developing countries. The World Bank, Emena Technical Department, Agriculture Division. Washington, D.C. pp.68.

Skřivan M., Skřivanová V., Dlouhá G., Brányiková I., Zachleder V., and M. Vítová, 2010. The use of selenium-enriched alga *Scenedesmus quadricauda* in a chicken diet, Czech. J. Anim. Sci. 55(12): 565–571.

Slade R., and A. Bauen, 2013. Micro-alga cultivation for biofuels: Cost, energy balance, environmental impacts and future prospects, Biomass and Bioener. 53: 29-38.

Smith D.S., Kramer J.R., and R.A. Bell, 2002. Metal speciation with organic matter. Cormp. Biochem Physiol. C 133: 65-74.

Smith V.M, 2007. Using primary productivity as an index of costal eutrophication: the unit of measurement matter, J. Plant Res. 29: 1-6.

Smith V.H., Sturm B.S.M., de Noyelles F.J., and S.A. Billings, 2010. The ecology of algal biodiesel production, Trends Ecol. Evol. 25: 301–309.

SolovchenkoA., Lukyanov A., Lukyanov A., Solovchenko O.,Cohen S.D., Boussiba S., and I. Khozin-Goldberg, 2014. Interactive effects of salinity, high light, and nitrogen starvation on fatty acid and carotenoid profiles in *Nannochloropsis oceanica* CCALA 804, Eur. J. Lipid Sci. Technol. 116(5): 635–644. DOI: 10.1002/ejlt.201300456

Soltis D.E., Soltis P., Bennet M., and I. Leich, 2003. Evolution of genome size in the angiosperms, Am. J. Bot. 90(11): 1596. doi:10.3732/ajb.90.11.1596-1603.

Sorensen P.W., Scott A.P., Stacey N.E., and L. Bowdin, 1995. Sulfated 17, 20β-dihydroxy-4-pregnen-3-one functions as a potent and specific olfactory stimulant with pheromonal actions in the goldfish, Gen. Comp. Endocrinol. 100: 128–142.

Sorensen P.W., and A.P. Scott, 1994. The evolution of hormonal sex pheromones in teleost fish: Poor correlation between the pattern of steroid release by goldfish and olfactory sensitivity suggests that these cues evolved as a result of chemical spying rather than signal specialization, Acta Physiol. Scand. 152: 191–205.

Spolaore P., Joannis-Cassan C., Duran E., and A. Isambert, 2006. Commercial applications of microalgae, J. Biosci. Bioeng. 101: 87–96.

Stein J., 1973. (ED.) Handbook of phycological methods. Culture methods and growth measurements, Cambr. Univ. Press. 448p.

Steinman A.D., 2000. Algae, Online Encyclopedia, http://encarta.msn.com

Stirk W.A., and J. Van Staden, 1996. Comparison of cytokinin-and auxin-like activity in some commercially used seaweed extracts, J. Appl. Phycol. 8: 503–508.

Stirk W.A., Ördög V., Novák O., Rolčík J., Strnad M., Bálint P., and J. van Staden, 2013. Auxin and cytokinin relationships in 24 microalgal strains, J. Phycol. 49: 459–467.

Stohs S.J., and M.M. El-Olemy, 1972. Metabolism of progesterone by *Dioscorea deltoidea* suspension cultures, Phytochem. 11: 1397-1400.

Sun Z., Li T., Zhou Z., and Y. Jiang, 2016. Microalgae as a source of lutein: Chemistry, Biosynthesis, and Carotenogenesis, Adv. Biochem. Eng. Biotechnol. 153: 37–58. doi:10.1007/10_2015_331.

Svistoonoff S., Gherbi H., and fourteen others, 2013. The independent acquisition of plant root nitrogen-fixing symbiosis in fabids recruited the same genetic pathway for nodule organogenesis, PLoS ONE 8(5): 64515. doi:10.1371/journal.pone.0064515

Sydney E.B., Sturm W., de Carvalho J.C., Thomaz-Soccol V., Larroche C., Pandey A., and C.R. Soccol, 2010. Potential carbon dioxide fixation by industrially important microalgae. Biores. Technol.101: 5892-5896.

Sydney E.B., da Silva T.E., Soccol C.R. et al., 2011. Screening of microalgae with potencial for biodiesel production and nutrient removal from treated domestic sewage, Appl. Ener. 88(10): 3291-4.

Szamrej I.K., and R. Czerpak, 2004. The effect of sex steroids and corticosteroids on the content of soluble proteins, nucleic acids and reducing sugars in *Wolffia arrhiza* (L.) Wimm. (Lemnaceae), Polish J. of Environmental Studies 13(5): 565-571.

Takeda F., Adler P.R., and D.M. Glenn, 1997. Strawberry production linked to aquaculture wastewater treatment, Acta Hort. 439: 673-677.

Talarek-Karwel M., Bajguz A., Piotrowska-Niczyporuk A., and I. Rajewska, 2018. The effect of 24-epibrassinolide on the green alga *Acutodesmus obliquus* (Chlorophyceae), Plant Physiol. Biochem. 124: 175–183. http://dx.doi.org/10.1016/j.plaphy.2018.01.016.

Tallarida R.J., 2011. Quantitative methods for assessing drug synergism, Genes & Cancer 2(11): 1003-1008.

Tarakhovskaya E.R., Maslov Y.I., and M.F. Shishova, 2007. Phytohormones in algae, Russ. J. Plant Physiol. 54: 163–70.

Tat W.H., 2011. The dwarves on the shoulders of a giant – Duckweeds growing on Tai Mo Shan, Searching for natural stories 2011.

Tate J., Gutierrez-Wing M.T., Rusch K., and M. Benton, 2013. The effects of plant growth substances and mixed cultures on growth and metabolite production of green algae *Chlorella* sp.: a review, J. Plant Growth Regul. 32: 417–28.

Tatkowska E., and J. Buczek, 1980. Effect of phytohormones on the growth of *Scenedesmus quadricauda* (Turp.), Breb. Acta Soc. Bot. Pol. 49: 211–20.

Tatsuki M., Nakajima N., Fujii H., Shimada T., Nakank M., Hayashi K.I., Hayama H., Yoshioka H., and Y. Nakamura, 2013. Increased levels of IAA are required for system 2 ethylene synthesis causing fruit softening in peach (*Prunus persica* L. Batsch), J. Exp. Bot. 64: 1049-1059.

Tatsuzawa H., and E. Takizawa, 1995. Changes in lipid and fatty acid composition of *Pavlova lutheri*. Phytochem. 40(2): 397–400.

Taylor T.C., Backlund A., Bjorhall K., Spreitzer J., and I. Andersson, 2001. First crystal structure of RuBisCo from a green algae *Chlamydomonas reinhardtii*, The J. of Biol. Chem. 276(51): 48159-48164. doi:10.1074/jbc.M107765

Thaler J.S., Humphrey P.T., and N.K. Whiteman, 2012. Evolution of jasmonate and salicylate signal crosstalk, Trends Plant Sci. 17(5): 260-270. http://dx.doi.org/10.1016/j.tplants. 2012.02.010.

Thompson G.A., 1996. Lipids and membrane function in green algae, Biochim. Biophys. Acta 1302: 17–45.

Tornabene T.G., Holzer G. and S.L. Peterson, 1980. Lipid profile of the halophilic alga, *Dunaliella salina*, Biochem. and Biophys. Res. Communicat. 96(3): 1349-1356.

Toyamaa T., Hanaokaa T., Tanakaa Y., Morikawa M., and K. Mori, 2018. Comprehensive evaluation of nitrogen removal rate and biomass, ethanol, and methane production yields by combination of four major duckweeds and three types of wastewater effluent, Biores. Technol. 250: 464-473.

Tsavkelova E.A., Klimova S.Y., Cherdyntseva T.A., and A.I. Netrusov, 2006. Microbial producers of plant growth stimulators and their practical use: a review, Appl. Biochem. Microbiol. 42: 117–126.

Tuteja N., 2007. Abscisic acid and abiotic stress signaling, Plant Signal Behav. 2(3): 135-138.

Um B.H., and Y.S. Kim, 2009. Review: a chance for Korea to advance algal-biodiesel technology. J. of Indust. and Engin. Chem. (15): 1–7.

Umysová D., Vítová M., Doušková I., et al., 2009. Bioaccumulation and toxicity of selenium compounds in the green alga *Scenedesmus quadricauda*, BMC Plant Biol. 9: 58–74. https://doi.org/10.1186/1471-2229-9-58

Upadhyay P., and C. Maier, 2016. Effects of 17β-estradiol on growth primary metabolism,phenylpropanoid-flavonoid pathways and pathogen resistance in *Arabidopssis thaliana*, Am. J. Plant Sci.1693–1710. https://doi.org/10.4236/ajps.2016.713160.

USA Department of Energy report: DOE (Department of Energy). (2011). National Algal Biofuels Technology Roadmap. Washington, pp. 153.

Van der Does D., Leon-Reyes A., Koornneef A., Van Verk M.C., Rodenburg N., Pauwels L., Goossens A., Körbes A.P., Memelink J., Ritsema T., Van Wees S.C.M., and C.M.J. Pieterse, 2013. Salicylic acid suppresses jasmonic acid signaling downstream of SCFCOI1-JAZ by targeting GCC promoter motifs via transcription factor ORA59, Plant Cell tpc.112. http://dx.doi.org/10.1105/tpc.112.108548.

Vance B.D., 1987. Phytohormone effects on cell division in *Chlorella pyrenoidosa* Chick (TX-7-11-05) (Chlorellaceae), J. Plant Growth Regul. 5: 169–173. http://dx.doi.org/10.1007/BF02087185.

Van Weerd J.H., Sukkel M., Lambert J.G.D., and C.J.J. Richter, 1991. GCMS-identified steroids and steroid glucuronides in ovarian growth stimulating holding water from adult African catfish, *Clarias gariepinus*, Comp. Biochem. Physiol. B. 98: 303–311.

Vardon D.R., Sharma B.K., Blazina G.V., Rajagopalan K., and T.J. Strathmann, 2012. Thermochemical conversion of raw and defatted algal biomass via hydrothermal liquefaction and slow pyrolysis, Bioresour. Technol. 109: 178-187.

Verhage A., van Wees S.C.M., and C.M.J. Pieterse, 2010. Plant immunity: It's the hormones talking, but what do they say? Plant Physiol. 154: 536-540.

Vermeirssen E.L.M., and A.P. Scott, 1996. Excretion of free and conjugated steroids inrainbow trout (*Oncorhynchus mykiss*): evidence for branchial excretion of thematuration-inducing steroid, 17, 20-dihydroxy-4-pregnen-3-one, Gen. Comp. Endocrinol. 101: 180–194.

Vert G., Walcher L.C., Chory J., and J.L. Nemhauser, 2008. Integration of auxin and brassinosteroid pathways by auxin response factor 2, PNAS, 105(28): 9829–9834.

Vidhyasekaran P., 2015. Plant hormone signaling systems in plant innate immunity, Springer Sci. Busi.Media. 458.

Vijayakumar S., and M. Menakha, 2015. Pharmaceutical applications of cyanobacteria—a review, J. Acute. Med. 5: 15–23.

Wan M., Liu P., Xia J., Rosenberg J.N., Oyler G.A., Betenbaugh M.J., Nie Z., and G. Qiu, 2011. The effect of mixotrophy on microalgal growth, lipid content, and expression levels of three pathway genes in *Chlorella sorokiniana*, Appl. Microbiol. Biotechnol. 91: 835–844.

Wang L., and Y.J. Wang, 2001. Structures and physico-chemical properties of acid-thinned corn, potato and rice starches. Starch/Starke. 53: 570–576. doi: 10.1002/1521-379X(200111)53:11<570::AID-STAR570>3.0.CO;2-S.

Wang S.B., Hu Q., Sommerfeld M., and F. Chen, 2004. Cell wall proteomics of the green alga *Haematococcus pluvialis* (Chlorophyceae), Proteom. 4: 692-708.

Wang D., Sentorun-Shalaby C., Ma X., and C. Song, 2011a. High-capacity and low-cost carbon-based "Molecular Basket" sorbent for CO_2 capture from flue-gas. Energy Fuels 25: 456–458.

Wang Y., Duanmu D., and M.H. Spalding, 2011b. Carbon dioxide concentrating mechanism in *Chlamydomonas reinhardtii*: inorganic carbon transport and CO_2 recapture. Photosynth. Resear. 109: 115-122.

Wang W., and J. Messing, 2011. High-throughput sequencing of three *Lemnoideae* (Duckweeds) chloroplast genomes from total DNA. PLoS One. 6(9): 24670. doi:10.1371/journal.pone.0024670.

Wang P., Wong Y.S., and F.Y. Tam, 2017. Green microalgae in removal and biotransformation of estradiol and ethinylestradiol, J. Appl. Phycol. 29: 263–273.

Ward OP, Singh A 2005. Omega-3/6 fatty acids: alternative sources of production, Process Biochem. 40(12): 3627–3652.

Wee K.L., 1991. Use of non-conventional feedstuffs of plant origin as fish feeds: Is it practical and economically feasible? S.S. De-Silva (Ed.), Fish Nutrition Research in Asia.

Widjaja A., Chien C.C., and Ju.Yi-H, 2008. Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*, J. of the Taiwan Instit. of Chem. Eng. 40: 13–20.

Wilde K.L., Stauber J.L., Markich S.J., Franklin N. M., and P.L. Brown, 2006. The effect of pH on the uptake and toxicity of copper and zinc in a tropical freshwater alga (*Chlorella sp*.), Arch. Environ. Contam. Toxicol. 51: 174–185. DOI: 10.1007/s00244-004-0256-0

Williams C., and G. M. Stancel, 1996. Estrogens and progestins, p. 1411– 1426. In J. G. Hardman, L. E. Limbird, P. B. Molinoff, and R. W. Ruddon (ed.), The pharmacological basis of therapeutics, 9th ed. The McGraw-Hill Book Co., New York, N.Y.

Wrolstad R.E., Acree T.E., Decker E.A., Penner M.H., Reid D.S., Schwartz S.J., Shoemaker C.F., Smith D.M., and P. Sporns, 2005. Handbook of food analytical chemistry, Wiley Interscience V. 1.

Xu J., Cui W., Cheng J.J., and A. Stomp, 2011. Production of high-starch duckweed and its conversion to bioethanol, Biosys. Eng. 110: 67-72.

Xu J., Zhao H., Stomp A-M., and J.J Cheng, 2012. The production of Duckweed as a source of biofuels, Biofuels 3(5): 589-601.

Xu W., Jia L., Shi W., Liang J., Zhou F., Li Q., and J. Zhang, 2013. Abscisic acid accumulation modulates auxin transport in the root tip to enhance proton secretion for maintaining root growth under moderate water stress, New Phytol. 197(1): 139-150.

Xu J., and G. Shen, 2011. Growing Duckweed in swine wastewater for nutrient recovery and biomass production, Biores. Technol. 102: 848-853.

Yamaga F., Washio K., and M. Morikawa, 2010. Sustainable biodegradation of phenol by *Acinetobacter calcoaceticus* P23 isolated from the rhizosphere of Duckweed *Lemna aoukikusa*, Environ. Sci.

Technol. 44(16): 6470-4. doi: 10.1021/es1007017.

Yamamoto Y.T., Rajbhandari N., Lin X., Bergmann B.A., Nishimura Y., and A.M. Stomp, 2005. Genetic transformation of duckweed *Lemna gibba* and *Lemna minor*, In Vitro Cellular & Developm. Biol. Plant 37(3): 349-353. DOI 10.1007/s11627-001-0062-6

Yang C., Hua Q., and K. Shimizu, 2000. Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark heterotrophic conditions, Biochem. Eng. J. 6: 87–102.

Yang D.J., Lu T.J., and L.S. Hwang, 2003. Isolation and identification of steroidal saponins in Taiwanese yam cultivar (*Dioscorea pseudojaponica* Yamamoto), J. of Agricul. and Food Chemist.. 51(22): 6438–44. doi:10.1021/jf030390j

Yang P., Diener D.R., Yang C., Kohno T., Pazour G.J., Dienes J.M., Agrin N.S., King S.M., Sale W.S., Kamiya R., Rosenbaum J.L., and G.B. Witman, 2006. Radial spoke proteins of *Chlamydomonas flagella*, J. of Cell Sci. 119: 1165-1174. doi: 10.1242/jcs.02811

Yang D.L., Yang Y., and Z. He, 2013. Roles of plant hormones and their interplay in rice immunity, Mol. Plant 6: 675-685.

Ye R.W., Stead K.J., Yao H., and H. He, 2006. Mutational and functional analysis of the beta-carotene ketolase involved in the production of canthaxanthin and astaxanthin, Appl. Environ. Microbiol. 72(9): 5829-37.

Ye Z.W., Jiang J.G., and G.H. Wu, 2009. Biosynthesisand regulation of carotenoids in *Dunaliella*: Progresses and Prospects, Biotechnol. Adv. 26: 352–360.

Yılmaz E., Akyurt H., and G. Güna, 2004. Use of Duckweed, *Lemna minor*, as a Protein Feedstuff in Practical Diets for Common Carp, *Cyprinus carpio*, Fry Turkish J. of Fisheries and Aqua. Sci. 4: 105-109.

Yilmaz H.K., Ayas D., Yilmaz H., and Y. Ozogul, 2010. The effects of different salinity rates on fat and fatty acid composition of *Spirulina platensis*. J Fisheries Sci, 4(3): 282-286.

Yin G.G., Kookana R.S., and Y.J. Ru, 2002. Occurrence and fate of hormone steroids in the

environment, Environ. Int. 28(6): 545-51.

Ying G.G., Kookanaa R.S., and Y.J. Ru, 2002. Occurrence and fate of hormone steroids in the environment, Envir. Intern. 28: 545–551.

Ylstra B., Touraev A., Brinkmann A.O., Heberle-Bors E., and A. Tunen, 1995. Steroid hormones stimulate germination and tube growth of in vitro matured tobacco pollen. Plant Physiol. 107: 639-643.

Yopp J.H., Mandava N.B., and J.M. Sasse, 1981. Brassinolide, a growth-promotingsteroidal lactone: I. Activity in selected auxin bioassays, Physiol. Plant 53: 445–452.

Yu C., Sun C., Yu L., Zhu M., Xu H., et al., 2014. Comparative analysis of Duckweed cultivation with sewage water and SH media for production of fuel ethanol, PLoS ONE 9(12): 115023. doi:10.1371/journal.pone.0115023

Zeevaart J.A.D., and R.A. Creelman, 1989. Metabolism and physiology of abscisic acid, Annu. Rev. Plant Physiol. Plant Mol. Biol. 39: 439-473.

Zhang J.S., Yang Z.H., and T.H. Tsao, 1991. The occurrence of estrogens in relation to reproductive processes in flowering plants, Sex Plant Reprod. 4: 193-196.

Zhang X., GaoY., Li Q., Li G., Guo Q., and C. Yan, 2011. Estrogenic compounds and estrogenicity in surface water, sediments, and organisms from Yundang lagoon in Xiamen, China. Arch. Environ. Contam. Toxicol. 61: 93–100.

Zhao, B. and Y. Su, 2014. Process effect of microalgal carbon dioxide fixation and biomass production: A Review. Renewable Sustainable Energy Rev. 31: 121-132.

Zhao Y., 2010. Auxin biosynthesis and its role in plant development, Annu. Rev. Plant Biol., 61: 49–64. doi: 10.1146/annurev-arplant-042809-112308

Zhekisheva M., Boussiba S., Khozin-Goldberg I., Zarka A., and Z. Cohen, 2002. Accumulation of oleic acid in *Haematococcus pluvialis* (Chlorophyceae) under nitrogen starvation or high light is correlated with that of astaxanthin esters, J. Phycol. 38, 325–331. 10.1046/j.1529-8817.2002.01107.x

Zhekisheva M., Zarka A., Khozin-Goldberg I., Cohen Z., and S. Boussiba, 2005. Inhibition of astaxanthin synthesis under high irradiance does not abolish triacylglycerol accumulation in the green alga *Haematococcus pluvialis* (Chlorophyceae). J. Phycol. 41, 819–826. 10.1111/j.0022-3646.2005.05015.x

Zhang J., Zeng G., Chen Y., Yu M., Huang H., Fan C., Zhu Y., Li H., Liu Z., Chen M., and M. Jiang, 2013. Impact of *Phanerochaete chrysosporium* inocula-tion on indigenous bacterial communities during agricultural waste composting, Appl. Microbiol Biotechnol. 97(7): 3159–3169.

Zhong-han Y., Yin T., Zong-xun C., and T.H. Tsao, 1994. The changes of steroidal sex hormone - testosterone contents in reproductive organs of *Lilium davidii* Duch., Acta Bot. Sin. 36: 215-220.

Zhou W., Li Y., Min M., Hu B., Chen P., and R. Ruan, 2011. Local bioprospecting for high-lipid producing microalgal strains to be grown on concentrated municipal wastewater for biofuel production, Biores. Technol. 102(13): 6909–6919.

Zhou G.J., Peng F.Q., Yang B., G.G. Ying, 2013. Cellular responses and bioremoval of nonylphenol and octylphenol in the freshwater green microalga *Scenedesmus obliquus*, Ecotoxicol. Environ. Saf. 87: 10–6.