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AL MAGNIFICO RETTORE DELL'UNIVERSITA'
DEGLI STUDI DI NAPOLI FEDERICO II
UFFICIO DOTTORATO, ASSEGNI E
BORSE DI STUDIO

Io sottoscritto/a MARINA MASSA

Autore/autrice della tesi di dottorato dal titolo SUSTAINABLE PRODUCTION OF MICROALGAE BIOMASS IN LIQUID DIGESTATES AND BY PRODUCTS FROM AGRO-FOOD INDUSTRIES

Parole chiave (*indicare* ***da una a tre parole*** *che richiamino il contenuto della tesi*) LIQUID DIGESTATE, MICROALGAE, BY PRODUCTS

Dipartimento sede del dottorato AGRARIA

Tutor VINCENZO FOGLIANO Coordinatore GIANCARLO BARBIERI

Denominazione dottorato SCIENZE E TECNOLOGIE DELLE PRODUZIONI AGROALIMENTARI
INDIRIZZO ACQUACOLTURA

Ciclo XXVIII

Settore scientifico-disciplinare del MIUR (*indicare il settore della disciplina di afferenza del tutor*) CHIM/10

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Autore/autrice della tesi di dottorato dal titolo

SUSTAINABLE PRODUCTION OF MICROALGAE BIOMASS IN LIQUID DIGESTATES AND BY-PRODUCTS FROM AGRO-FOOD INDUSTRIES.

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ALCUNI CAPITOLO SONO STATI SOTTOMESSI E SI ATTENDE RISPOSTA DALLE RIVISTE SCIENTIFICHE.

Data 30/03/2016 Firma Marina Massa

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UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II

DOTTORATO DI RICERCA IN
SCIENZE E TECNOLOGIE DELLE PRODUZIONI AGRO-ALIMENTARI

VERBALE DEL 07/03/2016

Il giorno 07/03/2016, alle ore 12.00, si è riunito nella Sala Giunta del Dipartimento del Dipartimento di Agraria il Collegio dei Docenti del Dottorato di Ricerca in "Scienze e tecnologie delle produzioni agro-alimentari" per discutere del seguente o.d.g.:

1) Dottorandi 28° e 27° ciclo: approvazione relazione finale e lavoro di tesi
2) Commissioni Esami finali ed afferenza dottorandi alle varie commissioni
3) Eventuale proposta di rilasciare il titolo di dottore di ricerca con o senza la denominazione dell'indirizzo
4) Varie ed eventuali

Sono presenti i proff.: Barbieri Giancarlo, Cavella Silvana, Villani Francesco, Barone Carmela, Sansone Giovanni
Sono assenti giustificati i proff.: Fogliano Vincenzo, Moio Luigi, Di Vaio Claudio, Masi Paolo
Sono, inoltre, assenti i proff.: Ferranti Pasquale, Sacchi Raffaele
Presiede il prof. Barbieri e funge da segretario la prof. Cavella.

1) Dottorandi 28° e 27° ciclo: approvazione relazione finale e lavoro di tesi
Il prof. Barbieri comunica che all'inizio di febbraio 2016 aveva invitato a produrre la necessaria documentazione e che sono pervenute le relazioni conclusive, riportanti anche una significativa sintesi della tesi, dei seguenti dottorandi:

XXVIII CICLO	Tutor	Indirizzo
1. BIONDI LOREDANA	Raffaele Romano	Scienze e tecnologie prod. alimentari
2. CECERE BIANCA	Barbieri, IZSM	Scienze e tecnologie prod. alimentari
3. ESPOSITO FRANCESCO	T. Cirillo	Scienze e tecnologie prod. alimentari
4. IACCARINO DORIANA	Barone	Acquacoltura
5. KONG XIANGHUI	Fogliano, Vitaglione	Scienze e tecnologie prod. alimentari
6. LI JIJI	Fogliano, Dott.ssa Manzo Enea	Acquacoltura
7. MASSA MARINA	Fogliano	Acquacoltura
8. SCHIAVO SIMONA	Fogliano, Dott.ssa Manzo Enea	Scienze e tecnologie prod. alimentari
9. STELLATO GIUSEPPINA	Ercolini	Scienze e tecnologie prod. alimentari
10. VOLPE STEFANIA	Cavella, Torrieri	Scienze e tecnologie prod. alimentari
11. ZHOU CHAO	Fogliano, Buttino – ISPRA Livorno	Acquacoltura
XXVII CICLO		
12. CAPORASO NICOLA	Sacchi	Scienze e tecnologie prod. alimentari
13. D'ANTUONI ISABELLA	Sacchi	Scienze e tecnologie prod. alimentari
14. DI MARTINO VERONICA	Villani	Scienze e tecnologie prod. alimentari
15. MENNELLA ILARIO	Fogliano, Vitaglione	Scienze e tecnologie prod. alimentari
16. TROISE ANTONIO DARIO	Fogliano, Vitaglione	Scienze e tecnologie prod. alimentari

1.5 dott.ssa MASSA MARINA
La dott.ssa Massa Marina presenta una tesi dal titolo "Sustainable production of microalgae biomass in liquid digestates and by products from agro-food industries" (Tutor Vincenzo Fogliano).

Oltre alle previste attività formative del dottorato ed alla partecipazione a progetti di ricerca dipartimentali, la dott.ssa Massa ha preso parte ad alcune manifestazioni scientifiche ed alle seguenti attività di formazione:
Seminari seguiti e tenuti da Docenti stranieri: How to write a scientific paper and present experimental results – Prof. Walters
Seminario nell'ambito della manifestazione ALI-MENTI PhD Rosa Muoio
Seminario Alghe e Licheni estremofili in Antartide Prof. Milos Bartak
Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology Bari, 24-26 Settembre, 2014
Convegno"Università e impresa in sinergia per la crescita del settore alimentare", Napoli , 4 Novembre 2014
I Congresso Nazionale della Società Italiana di Nanotossicologia, 27-28 Giugno 2014
International Algae Congress, 1st-3rd December 2014 Firenze

Il Segretario
(Prof. Silvana Cavella)
Silvana Cavella

Il Coordinatore
(prof. Giancarlo Barbieri)

XX Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology, 23rd-25th September 2015

E' autore di 9 lavori di cui 1 su riviste internazionali e 8 comunicazioni a convegno come risulta dall'elenco seguente

Pubblicazioni in riviste

1. Silvia Buono, Antonella Colucci, Antonella Angelini, Antonio Luca Langelotti, Marina Massa, Anna Martello, Vincenzo Fogliano, Angela Dibenedetto. 2015. Productivity and biochemical composition of *Scenedesmus obliquus* and *Phaeodactylum_tricornutum*: effects of different cultivation approaches. Journal of Applied Phycology. JAPH-SB 50115submitted

Comunicazioni a Convegni

1. MASSA M, Buono S., Langellotti A.L., Martello A., Fogliano V. (2014). Production of Galdieria sulphuraria on cherry-blanching wastewater. In: Proceedings book XIX Workshop on Devolopments in the italian PhD Research on Food Science, Technology and Biotechnology. p. 173-174, ISBN: 978-88-88793-48-1, Bari, 24-26 Settembre, 2014
2. E. Bocci, S. Buono, C. Gambardella, V. Vitiello, M. Vannuccini, F.S. Freyria, MASSA M, B. Bonelli, M. Faimali, I. Buttino, I. Corsi. (2014). Nanomateriali ecofriendly per la bonifica di acque marine: il progetto di ricerca nanoremeco. . In: I congresso Nazionale della società italiana di nanotossicologia. vol. I, p. 20, Napoli, 27-28 giugno 2014
3. E. Bocci, S. Buono, C. Gambardella, V. Vitiello, M. Vannuccini, F.S. Freyria, MASSA M, B. Bonelli, M. Faimali, I. Buttino, I. Corsi. (2014). Titania mesoporosa per la bonifica in ambiente marino: studio ecotossicologico per la definizione di eco-friendliness. In: I congresso nazionale della società italiana di nanotossicologia. p. 22, Napoli, 27-28 giugno 2014
4. MASSA M, Buono S., Langellotti A.L., Martello A., Fogliano V. (2015). Sustainable production of microalgae for feed purposes: the cases of *A.maxima and S.obliquus.*
5. MASSA M, Buono S., Langellotti A.L., Martello A., Fogliano V. (2014). Production of Galdieria sulphuraria on cherry-blanching wastewater.. In: XIX Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology. Bari, 2014
6. S. Buono, MASSA M, A. L. Langellotti, A. Martello, V. Fogliano (2014). Sustainable fertilizer for microalgae culture. In: EABA . Firenze, 1-3 dicembre 2014
7. L. Langellotti, S. Buono, A. Martello, G. Mariano, MASSA M, V. Fogliano (2014). Extraction and drying of blu aqueous extract of *Arthrospira maxima*. In: EABA . Firenze, 1-3 dicembre 2014
8. E. Bocci, S. Buono, C. Gambardella, V. Vitiello, M. Vannuccini, F.S. Freyria, MASSA M, B. Bonelli, M. Faimali, I. Buttino, I. Corsi. (2014). Titania mesoporous cleaning up in the marine environment: ecotoxicological study for the definition of eco-friendliness. In: I congresso nazionale della società italiana di nanotossicologia. Napoli, 27-28 giugno 2014

Giudizio sulla tesi

Il lavoro di tesi ha avuto come obiettivo la valutazione del potenziale di diverse microalghe marine e di acqua dolce (*S. obliquus, P. tricornutum, B. braunii*) e di un cianobatterio fotosintetico (*A. maxima*), allevate su digestati liquidi e sottoprodotti provenienti da industrie alimentari, utilizzati come medium alternativi per la crescita di microalghe. Inoltre, è stata valutata la loro capacità di metabolizzare le sostanze nutritive presenti in tali digestati o sottoprodotti. I campioni provengono da un processo di digestione anaerobica di diversi rifiuti organici: zootecnici, biomasse vegetali, Frazione Organica del Rifiuto Solido Urbano (FORSU); e un sottoprodotto proveniente da un'industria dolciaria. Sono stati valutati: la capacità di bioremediation (rimozione dei nutrienti) degli organismi scelti, l'ecotossicità dei campioni utilizzati come medium alternativi per la crescita delle microalghe, i tassi di crescita (growth rate) e le produttività delle biomassa algali, la composizione biochimica delle biomasse ottenute in termini di proteine, carboidrati, contenuto e profilo lipidico, ceneri, antociani, ficobiliproteine, attività antiossidante e digeribilità delle biomasse.
La tesi è valutata positivamente.

Giudizio complessivo

Il Collegio, considerati l'attività scientifica della dott.ssa Massa Marina, i risultati ottenuti e l'attività di formazione svolta e valutato positivamente il lavoro di tesi presentato, approva la relazione sull'attività svolta durante il corso e ritiene il candidato meritevole dell'ammissione all'esame finale per il conseguimento del titolo di Dottore di Ricerca in Scienze e Tecnologie delle Produzioni Agro-Alimentari, 28° ciclo.

OMISSIS

Non essendovi altro da deliberare, la seduta è sciolta alle ore 14.00.
Letto e approvato

Il Segretario
(Prof. Silvana Cavella)

Il Coordinatore
(prof. Giancarlo Barbieri)

Università degli Studi di Napoli "Federico II"

Scuola di dottorato in Scienze e tecnologie delle produzioni agroalimentari

Ciclo XVIII

Sustainable production of microalgae biomass in liquid digestates and by products from agro-food industries

Tutor:
Vincenzo Fogliano

Candidate:
Marina Massa

Year 2015/2016

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Abstract (Ita)

A livello globale, vi è un crescente interesse per la produzione di microalghe come biomasse vegetali innovative, essendo ricche di sostanze fitochimiche ad alto valore aggiunto da applicare in diversi settori commerciali (alimentare, mangimistico, nutraceutico, cosmetico e di depurazione dei reflui) e come fonti future di biodiesel, per il loro contenuto lipidico e il profilo in acidi grassi caratteristico di alcune specie. Fino ad oggi il mercato delle microalghe destinate al settore alimentare e mangimistico è stato un mercato di nicchia, ma si sta incrementando grazie alla loro composizione nutrizionale (in particolare per il contenuto in lipidi, grassi, proteine, carboidrati, pigmenti e vitamine), sottolineando che tali biomasse hanno un buon potenziale per essere considerate competitive rispetto alle altre fonti vegetali convenzionali. Dal punto di vista della produzione di biodiesel dalle microalghe,il processo risulta ancora troppo oneroso da un punto di vista economico, sia per il costo dei fertilizzanti sia per il consumo di energia impiegata per la raccolta e l'essiccazione della biomassa e per l'estrazione di "*phytochemicals*" dalla biomassa stessa.
Per migliorare la competitività dei prodotti a base di microalghe devono essere considerati alcuni aspetti tecnici ed economici, che giocano un ruolo chiave nei costi di produzione della biomassa e nella sostenibilità ambientale del processo di produzione. Nel presente lavoro di tesi, digestati liquidi provenienti da digestione anaerobica di rifiuti organici di diversa origine e natura e le acque reflue di una industria dolciaria sono stati analizzati come fertilizzanti alternativi ed economici per la produzione di microalghe autotrofe ed eterotrofe.
La ricerca è stata suddivisa in quattro capitoli:
1) Una mini-review sull'utilizzo dei digestati liquidi e dei sottoprodotti dell'industria alimentare come fertilizzanti per le microalghe sulla base della legislazione europea;
2) Produzione eterotrofica della microalga *Galdieria sulphuraria* utilizzando come medium alternativo un sottoprodotto proveniente dal processo produttivo delle ciliegie candite e il conseguente effetto sulla biomassa prodotta in termini di produttività e composizione della biomassa stessa;
3) La crescita e la capacità di metabolizzare i nutrienti presenti nei diversi digestati liquidi utilizzati di due specie microalgali di acqua dolce (*S. obliquus, B. braunii*), una diatomea marina (*P. tricornutum),* e un cianobatterio fotosintetico (*A. maxima*);
4) La valutazione della produttività in termini di biomassa, di tasso di fissazione di anidride carbonica e della composizione biochimica di due specie microalgali, *P. tricornutum* e *S. obliquus,* coltivate indoor in fotobioreattori ad alta tecnologia e in open pond e fotobioreattori a bassa tecnologia sotto serra nel sud Italia. Le microalghe sono state accresciute utilizzando standard medium e due digestati liquidi ottenuti dalla digestione anaerobica di biomasse vegetali e agro-zootecniche.
In conclusione i medium alternativi applicati hanno mostrato la capacità di sostenere la produzione microalgale, inducendo con l'utilizzo di sottoprodotti di industria dolciaria una riduzione della produttività per *G. sulphuraria* di circa il 25%, e una produttività in linea con gli standard medium con l'utilizzo dei digestati liquidi per *S. obliquus* e *P. tricornutum*.
Inoltre sono state evidenziate variazioni del profilo biochimico delle biomasse prodotte con l'utilizzo dei digestati liquidi in termini di lipidi (aumento medio del 55%) e di carboidrati (diminuzione media di circa il 5%).
Anche con l'utilizzo dei sottoprodotti dell'industria dolciaria si è osservata una variazione nella composizione nutrizionale della biomassa, con una riduzione media di circa il 35% del contenuto proteico e lipidico e, con un aumento di circa il 70% dei polifenoli, in particolare gli antociani e con un aumento del 12%dei carboidrati.
Anche il profilo lipidico ha mostrato delle variazioni, con una riduzione di circa il 70% degli acidi grassi saturi e un aumento del 10% degli insaturi sia per i digestati liquidi che per il sottoprodotto dell'industria dolciaria.
Le diverse specie microalgali hanno evidenziato una buona capacità di "*phyto-remediation"* con una riduzione dell' NH_4^+-N presente nei digestati di circa il 90% e una riduzione di circa l'86% di PO_4. La coltivazione con i digestati liquidi e le acque reflue del processo produttivo delle ciliegie candite permetterebbe oltre ad una riduzione dei costi dei fertilizzanti, anche interessanti modifiche sulla composizione della biomassa prodotta e in alcuni casi l'arricchimento delle biomasse di alcuni composti ad elevata attività biologica.

Abstract (Eng)

Globally, there is growing interest in microalgae production as innovative vegetable biomass rich in phytochemicals at high added value to apply in different commercial sectors (food, feed, nutraceutical, cosmetician and wastewaters depuration) and as future biodiesel source for the high lipid content and fatty acid profile of some species. To date the microalgae market for food and feed is a niche market but it is increasingly relevant and strictly correlate to their chemical composition (in particular lipids, fatty acids profile, proteins, carbohydrates, pigments and vitamins), underlying that microalgae biomass has the potential to be competitive respect the others conventional vegetable sources. From a point of view of biodiesel source the microalgae production is yet too expensive, both for fertilizers cost both for energy consumption for harvesting, drying and extraction. To develop the competitiveness of microalgae based products, some technical and economic aspects, that play a key role in biomass production costs and in production environmental sustainability, have to be considered.
In the present research liquid digestates from anaerobic digestion of organic waste and food industry by-products were analyzed as alternative cheap fertilizer for autotrophic and heterotrophic microalgae production. An overview in terms of possible application of produced biomass in commercial sector on the basis of EU legislation was also considered.

The thesis work consists of four experiments:
1) An overview of the use of liquid digestates and food industry by-products as microalgae grow media;
2) Heterotrophic production of *Galdieria sulphuraria* in spent cherry brines and its effects on the biomass productivity and composition;
3) The growth and the ability to metabolize the nutrients presents in different liquid digestates of two species of freshwater microalgae (*S. obliquus, B. braunii*), a marine diatom (*P. tricornutum*), and a photosynthetic cyanobacterium (*A. maxima*);
4) The evaluation of biomass productivity, the carbon dioxide fixation rate and the biochemical composition of two microalgal species, *P. tricornutum* and *S. obliquus*, grew indoors in high-tech photobioreactors and open ponds and low photobioreactors technology in greenhouses in southern Italy. Microalgae were cultivated using standard medium and two liquid digestates obtained by anaerobic digestion of vegetable biomass and zootechnical wastes.

In conclusion the alternative media applied showed the ability to support sustainably the microalgae production, leading a reduction of productivity of about 25% with the use of by-products of confectionary industry, and a productivity in line with the standard medium with the use of liquid digestates for *S. obliquus* and *P. tricornutum*.
Furthermore, the biochemical profile changes of biomass produced with the use of the liquid digestates were highlighted, in terms of lipids (average increase of 55%) and carbohydrates (average decrease of about 5%). With the use of by-products from the confectionary industry was observed a variation in the nutritional composition of the biomass, with an average reduction of about 35% of the protein and lipid content and, with an increase of about 70% in terms of polyphenols, in particular anthocyanins, and with an increase of 12% of carbohydrates.
Also the lipid profile showed the variations, a decrease of about 70% of saturated fatty acids and an increase of 10% of unsaturated FA for liquid digestates and by products from confectionary industry utilized. The different microalgae species utilized were metabolized and reduced by about 90% the NH_4^+-N The different microalgae species were highlighted a good capacity of phyto-remediation with a reduction of about 90% the NH_4^+-N and a reduction of PO_4 of about 86%.
The cultivation with liquids digestates and the wastewater of the preserved cherries production process allow in addition to a reduction in costs of fertilizer, also interesting changes on the composition of the biomass produced and in some cases the enrichment of the biomass with compound with high biological activity.

Sustainable production of microalgae biomass in liquid digestates and by products from agro-food industries

Introduction and thesis aims

Chapter 1

Chapter 1: Sustainable production of microalgae biomass in liquid digestates and by products from agro-food industries

Abstract
Globally, there is growing interest in microalgae production as innovative vegetable biomass rich in phytochemicals at high added value to apply in different commercial sectors (food, feed, nutraceutical, cosmetician and wastewaters depuration) and as future biodiesel source for the high lipid content and fatty acid profile of some species. To date the microalgae market for food and feed is a niche market but it is increasingly relevant and strictly correlate to their chemical composition (in particular lipids, fatty acids profile, proteins, carbohydrates, pigments and vitamins), underlying that microalgae biomass has the potential to be competitive respect the others conventional vegetable sources. From a point of view of biodiesel source the microalgae production is yet too expensive, both for fertilizers cost both for energy consumption for harvesting, drying and extraction. To develop the competitiveness of microalgae based products, some technical and economic aspects, that play a key role in biomass production costs and in production environmental sustainability, have to be considered. In the present minireview liquid digestates from anaerobic digestion of organic waste and food industry by-products were analyzed as alternative cheap fertilizer for autotrophic and heterotrophic microalgae production. An overview in terms of possible application of produced biomass in commercial sector on the basis of EU legislation was also considered.

Keywords: Microalgae; Biomass; Liquid digestate, food industry by-product

1. Introduction

Microalgae are a huge group of unicellular eukaryotic photosynthetic microorganisms from freshwater, brackish and marine systems. Although photosynthetic cyanobacteria (blue green algae) belong to the domain of bacteria, often they are considered and indicated as "microalgae" (Medlin *et al*., 2007).
Different metabolism can be observed in microalgae: autotrophy, heterotrophic and mixotroph.
For microalgae autotrophic production only inorganic compounds such as CO_2, N, S, P and light as energy source are essential for their growth and development. The solar energy is captured into biomass (photosynthesis) with an efficiency higher (3%) than terrestrial plants (0.2–2%)(Stephenson *et al*., 2011). Inorganic nutrients and water significantly affect the cost and the environmental sustainability of microalgae production. For algae culture systems, the total cost vary between 10-20 % for the cost of nutrients (Benemann and Oswald, 1996), but some authors as Xia and Murphy (2016) asserted in a recent Life-Cycle Assessment (LCA), that the use of artificial fertilizers (e.g., nitrogen, phosphorus) can account for half of the cost and energy input in microalgae cultivation. The recent hike in fertilizer price increased the microalgae production cost substantially and the environmental impact of chemical processes to their synthesis is not negligibile.
Some microalgae strains are mixotrophic performing at the same time photosynthesis and catabolising organic nutrients, but some species are able to change their metabolisms on the basis of environmental conditions in terms of nutrients availability and light exposition (Kaplan *et al*., 1986; Perez-Garcia *et al*., 2011).
The heterotrophic growth of microalgae can be an effective cheap option to photoautotrophic cultivation, that suffers from the light limitation due to mutual shading of cells (Chen, 1996) and by low concentration achieved, that increase the harvesting cost for the high energy consumption. Many microalgae have been shown to be able to grow rapidly in heterotrophic conditions, reaching high cellular density and biomass productivity. Although the heterotrophic production has several advantages over photosynthetic production including also a high degree of process control and a lowering for harvesting costs, it involves high capital costs for a large-scale fermentation facility.
Heterotrophic culture, requiring organic carbon source, can grow on different sugars or organic acids, which represent the main cost in medium formulation.
From an economic point of view the organic carbon source contributes 45.4%; inorganic chemicals, 3.2%; electricity 30.6 %; steam 14.2 %; and aseptic air 6.6% of the total production cost. The importance to reduce the cost of the organic carbon source is highlight of the glucose cost, about 80% of the total medium cost (Ogbonna and Moheimani, 2015).
With these simple nutritional requirements, microalgae are a promising environmentally friendly source of lipids, proteins and carbohydrates at a large scale, representing a good alternative respect other conventional vegetable source.
Microalgae showed some important advantages respect to conventional terrestrial plants: they present an higher biomass productivity and CO_2 assimilation, and moreover arid or low quality agricultural land is necessary for their cultivation (Singh and Gu, 2010; Scott *et al*., 2010). Even if the microalgae cultivation is carried out in aquatic environment, they required less water than terrestrial crops, showing a reduced fresh water consumption. Microalgae may be cultivated in brackish and sea water without herbicide or pesticide application, and reducing the need of external nutrients (NH_4, NO_3 and P)(Aslan and. Kapdan, 2006; Pratoomyot *et al*., 2005).
At the present the production of microalgae biomass is still in an advancing
phase and further work is necessary to improve the productivity and to decrease the cost of production.

Strategies to reduce production costs include cultivation in wastewaters (Wu *et al*. 2013; Holbrook *et al*. 2014), cultivation under stress conditions (Alvarez-Dıaz *et al*., 2014) and bio-prospecting for new strains.
In the present mini-review the microalgae growth on different kind of wastewaters, in particular liquid digestate from anaerobic digestion of organic waste (municipal, zootechnical and vegetable wastes) and food industry by-products was analysed. An overview of EU regulation on their application as fertilizer was reported together with possible microalgae biomass utilization.

2. Anaerobic digestion

The biogas process, known as anaerobic digestion (AD), happens naturally in different environments (the stomach of ruminants, landfills, volcanic hot springs, submerse rice fields, etc). In biogas plants the AD process is highly controlled to reach the maximum methane production. In this process the organic matter is digested in O_2 absence by a complex microorganism community comprising different metabolic phases: hydrolysis, acidogenesis, acetogenesis, and methanogenesis.
This result in two valuable products: methane and digestate. The produced biogas is a very useful source of renewable energy, whilst digestate is a highly valuable bio-fertiliser (IEA Bioenergy, 2015).

2.1 Biogas Industry: products applications

In the past 10 years the biogas global industry has developed significantly (Xia and Murphy, 2016) and to date the most of biogas is utilized to produce heat and electricity. Until now only in Sweden the use of up graded biogas as a transport fuel is applied and as indicated in 2013 the 54 % of biogas was used as transport biofuel (IEA Bioenergy, 2015), while in other countries (such as Switzerland, Germany, France, Chinaand the USA) biogas-fuelled transport systems are developing (Borjesson and Mattiasson, 2008).
In Europe, Germany is leader in the biogas industry, with about 10020 biogas plants, that in 2014 produced over 144PJ of energy, applied as electricity satisfying the 4.7% of demand and as heat satisfying the 1% of demand (IEA Bioenergy, 2015).
Another important by-product of AD is the digestate (Nkoa, 2014), which processing and treatment often represent the major "*problem*" in the development of a biogas industry (Fuchs and Drosg, 2013). Digestates may be separated into solid with a value ranging from 10 to 20% by mass and liquid with a value ranging from 80 to 90% by mass fractions by screw press or decanter centrifuge (Fuchs and Drosg, 2013). Solid digestate is more stable because containing less water and it can easily be transported and stored. It can either be used as agricultural biofertiliser or be further converted to heat and/or value-added products (e.g., pyrochar, nanocellulose) via thermal processes (Fuchs and Drosg, 2013; Monlau *et al*., 2015).
By contrast, liquid digestate processing is more difficult. The simplest treatment method is to directly spread on local agricultural land showing, however, some disadvantages.
In fact the most of the nitrogen is in the ammoniac form (up to 80%) (Uggetti *et al*., 2014), that volatilises causing nutrient loss, which may induce eutrophication of near water systems (Levine *et al*., 2011).
Furthermore it may induce different kind of soil contamination which reduces the long-term crop productivity of soil: chemical (e.g., heavy metals, PAH), biological (e.g.,pathogens), physical (e.g.,plastics) (Nkoa, 2014; Lukehurst *et al*., 2010).
It is important to underline that the fast developing of biogas plants in terms of number and capacity, leads to an over production of digestate for local agricultural land, considering also that the value of liquid digestate after long-distance transport may become negative.
Land application is strictly correlate to different aspects as the crop growth stage, soil type, and time of year (not winter application). Thus, digestate needs to be stored and often the storage can allow additional greenhouse gases release (e.g.,CH_4, N_2O) (Monlau *et al*., 2015; Lukehurst *et al*. 2010) because the digestion process rarely destroys all volatile compounds.
Technologies, such as membrane separation and evaporation, can efficiently concentrate the nutrients of liquid digestate but they require high energy consumption (Fuchs and Drosg, 2013). There are huge demands on agricultural land for application of digestate (in countries such as Germany) and in a number of countries (such as Ireland) authorisation for the application of digestate produced from the digestion of animal by-products and wastes is very difficult to obtain.
In **Germany**, usually the digestate is used without further treatment and only about 10% of the plants treating waste produce compost from the output of the digestion process. The liquid phase is separated after digestion and the separated fibre is generally post-composted. Only 6% of the quality assured digestate (BGK label) is produced as solid digestate in Germany. Liquid digestate (94% of whole digestate) is used directly as fertiliser in agriculture.
Others solutions where digestate is not land applied are extremely beneficial to a biogas system.
The European Nitrates Directive (91/676/EEC) regulates the use of nitrogen in organic materials to agricultural land (Fuchs *et al*., 2010). As a consequence the spreading of digestate to land is controlled (based on nitrogen content) and dependent on location and crop demand.
An alternative solution for digestate treatment could be aquatic microalgae (Monlau *et al*., 2015; Cheng *et al*. 2015), but an overwiev of EU regulation on digestate application is needed to define the possible application and utilization of microalgae biomass grown on diluted liquid digestate.

2.2 EU Legislation: new procedure for defining end-of-waste (EoW) criteria

Use of digestate as fertiliser requires to put great attention to the "*quality*" of digestate that is strictly correlate to the feedstock supplied to biogas plants. Quality management of digestate used as fertiliser should be integrated into overall national environmental protection and nutrient management policies as yet done in some countries like Austria, Canada (Ontario), Denmark, Germany, Netherlands, Sweden, Switzerland and the United Kingdom. National regulatory frameworks for digestate quality management and certification for use enhance its use as fertiliser in a safe and sustainable way.

In order to use digestate as a fertiliser, certain regulatory requirements, both on national and European level, must be applied. In this regard, the existing national regulations which guarantee high-quality digestate, as well as the ongoing European harmonisation procedure designed to facilitate cross-border trading with digestate, are to be mentioned.

In the EU final report the digestate definition was:"*digestate is the semisolid or liquid product that has been sanitised and stabilised by a biological treatment process of which the last step is an anaerobic digestion step. It can be presented as whole digestate or separated in a liquor phase and a semisolid phase".*

Taking a look at the EU regulation is essential, as it reveals important gaps but also areas of great improvement. Digestate is currently classified as 'waste' in the European legislation and as waste is not useful for other purposes if not treatment and disposal. The Waste Framework Directive (2008/98/EC, or WFD) among other amendments introduces a new procedure for defining end-of-waste (EoW) criteria for digestate in such a way as to allow the use as a fertilizer for human vegetable cultivation systems.

These criteria are yet to be defined for each specific waste stream, but the general conditions that a waste material has to follow are defined by Article 6 of the WFD in the following terms:

'certain specified waste shall cease to be waste [within the meaning of point (1) of Article 3] when it has undergone a recovery, including recycling, operation and complies with specific criteria to be developed in accordance with the following conditions:

a*) The substance or object is commonly used for a specific purpose;*

b) A market or demand exists for such a substance or object;

c) The substance or object fulfils the technical requirements for the specific purpose referred to in (a) and meets the existing legislation and standards applicable to products;

d) The use of the substance or object will not lead to overall adverse environmental or human health impacts.'

In the Communication from the Commission on future steps in bio-waste management in the European Union (COM(2010) 235)[3], the European Commission states that digestate from bio-waste are under-used materials. Furthermore, it is mentioned that the end-of waste procedure under the Waste Framework Directive could be the most efficient way of setting standards for compost and digestate, that enable their free circulation on the internal market and to allow using them without further monitoring and control of the soils on which they are used.

"Bio-waste" is defined in the Waste Framework Directive (WFD) as "*biodegradable garden and park waste, food and kitchen waste from households, restaurants, caterers and retail premises, and comparable waste from food processing plants*". It does not include forestry or agricultural residues, manure, sewage sludge, or other biodegradable waste (natural textiles, paper or processed wood).

The European Compost Network has provided a summary of the different aspects of quality assurance systems for digestate in different European countries, which consider input materials, process requirements and animal by-products regulations aspects (ABPR), physical contaminants, stability/maturity/fermentation degree, organic matter and dry matter requirements, heavy metal limits (mg/kg d.m.).

Other important framework regulations are the Regulation (EC) N°1069/2009 (Animal by-products Regulation) and the Regulation (EC) No 1907/2006 (REACH). The Animal by-product regulation distinguishes between the different input material categoriesand defines the exporting requirements for manure or digestate to other countries.

Digestate, unlike biogas and compost, is not exempt from the European chemical legislation, REACH. This means that if chemically modified feedstock is used in anaerobic digestion, the registration requirements under REACH will apply. This, in turn, could lead to an irrational situation where some plant operators might not apply to receive the EoW status for their digestate, to avoid the administrative burden related to REACH. In autumn 2013, EBA provided the European Commission with a position paper explaining the composition of digestate and the technology of anaerobic digestion, while delivering arguments for digestate's exclusion from REACH. The main argument of EBA's position is that digestate cannot be subject to REACH, as it is, in most cases, composed of either whole living or unprocessed dead organisms or, alternatively, waste. These types of feedstock do not fall under the scope of the regulation.

The big question remains, is digestate waste or a by-product? Until now, the question is still not answered properly.

2.3 Liquid digestate composition

The composition of digestate is strictly correlate to the type of feedstock processed (Chambers, 2011) and digestion process parameters and type applied. Different organic wastes can feed anaerobic digesters as organic municipal, zootechnical and vegetable waste. The variability of chemical profile of LD can be very high and also for the same digester feed with the same kind of feedstock it is possible to observe variability strictly correlate to the impossibility to apply the identic feedstock.

The application of LD as fertilizer both for plants and microalgae requires to know the chemical profile to ensure not only the absence of toxic compounds (as metals, PAH, plastics, ecc.) but also to know the right quantity to apply as fertilizer in terms of nitrogen, phosphorus and potassium. However, information about this aspect is still scarce, and evidences from the literature points out the need to consider the variability of digestate composition and the presence and concentration in pathogens and heavy metals as important factors. Therefore, further efforts are required to determine the operating conditions able to enhance fertilizer properties and pathogen reduction, as well as to promote the digestate nutrient recycling. The anaerobic digestion is a process that usually if conducted properly induces a significantly decrease of pathogens on the basis of microbial species, digester temperature, and retention time (Godfree and Farrell, 2005).

Uggetti *et al*. (2014) reported that the liquid digestates (LDs) usually are characterized by low level of organic matter and phosphorus, counter balanced by high potassium and nitrogen concentrations (up to 80% in the form of ammonium). They showed also that the micro-element composition of digestates can cover the nutrient requirements of a microalgae population. In table 1, adapted from Uggetti *et al*. (2014) and Pognani *et al*. (2009) the N, P and K content observed for liquid digestates from different anaerobic digester feedstock was reported.

The chemical composition of liquid digestates (Table 1) can cover properly the nutrient requirements of a microalgae population.

Table 1. Characteristics of liquid digestates from anaerobic digestion of different organic waste (adapted from Uggetti *et al*., 2014 and Pognani *et al*., 2009)

Feedstock of AD	**Total Nitrogen**	**Total Phosphorus**	**Total Potassium**
Dairy manure	125–3456 (mgL^{-1})	18–250 (mgL^{-1})	116 (mgL^{-1})
Poultry manure	1380–1580 (mgL^{-1})	370–382 (mgL^{-1})	592 (mgL^{-1})
Sewage sludge	427–467 (mgL^{-1})	134–321 (mgL^{-1})	12 (mgL^{-1})
Food waste and dairy manure	1640–1885 (mgL^{-1})	296–302 (mgL^{-1})	
Liquid Cattle Slurry	4.27 (%DM)	0.66 (% DM)	4.71 (% DM)
Energy crops, cow manure slurry and agro-industrial waste and OFMSW	105-110 ($g\ kg^{-1}$ TS)	10.9-11.8 ($g\ kg^{-1}$TS)	
Clover/grassor pea strawor cereal strawor silage maize and clover/grasssilage	0.253 (% FM)	0.62 (%DM)	18.5 (%DM)

DM= dry matter; TS= total solid; FM= fresh matter

2.4 Microalgae and liquid digestate

In the 1950s Golueke and Oswald (1959) conducted the first attempt to cultivate microalgae in digestate. This process has not been properly studied until the increasing demand for digestate processing from the biogas industry (IEA Bioenergy, 2015; Fuchs and Drosg, 2013).

Many studies report the use of digestate from urban wastewater treatment, manure or swine slurry for microalgal growth (Godfree and Farrell, 2005; Uggetti *et al*., 2014), considering both the microalgae biomass productivity, but also their ability to remove N, P from LDs (table 2). In fact microalgae with their ability to remove some compounds as Nitrogen (N), Phosphorus (P), Potassium(K) and metals present a high phytoremediation activity, that allow to recover the water reaching for some environmental toxic parameters values acceptable.

Table 2. Microalgae cultivated in diluted LDs and their performance in terms of nutrient removal efficiency and biomass concentration and productivity (Adapted from Xia *et al*., 2016)

Digestate Origin	**Species**	**Nutrient Removal Efficiency (%)**	**Biomass Productivity And/or Concentration**	**References**
Alcohol wastewater	*C. pyrenoidosa*	TN 91.6 TP 90.7	0.58 g $L^{-1}d^{-1}$ 3.01 g L^{-1}	Yang *et al*., 2015
Starch wastewater	*C. pyrenoidosa*	TN 83.1 TP 97.0	0.63 g $L^{-1}d^{-1}$ 2.05 g L^{-1}	Tan *et al*., 2014
Swine manure and sewage	*Chlorella PY-ZU1*	TAN 73 TP 95	0.601 g $L^{-1}d^{-1}$ 4.81 g L^{-1}	Cheng *et al*., 2015
Dairy manure	*Chlorella sp.*	TN 82.5 TP 74.5	1.71 g L^{-1}	Wang *et al*., 2010
Wastewater sludge	*Chlorella sp.*	TN 83.7 TP 94.2	0.45 g $L^{-1}d^{-1}$ 2.11 g L^{-1}	Akerstrom *et al*., 2014
Dairy manure	*C.vulgaris*	TN 93.6 TAN 100 TP 89.2		Wang *et al*., 2010
Poultry litter	*C. minutissima, C. sorokiniana, S. bijuga*	TN 60 TP 80	0.076 g $L^{-1}d^{-1}$ 0.612 g L^{-1}	Singh *et al*., 2011
Livestock waste	*C. vulgaris, S. obliquus, N.oleoabundans*	TN 76.0 TP 63.2		Zhao *et al*., 2015

Vinasse	*C. vulgaris*	TN 37 TP 71	11.8 g $m^{-2}d^{-1}$ 0.6 g L^{-1}	Serejo *et al*., 2015
Cattle slurry and raw cheese whey	*C. vulgaris,* *N.oleoabundans* *S. obliquus*	TAN 99.9 PO_4-P 97.3	0.26 g $L^{-1}d^{-1}$	Franchino *et al*., 2013
Pig manure	*Desmodesmus sp.*	TN 75.6 TAN 92.7 PO_4-P 100	0.385 g L^{-1}	Ji *et al*., 2015
Pig manure	*Desmodesmus sp.*	TN 100 TP 100	0.029 g $L^{-1}d^{-1}$ 0.412 g L^{-1}	Ji *et al*., 2014
Municipal wastewater	*N.salina*	TN 100 TP 100	0.092 g $L^{-1}d^{-1}$ 0.92 g L^{-1}	Cai *et al*., 2013
Municipal wastewater	*N. salina* *Synechocystis sp.*	TN 100 TP 100	0.151 g $L^{-1}d^{-1}$	Cai *et al*., 2013
Dairy manure	*N.oleoabundans*		0.088 g $L^{-1}d^{-1}$	Levine *et al*., 2011
Livestock waste	*S. accuminatus*		0.118 g $L^{-1}d^{-1}$	Park *et al*., 2010
Swine manure	*Scenedesmus sp.*	TAN >95 PO4-P >97	0.67 g $L^{-1}d^{-1}$	Dickinson *et al*., 2015
Livestock waste	*S. obliquus*	TN 74.6 TP 88.8	0.311 g $L^{-1}d^{-1}$	Xu *et al*., 2015
Dairy manure	*Chlorella sp.*	TAN 100 TP 34.3	1.71 g L^{-1}	Wang *et al*., 2010

Dairy manure	*N.oleoabundans*	TAN 95	0.0883 g $L^{-1}d^{-1}$	Levine *et al*., 2011
Sago starch factory wastewater	*A. platensis*	TAN99.9 TP 99.4	14.4 g m^{-2} day^{-1}	Pang *et al*., 2000
Wastewater	*Desmodesmus sp.*	TN 94.2 TP 95.6	1.039 g L^{-1}	Ji *et al*., 2015
Piggery wastewater	*A.platensis*	TN 80–93 TP 84–98	64.7 g·m^{-2}·d^{-1}	Liu *et al.,* 2015
Piggery wastewater	*O.multisporus,* *Nitzschiacf. pusilla,* *C.mexicana,* *S. obliquus,* *C. vulgaris,* *M. reisseri*	TN 62 TP 28	0.34 ±0.08gL^{-1} 0.37 ±0.13gL^{-1} 0.56 ±0.35gL^{-1} 0.53 ±0.30gL^{-1} 0.49 ±0.26gL^{-1} 0.35 ±0.08gL^{-1}	Reda *et al*., 2013

Furthermore the combining liquid digestate and microalgae cultivation can significantly reduce the nutrient cost of biomass production. For liquid digestate treatment, mixotrophic microalgae not only improve the biomass productivity and enhance nitrogen and phosphorus removal but also enable inorganic and organic carbon removal.
Mixotrophic cultivation induces higher biomass concentration and productivity and less photoinhibition/limitation than photoautotrophic cultivation; in particular mixotrophic microalgae can achieve from one to two magnitudes higher productivities than photoautotrophic cultures (Xia and Murphy, 2015).
Several experiments pointed out the existence of inhibitory effects on microalgal growth, especially with manure wastewater or digestate as substrate. Among the observed effects, high ammonia concentrations were often responsible for microalgal growth inhibition (Kallqvist and Svenson, 2003; Li *et al*., 2008). Indeed, although ammonia can be an excellent source of nitrogen for microalgal growth, free ammonia at high concentration is toxic for most strains of microalgae due to its uncoupling effect on photosynthetic processes in isolated chloroplasts. Another cause of microalgae growth inhibition is light limitation mainly due to the shading caused by LDs turbidity and color (Uggetti *et al*., 2013; Yuan *et al.,* 2011; Guieysse *et al*., 2002). No particular effect of digestate turbidity on microalgal growth has yet been reported in the literature. However, it should be noticed that the digestate is diluted in almost all the experiments reported in the literature (Becker, 2007; Godfree and Farrell, 2005; Wu and Pond, 1981) reducing the color and turbidity of LDs, and so the decreased input light.
Nevertheless, once the inhibitory factors have been identified, their effect can be easily overcome by substrate dilution or carbon dioxide addition (for pH and ammonia concentration control) (Uggetti *et al*., 2013).
In our study we observed also an increase in ash content of microalgae biomass cultivated in LDs respect artificial media, and variation in chemical profile of microalgae. The increase in ash hasn't be discussed properly in literature, but it can represent a problem in application of biomass produced or in extraction of phytochemicals, increasing also the harvesting cost.
The commercial application of microalgae biomass grown in LDs is yet low clear and strictly correlate to the nature of LDs as indicated in the Waste Framework Directive (2008/98/EC, or WFD) that introduces a new procedure for defining end-of-waste (EoW) criteria for LD.
When this criteria, still not properly developed, are respected in terms of high selected organic waste input in Anaerobic Digester and absence of

contaminants as pathogens, metals, PAH and others, the microalgae biomass grown in LDs is not consider a biomass of poor quality to utilizer as biofuel or biomass to treat for energetic purpose, but applicable on the basis of absence of contaminations for food and feed purpose in relation to EC Regulation 767/2009 (establishes the rules on the marketing of feed materials and compound to placing on the market and use of feed), to Regulation (EC) No 1774/2002 (Animal by-products not intended for human consumption) and to the EU Scientific Report of Enzing *et al*. (2014) on "Microalgae-based products for the food and feed sector: an outlook for Europe". This report analysed the production, markets and regulation of microalgae-based food and feed products, especially focusing on the European sector.
Also in this case the question on application in food and feed sectors of microalgae grown in "*selected and treated wastewaters*" is not enough clear and appearing applicable only for energetic purpose also if EU policy is extreme interest in nutrients and water recycling with great attention for phosphorus.

3. Wastewater from agro-food industry

The food and beverage industry is the largest manufacturing sector in the EU also respect the automobile, chemical, machinery, and equipment sectors (CIAA, 2004). Food processing activities produce in Europe large amounts of by-products and waste (about 2.5 10^8 ton $year^{-1}$) along with relevant amounts of high COD, BOD and SS effluents (Federici *et al*., 2008).
Water in fact is an essential input for the food and drink industry, as an ingredient, as a key processing element and as a cooling agent in many production processes, and wastewater is the most common waste in the food and drink industry, characterized by organic, chemical and microbial contamination.
In particular Food and Drink industry accounts for approximately 1.8% of Europe's total water use (excluding agriculture) and is considered one of the largest producers of wastewaters, often in base of its origin very rich in proteins, sugars, pigments and lipids along with particular aromatic and aliphatic compounds. The food industry wastewaters cannot be disposal in the environment before a depuration process to avoid negative environmental effects, but for its chemical composition as it is or enriched of some compounds could be applied as microalgae grow media, decreasing the fertilizers cost, reducing the disposal cost of wastewaters and producing a biomass to apply in different sectors on the basis of its quality and safety.

3.1 Food industry

The food wastewater produced is different from country to country on the basis of main market developed. For example in India about 65%–70% of the organic pollutants are produced from food and agro-product industries, such as distilleries, sugar factories, dairies, fruit canning, meat processing and pulp and paper mills (Rajagopal, 2008).

In Mediterranean countries the wine production is one of the main agro-food industries, and it has also reached importance in other parts of the world (Australia, Chile, the United States, South Africa and China) (Ganesh *et al*. 2010).

The wine industry releases large volumes of wastewaters due to the water involved in various washing operations.

In addition olive oil industries have gained an high economic importance for many Mediterranean countries (Meksi *et al*., 2012). Malaysia presently accounts for 39% of world palm oil production and 44% of world exports (MPOC, 2013), producing a huge amount of polluted palm oil mill effluent (POME).

Fia *et al.* (2012) in coffee-producing regions (e.g. Brazil, Vietnam and Colombia) showed that the industrial wastewaters released has become a large environmental problem, creating the need for low cost treatment technologies. In contrast Nieto *et al*. (2012) determined the potential for methane production from six agro-food wastes (beverage waste, milled apple waste, milk waste, yogurt waste, fats and oils from dairy wastewater treatment and cattle manure).

The annual worldwide production of olive oil is estimated to be about 1750 million metric tons, with Spain, Italy, Greece, Tunisia and Portugal being the major producers, and about 30 Mm^3 of oil mill wastes are produced annually in the Mediterranean regions during the seasonal extraction of olive oil (Meksi *et al*., 2012; Un *et al*., 2008).

3.2 Wastewaters/by-product composition from food industry

The composition and concentration of different agro-food wastewaters vary from low (wash water from sugar mill or dairy effluents) to high strength substrates (cheese, winery and olive mill wastewaters), particularly in terms of organic matter, acids, proteins, aromatic compounds, nutrients availability, etc. (Rajagopal, 2008; Ganesh *et al*., 2010; Zhao *et al*., 2012). The main parameters of the agro-food industrial wastewater, such as total solids (TS), total nitrogen (TN), total phosphorus (TP) and biological and chemical oxygen demand (BOD and COD) are given in Table 3.

Table 3. Characteristics of typical agro-food industrial wastewater (modified by Rajagopal et al. 2013)

Industry	**TS mgL^{-1}**	**TP mgL^{-1}**	**TN $mg\ L^{-1}$**	**BOD mgL^{-1}**	**COD mgL^{-1}**	**Ref.**
Palm oil mill	40	-	750	25	50	Rupani *et al*., 2010
Sugar-beet processing	6100	2.7	10	-	6600	Alkaya and Demirer, 2011
Dairy	250–2,750	-	10–90	650–6,250	400–15,200	Gotmare *et al*., 2011; Passeggi *et al*., 2009
Corn milling	650	125	174	3,000	4850	Ersahin *et al*., 2013
Potato chips	5,000	100	250	5000	6000	Senturk *et al*., 2010
Baker's yeast	600	3	275	-	6,100	Ersahin *et al*., 2011
Winery	150–200	40-60	310–410	-	18000-21000	Rajagopal, 2008 Ganesh *et al*.,2010
Cheese dairy	1,600–3,900	60-100	400–700	-	23,000-4,0000	Rajagopal, 2008
Olive mill	75,500	-	460	-	130,100	Gonçalves *et al*.,2012
Cassava starch	830	90	525	6300	10500	Sun *et al*., 2012
Wheat starch	12800-16400	170-190	500-600	9400-13200	18,750	Murayama, 2002
Beer	5100–8750	As PO_4 10-50	25–80	1200–3600	2000–6000	Simate *et al*., 2011
Tomato		47.5±46	151.4 ± 70		6953 ±3278	Gohil and Nakhla, 2006

canning waste						
Confectionary Industry	TSS 177-1050	8,6-65	33-100	1840-4910	2840-19900	El-Gohary *et al.,* 1999; Orhon *et al.*, 1995; Diwani *et al.,* 2000; Ozturk and Altinbas, 2008
Notes: [a]contains flour, soybean, tomato, pepper and salt. TS: total solids; TN: total nitrogen; TP: total phosphorus; BOD: Biochemical Oxygen Demand; COD: Chemical Oxygen Demand.						

Very interesting is also the sugar content of some food industry waste, in particular from vegetable and fruits (dried plum, sweet corn, olive oil, syrum fruit, winery, ecc.), where the most abundant are glucose and fructose, while maltose and saccharose were observed in wastewaters of syrum production and sweet corn cannery. Polyols, such as sorbitol, mannitol and other forms were respectively encountered from plum, sweet corn and olive oil processing.
The literature data reported that the food industry wastewaters are very rich in nutrients that can be recycled in different sectors from agriculture to energy, and presents a lot of nutrients for microalgae growth both in autotrophic production that in heterotrophic and mixotrophic conditions.
From our experience sometimes food wastewaters can be reach in compounds with interesting biological activity, e.g. as polyphenols, that are the primary source for coloration in flowers and fruits. The dark colour often associated with wastewater streams from the distillery and beverage industries is due to the polyphenolic compounds. Polyphenols are present in olive oil waste (Mulinacci, 2001) and in spent cherry-blanching liquid is observed the presence of anthocyanin (Chaovanalikit and Wrolstad, 2003). In our experience growing *G.sulphuraria* in spent cherry blanching liquid enriched the biomass harvested in polyphenols (anthocyanin), but it is not clear if they are introjected/absorbed, linked to the cell wall or simply dragged with biomass.

3.3 Microalgae and food industry wastewaters/by-product

Many microalgae have been shown to be able to grow rapidly in heterotrophic conditions with glucose as a carbon source to accumulate high amounts of lipids, which are one of the most promising feedstock for biodiesel production. However, glucose is one of the main contributors in the high cost of microalgae culture. Numerous studies have attempted to find less expensive organic carbon sources for biodiesel and other applications.
The application of food industry waste for microalgae growth can be preceded from others treatment (e.g. hydrolysis, enzymatic digestion) to improve the nutrients avaibility.
Xu *et al.* (2006) developed a corn powder hydrolysate instead of glucose as the carbon source to obtain favorable results in terms of biomass and lipid productivities (2 g L^{-1} day^{-1}, 932 mg L^{-1} day^{-1} respectively) for heterotrophic grow of *Chlorella protothecoides*.
Gao *et al.* (2010) used sweet sorghum juice as a substrate for the heterotrophic cultivation of *Chlorella protothecoides*, obtaining satisfactory

results for the biomass concentration (5.1 gL^{-1}) and the corresponding lipid content (52.5%). Yuqin *et al.* (2013) evaluated the feasibility of using potato starch hydrolysate (PSH) instead of glucose as a carbon source for *C. protothecoides* lipid production. Literature data are few as showed in table 4 and not complete in terms of microalgae productivity and phytoremediation activity.

Some agro-food industries wastewaters/by-products can be used as the sole carbon and energy sources with a high reduction of media costs for microalgae production and a recovery of nutrients and water.

The foregoing achievements indicate that the use of relatively inexpensive biomass materials for the production of biodiesel is feasible and economically viable.

Table 4. Microalgae Cultivated in food waste and food by-products and their performance

Food waste	Species	Nutrient Removal Efficiency	Growth rate, productivity and/or concentration	Ref.
Corn powder hydrolyzate	*Chlorella protothecoides*		3.92 gL^{-1}	Xu *et al.,* 2006
Sweet sorghum juice			5.1 gL^{-1}	Gao *et al*., 2010
Hydrolyzed food waste (containing rice, noodles, meat, and vegetables)	*S. mangrovei, C. pyrenoidosa*		2.1±0.3 day^{-1} 2.2 ±0.7 day^{-1}	Pleissner *et al*., 2013
Hydrolyzed food waste	*Cryptococcus curvatus*	TN= 29.7±1.0 mgL^{-1} TP= 16.9±0.3 mgL^{-1}	1.1 g L^{-1}	Chi *et al*., 2011
Hydrolyzed food waste (Bakery: cake and pastry; food: noodles, rice, meat and vegetables)	*C.vulgaris*		[a]0.8 day^{-1}	Lau *et al*., 2014
Hydrolyzed dairy waste	*C. vulgaris*		[a]0.4 day^{-1}	Abreu *et al*., 2012
Hydrolyzed food waste (restaurant food and bakery waste)	*C. pyrenoidosa*		1.4 day^{-1} 0.79 ± 0.1gg^{-1}	Pleissner *et al*., 2015
Potato starch hydrolysate	*C. protothecoides*		20.23 g L^{-1}	Yuqin *et al*., 2013

4. Graphical Conclusion

With the purpose of clearing all the possibilities and threats originated from the use of liquid digestates and food by-products/wastewaters for microalgae growth, two different SWOT (Strengths, Weaknesses, Opportunities, and Threats) analysis has been developed and are shown in Table 5 and Table 6, respectively.

Table 5. A SWOT analysis for liquid digestate to apply as microalgae growth media

	Positive	Negative
Internal	**Strengths** Rich in inorganic nutrients (N, P, K); Lowering of the pathogen load; Spore absence; Very abundant; Useful for microalgae grow; Reduction in fertilizers cost for microalgae production; Phytoremediation; Water and nutrients recovery; Reduction cost of LD disposal; Reduced release in environment (soil).	**Weaknesses** High cost transport Pure Stability Extremely variable composition Presence of impurities: Pathogen, PAH, metals, plastics, ecc. Turbidity Limited markets for biomass Reduced microalgae biomass production High ash content in biomass produced High microalgae harvesting cost Possible release in environment
External	**Opportunities** Environmental and economic sustainable production of microalgae; Water and nutrients recovery; Reduced the environmental impact of LD;	**Threats** Microalgae biomass low quality for presence of metals, PAH, pathogens and others impurities Lost of NH_3 for volatilization

	Promotes economic development	

Table 6. A SWOT analysis for food by-products and microalgae.

	Positive	Negative
Internal	**Strengths** Rich in inorganic and organic nutrients (N, P, K and sugars); Very abundant; Useful for microalgae grow; Reduction in fertilizers cost; Phytoremediation; Water and nutrients recovery; Reduction cost of food wastewater disposal Enriched of biological activity of microalgae biomass produced	**Weaknesses** High cost transport Pure Stability Extremely variable composition Presence of impurities: pathogen, pesticides, ecc. Color Limited markets for biomass Reduced microalgae biomass production High ash content in biomass produced High microalgae harvesting cost Possible release in the environment
External	**Opportunities** Reduction the organic matter that needs to bedisposed Sustainable production of microalgae Promotes economic development Effective food waste management	**Threats** Contamination (such as bacteria) of produced microalgae biomass The impurities may find themselves in the final biomass

5.Aim

The aim of this research was to improve the environmental and economic sustainability of different microalgae species production, utilizing cheap alternative media as Liquid Digestates and food by-products. In particular were analyzed LDs from different industrial Anaerobic Digesters, fed with various organic substrates (zootechnical, vegetable and municipal organic waste) and spent cherry blanching liquid from a confectionary industry.
Research was carried outat CAISIAL (Aquaculture section - University of Naples, Federico II, Portici (Na), Italy) in an aquaculture plant with different technology for microalgae production (indoor and outdoor).
The PhD thesis is organized in 4chapters.
First chapter is a general introduction (or minirewiev), followed from 3 experiments reported as scientific papers, that are submitted or in submission to scientific journals.
The title and aim of different experiments carried out are summarized below.

Chapter 2

Production of Galdieria sulphuraria on spent cherry-blanching liquid.

The research aim was to evaluate the use of spent cherry-blanching liquid as heterotrophic growth medium for *G.sulphuraria,*defining its biomass productivity, chemical composition, antioxidant activity and in vitro digestibility respect the biomass produced on artificial standard medium (SM). The antioxidant activity release during the digestion was studied at all phases on soluble and insoluble fractions. The microalgae phytoremediation ability in terms of effluent toxicity, before and after *G.sulphuraria* growth, were also assessed applying an acute and chronic ecotoxicity tests with organisms belonging to different trophic levels (a primary producer and consumer), due to the impossibility to have a complete chemical framework of the effluent, which is a complex mixture.

Chapter 3

Biological treatment of liquid digestate: the potential of microalgae.

The aim of the present study was to evaluate the potential of different marine and freshwater microalgae species to produce renewable biomass removing and recycling nutrients from LDs. Different strains of microalgae (*Arthrospira maxima*, *Scenedesmus obliquus, Pheodactylum tricornutum* and *Botryococcus braunii*) known for their productivity, phytoremediation activity and commercial interest were chosen.

Chapter 4

Influence of the growing conditions on large scale productivity and biochemical composition of two microalgae species. (article accepted and under revision by Journal of Applied Phycology)

The aim of present work was to evaluate the biomass productivity, carbon dioxide fixation rate and biochemical composition of two microalgae species, *P. tricornutum* and *S. obliquus,* cultivated indoor in high technology photobioreactors (HT-PBR) and outdoor both in pilot ponds and low technology photobioreactors under greenhouse in Southern Italy. Microalgae were grown in standard media and in two liquid digestates obtained from anaerobic digestion of agro-zootechnical and vegetable biomass.

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Production of *Galdieria sulphuraria* on spent cherry brine liquid

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Chapter 2

Chapter 2: Production of Galdieria sulphuraria on spent cherry brine liquid

Abstract

The candy fruit industry produce large amount of wastewaters that represent an added cost for the companies. In this paper the spent cherry-brine liquid (sCBL), used in the sweet cherry manufacturing to bleach, firm and preserve fruit, was considered. Because sCBL is reach of free sugar and SO_2 it could represent a suitable growing substrate for the microalgae *Galdieria sulphuraria* which needs these two components to grow.

The research aim was to evaluate the use of sCBL as heterotrophic growth medium for *Galdieria sulphuraria*, a unicellular rhodophyte, and to assess the quality of the microalgae biomass. For this reason biomass productivity was measured together with its chemical composition, antioxidant activity and in vitro digestibility. Data were compared with those obtained producing the biomass on standard medium (SM). The *G. sulphuraria* phytoremediation ability and the effluent ecotoxicity, before and after microalgae growth, were also assessed.

The biomass grown in sCBL has a nice red color and its productivity was 0.57±0.04 g $L^{-1}day^{-1}$ a value that is about 25% lower than in the SM. The biomass produced in sCBL showed a lower content in proteins (220±6.4 vs 320±5.5 mg g^{-1}) and lipids (46±3.2 vs 77±1.1 mg g^{-1}) and an increase in polyphenols (5.3 vs 1.6 mg g^{-1}). Carbohydrates content was 12% higher respect to the biomass produced in SM.

The growth in sCBL induced on microalgae biomass also a changing in the fatty acid profile with a reduction in palmitic acid of 12% and in linoleic acid of 8% and an increase in oleic acid of 25%. Also the total digestibility of entire biomass was decreased being of 63.4±8.1% and 79.3±5.8%, for sCBL and SM respectively.

As expected from the higher content in total polyphenols, in particular anthocyanin, the biomass grown in sCBL has a higher antioxidant activity (AA), that during in-vitro digestion presents the highest release in the soluble fraction of intestinal phases. In conclusion, the *G. sulphuraria* nutritional profile underline that this strain is an interesting source of added value phytochemicals, and cultivation on sCBL would allow the production process environmental and economically sustainable also enriching the biomass of anthocyanin.

Key words: *Galdieria sulphuraria*, Brine cherry liquid**,** Food industry by-product, Phytoremediation, Heterotrophic, Batch culture

Highlights

1) The spent cherry-brine liquid, enriched in nitrogen was used successfully as heterotrophic growth medium for *G. sulphuraria*.
2) The spent cherry-brine liquid as heterotrophic growth medium induced changing in biomass composition of *G. sulphuraria* respect to the standard medium.
3) *G. sulphuraria* biomass was enriched in anthocyanin presented in spent cherry-brine liquid and released by cherries.
4) The intestinal digestion phase for soluble fraction of sCBL showed the highest antioxidant activity release for *G. sulphuraria*.
5) *G. sulphuraria* showed a good nutrient removal efficiency from spent cherry-brine liquid.

1. Introduction

Globally, there is growing interest in microalgae production as innovative vegetable biomass rich in phytochemicals at high added value to apply in different commercial sectors (food, feed, nutraceutical, cosmetician, energetic and wastewaters depuration). The microalgae market for food and feed is increasingly relevant and strictly correlate to their chemical composition (in particular lipids, fatty acids profile, proteins, pigments and vitamins), underlying that microalgae biomass has the potential to be competitive respect to the others conventional vegetable sources. To develop the competitiveness of microalgae based products, some technical and economic aspects, that play a key role in biomass production costs, have to be considered.

The autotrophic microalgae biomass production generally requires nutrients (e.g., N, P, C) and water, which significantly affect the cost and the environmental sustainability of its production. The cost of nutrients in algae culture systems vary between 10-20 % of the total cost (Benemann and Oswald, 1996), but some authors as Xia and Murphy (2016) asserted in a recent Life-Cycle Assessment (LCA), that the use of artificial fertilizers (e.g., nitrogen, phosphorus) can account for half of the cost and energy input in microalgae cultivation. The recent hike in fertilizer price increased the microalgae production cost substantially and the environmental impact of chemical processes to their synthesis is not negligible.

In microalgae culture, heterotrophic growth can be a cost-effective alternative to photoautotrophic, that is often limited by insufficiency of light caused by mutual shading of cells (Chen, 1996) and by low concentration achieved, that increase the harvesting cost for the high energy consumption. Many microalgae have been shown to be able to grow rapidly in heterotrophic conditions, reaching high cellular density and biomass productivity. Although the heterotrophic production has several advantages over photosynthetic including also a high degree of process control and a lowering for harvesting costs, it involves high capital costs for a large-scale fermentation facility.

To date few heterotrophic microalgae species are commercially produced mainly for PUFA extraction to be used in infant food formulation or as health food and feed ingredient. The main species are *Crypthecodinium cohnii* for DHA production (Martek, USA), *Schizochytrium sp*. for food and feed supplement (Omegatech and Bio-Marin, USA) and *Ulkenia* (Nutrinova, Germany) for DHA-rich oil. It is important to remember also the studies on heterotrophic production of *Chlorella protothecoides* for biodiesel production (Xu *et al*., 2006, 2007; Eredia-Arroyo *et al*., 2010; Espinosa-Gonzalez *et al*., 2014).

The heterotrophic biomass productivity can be variable between the microalgae species and for the same strain on the basis of the concentration and form of organic carbon source utilized and growth parameters applied: a biomass productivity ranging from 9.5-50 $gL^{-1}day^{-1}$ can be assumed for *G. sulphuraria* cultivated in different conditions as reported by Graverholt and Eriksen (2007) and confirmed by Barclay *et al*. (1994) and Behrens (2005) for Martek operations with others microalgae species (Alabi *et al*, 2009).

Heterotrophic culture require organic carbon sources such as sugars or organic acids, which represent the main cost in medium formulation, and some agro-food industries wastewater, can be used as the sole carbon and energy sources with a high reduction of media costs and water consumption.

From an economic point of view the organic carbon source contributes 45.4%; inorganic chemicals, 3.2%; electricity 30.6 %; steam 14.2 %; and aseptic air 6.6% of the total production cost. The cost of glucose has also been estimated to be about 80% of the total medium cost, underlying the importance to drastically reduce the cost of the organic carbon source (Ogbonna and Moheimani, 2015).

In the present research, the spent cherry-brine liquid (sCBL) from an Italian local confectionery industry was evaluated as possible heterotrophic growth medium for *G. sulphuraria*. This food industry by-product is produced starting from sodium metabisulphite brines, used by the sweet cherry processing industry to bleach, firm and preserve cherries, which will be used into confectionery industries and applied in different products.

The disposal of sCBL is mandatory for its environmental toxicity strictly correlate to its chemical composition (e.g. several thousand ppm SO_2, high COD level) and acid pH (~4) and implicates added costs for confectionery industries.

One possible solution to the disposal problem was its use as heterotrophic microalgae growth medium.

G. sulphuraria (Cyanidiales), a thermo-acidophilic (extremophile) unicellular red algae is one of the best candidate to be used in sCBL for its ability to grow in an environment rich in sulfites with high concentration of sugars and low pH, making this strain very resistant to contamination. *Galdieria* is too a promising microalgae in the food and feed sector both for protein and carbohydrates production and a particular attention from the scientific community was for the production of C-phycocyanin, a potent antioxidant protein, suitable also for blue food dye and already authorized as an additive color in Japan. Interestingly also the skin care market require *Galdieria* extracts and an existing patent (MI2014A 000186), on its use as active ingredient to reduce the effects caused by acne, is owned by an Italian company (Vitalab srl) in agreement with our research group.

The research aim was to evaluate the use of sCBL as heterotrophic growth medium for *G.sulphuraria*, defining its biomass productivity, chemical composition, antioxidant activity and in vitro digestibility respect the biomass produced on artificial standard medium (SM). The antioxidant activity release during the digestion was studied at all phases on soluble and insoluble fractions. The microalgae phytoremediation ability in terms of effluent toxicity, before and after *G. sulphuraria* growth, were also assessed applying an acute and chronic ecotoxicity tests with organisms belonging to different trophic levels (a primary producer and consumer),due to the impossibility to have a complete chemical frameworkof the effluent, which is a complex mixture.

2. Materials and methods

2.1. Organism and cultivation

G. sulphuraria (SAG 107.79) was obtained from Culture Collection of Algae at the University of Göttingen (SAG), Germany. Stock culture of axenic microalgae strain in photoautotrophic condition was maintained routinely by regular sub-culturing at 4 weeks intervals on both liquid and agar slants of Cyanidium Medium (Troxler, 1972) at pH 4.

The transition from autotrophy to heterotrophy was performed in conical glass flasks of 1 L using the standard medium Allen (1968) whit 30 $gL^{-1}$of glucose as organic carbon source and placing the cultures in the dark. The pH was set at 1.5, while temperature was maintained at 26 ± 2 °C. The culture oxygenation and mixing was provided through an air bubbling system equipped with a filter of 0.22 µm to avoid culture contamination, while the exit gas passed through a condenser at 4°C to reduce evaporation.

2.2 Production and chemical characterization of spent cherry-brine liquid (sCBL)

The sCBL was collected from an Italian confectionery industry, that produced candied cherries, and transported in lab at 4°C. spent brine contains about 10% glucose and fructose, 0.5–1.5% $CaCl_2$, up to 0,4 % sulfur dioxide, citric acid, and lesser amounts of other of soluble constituents leached from the fruit (data from confectionery industry). After cherries processing the sCBL was enriched by leach sugars, organic acids, bleached anthocyanin pigments and other water-soluble compounds from the fruit (Wrolstad, 2009), showing a yellow color induced by yellow carotenoids.

For chemical and ecotoxicity analysis the sCBL was filtered on 20 µm filter and centrifuged at 5000 g for 15 min at 10°C.

Measurements of pH, dry matter (gL^{-1}), Biological Oxygen Demand (BOD5) (mg O_2 L^{-1}), Chemical Oxygen Demand (COD) (mg O_2 L^{-1})(following APAT-IRSA 5120 and 5130 methods, respectively) and Total Suspended Solid (TDS)(gL^{-1}) (according to APHA, Standard Methods, 1995) were performed. The sulfate content was evaluated applying APAT-IRSA 4020 procedure.

Before and after the *G. sulphuraria* growth, sCBL was analyzed for ammonium ($N\text{-}NH_4$) (Salicylate Method), nitrate ($N\text{-}NO_3$) (Cadmium Reduction), nitrite ($N\text{-}NO_2$) (Diazotization Method) and phosphate (PO_4) (Acid digestion Method,) (mg L^{-1}) content using a spectrophotometer test kit (HACH –Milano- DR 2400) and for BOD5 and COD level to define the *Galdieria* nutrient removal efficiency.

2.2.1 Ecotoxicity test on spent CBL

Before and after the *G. sulphuraria* growth, the ecotoxicity of sCBL was evaluated by performing ecotoxicity tests with organisms belonging to different trophic levels in particular a primer producer (*Pseudokirchneriella subcapitata),* as a chronic ecotoxicity test, and a primer consumer (*Daphnia magna)* as acute ecotoxicity test, following ISO standards methods: UNI-EN ISO 6341:2011 and UNI-EN ISO 8692:2005, respectively. Before the running the ecotoxicological tests the pH of the sample was adjusted to 7.5, to prevent the intrinsic toxic effects associated with the low pH-value. The experiments were carried out in triplicate for each treatment. The EC50 was calculated using the Linear Interpolation Method (Inhibition Concentration procedure or ICp) (Cesar *et al.*, 2004, US EPA 1993). The bootstrap method is used to obtain the 95% confidence interval. Antocyanin analysis was also performed on sCBL as described in paragraph 2.4.

Analysis of variance (ANOVA) was applied, using raw data, to test for significant differences in effects among treatments (significance level was always set at a = 0.05).

2.3 *G. sulphuraria* growth tests on spent CBL

For growth test and batch production the pH of sCBL was adjusted at 1.5 with H_2SO_4 (96-98%), as for SM, and the samples were enriched in N content (due to very low level in sCBL) until the concentration of Allen medium (280 mg L^{-1}) with NH_4Cl and then sterilized by autoclaving. The culture oxygenation and mixing was provided through an air bubbling system equipped with a filter of 0.22 µm and the temperature was maintained at 26 ± 2 °C.

2.3.1 Preliminary lab scale growth test

In a preliminary test to define the best sCBL dilution to apply for *G. sulphuraria* growth and its possible toxicity, the microalgae were pre-cultured and inoculated with a final inoculum concentration of about 4.2±0.2 g L^{-1} in 250 mL erlenmeyer flasks containing sCBL at five different dilutions (20, 40, 60, 80 and 100 % v/v); the dilution were carried out using as diluent the SM (Allen, 1968), that was utilized also as standard control.

The culture growth was assessed experimentally by measuring daily the corresponding dry weight (g L^{-1}) estimated gravimetrically following APHA Standard Methods (1995).

The experiments were carried out in triplicates for each growth medium dilution applied for 16 days, to define the growth-curves. Growth-curve functions were statistically determined by applying a best-fit procedure. With this approach, different regression models, provided by Origin1 8 SR2 (Northampton, MA) statistical software, were applied to each data set in order to determine, on the basis of statistical criteria (the least-square method and the analysis of residuals), the regression model that best described the observed data. At this stage, the most appropriate model was chosen by applying a goodness-fit of criterion (Scholze et al., 2001).

N and P removal efficiency and BOD and COD reduction by microalgae was evaluated measuring their variations in concentration at time 0 and after 16 days of growth and expressed as mean % of N and P removal efficiency and as % reduction of BOD and COD.

2.3.2 Pilot scale production

To evaluate the batch productivity of *G. sulphuraria*, 100% sCBL, that in the preliminary test showed a good growth performances no statistically different from the control, was utilized as growth medium. As control medium in addition to the Allen medium with 30 g L^{-1}glucose was tested also Allen with citric acid, added at a concentration equal to sCBL initial content (0.75%). The microalgae were pre-cultured and inoculated with a final inoculum concentration of about 3.7±0.2 g L^{-1} for all the treatments (day 0).The production volume was of 5 L in glass flaskes and the experiments were carried out in triplicates for each growth medium applied for 12 days. The growth was monitored counting the numbers of cells for mL with an optical microscope at 40X with Burker chamber and measuring daily the corresponding dry weight (g L^{-1}).

The produced biomass was harvested with a continuous centrifuge at 4000 rpm and plenty rinsed with distilled water to remove culture medium constituents. Each supernatant was collected for chemical analysis and ecotoxicology tests (as described in par. 2.2), while the wet microalgae biomass was frozen and dried in lyophilizer to evaluate the dry weight and measure the productivity (mg DW $L^{-1}day^{-1}$).

Mean productivity P (g DW L^{-1} day^{-1}) for cultivation cycle lasting n days was calculated as reported in equation 1:

$$P = \frac{X/L}{n} \quad (1)$$

where X is the dried harvested biomass weight, L the total liters centrifuged and n the number of production days.

Analysis of variance (ANOVA) was applied, using raw data, to test for significant differences in growth among sCBL and the two control media (significance level was always set at p = 0.05).

2.4 Chemical characterization of *G. sulphuraria* biomass composition

The biomass ash content was measured in a muffle furnace at 550° until constant weight following the standard procedure describe by IRSA-CNR methods (1994).

Carbohydrate determination was performed on 1 g of freeze dried samples treated with hydrochloric acid (0.2 M) at 85°C for 1 h. After neutralization by sodium hydroxide, reduced sugars were determined using the Fehling test.

The lipid content was evaluated by Bligh and Dyer method (1959) briefly: 20 mL of a mixture chloroform: methanol (2 : 1, v/v) were added to 100 mg of the lyophilized sample followed by 20 min of mixing. This mixing was repeated for 10 min both after adding the second portion of 10 mL of chloroform and after addition of 20 mL of water. After centrifugation, the organic phase was evaporated to dryness and the weight of the residue was determined after 30 min at 105 °C. The lipid extract prepared for total lipid determination was suspended in hexane and used for the fatty acid methylation. Fatty acids were converted to their methyl esters before analysis. Hexane extract (1 mL) was added with 200 µL of KOH 2 N in methanol for 30 s at room temperature, and 1 mL was injected directly in the GC apparatus. The analysis of fatty acid methyl esters was carried out using a Shimadzu 17A gas chromatograph equipped with a fused silica capillary column (Phenomenex ZB-WAX, 0.50 µm film thickness, 60 m x 0.32 mm i.d.) and a FID detector. Helium was the carrier gas with a flux of 2 mL min^{-1}. The temperature program was 200 °C x 5 min, 200 °C until 230 °C in 15 min (2 °$Cmin^{-1}$), constant at 230 °C for 30 min. The column, the injector and FID temperatures were 200, 240 and 240 °C, respectively. Identification of fatty acid was carried out using reference fatty acids methyl esters (FAME) from Merck (Merck, Darmstadt, Germany).

Samples were analyzed for protein concentration by the Kjeldahl method (AOAC Official Method 985.29, 1995) using a nitrogen conversion factor of 6.25.

Polyphenols were determined using a Folin metod (1927) with the Folin-Ciocalteu reagent (Obanda & Owuor, 1997). A calibration curve of gallic acid (ranging from 0.0125 to 0.1 mg mL^{-1}) was elaborated and the results, determined from regression equation of the calibration curve (y=6.781x, R^2=0.997), were expressed as mg gallic acid equivalents per g of the sample. In this method, 125 µL of the sample extract diluted 10–75 times with deionized water (to obtain absorbance in the range of the prepared calibration curve) was mixed with 0.5 mL of distilled water with the addition of 125 µL of 3-fold-diluted Folin–Ciocalteu phenol reagent and it was shake. After 6 min, 1.25 mL of 7.5% sodium carbonate solution and 1 mL of distilled water were added to the mixture. The mixture is allowed to stand for 90 min and blue color formed is measured at ƛ 760 nm using a spectrophotometer. The blank was prepared at the same way but with the distilled water (125 µL).

The detection of anthocyanins was performed according to previously paper from Troise *et al*., 2014. The detection of anthocyanin was performed by extraction of 400 mg lyophilized sCBL and lyophilized *Galdieria* biomass grown on sCBL and in Allen medium in 12 mL of MetOH / water (70:30 v /v). After 30 min of mixing, the samples were centrifuged (4000 g at 4° C for 10 min). The supernatant filtered with a 0.45µm filter was centrifuged for a second time at 14.800g for 10 min at 4° C. Finally, 10 µL was injected into the LC-HRMS system. Anthocyanins separation was performed on a U-HPLC Accela system 1250 (Thermo Fisher Scientific, Bremen, Germany) consisting of a degasser, a quaternary pump, a thermostated autosampler (10 °C) and a column oven set at 30 °C. Mobile phase A was 0.1% formic acid and mobile phase B was 0.1% formic acid in acetonitrile; the separation was achieved by using a Gemini C18 column (150 x 2.0 mm, 5.0 µm; Phenomenex, Torrance, CA) and the following gradient flow (300 µL/min) of

solvent B (min/%B): (1/5), (2/15), (4/15), (8/90), (12/90) was used. The autosampler needle was rinsed with 800 µL of acetonitrile/ water (70/30, v/v) before each injection. The Exactive Orbitrap HRMS system (Thermo Fisher Scientific, Bremen, Germany) was equipped with a heated electrospray interface operating in the positive ion mode and scanning the ions in the *m/z* range of 60−1200. The resolving power was set to 50000 full width at half-maximum (FWHM, *m/z* 200), resulting in a scan time of 1 s. The automatic gain control was used to fill the C-trap and gain accuracy in mass measurements (balanced mode, 10^6 ions); maximum injection time was 100 ms. The interface parameters were as follows: spray voltage, 3.5 kV; capillary temperature 275 °C and heather temperature at 200 °C, capillary voltage, 30 V ; skimmer voltage, 14 V; sheath gas flow, 30 (arbitrary units); and auxiliary gas flow, 15 (arbitrary units). Prior anthocyanins determination, the instrument was externally calibrated by infusion with a positive ions solution that consisted of caffeine, Met-Arg-Phe-Ala (MRFA), Ultramark 1621, and acetic acid in a mixture of acetonitrile/methanol/water (2:1:1, v/v/v) in order to guarantee the best analytical conditions. Reference mass (lock mass) of diisooctyl phthalate ($[M + H]^+$, exact mass = 391.28429) was used as recalibrating agent for positive ion detection. Anthocyanins were detected by using an in-house database developed according to compounds list reported in phenol-explorer www.phenol-explorer.eu (Rothwell *et al*., 2013; Neveu *et al*., 2010). Specific *m/z* ratios were included in Exact Finder (Thermo Fisher Scientific, Bremen, Germany) and the following parameters were selected: signal to noise ratio higher than 5, area threshold higher than 10000, mass tolerance up to 5 ppm.
Chemicals for the Anthocyanins detection: Acetonitrile and water for LC/HRMS determination were obtained from Merck (Darmstadt, Germany); formic acid was purchased from Sigma-Aldrich (St. Louis, MO). The calibration solutions (see "Anthocyanins detection" section) were obtained from Thermo Fisher Scientific (Bremen, Germany).

2.5 In vitro digestibility of *G. sulphuraria* biomass

The total digestibility of dried biomass produced was assessed following the method described in Minekus *et al.* (2014), that in vitro simulated the conditions of *in vivo* digestion (enzymes involved, pH, temperature, incubation time, fluids of the gastrointestinal tract)carrying out the digestion three phases: oral, gastric and intestinal. The fluids of the gastrointestinal tract such as salivary (pH 7), gastric (pH 3) and intestinal (pH 7.5) have been formulated as previously described (Minekus et al.,2014).The experiment were carried out in triplicate on 2.5 g of lyophilized biomass of *G. sulphuraria* grown in SM and sCBL. Firstly, 1.75 mL of the Simulated Salivary Fluid (SSF) solution was added to the sample; the mix was minced. 0.25 mL of α-amylase solution (1500 U/mL di SSF), 12.5 µL of $CaCl_2$ 0.3 M and 487.5 µL of water were added. The samples were incubated for 2 min at 37 ° C under stirring. Subsequently, 5 mL of the sample from the salivary phase, were added to 375 mL of Simulated Gastric Fluid (SGF), 0.8 mL pepsin solution (25000U mL^{-1}SGF), 2.5 µL of $CaCl_2$ 0.3 M, 0.1 mL hydrochloric acid to reach pH 3, 0.35 mL of water and exposed to gastric phase.The solution have been mix to the dark at 37° C for 2 hours. Then 10 mL of the sample from the gastric phase, were exposed to the intestinal phase. To 10 mL of gastric chyme (partially digested food) were added 5.5 mL of Simulated Intestinal fluid(SIF), 2.5 mL of pancreatic solution (800 U $mL^{-1}$of SIF), 1.25 mL of bile solution (6 $mgmL^{-1}$of SIF), 0.02 mL of $CaCl_2$ 0.3 M, 0.075 ml of NaOH 1M (to reach pH 7) and 0.65 mL of water. The solution have been in agitation in the dark at 37° C for 2 hours. At this point, the solution (20 mL) was centrifuged at 4000 rpm for 10 min at 4°C. The soluble and insoluble fractions were divided, weighed, frozen at -40 ° C for subsequent analysis of antioxidant activity (AA) described in the next paragraph.
The total digestibility was evaluated utilizing the gravimetric method for the determination of the soluble fraction (g) after digestion in vitro, and the quantity (g) of the initial sample.
The quantities were expressed as dry weight:

$$D = \frac{\%\ \text{soluble fraction (g)}}{\text{sample start (g)}} * 100 \qquad (2)$$

2.6 Antioxidant Activity

2.6.1 ABTS method

The AA of the soluble fractions, after *in vitro* digestion, and on water extract of whole biomass was determined using a direct measurement of ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6- sulfonic acid). Antioxidant capacity assay was carried out using an UV–VIS recording spectrophotometer (HACH –Milano- DR 2400) by the improved $ABTS^+$method as described by Re *et al*. (1999). $ABTS^+$ radical cation was generated by reacting 7 mM ABTS and 140 mM potassium persulfate after incubation at room temperature (23°C) in dark for 16h.
The $ABTS^+$solution was diluted with ethanol to an absorbance of 0.70±0.05 at ʎ 734nm. The filtered sample was diluted with 70% methanol soas to give 20–80% inhibition of the blank absorbance with 0.1mL of sample. 1mL of $ABTS^+$solution (with absorbance of 0.70±0.05) was added to 0.1mL of the tested samples and mixed. The reactive mixture was allowed to stand at room temperature for 2.5 min and the absorbance was immediately recorded at 734nm. Trolox standard solution (final concentration 0–15µM) in methanol was prepared and assayed at the same conditions. The absorbance of the resulting oxidized solution was compared to that of the calibrated Trolox standard. Results were expressed in terms of Trolox equivalent antioxidant capacity (TEAC, mmol Trolox equivalents per 100g dry weight of sample). On the in soluble fractions AA was assessed with ABTS Quencher method (Serpen *et al*., 2007; Vural *et al*., 2009).

2.6.2 DPPH method

The AA of the soluble fractions, after *in vitro* digestion, was measured also through the DPPH method (Brand-Williams, 1995).The stock solution was prepared with 20 mg of DPPH (2,2-diphenyl-1-picrylhydrazyl) and 50 mL of methanol. The "working solution" (ws) was obtained by combining the stock solution (DPPH/methanol 20mg/50mL) to methanol, 1:10 ratio. The absorbance of the ws was at ƛ 517 nm equal to 0.9±0.2. The ws was utilized for the test: 1 mL of ws was added to 0.2 mL of sample, for the blank was used the same quantity (assay with distilled water). The reactions were carried out in triplicate for each sample and for each blank. After 10 min, the absorbance at 517 nm was measured for each sample. The percentage of inhibition was calculated:

% Inhibition = [(1-Abs sample) / Abs blank]*100

The AA was expressed in Trolox equivalent, used for the system calibration. The values of inhibition percentage were replaced in the equation of the calibration curve, obtained by measuring the absorbance of solutions of Trolox a known concentration, so can obtain values expressed in µmol Trolox equivalent L^{-1} of the sample. On the insoluble fractions the DPPH AA was assessed following the Quencher modification of Gokmen *et al*. (2009).

3. Results and discussion

3.1 Spent brine cherry liquid composition and ecotoxicity

The chemical composition of sCBL were showed in table 1. It was characterized by low pH and a high dry matter content of 88.4 g L^{-1}. The higher N content was in the form of NO_3-N and the total P was 30.10 mg L^{-1}, with an N:P ratio of 0.4, very low and not sufficient to sustain the microalgae growth. To utilize sCBL as microalgae growth medium before preliminary tests and batch production the N concentration was adjust adding NH_4Cl until to reach a N concentration as in SM.

The sulphates SO_4^{2-} concentration was of 162 mg L^{-1} with a S content of 54.1 mg L^{-1}. The TOC was 10.8 g L^{-1} showing an increase of 3.8 times respect the initial concentration in cherry-brine liquid (2.81 g L^{-1} due to acid citric) and strictly correlate to the sugars release from cherries (essentially as glucose, fructose and sorbitol) (Park *et al*., 1997). In the SM the N and S source was represented by $(NH_4)_2SO_4$ applied at a final concentration of 1.32 g L^{-1}, with a [N] of 280 mg L^{-1}, and a [S] of 320 mg L^{-1}, values higher respect sCBL composition (table 1). Also the [P] was higher (110 mg L^{-1}), showing a N:P ratio of 2.5, lower than sCBL after NH_4Cl addition (9.3), where P was a limiting nutrient. The SM organic carbon source was the glucose at a concentration of 30 g L^{-1} with a TOC of 12g L^{-1}. Both in sCBL that in SM for the pH adjustment it was added sulfuric acid, which induced an addition in S content equal to 1.34g L^{-1}. After pH adjustment at 1.5 the sCBL color changed from yellow to red due to anthocyanin presence, released by cherries.

Table 1. Chemical and physical parameters of raw sCBL

Parameters	Value
pH	4.2
Total Suspended Solid (gL^{-1})	0.64
Dry matter (gL^{-1})	88.4
NH_4-N(mgL^{-1})	1.15
NO_3-N(mgL^{-1})	11.0
NO_2-N(mgL^{-1})	0.3
P total(mgL^{-1})	30.10
TOC (gL^{-1})	10.8
Sulphates SO_4^{2-}(mgL^{-1})	162
BOD (mgL^{-1} O_2)	1200
COD (mgL^{-1} O_2)	65000

Given the impossibility to perform a complete chemical characterization of sCBL and to evaluate the microalgae phytoremediation activity, it was decided to perform ecotoxicity tests before and after the *Galdiera* growth on sCBL. Before growth the sample showed an EC50 value ranging from 5.49 to 5.56% (v/v) for *P. subcapitata*, that was the most sensible organism, while *D. magna* showed an EC50 of 18.86 % (v/v) ranging from 17.5 to 19.37.

The sCBL chemical composition for the parameters analyzed resulted adapted to grow in heterotrophy *G. sulphuraria*, when compared to the SM, with the only exception of N concentration. It is important to underline that the sample showed a very high COD and a BOD5 of 1200 mg L^{-1} O_2 (table 1), that prohibit it disposal into surface water bodies and sewage (D.L.152/06) as also confirmed by ecotoxicity results obtained in the present research.

The high COD value, respect BOD, showed very low biodegradability of sCBL, strictly correlated to the low pH and high sulfur dioxide content, acting as an inhibitor of most yeasts, molds, and bacteria, avoiding the natural sugar oxidation.

3.2 Preliminary lab scale growth test and pilot scale production

The results obtained from the preliminary test showed that at all dilutions of sCBL tested, the growths were not statistically different respect the SM (Fig. 1), underlying the no-toxic chemical profile of sCBL against *Galdiera* and its

reuse in terms of nutrients and water. The microalgae reached a concentration of 10.2±0.6 g L^{-1} DW on the 16th day of cultivation in SM and values ranching between 9.5-9.9 g L^{-1} DW in sCBL at different dilutions tested. Graverholt and Ericksen (2007) reported, for different strains of *G. Sulphuraria* growth in batch and fed-batch, maximal biomass dry weight concentration between 4.6-109 g L^{-1} at different glucose concentration (ranging from 10 to 500 g L^{-1}) and at temperature higher than our (42°C). Our results are in line with literature data if it was considered the lower temperature applied during the growth experiments (26°) and selected to reduce the energy heating input and the evaporation process of culture. The production process sustainability has been a key point of this research. Differently from what reported in literature for microalgae heterotrophic production in the preliminary test was evidenced a long lag phase of about 6 days (fig.1) before start the exponential phase as yet observed by Graverholt & Eriksen (2007) (4 days), probably due to the reproductive process of this heterogenic size strain, where an increase in little cells number not correspond an weight increase.

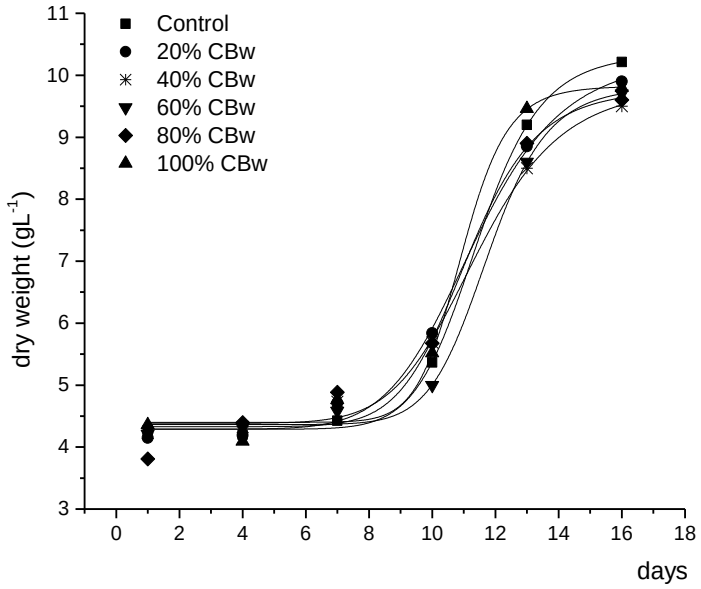

Figure 1. *G. sulphuraria* growth curves obtained in the preliminary test at different dilution factors of sCBL (20, 40, 60, 80 and 100% v/v) in SM. Increase in dry weight (g L^{-1}) in the cultivation days. The function that best fit raw data was a Growth/Sigmoidal: Logistic $y = \frac{A_1 - A_2}{1 + \left(\frac{x}{x_0}\right)^P} + A_2$

The N and P removal efficiency in sCBL, evaluated after 16 days of culture, were respectively of 97.85% and 98.23%, with a final concentrations of 8.02 mg $L^{-1}$for N and of 1.05 mg $L^{-1}$for P. A BOD5 reduction of 80% (240 mg L^{-1} O_2) and a COD reduction of 60% (26.000 mg L^{-1} O_2) were evaluated. Whit microalgae growth some sCBL parameters as N and P content and BOD5 and COD decreased, but only for N and P concentrations until values that make the wastewater downloadable in sewerage system and into surface waters. A 240 mg L^{-1} O_2 of BOD5 is enough only to discharge in sewer, but the COD is yet enough high requiring others treatment before its disposal as required by Italian D.L. 152/06. The ecotoxicity tests repeated after *Galdieria* growth showed a reduction of EC50 for both species involved in the test: the EC50 of chronic test with *P. subcapitata* decreased of 50%, whilethe EC50 of acute test with *D. magna* decreased of 45% underlying the phytoremediation activity of microalgae.

Data on *G. sulphuraria* grown on wastewaters are very scarces. Selvaratnam *et al*. (2014) evaluated the growth and nutrient removal efficiency of *G. sulphuraria* grown on primary wastewater effluent in mixotrophic condition in seven days assessing an NH_3-N removal efficiency of 88.3% and for PO_4 of 95.5% value in line with our results.

For batch production, the dilution of sCBL chosen was 100% (v/v) because in the preliminary test it induced a microalgae growth not statistically different from the control and the others dilutions tested and in the way to obtain a complete reuse of water. Two different organic carbon sources in SMs (Allen) were applied: one was glucose (30g L^{-1}) and the other citric acid (7.5 g L^{-1}). In this case, the microalgae growth was expressed in terms of dry weight and cell density (cell mL^{-1}), due to the heterogeneity of cell dimension, characteristics of this strain. Kuroiwa *et al.*(1989) described the life cycle of *Cyanidium caldarium*, to date reclassified as *G. sulphuraria* (SAG strain information): a large mother cell of the I-type divides to form 16 endospore cells; the young cell increases in volume after the initiation of synchronous culture and reaches about 16 times the initial volume of the young cell. As the volume of the I-type cell does not change 48 h after the initiation of synchronous culture, the daughter cells decrease stepwise in volume after

each endospore division. Patterns of quantitative changes similar to that in cell volume are observed in the size of the chloroplasts and the spindle of the cell nucleus, and in contents of chloroplast DNA and pigments (Kuroiwa *et al*., 1989). The cells size reported in literature (Albertano *et al*., 2000) were between 3-11µm.

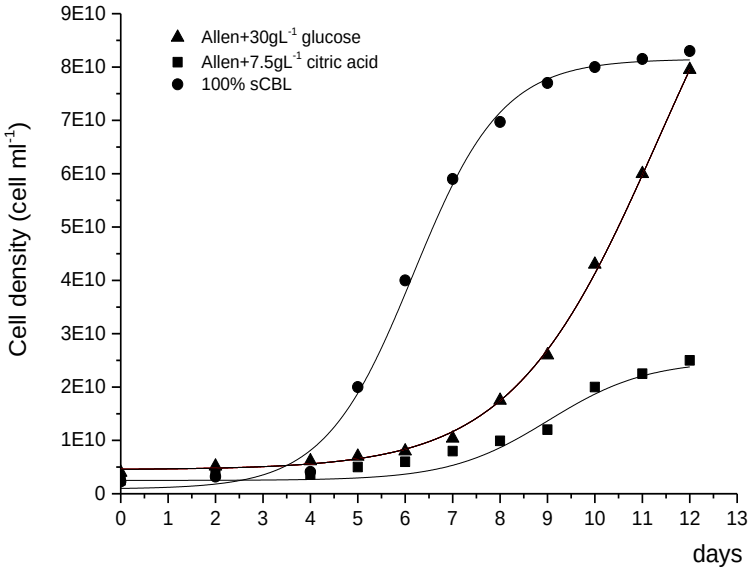

Figure 2. *G. sulphuraria* batch production in sCBL 100% (v/v) and in two SM (Allen+glucose 30gL^{-1}and Allen +citric acid 7.5gL^{-1}): increase in cell density(cell mL^{-1}) in the cultivation days.

In the SM (Allen medium + glucose), the productivity was 0.76±0.03 g L^{-1}day^{-1}, value statistically different and higher respect sCBL productivity (0.57±0.04 g L^{-1}day^{-1}), also if the cell numbers for mL was higher in sCBL respect SM. In fact under microscope 40X in sCBL the cells were very little respect the control and as indicated by results also lighter (photo 1). In Allen medium with citric acid (0.75%), the productivity was the lowest with a value of 0.03±0.01 g L^{-1}day^{-1}, indicating that the microalgae growth in sCBL was supported not only by acid citric utilized in the preparation of cherry-brine liquid but also sugars released by cherries during their permanence time and that contributes to the BOD of the brine and the problems of waste disposal (Wrolstad, 2009).

The C:N ratio is an important parameter in microalgae production and lowest ratio was measured for Allen + 7.5 g L^{-1}citric acid (10) respect the SM (43) and sCBL (39), indicating organic C limitation, while in sCBL it was observed a very high N:P ratio of 9.3 respect SM (2.5) underlying P limitation.

Galdieria showed a good growth performance despite the heterogeneity of cellular dimension (fig. 2). Literature data on heterotrophic biomass productivity of *G. sulphuraria* are variable on the basis of the concentration and form of organic carbon source utilized and growth parameters applied in particular temperature: a biomass productivity ranging from 9.5-50 g L^{-1}day^{-1} can be assumed for *G.sulphuraria* cultivated in different conditions as reported by Graverholt and Eriksen (2007) and confirmed by Barclay *et al*. (1994) and Behrens (2005) for Martek operations with others heterotrophic microalgae species (Alabi *et al*, 2009).

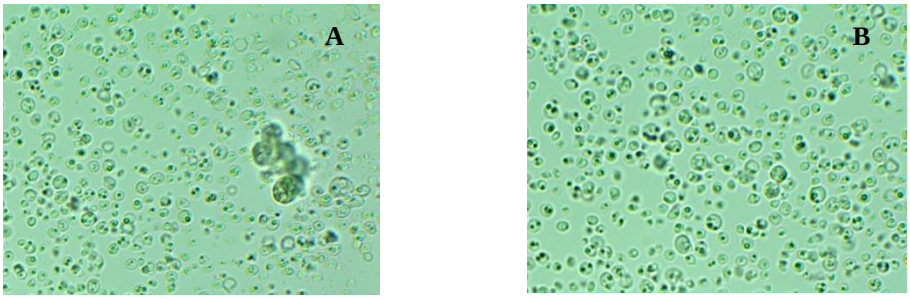

Photo 1. Cellular dimension of *G. sulphuraria* grown in sCBL (A) and in SM (B)

3.3Macronutrient composition

The chemical composition of *Galdieria* biomass growth in SM and sCBL was showed in table 2 and demonstrated to be influenced from medium growth composition. The biomass obtained in SM presented as main component, the carbohydrates' fraction (52.3%), followed by protein content (32%) in line with data reported also for another strain of *G. sulphuraria* by Graziani *et al.* (2013) with a higher carbohydrates' fraction of 69% and a lower protein content of 26.5%. The carbohydrates' fraction and protein content were also in agreement with value reported by Harun *et al.* (2010) on another rhodophyta microalgae species (not Cyanidiales) as *Porphyridium cruentum*, that showed a mean protein content of 28-39% and 40-57% of carbohydrates fraction.

The lipid % assessed in this research is slightly higher respect literature data for this species (Graziani *et al*., 2013) but generally lower for that reported for other microalgae, indicating also as main storage nutrient the carbohydrate fraction. The sCBL biomass respect SM showed adecrease in proteins (of about 31%) and in lipids (of 40%), with an increase in carbohydrate content of about 12% and in polyphenols (5.3 vs 1.6 mg g^{-1}).

It is known that *Galdieria* accumulate storage glycogen (α-glucan) (Stadnichuk *et al.*, 2007; Shimonaga *et al*., 2008), which is distinguished from the semi-amylopectin type of α-glucan, called floride an starch, in red algae (Sheath et al., 1979; Nakamura *et al*., 2005; Shimonaga *et al*., 2008, Sakurai *et al*., 2016). Sakurai *et al*. (2016) observed that in heterotrophic cultures of *G. sulphuraria*, cellular glycogen content increased with the glucose addition in the culture medium. Our results of increase carbohydrates' fraction when cells were grown in sCBL were strictly correlate to the sugars released from cherry in sCBL. In fact previous studies showed that *G. sulphuraria* can use various carbon sources for its growth (Seckbach and Baker, 1970; Gross and Schnarrenberger, 1995; Kurano *et al*., 1995; Graverholt and Eriksen, 2007).

The data showed that the amounts of carbohydrates and lipids are largely regulated by growth conditions. It is known for autotrophic microalgae, that stress conditions due to N and P deprivation induced a reduction in protein content and promote the conversion of carbon dioxide fixed into energy-rich compounds such as lipids and carbohydrates (Ho *et al*. 2013). An important role in heterotrophic growth was represented by the C:N ratio, that was 43 and 39 respectively in SM and sCBL.

Different authors reported that the C:N ratio can influence cellular lipid content by controlling the switch between protein and lipid syntheses (Gordillo *et al*., 1998, Wen and Chen, 2003). A high C:N ratio induced an increase in lipid content, which is caused by nitrogen depletion in the culture. Wen and Chen (2003) reported that in heterotrophic cultivation of the green microalga *Chlorella sorokiniana*, a C/N ratio of 20 was found to indicate a change from carbon to nitrogen limitation (Chen and Johns, 1991). Cellular lipid content was at a minimum at this value and increased at both higher and lower C/N ratios (Chen and Johns, 1991). For some species as *Cryptheconidium conhii* (marine) and *C. sorokiniana* the accumulation of lipids may not be dependent on nitrogen exhaustion but on an excess of carbon in the culture media. Hence in heterotrophic cultures, lipid accumulation was attributed to consumption of sugars at a rate higher than the rate of cell generation, leading to conversion of excess sugar into lipids (Ogbonna and Mohemani, 2015). In our case the C:N ratio in sCBL was little lower respect SM but the P limitation was more evident inducing in *G. sulphuraria* biomass an increase in carbohydrates as storage component and not in lipid content that in this strain generally was very low.

The fatty acid profile of *G. sulphuraria* was characterized by palmitic, oleic and linoleic acids and a % of unsaturated fatty acids of 56% was assessed (table 2). In sCBL biomass was observed a reduction in palmitic acid of 12% and linoleic acid of 8% and an increase in oleic acid of 25% respect the biomass growth in SM. In both growth condition was observed a high prevalence of monounsaturated fatty acid (C18:1), that in microalgae usually was induced in heterotrophic growth condition as also reported for *Chlorella zofingiensis* (Liu *et al*., 2011), and probably related to a decrease of thylakoid structural lipids, occurring in the algae grown in the dark. In agreement with Sakurai *et al*. (2015), Lang *et al*., (2011) and Graziani *et al* (2013) long-chain fatty acids were not found in *G. sulphuraria*.

The assessed ratio of mono/polyunsaturated in sCBL was about 3:1, while in SM was 2.2:1.

In *G. sulphuraria* under heterotrophic conditions in SM, the unsaturated/saturated ratio reported in literature was about five (Graziani *et al*., 2013), while we assessed a different ratio of 2:1 in SM and 2.6:1 in sCBL due to the different growth conditions applied. Although, the data of *G. sulphuraria* fatty acid profile, are very few, our results confirm the ratio unsaturated/saturated reported for other microalgae (Gouveia and Oliveira, 2009). A C/N ratio also affects fatty

acid composition. Chen and Johns (1991) found for *C. sorokiniana* that a low C/N ratio favoured a high proportion of unsaturated fatty acids, as observed also from our results (tab. 2).
These results confirmed that microalgae are metabolically very flexible and their total lipid concentration as well as fatty acid composition can change significantly depending on the strain, on culture medium composition and on the growth conditions applied (Buono *et al*., 2014).
Regarding the possible use of the whole *G. sulphuraria* biomass, the low amount of total lipids and the limited percentage of polyunsaturated fatty acids can positively influence the storage time, limiting the oxidative degradation, which is usually a relevant problem when microalgae are incorporated in foods (Ryckebosch *et al*., 2011).

Table 2. Main fatty acids (%) profile of *G. sulphuraria* cultivated in SM and sCBL

Fatty acid (%)	SM	sCBL
C14:0	0.32±0.1	0.52±0.03
C16:0	19.86±0.02	17.46±0.16
C18:0	7.77±0.03	7.07±0.5
C18:1	38.34±1.05	47.84±1.12
C18:2	17.83±1.13	16.41±1.09
% Saturated	27.95	25.05
% Unsaturated	56.47	64.25

A qualitative analysis of anthocyanin was performed both on *Galdieria* biomass grown in sCBL and SM, and on lyophilized sCBL before growth experiment.
In *Galdieria* grown on SM was observed the absence of anthocyanin, while in sCBL before growth experiment and in *Galdieria* growth in sCBL the presence of different anthocyanins was assessed (figure 3). The anthocyanin identified were showed in Table 3 with the respective mass error.

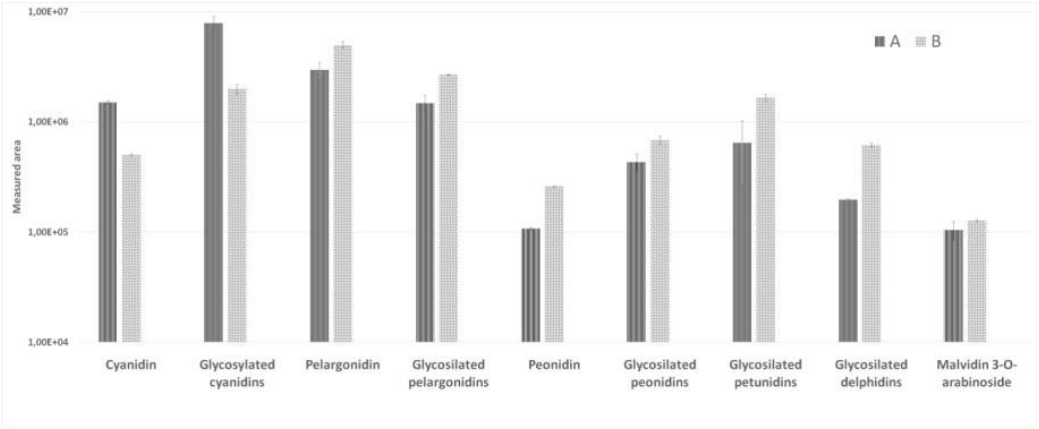

Fig. 3 Measured areas of antocyanin after methanol/water extract in sCBL (A) and *Galdiera* biomass grown in sCBL (B)

Table 3: Compounds identified by using Exact Finder and phenol-explorer. The mass accuracy (Δppm) was calculated dividing the mass error (i.e.: the difference between the theoretical mass and the experimental mass) by the theoretical mass. The results were reported in ppm by multiplying by 10^6.

Compound Name	Formula	m/z (Expected) $[M]^+$	m/z (Measured) $[M]^+$	m/z (Δppm)
Cyanidin	C15H10O6	287.0550	287.0550	-0.07
Cyanidin3-O-(6''-acetyl-galactoside)	C23H22O12	491.1184	491.1164	-4.0892
Cyanidin 3-O-(6''-acetyl-glucoside)	C23H22O12	491.1184	491.1164	-4.0892
Cyanidin 3-O-(6''-succinyl-glucoside)	C25H24O14	549.1239	549.1224	-2.7427
Cyanidin 3-O-arabinoside	C20H18O10	419.0973	419.0962	-2.5807
Cyanidin 3-O-galactoside	C21H20O11	449.1078	449.1078	-0.1104
Cyanidin 3-O-glucoside	C21H20O11	449.1078	449.1078	-0.1104
Cyanidin 3-O-rutinoside	C27H30O15	595.1657	595.1648	-1.5986
Cyanidin 3-O-xyloside	C20H18O10	419.0973	419.0962	-2.5807
Delphinidin 3-O-galactoside	C21H20O12	465.1028	465.1032	1.0508
Delphinidin 3-O-glucoside	C21H20O12	465.1028	465.1032	1.0508
Malvidin 3-O-arabinoside	C22H22O11	463.1235	463.1230	-0.9519
Pelargonidin	C15H10O5	271.0601	271.0604	0.9740
Pelargonidin 3-O-galactoside	C21H20O10	433.1129	433.1125	-0.9345
Pelargonidin 3-O-glucoside	C21H20O10	433.1129	433.1125	-0.9345
Pelargonidin 3-O-sambubioside	C26H28O14	565.1552	565.1570	3.2943
Pelargonidin 3-O-sophoroside	C27H30O15	595.1657	595.1669	1.9907

Peonidin	C16H12O6	301.0707	301.0708	0.3516
Peonidin 3-O-arabinoside	C21H20O11	449.1078	449.1075	-0.7899
Peonidin 3-O-galactoside	C22H22O11	463.1235	463.1230	-0.9519
Peonidin 3-O-glucoside	C22H22O11	463.1235	463.1230	-0.9519
Peonidin 3-O-rutinoside	C28H32O15	609.1814	609.1828	2.3045
Petunidin 3-O-arabinoside	C21H20O11	449.1078	449.1078	-0.1104
Petunidin 3-O-rutinoside	C27H30O15	595.1657	595.1669	1.9907
Vitisin A	C26H24O14	561.1239	561.1255	2.8634

In fact Chaovanalikit and Wrolstad (2004) observed that about half of the polyphenolics of cherries were leached into the brine solution, suggesting the application of cherry-processing wastewaters as potential sources for natural colorants, nutraceuticals and natural antioxidants and anti-inflammatory (Wang and Stoner, 2008; Blando *et al*., 2004). In line with our results Mozetic and Trebse (2004) showed as main anthocyanins in cherries Cyanidin 3-O-glucoside (Cy 3-glc) and Cyanidin 3-O-rutinoside (Cy 3-rut).

In figure 3 the relative measured areas of anthocyanins for sCBL and *Galdieria* grown in sCBL were reported. The measured area of glycosylated derivatives of cyanidin, pelargonin, petunidin, peonidin were summed to assess the relative amount in the two samples. The anthocyanin presence in *Galdieria* biomass grown in sCBL is a very interesting aspect even if the absorption was not clear (inside cells, outside on surface attachment or only for dragging during biomass harvesting), but as indicated in the graph the anthocyanin profile in the two samples is equal indicating an bio-accumulation in microalgae biomass. In this way the biological activity of *G. sulphuraria* and its possible application in food, feed and cosmetician can be improved and enriched. Literature data on microalgae anthocyanin assimilation are scarce.

Jelinek *et al.* (2015) observed the biosorption of polyphenols on the surface of *Chlorella vulgaris* non living cells apparently hardly correlated by molecular polyphenol structure. Other authors as Sherman *et al.* (1991) and Ismail *et al.* (2013) reported that some microalgae (Chlorella, Stigeoclonium, Microspora, Ulva and Spirogyra) metabolized polyphenols through polyphenol oxidase enzyme, while Kunamneni *et al.* (2007) observed that white rot fungi like *Pleurotus* present a group of enzymes (e.g. laccase, manganese, peroxidase, veratryl alchol oxidase), that have the ability to metabolize and degrade polyphenolic compounds.

3.4 In vitro total digestibility and antioxidant activity (with DPPH and ABTS methods)

About microalgae digestibility, it is important to underline that many microalgae species showed a thick polysaccharide/cellulosic cell wall, representing about 10% of the algal dry matter. The intact cell wall posed serious problems in the downstream process as well as in the use as food/feed, since they are difficult to digest for humans and other non-ruminants (Buono *et al*., 2014) and the technological system applied for drying can act on wall cells disrupt and microalgae digestibility. Digestibility improved through the introduction of the technology of spray-

drying, and also quick blanching at over 100°C. Digestibility of *Chlorella* powder in the human body is presently assumed to be ca. 80%. Chlorella cells broken by a mill are known to have a slightly higher digestibility than non-broken *Chlorella*, but the difference may not be significant (Becker et al., 2004). Our sample were lyophilized and the total digestibility of *Galdieria* grown in SM and sCBL assed using INFOGEST protocol was 79.3±5.8% and 63.4±8.1%, respectively. *Galdieria* total digestibility was in line with literature data for other microalgae strains(Becker &Venkataraman,1982) and the reduction digestibility for sCBL can be attribute to major ash and carbohydrates content (table 4).

Table 4. Chemical composition of *Galdiera* biomass growth in SM (Allen + 30gL-1) and in sCBL

Nutrients (mg g^{-1})	SM	sCBL
Proteins	320±5.5	220±6.4
Lipids	77±1.1	46±3.2
Carbohydrates	523±6.3	584±1.3
Polyphenols	1.6	5.3
Ash	80	150

The *in vivo* digestibility of algae is not well documented, and the available studies on their assimilation by humans have not provided conclusive results. However, several authors have described a high rate of algal protein degradation *in vitro* by proteolytic enzymes such as pepsin, pancreatin, and pronase (Mabeau and Fleurence 1993).

Mišurcová *et al*., (2010) evaluated the total digestibility (TD) of different kind of microalgae simulating in vitro the human digestion applying different methods in which used as enzyme only pepsin, only pancreatin and pepsin and pancreatin. They observed an efficiency of digestibility in the following order: pancreatin>pancreatin/pepsin > pepsin.

In the blue-green microalgae they assessed a TD between 71.4 and 81.9 %, for the green microalgae the TD was 78.6 - 60.9 %, for the red microalgae the TD was between 77.6- 80.3% and for the brown microalgae the TD was between 58.4 and 60.3 %. The TD of red macroalgae analyzed ranging between 87.4-84.9% for *Porphyra tenera* and 65.9-73.2 % for *Palmaria palmate,* data in line with our results.

The innovative material and methods applied to evaluate TD was INFOGEST (Minekus *et al*., 2014), that received international consensus, and was developed to overcome some difficulties in the digestibility evaluation of different matrices, as expensive human trials and the high variability of "*in vitro*" test utilized, that usually obstruct to compare results across research teams.

The polyphenol content for the control was 1.6 mg gallic acid g^{-1} and for the *G. sulphuraria* grown in sCBL was 5.3 mg gallic acid g^{-1}. At the moment, there are no data in the literature on the total phenolic content of *G.sulphuraria*.

Goirisi *et al.* (2012) evaluated the phenolic content of 32 different microalgae strains evaluating a mean phenolic content of 2.11 mg GAE g^{-1} DW; in particular they observed the highest content of 4.57 mg GAE g^{-1} DW for *Isochrysis sp*. and the lowest 0.54 mg GAE g^{-1} DW for *Haematococcus pluvialis* in red phase. The only red microalgae phenolic content reported was of 1.22 GAE g^{-1} DW for *Porphyridium cruentum* with a value in line with our results for biomass grown in SM. Clearly, the higher polyphenol content observed for *G. sulphuraria* biomass produced in sCBL is strictly correlate to the raw composition in anthocyanin of sCBL as before discussed.

There are only few published studies regarding the identification and quantification of phenolic composition in microalgae species and very interesting was the research carried out by Safafar *et al*. (2015) that analysed the phenolic compounds and quantity in microalgae grown in municipals wastewaters autotrophically.

In UPLC-MS/MS study, simple phenolics and hydroxycinnamic acids (ferulic acid and p-coumaric acid) were detected in *Chlorella vulgaris, Haematococcuspluvialis, Diacronema lutheri, Phaeodactylum tricornutum, Tetraselmis suecica*, and *Porphyridium purpureum* microalgae species (Goiris *et al*., 2014).

The Antioxidant capacity (TEAC) of *G. sulphuraria* biomass grown in SM and in sCBL was of 29.0 mmol kg^{-1} and of 42.2 mmol kg^{-1}, respectively. The high value observed in biomass growth in sCBL is strictly correlate to the higher concentrations of polyphenols (table 4) (in particular anthocyanin), molecules well known in the literature for their antioxidant activity.

Graziani *et al*., 2013 reported an antioxidant activity absolute value for *G. Sulphuraria* biomass of 5.6 $mmolkg^{-1}$, very low respect our data. At the moment, there are no data in the literature on the antioxidant capacity of *G. sulphuraria* or other Cyanidiales.

Goirisi *et al*. (2012) evaluated the antioxidant capacity of antioxidant extracts of 32 different microalgae strains applying ABTS assay and observing data from 4.55 to 69.4 μmol trolox eq. g^{-1} DW, with a value of 5.14 for *Porphyridium cruentum*, very low respect our results.

The release of AA during the digestion was evaluated applying two different methods. In the soluble fraction, the release of the antioxidant activity, according to the DPPH method, was significantly higher during the intestinal phase for sCBL and salivary phase for SM (fig. 3A), while the release of the antioxidant activity according to the method ABTS is significantly higher during the gastric phase for SM and intestinal phase for sCBL (fig. 3B). In the insoluble fraction, the release of AA according to the DPPH Quencher method was significantly higher during the intestinal phase for both samples, while there were no statistically differences in the release of the antioxidant activity according to the method ABTS during the three digestion phases for insoluble fractions.

In the salivary and gastric phases, the antioxidant activities of the soluble and insoluble fraction of *Galdieria* grown in SM and in sCBL, evaluated with DPPH and ABTS methods, were no statistically different. In the intestinal phase, with DPPH method, the values were statistically different both for the soluble fraction (SM 24.8 ± 0.5; sCBL 271±5.3 mmol TE kg^{-1}) and for the insoluble fraction (SM 48±7.0; sCBL 40±0.9 mmol TE kg^{-1}) (fig. 4).

The highest values generally observed with ABTS methods respect DPPH were also found by Shalaby and Shanab (2013) for *S. platensis* and by Thaipong (2006) for guava fruit extracts.

At the moment, there is no data on *Galdieria* biomass antioxidant activity release during in vitro digestion. Carfagna *et al.* (2014) reported the effect induced by a diet of 10% of *Galdieria* on oxidative damage and metabolic changes elicited by acute exercise in rats, observing a reduction of oxidative damage and mitochondrial dysfunction, made it potentially useful even in other conditions leading to oxidative stress, including hyperthyroidism, chronic inflammation, and ischemia/reperfusion.

The high release of AA at the end of in vitro digestion (intestinal phase) can be strictly related to the higher total polyphenol content in Galdieria grown in sCBL, as reported for digested jackfruit and araticum by Pavan *et al.* (2014).

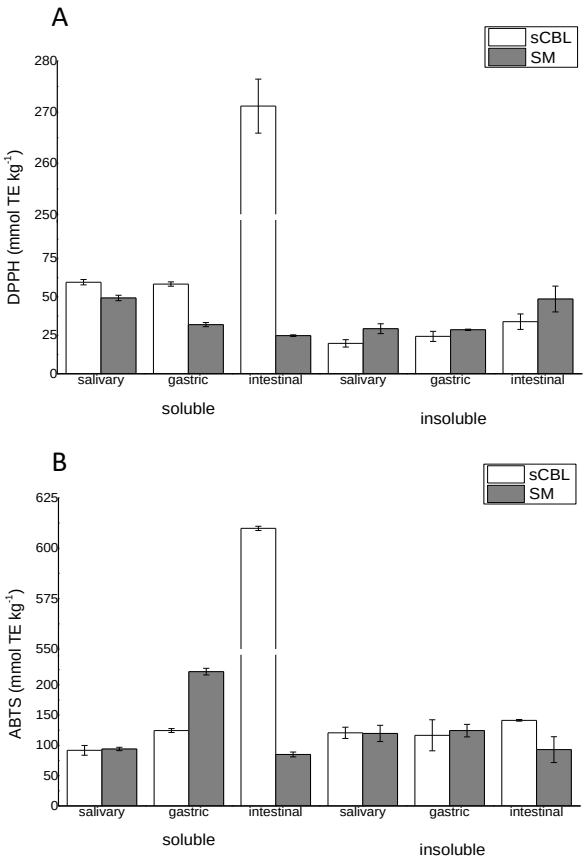

Figure 4. Antioxidant activity (mmol TE kg^{-1}) evaluated with DPPH (**A**) and ABTS (**B**) methods of soluble and insoluble fraction of salivary, gastric and intestinal digestion

4. Conclusion

G.sulphuraria showed a good growth performance in sCBL, that was found to be a good and cheap alternative fertilizer for this microalgae, allowing a complete water recovery. The produced biomass presented a lower productivity respect the control for the different ratio of C:N:P of sCBL and the chemical composition of biomass showed to be influenced by chemical composition of growth medium. Very interesting was the higher polyphenol content and antioxidant activity of sCBL biomass.

The highest antioxidant activity of biomass produced in sCBL is due to the high content of polyphenols, in particular anthocyanins, that were assimilated in a way not known from sCBL as indicated also by red color of biomass.

Galdieria grown in SM showed a high digestibility (79%) in line with value indicated for *Chlorella*, while sCBL biomass probably also for its ash and carbohydrates content showed a total digestibility of 63%. The max release of antioxidant activity was observed for sCBL biomass for soluble fraction at intestinal phase with both determination methods applied and for SM biomass with ABTS method in gastric phase for soluble fraction, and with DPPH Quencher method at intestinal phase for insoluble fraction and at salivary phase for soluble fraction. These data are very interesting in the elaboration of nutraceutical products with specific activity to undress to target organ.

According to data of this paper, the production cost of Galdieri with sCBL can be strongly reduced avoiding the use of expensive C organic source.

The environmental toxicity of sCBL as indicated from N and P removal efficiency and BOD5 and COD reduction was interesting, but the COD is yet enough high requiring others treatment before its disposal in surface water bodies or sewage as required by Italian D.L. 152/06. The ecotoxicity tests repeated after *Galdieria* growth showed a reduction of ecotoxicity of about 45% for both species involved in the test.

The growth in heterotrophy not reached high cell density as reported in other research for low culture temperature (26°) applied with the aim to reduce at minimum the heating energy cost and the evaporation process of culture, that substantially increase the environmental and economic sustainability of biomass production. Furthermore at this growth temperature the *G sulphuraria* aqueous extract showed interesting cosmetician activity (anti-acne) (data not shown) as indicated by an existing patent (MI2014A 000186), developed by Italian company and our research team, and that has aroused the interest of many international cosmetic companies.

G. sulphuraria obtained in sCBL can be used to develop new food ingredients, and thanks to its macro and micronutrient profile, it can be used to design food preparations and to extract some high value phytochemicals (pigments, anthocyanin). Additionally microalgae cultivation on food industry by-product would be a socially responsible way to reintroduce food wastes in the food chain.

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Biological treatment of liquid digestate: the potential of microalgae.

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Chapter 3

Chapter 3: Biological treatment of liquid digestate: the potential of microalgae.

Abstract

The present work evaluated the potential of two freshwater microalgae (*S. obliquus, B. braunii*), a marine diatom (*P. tricornutum),* and a photosynthetic cyanobacterium (*A. maxima*) to grow metabolizing the nutrients from Liquid Digestates (LDs). The LDs were obtained from anaerobic digestion of different organic wastes: zootechnical (LDA), vegetable biomass (LDB) and organic fraction of municipal solid wastes (LD C). In bacth experiements LD A induced the best growth performance with a growth rate not statistically different from the control using standard media (SM) for *S. obliquus* (0.14±0.02 vs 0.15±0.02 g/L/day) and *B. braunii* (0.24±0.01 vs 0.2±0.04 g/L/day), while *A. maxima* and *P.tricornutum* reached a growth rate of 0.2±0.03 and 0.15±0.02 g/L/day, respectively in LDB. LDC gave good performances as a growth medium only using *A. maxima*.
A.maxima and *S. obliquus* showed the best NH_4^+-N removal efficiency with value ranging between 98.9-99.8 %, while *P. tricornutum* and *B. braunii* exhibited values of 79.0 and 88.5 % respectively. Biochemical composition of *A. maxima* and *S. obliquus grown* in repeated batch cultivation using photobioreactor using LDA and LDB as a medium replacement showed an increase of lipid, carbohydrates and ash in both microalgae. The produced biomass showed an interesting biochemical composition, suggesting its application in feed, chemicals and energy sectors.

Keywords: microalgae, digestate, phytoremediation, biomass composition

Highlights

1. Liquid digestates, coming from anaerobic digestion (LDs) of different organic wastes, were used successfully as diluted microalgae growing media.
2. A photosynthetic Cyanobacterium, two Chlorophyceae and one diatom showed good growth performances in liquid digestates from agro-zootechnical waste.
3. About 100% of N from liquid digestate were removed by *S. obliquus* and *A. maxima.*
4. Cultivation using liquid digestates gaves microalgae biomass with valuable chemical composition although they induced an increase in ash content up to 20%.

1. Introduction

Organic streams such as urban organic waste, vegetable by-products and animal wastes are largely used to produce energy by Anaerobic Digestion (AD). In AD acid and methane-forming microorganisms in absence of oxygen and in stable conditions, convert the organic components to methane, carbon dioxide and other compounds (Demirel and Yenigün, 2002). The spent by-product of AD is usually separated in a liquid (LD) and a solid digestate phases. Chemical composition of LD depends largely from the input biomass and often it shows high content of potassium and nitrogen (mainly in form of ammonium) and low levels of phosphorus and organic carbon. (Uggetti *et al*., 2014). LDs represent a treat for the environment for their eutrophication potential and require expensive treatments to lower toxicity and to safely dispose them.
LDs represent a useful source of nutrients in microalgae culture lowering the needs for chemical fertilizers and consequently production costs. As a renewable biomass feedstock, microalgae provide several benefits respect to terrestrial plants such as: (1) higher biomass productivities and CO_2 fixation rate, (2) possibility to be grown in arid or low quality lands, (3)less water consumption with possibility to use brackish and sea water, (4) no needs of herbicides or pesticides, (5)accumulation of lipids which can be extracted and converted to biodiesel (Chisti, 2007), (6) production of different high value phytochemicals, (7) potential for phytoremediation.
Benemann and Oswald, (1996) were reported the cost of nutrients in algae culture systems, affect between 10-20% of the total cost; but several authors as Xia and Murphy (2016) affirmed that the half of the cost and energy input in cultivation of microalgae is represented of the use of artificial fertilizers (e.g., nitrogen, phosphorus). An increase in fertilizer price has immediate consequences on the microalgae production cost thus making any long-term investment plan highly vulnerable to unpredictable factors.
Several studies, regarding the use of wastewaters as algae growth media, have been published (Wilkie and Mulbry, 2002; Kebede-Westhead *et al*., 2006; Chinnasamy *et al*., 2010) showing high efficiency in N and P removal and also of some heavy metals and toxic compounds (Mallick, 2003; Priya *et al*., 2007). For these reasons the wastewaters represent potential sustainable sources of fertilizers and water for microalgae culture especially when industrial scale up of the productions are designed.
Unfortunately, the use of LDs as a nutrient source for microalgae growth may affect growth rates, biomass chemical composition and the growth of undesired organisms, when compared to levels achieved with expensive defined artificial growth media (Gao and Li, 2011; Vasseur *et al*.,2012; Xin *et al*., 2010). For LDs the ammonia content often cause an inhibition of microalgae growth (Cho *et al*., 2013; Källqvist and Svenson, 2003). In fact, the ammonia have the ability to restrict the electrons usable for photosynthesis, causing a inhibition of growth (Konig *et al*., 1987). A problem solving can be the use of an optimal LD concentration and dosing in a way able to support the growth of microalgae avoiding inhibition effects (Uggetti *et al*., 2014; Erkelens *et al*. 2014).

Despite the overwhelming interest in the treatment of wastewaters by microalgae, few research works have been done on the use of LD as nutrient source, highlighting several hurdles that must be solved for their efficient use in industrial plant (Khanh *et al*., 2013; Bjornsson *et al*., 2013; Ras *et al*., 2011; Wang *et al*., 2010; Ugetti *et al*., 2014; Veronesi *et al*., 2015).

The aim of the present study was to evaluate the potential of three different LDs to be used as a fertilizer substitute in the cultivation of the cyanobacterium *Arthrospira maxima*, of the freshwater microalgae *Scenedesmus obliquus* and *Botryococcus braunii* and of the marine diatom *Pheodactylum tricornutum*. Nutrient removal efficiencies from LDs and chemical composition of final biomass were evaluated and compared with standard culture conditions.

2. Materials and methods

2.1. Microalgae strains

The starting cultures of *A. maxima (*SAG 84.79), *S. obliquus (*SAG 276-5*), P. tricornutum (*SAG 1090-1a*)*and *B. braunii* (SAG30.81) were routinely maintained in Erlenmayer flasks of 500 mL using standard media (SMs), respectively Zarrouk (Zarrouk,1966), BG11 (Rippka *et al*., 197), 1/2 SWES (Schlösser, 1993) and CHU13 (Chu, 1942), at 20 ± 2 C°, 100 µmol m^{-2} s^{-1} light by fluorescent lamps and mixing through orbital shaker at 50 rpm.

2.2 Liquid Digestates

The LDs were collected from industrial anaerobic digestors fed with different organic substrates. The zootechnical LD came from AD of water buffalo farming waste enriched with whey coming from a mozzarella cheese factory. The vegetable LD was made by AD of corn, rye and wheat grown on agricultural soil not permitted for food commodities (some hectares around a landfill). The third LD tested in the present study was produced by AD of organic fraction of municipal solid wastes in a plant having a capacity of about 40.000 tonn/year. The LD samples were transported in laboratory at 4°C and measurements of pH, conductivity ($mScm^{-1}$), dry matter (gL^{-1}), BOD5 (mg O_2L^{-1}), COD (mgO_2L^{-1}), Total Solid (TS) and Dissolved Solid (DS) (gL^{-1}) were performed according to APHA, Standard Methods, 1995.
The samples were labeled as: LDA= zootechnical wastes; LDB = vegetable biomass; LDC = organic fraction of municipal solid wastes.
To reduce the shadow effect due to the presence of solid particulate, LDs were filtered progressively from 250 to 50 µm and centrifuged at 2500 g for 20 min. Finally LDs were autoclaved to inactivate any contaminating organism. LDs were analyzed for ammonium ($N\text{-}NH_4$) (Salicylate Method), nitrate ($N\text{-}NO_3$) (Cadmium Reduction), nitrite ($N\text{-}NO_2$) (Diazotization Method) and phosphate (PO_4) (Acid digestion Method,) content using spectrophotometric test kits (HACH –Milano- DR 2400).N-NH4 was analized before and after autoclaving to assess the amount of ammonia lose by stripping.
The LDs were used as fertilizers for microalgae at a variable dilutions on the basis of their NH_4^+-N content and the microalgae species-specific requests. The dilutions were performed in distilled water for freshwater strains (*S. obliquus* and *B. braunii*) and in artificial sea water (Instant Ocean® Sea Salt) at 20‰ for the marine diatom *P. tricornutum*. The LDs for *A. maxima* growth were diluted in tap water enriched with sodium bicarbonate at a concentration of 14 gL^{-1}.

2.3 Experimental design

2.3.1 Batch culture experiments

A first set of experiments was performed in order to evaluate algal growth and microalgae ability to remove N and P in diluted digestates. The selected strains were inoculated at an inoculum:medium ratio of 1:3in 250 mL Erlenmeyer flasks containing LDs at different dilutions on the basis of species-specific microalgae NH_4^+-N tolerance and requests in terms of N, considering the N concentration in SMs. The starting inoculum concentration was from 85 to 100 mg $L^{-1}$and pH was adjusted at 7.5 for all the media.
The NO_3-N concentration of each artificial standard control medium (SM): 410 mgL^{-1}in Zarrouk; 250 mgL^{-1} in BG11; 28 mgL^{-1} in ½ SWESS; 60 mgL^{-1} in CHU13. The LDs were added daily until the achievement of a final N concentration equal to the respective SM as reported in table 1. All the experiments were carried out in triplicate.
The cultures were carried out at 24 ± 1 C°, under continuous illumination of 200 µmol m^{-2} s^{-1}(Light Emitting Diode) and mixing provided by orbital shaker at 150 rpm.
The growth curves were estimated by daily measurements of absorbance at species-specific wavelength (*A. maxima* and *S. obliquus* at 560 nm; *P. tricornutum* at 625 nm and B. *braunii* at 680 nm) until 14 days.
The specific growth rates (µ) of the cultures were calculated using Eq. (1), where *Xt* is the algal dry weight at time *t*, *X0* is the algal dry weight at t=0 and *t-t*0 are days spent to achieve the end of exponential phase.

$$\mu = [\ln(Xt/X0)]/(t - t0) \qquad \text{(Eq. 1)}$$

Growth-curve functions were statistically determined by applying a best-fit procedure: different regression models, were applied to each data set in order to define, on the basis of statistical criteria, the regression model that best described the observed data. Regression curves were obtained and analyzed using the least-square method and the analysis of residuals. At this stage, the most appropriate model was chosen by applying a goodness-fit of criterion (Scholze *et al*., 2001). Statistics were made with the help of Origin1 8 SR2 software (Northampton, MA).
N and P removal efficiency by microalgae was evaluated. At the end of culture growth the media samples were centrifuged at 3500 rpm for 35 min and supernatants, filtered at 0,22 micron analized for NH_4^+-N, NO_3-N, PO_4^{3-}P calculating the percentage of removing dividing the results with the starting values.

2.3.2 Semi-continuous culture experiments

The microalgae, that showed better growth performances in the bacth test (*S. obliquus* and *A. maxima*) in terms of growth rate, % N and P removal efficiency, were cultivated using LDA and LDB in bubble column photobioreactors (PBRs) with a working volume of 10 L with a semicontinuous protocol at 24±1C°. The PBRs were continuously

illuminated by florescent lamps at 200 mmol/m^2/s and the mixing and gas exchange provided through air bubbling at 200 L/h.
Daily assessment of pH, temperature, dissolved O_2 and conductivity were performed. The N and P concentrations in the medium were assessed at harvesting times by spectrophotometric analysis previously described. Microalgae growth was routinely monitored through optical density measures: a linear relationship between OD and dry weight (DW) estimated gravimetrically (APHA, Standard Methods, WPCF 1995) was determined for each strain.
On the basis of growth curves, when the microalgae culture arrived near to the end of the exponential phase, the harvest of 1/3 of culture was carried out (about three times a week) using a continuous centrifuge (4.000 g).After harvesting the supernatant was discharged and diluted LDs were added to each photobioreactor, re-establishing in 20h the starting N concentration at the different SMs levels through a peristaltic pump. An aliquot of each supernatant was collected for chemical analysis, while the wet microalgae biomass was freeze dried to calculate the productivity expressed in mg biomass produced daily for culture liter. Mean productivity P (g DW l^{-1} d^{-1}) for cultivation cycle lasting n days was calculated as:

$$P = \frac{(\sum X/L)}{n} \qquad \text{(Eq. 2)}$$

where X is the dried biomass harvested, L the harvested total volume and n the number of production days.
The ash content of harvest biomass was evaluated following the IRSA-CNR methods (1994) and utilized to evaluate the ash free productivity and biochemical composition.
Culture contaminations were evaluated accurately with an optical microscope at 100X and 400X magnifications. In the presence of fungi, protozoa, rotifers or unwanted algae the cultures were discharged.
All the experiments were carried out in triplicate and average values with standard deviation were reported in the results. Analysis of variance(ANOVA) was applied, using raw data, to test for significant differences in growth among LDs and control (significance level was always set at p = 0.05).

2.4 Chemical analysis
Microalgae biomass was analyzed for chemical composition in terms of lipids, proteins, carbohydrates, pigments and fatty acid profile. The lipid content was evaluated by Bligh and Dyer method(1959): 20 mL of a mixture chloroform : methanol (2 : 1, v/v) were added to 100 mg of the lyophilized sample followed by 20 min of mixing using 1 g of 1 mm glass beads. This mixing was repeated for 10 min after adding 10 mL of chloroform and 20 mL of water. After centrifugation, the organic phase was evaporated and the weight of the residue was determined. The lipid extract prepared for total lipid determination was suspended in hexane and used for the fatty acid methylation. Fatty acids were converted to their methyl esters before analysis. Hexane extract (1 mL) was added with 200 µL of KOH 2 N in methanol for 30 s at room temperature, and 1 mL was injected directly in the GC apparatus. The analysis of fatty acid methyl esters was carried out using a Shimadzu 17A gas chromatograph equipped with a fused silica capillary column (Phenomenex ZB-WAX, 0.50 µm film thickness, 60 m x 0.32 mm i.d.) and a FID detector. Helium was used as carrier gas at 2 mL min^{-1}. The temperature program was 200 °C x 5 min, 200 °C until 230 °C in 15 min (2 °C/min), constant at 230 °C for 30 min. The column, the injector and FID temperatures were 200, 240 and 240 °C, respectively. Identification of fatty acid was made using reference fatty acids methyl esters (Merck, Darmstadt, Germany).Protein content was estimated by the Kjeldahl method using a nitrogen conversion factor of 6.25(AOAC, 1995). Carbohydrate determination was performed on 1 g of samples treated with hydrochloric acid (0.2 M) at 85°C for 1 h. After neutralization by sodium hydroxide, reducing sugars were determined using the Fehling test (AOAC, 1995).
C-Phycocyanin (C-PC) concentration in *A. maxima* was assessed with a spectrophotometric assay developed by Yoshikawa and Belay (2008) with PBS (5 mM - pH 7) as extraction medium. Chlorophyll-a was evaluated according to AOAC (1995) using an acetone/water (85% v/v) solution and centrifuging, the pellet subjected to five extraction and final collected supernatants mixed and reading the absorbance at666 and 642 nm using Jasco V530 spectrophotometer.
Total chlorophyll content in *S.obliquus* was measured with N-N dimethylformamide. 1 g of the lyophilized biomass was diluted in 10 mL of distilled water, followed centrifugation at 4000rpm for 5 min. After centrifugation, the biomass was suspended in N-N dimethylformamide at 4°C for 24h. The supernatant absorbance was evaluated spectrophotometrically at 664 and 647 (Inskeep and Bloom, 1985). The chemical composition in lipids, carbohydrates, proteins and pigments was reported as mean value of biomass and relative standard deviation.

3. Results and discussion
3.1Wastewaters chemical characterization
Chemical analysis after sterilization of the digestates in autoclave, showed a decrease in ammonia concentration of about 50-60% due to a stripping effect as yet observed by several authors (Hansen *et al*., 1998; Gonzàlez-Fernàndez *et al*., 2011; Franchino *et al*., 2013). In table 1 the results of chemical analysis after autoclaving of the three different LDs. LDC showed the highest N-NH_4 concentration and the lowest of PO_4^{-2}, respect to LDA and LDB. LDA exhibited the highest PO_4^2 content and a pH around 7.7.

Table 1. Chemical characteristics of LDs			
Parameters	LD A	LD B	LD C
TS (gL^{-1})	23.4	20.5	52.8
N-NH_4 (mg L^{-1})*	1400	2000	2650
N-NO_3 (mg L^{-1})*	230	890	720
PO_4-P (mg L^{-1})*	716	66	24
BOD5 (mgO_2 L^{-1})	4000	6410	5000
COD (mg L^{-1})	14100	22120	19800
*Analysis carried out on autoclaved liquid digestates			

Although microalgae prefer to use NH_4^+-N rather than NO_3-N (Wang *et al*., 2014; Veronesi *et al*., 2015; Khalaf and Zeinab, 2007; Jongkon *et al*., 2008) with an optimal nitrogen concentration in the range 5 - 110 mg L^{-1}, an high concentration of ammonium can inhibit the microalgae growth in a species-specific concentration range.
Collos and Harrison (2014) observed the effects of high ammonium concentrations on the growth of different microalgae strains and identified for different microalgae families the mean optimal ammonium concentrations, that were 140 mg L^{-1}, 45 mg L^{-1}, 6 mg L^{-1} for Chlorophyceae, Cyanophyceae and Diatomophyceae, respectively and their tolerance to high toxic ammonium levels was 702, 234, 65 mg L^{-1} underlying Chlorophytes as significantly more tolerant to high ammonium than diatoms. The toxicity of ammonium and its suitability as microalgae growth media was evaluated by different researches, that reported data with a great variability on different microalgae species (Uggetti *et al*., 2014; Park *et al*., 2010; Akerstrom *et al*., 2014). In fact, the ammonium concentration is correlate to the free ammonia (FAN), that changed on the base of pH and temperature. For example Akerstrom *et al*. (2014) reported that at 25 °C, FAN accounts for only 0.1% of TAN at pH 6; this significantly increases to 0.6%, 5%, and 36% at pH 7, 8 and 9 respectively. Therefore, pH control is considered an important strategy for reduction of FAN inhibition.

Table 2. Dilution and daily dosing of LDs on the basis of their NH_4-N content for each microalgae strains analyzed

Strain	LD A		LD B		LD C		
	Dil* (%)	Dosing time (days)	Dil* (%)	Dosing time (days)	Dil* (%)	Dosing time (days)	[NH_4^+-N] daily added (mgL^{-1})
P. tricornutum	0.5	4	0.28	4	0.26	4	7
S. obliquus	6	3	3.4	3	3.1	3	84
A. maxima	7.3	4	4.1	4	3.9	4	100.2
B. braunii	1.4	3	0.8	3	0.75	3	20

*Dil= Dilution

To avoid the toxic effect caused by a high concentration of ammonium, and also to reduce the shading effect due to the brown color of the effluents, LDs were progressively diluted until the N concentration of the respective SMs as reported in table 2 with a daily NH_4^+-N provision of 7, 20, 84 and 100 mg L^{-1} for *P. tricornutum, B. braunii, S. obliquus* and *A. maxima* respectively.
growth was the N:P ratio. The N:P ratio was different between the samples with a value of 2.3, 43.8 and 134.2 for LD A, LD B and LD C respectively, indicating that P is the limiting growth macronutrient. The N:P ratio observed on our LD B and C are in line with value reported by Marcilhac *et al*. (2014) for different LDs (ranging between 38-135 N:P).Chemical composition of tested LDs in terms of N and P observed was in line with data published by Uggetti et. (2014).
LD A and LD B showed values in line with their respective SMs: 4,5:1 in Zarrouk, 25:1 in BG11, 3.9:1 in CHU13 and 8:1 in ½ SWES.
Usually, the culture media for microalgae and cyanobacteria have a N:P ratio species-specific between 100:1 to 2:1 but the range of 5.5:1 to 30.5:1 are the most desirable for green algae biomass production. The wide variability in terms of LDs concentration of nutrients highlights the needs of chemical and physical checks before use (even with quick and inexpensive methods).

3.2 Batch cultures

For each strains were reported the growth curves (Fig 1) represented by Logistic function (Growth-Sigmoidal). For all the strains the worst growth performances were observed applying the LD C probably due to its P content, that at the dilution factor applied was always the lowest, ranging between 2 to 0.2 mg L^{-1} and representing the limiting nutrient also respect to the SMs, in which the P concentration was between 4 in 1/2 SWES to 89 mg L^{-1} in Zarrouk.

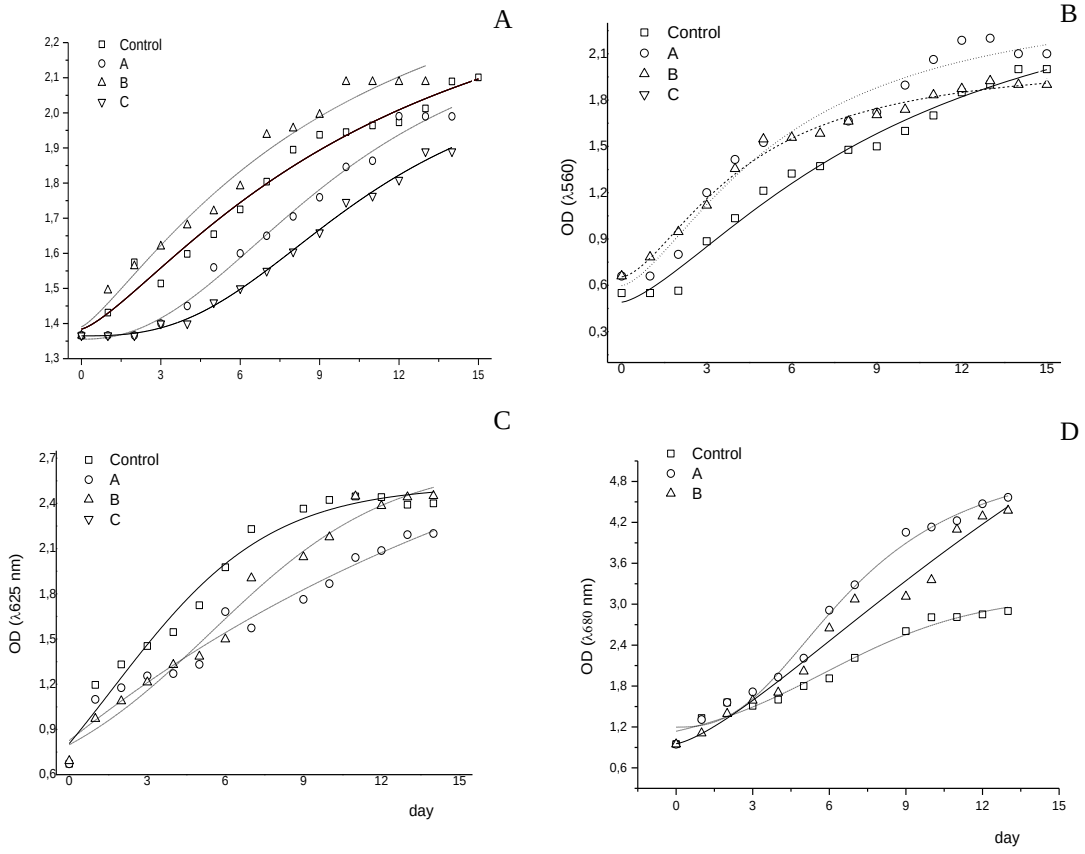

Figure 1. Optical density (OD) variations during cultivation days for **A**) *A. maxima*at ʎ 560 nm, **B**) *S. obliquus*at ʎ 560 nm, **C**) *P. tricornutum*at ʎ 625 nm and **D**) *B. brauniiat* ʎ 680 nm growth on Standard Media and on LDs diluted at the SM N concentration.

Moreover, respect the others LDs it may contain toxic compounds due to the quality of the municipal organic waste input to anaerobic digester, that often presents some impurity which give to the LD a difficult to control and to predict.
In figure 1 **A** the growth curves of *A. maxima* on Zarrouk medium and on LDs media were showed: the best growth performance was obtained with LD B**,** that induced a specific growth rate of 0.2 d^{-1} reaching plateau after 9 days, while the lowest from 1 to 14 days was observed using LD C. The specific growth rate for LD A was obtained from 1 to 14 days like into control. The value of LD A was statistically lower than control (Zarrouk medium), that was 0.18±0.02 d^{-1}, result in line with literature data (Barrocal *et al*. 2010) and not statistically different from LD B (table 3). In Zarrouk medium the N:P ratio was 4.5:1, while the optimum value reported in literature was 7-11:1 (Kebede and Ahlgren, 1996); LD B showed an N:P ratio of 43.8:1 and the growth rate was not statistically different from the control due to the N content in the form of ammonium that speeding the *A. maxima* growth in 9 days respect 14 days of control, however it not overcome the control growth probably for limiting P content, that was 20 times lower respect Zarrouk medium.
The growth curves of *S. obliquus* was reported in figure 1**B**; LD A and LD B showed specific growth rates not statistically different to the control (0.16±0.05 vs 0.15±0.02 d^{-1}). Also in this case for LD B the time to achievement of plateau was lower than the control. For the sample C the growth rate was significantly lower than the control and the others LDs (data not plotted) (table 3).

Table 3. Specific growth rate(day^{-1})to the preliminary test for *A. maxima*, *S. obliquus*, *P. tricornutum* and *B. braunii* utilizing different liquid digestates as growth media

Species	Control (day^{-1})	Growth rate (day^{-1}) **LD A**	Growth rate (day^{-1}) **LD B**	Growth rate (day^{-1}) **LD C**
A. maxima	0.18±0.02	0.1±0.02	0.2±0.03	0.04±0.01
S. obliquus	0.15±0.02	0.14±0.03	0.12±0.05	0.021±0.03
P. tricornutum	0.18±0.04	0.088±0.04	0.15±0.02	0.03±0.01
B. braunii	0.20±0.04	0.24±0.01	0.22±0.05	0.06±0.01

P. tricornutum presented a growth rate in the control of 0.18±0.04 d^{-1}(as showed in figure 1 C). LD B induced a growth rate not statistically different from the control (table 3). Cavounise *et al*. (2015) reported for *P. tricornutum* a growth rate between 0.072±0.04 $day^{-1}$and 0.12±0.02 day^{-1} in standard conditions, data in line with our results. To the best of our knowledge to date only Veronesi *et al.* (2005) reported data on the growth of *P. tricornutum* grown on liquid digestate showing at 1:10 dilution a specific growth rate of 0.24 d^{-1}, a value higher than into control using Guillard f/2 medium (0,10 d^{-1}).

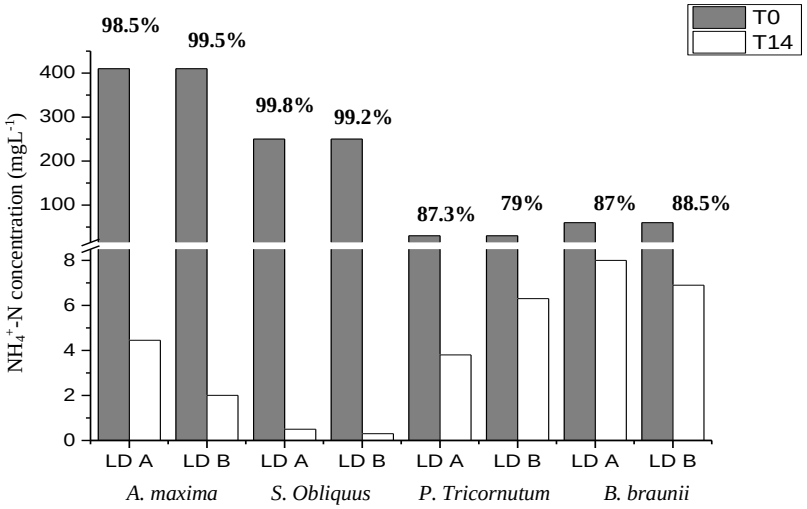

Figure 2. NH_4^+-N concentration evaluated at T=0 and after 14 cultivation days for each strains growth on LD A and LD B. The percentage of removal efficiency (%) are reported.

The control *B. braunii* specific growth rate was 0.20±0.04 d^{-1}, value higher respect to the ones obtained in LD C (data not plotted). LD A and LD B (fig. 1 **D**) showed a specific growth rates not statistically different from the control (table 3).

For *A. maxima* and *P. tricornutum* the LD B represented a good growth media also if the P concentrations were lower respect the respective. For diatoms, the optimum N:P ratio is reported to be of 16:1 (Harrison *et al*. 1977) and they usually show low affinity for P.

P.tricornutum did not divide the cells when the intracellular P concentration dropped below 2 fmol $cell^{-1}$ as described by Chauton *et al* (2013).

For photosynthetic cyanobacteria as opposed to microalgae, the absolute quantity and quality of nutrients influencing more than N:P ratio (Schreurs, 1992). LD B showed an high N:P ratio (43.8), but also the highest BOD5 (6410 mg O_2 L^{-1}) that probably induced a mixotrophic metabolisms by *A. maxima* and *P. tricornutum* and resulting in a better growth performance respect to LD A, that at the dilution factor applied presented a higher P content (13 mg $L^{-1}$for *P. tricornutum* and 181 mg L^{-1} for *A. maxima*) than LD B. For *S. obliquus* and *B. braunii* (Chlorophyceae) the LD A and LD B showed growth rates not statistically different from the control; the highest value was observed for LD A, that showed a N:P ratio of 2.3 value close to CHU13 (3.9), but lower than BG11 (25). Usually ratios within the range of 5.5:1 to 30.5:1 are the most desirable for green algae (Schweitzer *et al*. 2014). LD A at dilution factor applied showed the highest P concentration for *B. braunii* (26 mg L^{-1}vs 14 mg L^{-1}in CHU13) and for *S. obliquus* (110 mg L^{-1} vs 7 mg L^{-1} in BG11).

The N and P removal efficiency in percentage, from t0 to day 14, evaluated for each strains are showed in figure 2 and 3 respectively, where the nutrients concentration at T=0 and after 14 days are reported in bars with respective removal percentage (%). *A. maxima* and *S. obliquus* showed the best NH_4^+-N removal efficiency with value near to 100% for both LDs, while *P. tricornutum* and *B. braunii* exhibited values ranging from 79 to 88.5 % (Fig. 2). The P removal efficiency was high for all the strains with value up 90%, only *S. obliquus* showed a value of 86.1 % in LD A, that presented the highest P content of 110 mg L^{-1} vs 7 mg L^{-1} of the SM BG11. Results achieved showed the good ability of microalgae to remove N and P from liquid digestates until values ranging between 0.3-8 mg L^{-1} for NH_4^+-N and 0.01-17 mg L^{-1} for PO_4^{3-}P.

In Italy the DL 152/06 defines the values of emission limits in surface water and sewage for NH_4^+ (≤15 mg L^{-1} for surface waters and ≤30 mg L^{-1} for sewage) and total P (≤10 mg L^{-1}both in drain into water surface and discharge into the sewage system), this values are in line with our

data for the NH_4^+-N but lower for the P at dilution applied for LDA for

A. maxima, that can be improved changing the dilution applied or adding nitrate to liquid digestate in such a way also to increase the assimilation of the P.
A critical point is to define the correct dilution of digestates for the microalgae growth that make sustainable the microalgae production, considering the quality of digestates and the quantity of LD produced by the anaerobic digesters.

Our results on nutrients removal efficiency are in line with some literature data. Franchino *et al* (2013) reported for *S. obliquus* grown on agro-zootechnical digestate a removal percentage for N ranging from 84 to 94% and for P of about 95 % at different LD dilutions, Marcilhac *et al.* (2014) using the same species, reported a N removal rate from 3.4 to 36.5 mg N L^{-1} d^{-1} with different agro-zootechnical LDs diluted at 10%.

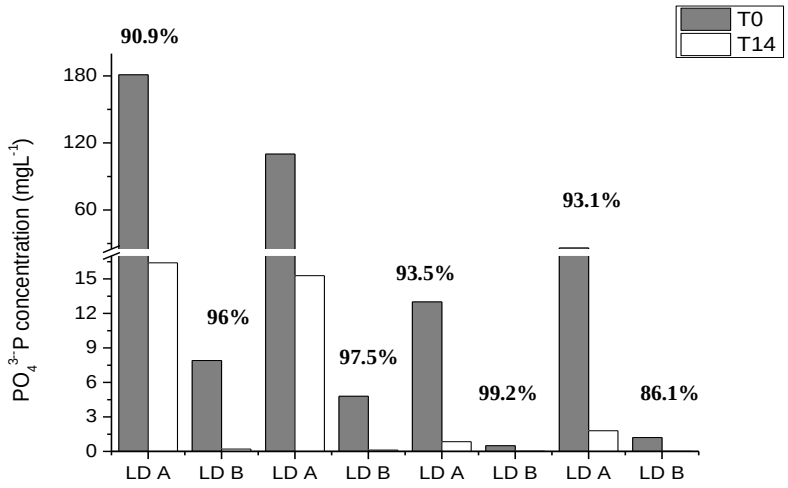

Figure 3. PO_4^{3-}-P concentration evaluated at T=0 and after 14 cultivation days for each strains growth on LD A and LD B. The percentage of removal efficiency (%) are reported.

Kaushik *et al.* (2006), growing *A. platensis* on anaerobically digested distillery effluent diluted at 50% observed a decrease of total N of about 35.4-58%, value lower than our probably for reduced dilution of samples.
The main species studied in literature are *Chlorella spp* and *S. obliquus*, Xia and Murphy (2016) reported the recent trends and performances of microalgae cultivated in LDs with value of Nutrient Removal Efficiency varying from 73 to 100% for total N (except a value of 37% per *Chlorella vulgaris* grown on treated LD) and from 63 to 100% for P.

Table 4. *S. obliquus* and *A. maxima* productivity ($gL^{-1}day^{-1}$) in standard media and in LDs samples in a 10L photobioreactor

	S. obliquus	*A maxima*
Growth medium	Productivity ($mgL^{-1}day^{-1}$)	
Control	70.4±7.4	98.3±5.2
LD B	64.3±4.1	120.2±8.3
LD A	73.4±5.6	101.7±8.0

3.3 Semicontinuous productions using LDs
On the basis of the results in the batch experiments and their low susceptibility to contamination, *A. maxima* and *S. obliquus* were chosen to perform cultivations in semi-continuous using photobioreactors and the digestate LD A and LD B. The productivity values obtained were shown in table 4.
A.maxima reached the highest average biomass productivity in LD B, while using LD A the productivity was not statistically different from the control. For *S. obliquus*, both LD A and LD B showed not significant differences respect to the control.
Data on microalgae productivity grown on LDs are scares and very difficult to compare due to the variable LD composition, % of LD tested, and microalgae strain assessed. Morais and Costa (2007) reported a biomass productivity

for *S. obliquus* between 40 and 140 mg $L^{-1}d^{-1}$ in column photobioreactors of 2 L maintained at 30°C under a 12 h dark/light photoperiod on SM without extra CO_2 supply.
Kaushik *et al. (*2006)studied the growth of *A. platensis* on anaerobically digested distillery waste and the biomass produced, at 50% diluted sample, was significant higher (1.23 mg ml^{-1}) than the standard medium (0.78 mg ml^{-1}) and the others dilutions tested. Phang *et al.* (2000) cultivated *A. platensis* in digested sago starch factory wastewater and observed an average specific growth rate of 0.51 d^{-1}. Xu *et al.* (2015) studied the growth of *S. obliquus* with piggery LD at different dilutions reporting a biomass productivity of 310 mg $L^{-1}d^{-1}$, a very high value respect to our results. Xia *et al.* (2016) reviewed the literature data on the biomass productivities and maximum concentrations of some microalgae cultivated in liquid digestates reporting ranges of 30–670 mg $L^{-1}d^{-1}$ and 400–4800 mg L^{-1} respectively. These values are comparable with or slightly higher than those of photoautotrophic cultivation in synthetic medium. However, the performance can be further improved by removing inhibiting compounds or by adding limiting factors.
The chemical composition of *A. maxima* and *S. obliquus*. cultivated in LD A, LD B and SMs was reported in table 5. Proteins are the main component in *A.maxima* and the values observed in biomass grown in LD A and LD B were not statistically different from value observed in SM. Data of protein content in SM are in line with data reported in literature (50-70% DW) (Devi *et al.,* 1981; Becker, 2007). Our data suggest that protein content, at constant levels of nitrogen into the culture medium and following our production protocol, was not influenced by LDs. Maximum carbohydrate levels in *A. maxima* was reported in SM and the highest lipid content in LD B and the lowest in SM.

Table 5. Biomass compositon (mean %± SD) of *A. maxima* and *S.obliquus* grown on SM and LDs

Components (mg/g)	SM	LD A	LD B	**Components (mg/g)**	SM	LD A	LD B
Protein	587.5±16.2	607.2±18.4	601.8±19.2	Protein	566.5±15.2	571.2±18.1	533.9±19.3
Lipid	57.7±6.4	80.9±3.9	107.5±2.6	Lipid	165.6±9.3	234.1±9.3	263.6±6.3
Carbohydrate	351.5±11.7	311.2±12.4	289.7±10.6	Carbohydrate	173.5±11.1	192.4±7.7	184.3±12.3
Phycocianin	7.5±0.1	8.3±0.3	8.5±0.1	Chlorophyll	6.6± 0.1	7.2±0.3	8.5±0.4
Chlorophyll	13.8±0.2	11.5±0.2	12.4±0.2				

Kim *et al.* (2013) and Markou *et al*., (2012) reported a lipid content for *Arthrospira* sp. grown on SM ranging from 4 to 7% DW. The decrease in carbohydrates and increase in lipid content are strictly correlate with the N:P ratio of LDs: LD A showed a N:P ratio of 2.3 indicating respect SM the N as limiting nutrient. In LD B the N:P ratio was 43.8, so respect SM the limiting nutrient is P with a final concentration of 8 mg L^{-1}. In stress condition, such as macronutrient starvation, microalgae change their metabolism and usually accumulate carbohydrates and lipids, as energy storage. In Markou *et al*. (2012) *A. platensis* under P limitation showed an increase in carbohydrates up to 65% and in lipids up to 7.5% with a reduction in protein content. In LD B it was observed a lipid increase from 5.7 of SM to 10.7 %, but not an increase in carbohydrates that decreased from 35 to 29%. In LD A we observed an increase of lipids (8%) and a reduced carbohydrates content (31%).

Depraetere *et al.* (2015) observed under N deprivation an increase in carbohydrates and a reduction in proteins, but in our semi-continuous production experiment conducted for 30 days the N deprivation was avoided so as a result the same protein levels were achieved, altought different level of lipid and carbohydrates probably because of the different N:P ratios.

Phycocianin content was higher in LDs respect SM (table 5) but not different between LD A and LD B. Chaiklahan *et al.* (2011) reported a Phycocianin content of 6.17 mg g^{-1}, value lower than the our experimental data. Maurya *et al.* (2014) in lab-scale experiment reported a reduction in phycocyanin at increasing light intensity, so it is in line with our results, where the slightly yellow colour of diluted LDs lowering the light availability inducing a phycocyanin content increase.

The fatty acids profile of *A. maxima* cultivated on SM and LDs were showed in table 6.

Table 6. Fatty acids composition (mean %± SD) of *A. maxima* and *S.obliquus* biomass growth in SM and diluted LDs

Fatty acid (%)	SM	LD A	LD B	**Fatty acid (%)**	SM	LD A	LD B
C16:0	45.4±7.4	49.3± 9.9	43.2± 10.1	C16:0	19.2±8.4	16.5± 3.7	20.7± 4.2
C16:1	6.1±0.4	6.3± 1.9	4.7± 0.1	C16:1	2.6±1.9	4.1± 0.0	1.1± 0.5
C18:0	7.4±0.9	7.2± 2.97	6.9± 2.1	C18:0	6.1±2.9	4.9± 2.5	3.7± 1.9
C18:1	6.6±1.7	3.9± 0.6	11.4± 10.9	C18:1	15.1±1.4	30.7± 6.5	31.2± 5.4
C18:2	12.2±1.9	18.7± 0.8	18.8± 4.2	C18:2	7.8±0.4	4.8± 1.1	5.2± 2.7
C18:3 n-6	15.7±0.6	11.5± 5.6	11.7± 2.6	C18:3 n-6	18.0±3.6	11.4± 2.3	9.9± 4.2

The chlorophyll content ranging between 14-11.5 mg g^{-1} data statistically higher than the ones reported by Jiménez *et al.*(2003) where they achieved a chlorophyll content between 6 and 9 mg g^{-1}.
The main fatty acids are palmitic (PA), linoleic (LA) and γ-linolenic (GLA) acids. LA and GLA account for about 30 % of total fatty acids, GLA reported a mean value of about 15.7% in SM. In LDs was observed an increase in LA and a decrease in GLA respect to the SM.
The proximate biochemical composition of *S. obliquus* in complete medium reported in literature (Becker, 2007) was 50-56 % DW protein, 10-17 % DW carbohydrates and 12-14 % DW lipid, data in line with our results (table 5).
S. obliquus showed a lipid content higher in LDs respect SM (7 and 10% more in LD A and LD B respectively) and a protein content not statistically different between the treatments as reported for *A. maxima*. Furthermore in LDs also the chlorophyll content was higher than the control. The fatty acids profile of *S. obliquus* cultivated on SM and LDs were showed in table 6. Chromatographic profiles showed that the main fatty acids are PA, oleic acid (OA), LA and linolenic acid, data in line with Hakalin *et al*. (2014) and in agreement with the data of Shih-Hsin *et al*., (2010) the C16 and C18 fatty acid groups accounted for about 65–70% of the total fatty acids.
In LDs, PA were not statistically different from the control, while it was observed a decrease of linolenic acid and an increase in content of OA respect to the SM in both LDs.
In semi-continuous production a reduction of about 20±2% in N and P removal efficiency was observed for both species in LD A, while in LD B it was noticed a reduction of 23±4% for N. Furthermore it was observed an increase in ash content in *A. maxima* from 9% in SM to 16 and 18% in LD A and LD B respectively. For *Scenedesmus* in SM the ash content reached 7,5% instead of 13 and 16 of 20% in LD A and LD B respectively. Algal biomass harvested from wastewater typically contains 30–50% (dry weight basis) ash content (Yu, 2012). Some authors addressed the physical pretreatment of wastewater such as centrifugation to lower the content of ash into the final algae biomass. Chen *et al.* (2014) reported that the ash content of algae biomass harvested from wastewater was reduced from 28.6% to 18.6% with the pretreatment of centrifugation in line with the results of our work.

4. Conclusion

In conclusion the four microalgae strains (one photosynthetic Cyanobacterium, two Chlorophyceae and one diatom) showed a good growth performance in liquid digestates from agro-zootechnical waste. Liquid digestate from AD of municipal organic fraction waste supported efficiently the growth only of *A. maxima*, probably due to low P content and presence of some inhibiting compounds.

The LDs input in terms of total N resulted winning in biomass productivity of *A. maxima* and *S. obliquus* and on their ability to remove N and P. LDs used to as growth media for microalgae could contribute to a more sustainable production without the use of chemical fertilizers, however it is not a solution that can be applied generically to all kind of LD because of the different quality of input that determines the quality of final liquid digestate. To save water usage and maximize the phytoremediation effect the LD must be used as much as possible with minimum dilution to keep economically and environmentally sustainable the whole microalgae production process.

In the worst scenario if the LDs contains toxic compounds which are accumulated in the microalgae, the resulting biomass could be utilized as a biofuel for energy production via lipid extraction, pyrolysis, anaerobic digestion or for extraction of high value fine chemicals .

Although the biomasses produced with LDs in this study have a high ash content, some of them showed a valuable biochemical composition, suggesting a possible application in feeding sector. Further investigations on accumulation of toxic compounds coming from LDs in produced biomass is necessary to define the application in commercial sectors.

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Productivity and biochemical composition of *Scenedesmus obliquus* and *Phaeodactylum tricornutum*: effects of different cultivation approaches.

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Chapter 4

Chapter 4: Productivity and biochemical composition of *Scenedesmus obliquus* and *Phaeodactylum tricornutum*: effects of different cultivation approaches.

Abstract
The present work evaluated biomass productivity, carbon dioxide fixation rate and biochemical composition of two microalgae species, *P. tricornutum* and *S. obliquus*, cultivated indoor in high technology photobioreactors (HT-PBR) and outdoor both in pilot ponds and low technology photobioreactors under greenhouse in Southern Italy. Microalgae were grown in standard media and in two liquid digestates obtained from anaerobic digestion of agro-zootechnical and vegetable biomass. *P. tricornutum*, cultivated in semi-continuous in indoor HT-PBRs with standard medium, showed a biomass productivity of 21.0±2.3 g $m^{-2}d^{-1}$. Applying nitrogen starvation, the lipid productivity increased from 2.3 up to 4.5±0.5 g $m^{-2}d^{-1}$, with a 24% decrease of biomass productivity. For *S. obliquus* a biomass productivity of 9.1±0.9 $gm^{-2}d^{-1}$ in indoor HT-PBR was evaluated using standard medium. Appling liquid digestates as fertilizers in open ponds *S. obliquus* kept a not statistically different biomass productivity (10.8±2.0 g $m^{-2}d^{-1}$), such as *P. tricornutum* (6.5±2.2 g $m^{-2}d^{-1}$) respect complete medium. The biochemical data showed that the fatty acid composition of the microalgae biomass was affected by the different cultivation conditions for both microalgae.
In conclusion it was found that the microalgae productivity in standard medium were about doubled in HTPBR respect to open ponds for *P. tricornutum* and about 20% higher for *S. obliquus*.

Keywords: Microalgae, CO_2 fixation, biomass productivity, wastewater, photobioreactor, pond

1.Introduction

In the last decades microalgae have attracted a great attention for its huge potential applications in different commercial sectors as nutraceutical, pharmaceutical, cosmetic, food and feed (Buono et al. 2014; Dibenedetto 2013; Gouveia 2011). Because of their high productivity and CO_2-fixation efficiency respect to terrestrial plants, microalgae are considered a promising source of biofuel. To date microalgae represent a source of valuable products such as fats, in particular polyunsaturated fatty acids (EPA, DHA), pigments, protein and carbohydrates with remarkable biological activities. Microalgae are considered also an environmental friendly biomass because they can grow in low-quality, brackish, saline or seawater (Dibenedetto 2012; Dibenedetto and Colucci 2015; Guccione et al. 2014; Pérez- Martínez et al. 2010; Pittman et al. 2011) reducing the use of freshwater and avoid the use of arable land and pesticides for their cultivation. The use of municipal, industrial or agricultural wastewaters, as source of water and nutrients (N, P) is an attractive idea reducing both the microalgae cultivation and the organic wastewaters treatment costs and contributing to make the microalgae biomass production environmentally sustainable. Different microalgal cultivation systems can be applied for large-scale production: open ponds with different design (circular and raceway) and various types of closed photobioreactors (PBRs) e.g. tubular
(vertical, horizontal and helical), flat plate and fermenter (Chisti 2008; Dibenedetto 2011). To reduce the microalgae biomass costs a massive cultivation system with high biomass productivity, low production and downstreaming costs and ease of handling, has to be developed (Abomohra et al. 2014). To date, raceway ponds are the most widespread production systems applied for microalgae biomass production mainly for nutraceutical purpose and has been intensively investigated and optimized over the past three decades (Dibenedetto 2011; Jonker and Faaij 2013; Li et al. 2014) for some species such as *Arthrospira spp* and *Chlorella spp.* The microalgae growth and productivity are closely related to the growth medium composition, light, temperature, gas exchange and culture mixing. Respect to open ponds, PBRs, present a better control of cultivation conditions such as gas transfer, reduced contaminations and less water loss (Jorquera et al. 2010; Li et al. 2008; Posten 2009); on the other hand culture mixing and temperature control require large amount of energy and PBRs still showed high capital and management costs.
Problems related to mixing, harvesting and drying should be further investigated to reduce the energy consumption achieving sustainable productions (Shimamatsu 2004). The use of microalgae providing a combination of CO_2 fixation, biofuels and other chemicals production together with wastewater treatment is a promising alternative between the strategies applied for CO_2 mitigation. *P. tricornutum* is a marine pennate diatom considered an important and innovative potential source of eicosapentanoic acid (EPA) for human consumption and for aquaculture animal feeds and it was also included among potential candidates for biodiesel production (Benavides et al. 2013). *S. obliquus* is a genus of freshwater microalgae, specifically of the Chlorophyceae, that can grow efficiently on different wastewaters, showing good growth performances and it is a promising microalgae for biofuels production (Mata et al. 2013). While many attempts have been made to assess *Arthrospira* spp. outdoor large scale productivity in North Mediterranean countries as in Spain (Jiménez et al., 2003a 2003b) and Italy (Converti et al. 2006; Torzillo et al. 1986; Tredici et al. 1986; Tredici and Materassi 1992), few data exist about outdoor and large scale productivity of *P. tricornutum* and *S. obliquus* at our latitudes (Benavides et al. 2013; Miranda et al. 2012; Torzillo et al. 2012) and how the CO_2 fixation and biomass composition change applying different technologies and fertilizers.
In this research the biomass productivity, carbon dioxide fixation and biochemical composition of *P. tricornutum* and *S. obliquus* were assessed. Cultivations were carried out during two production years (2013-2014) using indoor high technology photo-bioreactors (HT-PBR) and outdoor pilot ponds and low cost/technology PBR (LT-PBR) under greenhouse in Southern Italy (Portici, Naples). The effects of nitrogen starvation and liquid digestates from anaerobic digestion on productivity and chemical composition of microalgae in terms of protein, carbohydrate, lipid content and fatty acids profile were evaluated.

2. Materials and methods

2.1 Microorganisms

Microalgae *S. obliquus* (SAG 276-5) and *P. tricornutum* (SAG 1090-1 a) were obtained from Culture Collection of Algae at the University of Göttingen (SAG), Germany. Stock culture of axenic microalgae strains were maintained routinely by regular sub-culturing at 4 weeks intervals on both liquid and agar slants of BG11 (Rippka et al. 1979) and ½ SWES (Schlösser 1993) standard growth media (SM), respectively for *S. obliquus* and *P. tricornutum*. Culture flasks were incubated at 24±1°C temperature, with continuous light intensity of 90 mol photons m^{-2} s^{-1}. To obtain the inocula for massive productions the cultures were inoculated in increasing volumes.

2.2 Growth media

The culture media BG11 and ½ SWES were applied as Standard control Media (SM), respectively for *S. obliquus* and *P. tricornutum* and the cultivations were carried out also in N Starvation condition (NS). Liquid Digestates (LD) were collected from industrial anaerobic digesters, fed with different organic substrates (vegetable biomass and zootechnical wastes) and were analyzed for chemical composition. Measurements of pH, conductibility (mS cm^{-1}), salinity (g L^{-1}), dry matter (g L^{-1}), Biological Oxygen Demand (BOD5) (mgO_2 L^{-1}), Total Solid (TS) and Total Dissolved Solid (TDS) (g L^{-1}) (according to APHA, Standard Methods 1995), were carried out. The samples were labeled as: LD A (zootechnical wastes) and LD B (vegetable biomass). LDs were analyzed for ammonium ($N\text{-}NH_4$) (Salicylate Method),

nitrate ($N\text{-}NO_3$) (Cadmium Reduction), nitrite ($N\text{-}NO_2$) (Diazotization Method) and phosphate (PO_4) (Acid digestion Method,) (mg L^{-1}) content using a spectrophotometer test kit (HACH –Milano- DR 2400). For biomass production in order to prevent interferences from other microorganisms and to reduce the shadow effect due to the presence of solid particulate, wastewaters were filtered through filters from 250 to 100 μm and then centrifuged at 2500 g and autoclaved. Preliminary tests were carried out at laboratory scale (tubular PBR 10 L) to verify the tolerance and nutrients removal efficiency of each strain to LDs diluted at the same N-concentration of SMs (BG11 and ½ SWESS N-concentration: 0.25 gL^{-1} and 0.028 gL^{-1}, respectively). Before and after the microalgae growth (until stationary growth phase), samples of the LD were analyzed to determine the microalgae macronutrient assimilation (N and P). On the basis of lab-scale results LD A was selected to be used as fertilizer for *S. obliquus* and LD B for *P. tricornutum* pilot scale cultivations in ponds.

2.3 Production technologies and culture conditions

The indoor high technology photobioreactor (HT-PBR) (Treelife ® Microlife, Padova - Italy) is an annular Plexiglas-made vessel with a diameter of 50 cm and a working volume of 250 liters, illuminated with internal light sources, made up of 6x58 W fluorescent lamps. The area occupied by the HT-PBR was about 0.2 m^2, and one PBR was placed in 1 m^2 to calculate the areal yield of the culture. The HT-PBR was equipped with a distribution airline (air flow of 1800 L h^{-1}) characterized by a circular designed submersed PVC tube and with systems of harvesting and filling. The cultivation was carried out in semi-continuous regime at a culture temperature of 23±2°C with a continuous artificial illumination of 174 μE m^{-2}. This technology can operate theoretically 365 days/year. The cultivations were carried out in triplicate for three times for each strain for each medium analyzed. The outdoor productions in LT-PBR and open ponds were carried out under two greenhouses, each of about 100 m^2 and located at a latitude and longitude of 40.816135 N and 14.350268 E. During the summer period, when the global radiation was high a shading of 50% was applied to reduce culture stress by overheating, photo-oxidation and photo inhibition (from June to August). The outdoor production technologies at our latitudes have an operation period of about 275 days/year, due to winter temperature and irradiance values, too low for microalgae profitable production. The low cost/technology PBR (LT-PBR) is a tubular polyethylene soft bag-made vessel with a diameter of 35 cm and a working volume of 100 L, supported by a metal mesh on a fiberglass base. The area occupied by the PBR was about 0.1 m^2, and two PBRs were placed in 1 m^2 to calculate the areal yield of the culture. PBRs were oriented respect natural light to minimize the shadow effect. The air distribution was provided by 220 V diaphragm pump of 40 W, at a continuous air flow of 2400 L h^{-1}, diffused through cylindrical ceramic diffusers (diameter 2 cm – length 7 cm). The cultivation was carried out in triplicate in batch regime, until maximum concentration achievement; in 2014 three runs were performed (in July, September and November) under natural photoperiod (sunshine duration max light 15 h, min 9 h), irradiance and temperature (Fig.1).

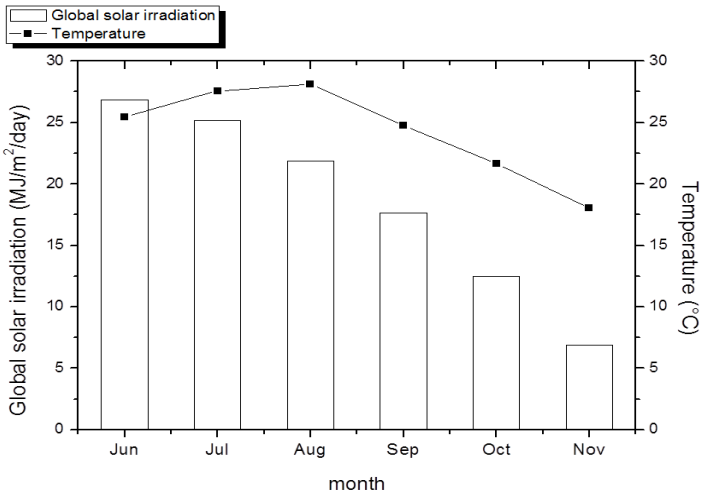

Fig. 1 Temperature culture (line - monthly means) and daily global radiations (bars - monthly means) from June to November (2013-2014) on horizontal surface (ENEA data).

Under each greenhouse, there are three pilot ponds: each concrete rectangular pond (4.6 x 2.4 m) was coated with a white food grade PolyVinyl Chloride (PVC) liner. The height of the culture was kept at 0.25 m with a working volume of about 2.5 m^3 for each pond. For the culture mixing and gas exchange an air bubbling system was used: 220 V diaphragm pumps of 40 W, at a continuous air flow of 2400 L h^{-1} for each pond, diffused through a square designed submersed PVC tube (10 m length, 10 mm diameter, 0.8 mm holes placed every 25 cm of pipe). The cultivation was carried out in semi-continuous regime in triplicate three times from June to November under natural photoperiod for two years (2013-2014). The *S. obliquus* strain was pre-cultured and inoculated into the PBRs and ponds with an inoculum size of about 90–100 mg L^{-1}, while the *P. tricornutum* strain with an inoculum size of about 85–100 mg L^{-1}.

2.4 Culture management and production estimation

Monitoring of the cultures was carried out daily measuring pH, temperature (°C), dissolved O_2 (%) and salinity (‰) and the N and P concentrations (mg L^{-1}) in the medium were assessed before harvesting through spectrophotometer measurements (as described at 2.2 paragraph). Microalgae growth was routinely monitored through optical density measures (OD ʎ 560 nm and 625 nm respectively for *S. obliquus* and *P. tricornutum*) using a spectrophotometer (DR 2400, HACH –Milano): a linear relationship between OD and dry weight (DW) was determined for each strain. The dry weight was estimated gravimetrically (APHA, Standard Methods, WPCF 1995). During harvesting, a pre-filtering phase of 100 μm, to eliminate gross material was performed before the flocculation step with 0.1 N NaOH until a pH of 10.5; after supernatant removing, the microalgae sludge was centrifuged by a continuous centrifuge (MSE 300). After adjusting pH the supernatant was recycled in the culture and the nutrients reintegration applied. On a monthly base, a substitution with fresh medium was made. On a weekly base, the evaporated water was replaced by adding fresh water until reaching the final culture volume.

An aliquot of collected wet biomass (about 3 g) was dried overnight at 105°C until reaching the dry weight, to calculate the final dry weight harvested. The volume of each harvesting for semi-continuous production regime (HT-PBR and ponds) was modulated following the different growth rate and keeping the concentration of microalgae in a range of density between 0.9 and 1.8 OD at ʎ of 560 nm for *S. obliquus* and between 0.7 and 1.8 OD at ʎ of 625 nm for *P. tricornutum*. Three harvests for week of about 1/3 of culture volume were carried out. Based on the total dry weight harvested from each pond, reintegration of fertilizers were carried out following the residual N and P concentration in the culture medium. For LDs the fertilizers reintegration was done on the base of their N total content. Culture contaminations were evaluated with an optical microscope at 10X and 40X because the cultures were not maintained in a strictly sterile environment. Daily global radiation data (MJ m^{-2} day^{-1}) on horizontal surface were taken from ENEA Research Center (National Agency for New Technologies, Energy and Sustainable Economic Development, Portici - NA) located near the cultivation site.

The productivity of the PBRs and ponds was expressed as mean productivity P (g DW m^{-2} d^{-1}) for cultivation cycle lasting n days as:

$$P = (\sum X/\mathrm{m2})/n \qquad (1)$$

where $\Sigma X/m^2$ is the sum of dried biomass harvested from 1 m_2 and n the number of production days.

All the experiments were carried out in triplicate and average values with standard deviation are reported in the results. Analysis of variance (ANOVA) was applied, using raw data, to test for significant differences in growth among production technologies and growth media applied (significance level was always set at $p = 0.05$).

2.5 Biochemical composition of microalgae

Ash content was determined according to the modified method CEN TS/14775 (2004) in particular, 1 g of powered dry biomass, in a porcelain capsule, was ignited and incinerated in a muffle furnace at about 800°C for 8 h. The total ash is expressed as percentage of dry weight (% DW).

Lipids were extracted using 5 g of dry biomass powder pretreated with liquid nitrogen. The biomass was subjected to two extraction cycles (8 h each at solvent boiling temperature) using a traditional Soxhlet apparatus: the first cycle was carried out using 100 mL of hexane as solvent, while for the second cycle a mixture of 100 mL chloroform-methanol 2:1 (v/v) was used in order to extract the remaining polar lipid fraction. At the end of each cycle the extraction solvent was removed by a rotatory evaporator at 60°C and then the residue was placed in an oven at 80°C in order to dry the lipid fraction for 20 min. Then the lipid fraction was cooled into a desiccator under nitrogen and weighted. Results are expressed as total lipids content (% DW ash free).

Carbohydrates in algal biomass were determined according to the NREL Laboratory Analytical Procedure (Wychen and Laurens 2013). In brief, 100 mg of lyophilized algal biomass were hydrolyzed in two-stage with sulfuric acid (2 h at 30°C in 72 wt % sulfuric acid, followed by 1 h at 121°C in 4 wt % sulfuric acid), after which soluble carbohydrates were determined by high-performance liquid chromatography with refractive index detection (HPLC-RID).

Protein content was analyzed by the Kjeldahl method (AOAC 1995) using a nitrogen conversion factor of 6.25. The total organic carbon (TOC) was determined using a TOC analyzer (Shimadzu 5000A instrument – suspension method).

Fatty acid methyl esters (FAMEs) compositions were determined in according to AOCS method - Ce 2-66 (1997). FAMEs were analyzed by using a FOCUS gas chromatograph with a FID detector. Helium was used as carrier gas. The oven temperature was set at 150°C: it was initially held for 13 min at such temperature, that was then increased to 210°C for 13 min and to 230°C for 15 min (at a rate of 5°C/min). Injector and detector temperature were set at 230°C and 250°C, respectively. Fatty acids identification was done by comparison of retention times with certified standards.

3. Results and discussion

3.1 Culture parameters

While the culture parameters in indoor production are strictly controlled and maintained at optimal values for the two strains produced, in outdoor production no control of culture temperature and irradiance was applied, excluding shading during summer season. Culture temperature and solar irradiance data during the production period are reported in Fig.1.

The max monthly mean temperature of the cultures was observed in August (28.1±1.7°C) while the min average temperature was registered in November (18.0±1.4°C). The global solar irradiance (monthly average) on horizontal surface showed a typical Gaussian trend increasing from January (6.8 MJ m^{-2} d^{-1}) to June (27 MJ m^{-2} d^{-1}) and then decreasing from August (22 MJ m^{-2} d^{-1}) to November (6 MJ m^{-2} d^{-1}). The period selected for outdoor microalgae production is expected to be specular for the other months (to the exclusion of December, January and February) in biomass productivity due to the Gaussian trend of temperature and irradiance. *P. tricornutum* requires an optimal growth temperature range of 20-25°C (Benavides et al. 2013). This range of temperature is usually difficult to maintain in open ponds, particularly in summer (Fig. 1), indicating this season as stressful production period in terms of contamination and growth rate. *S. obliquus* is able to grow in a relatively wider temperature range, since its growing rate has limited variation between 14 and 30°C (Xu et al. 2012). This ability is especially important for the outdoor cultivation, because temperature is one of the main environmental parameters, that affects the microalgae growth (Martinez et al. 1999; Voltolina et al. 2005), which usually changes between hot or cold seasons and day or night-time temperatures in species-specific way (Mata et al. 2013).

3.2 Liquid digestate composition

The original LDs colour (100 % raw LD) was dark brown and the most of the nitrogen was in the form of ammonium (Table 1) thus highly available to microalgae (Veronesi et al. 2015; Wang et al. 2014;). In fact, microalgae are able to assimilate more quickly NH_4-N rather than NO_3-N (Jongkon et al. 2008; Khalaf and Zeinab 2007) with an optimum nitrogen concentrations in the range of 1.3 - 6.5 mg L^{-1}. However, it is important to underline that too high concentration of ammonium can induce toxic effect on microalgae growth because being lipid-soluble it easily and very fast diffuses through membranes (Collos and Harrison 2014). Respect to LD A, LD B showed an higher $N\text{-}NH_4$ concentration (5000 mg L^{-1} vs 2800 mg L^{-1}) and the lower PO_4 content (200 mg L^{-1}vs 2170 mg L^{-1}). Both LDs had a pH around 7.7 (Table 1). As consequence the N:P ratio was very different between the samples with a value of 1.4 for LD A and about 29 for LD B indicating that phosphate would act as limiting growth factor for microalgae, that usually grown in lab at N:P ratio species-specific between 100:1 to 1:0.5. The ratio N:P is an important factor for microalgae biomass production and media with ratios within the range of 5.5:1 to 30.5:1 are the most desirable for green algae (Schweitzer et al. 2014). In our work, the N:P ratio in the standard media applied were 25:1 in BG11 (Rippka et al. 1979) and 8:1 in ½ SWES (Schlösser1993). Other parameters as BOD_5 level, salinity and EC have been quantified. The wide variability in terms of LDs concentration of nutrients highlights the needs of chemical and physical checks before use. In preliminary tests the P removal efficiency was about 100 % for $PO^{-2}{}_4$ using LD B for *P. tricornutum* and 32% using LD A for *S. obliquus*. N removal efficiency of about 97 % for *S. obliquus* growth in LD A and about 93 % for *P. tricornutum* growth in LD B. Ji et al. (2013) found for the removal of nitrogen and phosphorus from piggery wastewater effluent using *S. obliquus* values of about 95% and Shen et al. (2015) reported for *S. obliquus*, grown on artificial wastewater, a N removal efficiency of 97.8%, data in line with our results. Craggs et al. (1997) showed for *P. tricornutum* a removal efficiency of 100% in terms of ammonium and orthophosphate from the wastewater, a value slightly higher respect our results.

Table 1 Physico-chemical characteristics of LD A and LD B.

Parameters	**LD A**	**LD B**
pH	7.8	7.6
EC (mS cm^{-1})	9.8	5.4
Salinity (g L^{-1})	5.3	1.9
TDS (g L^{-1})	6.3	2.4
TS (gL^{-1})	23.4	5.0
$N\text{-}NH_4$ (mg L^{-1})[a]	2800	5000
$N\text{-}NO_3$ (mg L^{-1}) [a]	230	890
$N\text{-}NO_2$ (mg L^{-1}) [a]	1	0.5
PO_4 (mg L^{-1}) [a]	2170	200
BOD5 (mgO_2 L^{-1})	4000	6410

[a] samples analyzed after centrifugation

3.3 Productivity and CO2 assimilation

The biomass productivity data of *P. tricornutum* and *S. obliquus*, cultivated with different technologies and on different growth media, were showed in Table 2 and 3 respectively. *P. tricornutum* reached the highest average biomass productivity and CO_2 fixation rate, respectively 21.0±2.3 gm^{-2} d^{-1} and 35.5±4.3 gm^{-2} d^{-1}, during the indoor cultivation in HT-PBR on SM (Table 2). In outdoor systems, under greenhouse, the biomass productivity and the CO_2 fixation rate

observed in LTPBR and in open pond on ½ SWES, were lower than HT-PBR, most probably due to daily and seasonal changes of temperature and solar irradiation (in terms of intensity and sunshine duration), reaching 4.9±2.0 $gm^{-2}\,d^{-1}$ and 8.8±2.0 $gm^{-2}\,d^{-1}$ respectively in LT-PBR, and 10.9±3.7 $gm^{-2}\,d^{-1}$ and 19.8±2.7 $gm^{-2}\,d^{-1}$ respectively in open ponds (Table 2).

These data confirmed the sensitivity of *P. tricornutum* at temperature higher than 26°C: in fact, in outdoor cultivations, without a temperature control, this strain showed the worst growth performance from July to August (Fig. 1). Applying an enriched basal medium and a thermo regulated system (20°C) Benavides et al. (2013) showed from July to September in Florence for *P. tricornutum* a productivity of 11.7 $gm^{-2}\,d^{-1}$and 13.1 $gm^{-2}\,d^{-1}$ in open ponds (10 cm culture height) and in PBRs respectively, data in line with our results for pond production. Griffiths et al. (2009) showed a biomass productivity in open ponds of 0.07 g $L^{-1}d^{-1}$, value higher respect our data (0.04 g $L^{-1}d^{-1}$).

Table 2 Biomass productivity and CO_2 assimilation (mean value ± SD) of *P. tricornutum*

Production technology	**Growth medium**	**(g m^{-2} day^{-1})**	
		Biomass productivity	**CO_2 fixation rate**
250 L HT-PBR	½ SWES	21.0±2.3	35.5±4.3
	N-starvation	16.0±2.0	27.1±3.1
100 L LT-PBR	½ SWES	4.9±2.0	8.8±2.0
Open pond	½ SWES	10.9±3.7	19.8±2.7
	N-starvation	4.5±2.0	7.5±1.8
	LD B	6.5±2.2	10.7±2.3

The higher productivity observed in outdoor PBRs by Benavides et al. (2013) and by other researchers (Grima et al. 1996; Miron et al. 2003; Torzillo et al. 2012) was due to the different culture volume involved, PBR design and technology (thermoregulation system), that in a closed system are essential, and to the different natural condition of irradiance. Torzillo et al. (2012) applying the same system of Benavides et al. (2013) showed a *P. tricornutum* productivity of 14.8±1.6 g $m^{-2}d^{-1}$ in pond outdoor and of 12.5 and 10.8 g $m^{-2}d^{-1}$ in PBR outdoors. During nitrogen starvation (NS) the biomass productivity was reduced respect SM of about 5 g $m^{-2}d^{-1}$ in HT-PBR (24 % reduction) and about 6 g $m^{-2}d^{-1}$ in open ponds (59 % reduction). The literature data on *P. tricornutum* outdoor and indoor productivity under NS regime in large volumes are very scarce; Breuer et al. (2012) in 250 mL flasks registered an average biomass productivity of 486 $mgL^{-1}d^{-1}$ in standard medium and 122 $mgL^{-1}d^{-1}$ in NS condition (with a difference of 364 $mgL^{-1}d^{-1}$), value higher respect our results (67 $mgL^{-1}d^{-1}$in HT-PBR and 16 $mgL^{-1}d^{-1}$ in open pond) but not comparable for the different volumes involved and growth parameters. LD B in open ponds induced a *P. tricornutum* productivity of 6.5±2.2 g $m^{-2}d^{-1}$, value not statistically different than that observed with SM but lower of about 4 g $m^{-2}d^{-1}$ probably due to the high N:P ratio of LD B (28:1), respect to ½ SWES (8:1), while the optimum ratio is reported to be of 16:1 for diatoms (Harrison et al. 1977). In fact diatoms may show low affinity for P and P. *tricornutum* did not divide the cells when the intracellular P concentration dropped below 2 fmol $cell^{-1}$ as described by Chauton et al (2013). Few data underlined the ability of *P. tricornutum* to grow on different kind of wastewaters as described by Chinnasamy et al. (2010) and Craggs et al. (1995), that utilized respectively carpet mill effluents and primary sewage effluent but without assess the biomass productivity. Literature data on CO_2 fixation rate by *P. tricornutum* are scares: Ho et al. (2011) reported a carbon assimilation of 282 $mgL^{-1}d^{-1}$ and a biomass productivity of 150 $mgL^{-1}d^{-1}$ when it was cultivated in batch with 15% of CO_2. In our study the max CO_2 fixation was observed in HT-PBR in SM and at atmospheric CO_2 concentration with a value of 142 $mgL^{-1}d^{-1}$, and a biomass productivity of about 84 $mgL^{-1}d^{-1}$ showing that there is not a linear relation between the CO_2 added to the culture and the CO_2 assimilated by microalgae. This also underlying that a significant amount of CO_2, was lost in atmosphere when the gas is added to the culture, making this practice not environmental friendly. Closed systems respect to open ponds present higher ability to assimilate CO_2 as described by Ho et al. (2011), and this observation were confirmed by the data of our research for the two strains showed in Table 2 and 3. The highest biomass productivity and CO_2 assimilation for *S. obliquus* were recorded in indoor HT-PBR on SM: 9.1±0.9 g $m^{-2}d^{-1}$ (0.05±0.005 $gL^{-1}d^{-1}$) and 17.5±1.7 $gm^{-2}d^{-1}$ (0.07±0.01 $gL^{-1}d^{-1}$) respectively (Table 3). These values are in line with data reported by de Morais and Costa (2007), that measured a biomass
productivity between 0.04 – 0.06 $gL^{-1}d^{-1}$ in column photobioreactors of 2 L maintained at 30°C under a 12h dark/light photoperiod. Ho et al. (2012) in photobioreactors indoor of 1L with an addition of 2.5% of CO_2 evaluated the effect of N starvation, observing a biomass productivity of 626.6 $mgL^{-1}d^{-1}$ and a CO_2 fixation of 1058.9 $mgL^{-1}d^{-1}$, higher respect to those obtained in this paper likely because of little volume involved and the CO_2 addition. In open pond under greenhouse and on SM, *S. obliquus* showed a productivity of 7.5±2.5 $gm^{-2}d^{-1}$ (0. $gL^{-1}d^{-1}$), value lower respect to Gouveia and Oliveira (2009) data (0.09 $gL^{-1}d^{-1}$) obtained in outdoor raceways agitated by paddle wheels from May to August in Portugal. Gomez-Villa et al. (2005), cultivating *S. obliquus* in open ponds in Mexico at CO_2 atmospheric level reported a winter productivity of 9 $mgL^{-1}d^{-1}$ and in summer of 16 $mgL^{-1}d^{-1}$ with a respective CO_2 assimilation of 16 and 31 $mgL^{-1}d^{-1}$. In this case the data are significantly lower than that obtained by us. The great differences in

productivity observed in literature for outdoor productions are strictly correlated to the environmental variability of temperature and irradiance, to different management of culture and to microalgae species-specific growth performance. Appling a NS fertilizer regime a productivity reduction of about 3 $gm^{-2}d^{-1}$ in HT-PBR (36% reduction) and of about 1.5 $gm^{-2}d^{-1}$ in open pond (21.4% reduction) was observed in comparison to SM (Table 3).

Table 3 Biomass productivity and CO2 assimilation (mean value ± SD) of S. obliquus

Production technology	Growth medium	(g m-2 day-1)	
		Biomass productivity	CO2 fixation rate
250 L HT-PBR	BG11	9.1±0.9	17.5±1.7
	N-starvation	5.9±1.2	12.1±1.9
100 L LT-PBR	BG11	6.2±2.5	11.6±2.6
Open pond	BG11	7.5±2.5	14.2±2.1
	N-starvation	5.9±1.9	10.8±1.3
	LD A	10.8±2.0	19.3±0.9

Breuer et al. (2012) in 250 mL flasks registered indoor an average biomass productivity of 719 $mgL^{-1}d^{-1}$ in SM and 767 $mgL^{-1}d^{-1}$ in NS condition (with a difference of 48 $mgL^{-1}d^{-1}$), value higher respect our results (24 $mgL^{-1}d^{-1}$ in HT-PBR and in open pond), strictly correlated to the culture volume, that in our case was of 250L in HT-PBR and 2.5 m^3 in pond and being microalgae growth likely affected by light deficiency. Fertilizing the medium in open ponds with LD A, a productivity of 10.8±2.0 $gm^{-2}d^{-1}$ was assessed with a CO_2 assimilation of 19.3±0.9 $gm^{-2}d^{-1}$. These results were in agree with those proposed by others authors (Arbib et al. 2013; De-Godos et al. 2010; Gomez-Villa et al. 2005; Martinez et al. 2000). This confirms the good adaption ability to wastewaters coming from different industrial origins (Mata et al. 2013) and the good efficiency to assimilate N and P compounds (Martínez et al. 2000) of this strain also in open ponds. LD A showed a ratio N:P of 1.4, very low respect SM (28:1), but it not induced P limitation phenomenon as observed for LD B.

3.4 Biomass composition

P. tricornutum showed differences on biochemical composition (lipids, carbohydrates and proteins) between the indoor and outdoor production systems (Fig. 2) and between the growth media applied. It is worth to note that the *P. tricornutum* is characterized by high ash content (17-22%) (Rebolloso-Fuentes et al. 2007; Zamalloa et al. 2012) depending from its nature (marine diatom) and the cultivation methods applied. On SM the most important component was the protein fraction, which achieved 58.0±1.6 % in indoor HTPBR, 50.5±0.9 % in LT-PBR and 49.5±1.2 % in the open pond (Fig. 2), reaching the highest value of protein productivity in indoor system (12.2±1.3 $gm^{-2}d^{-1}$).

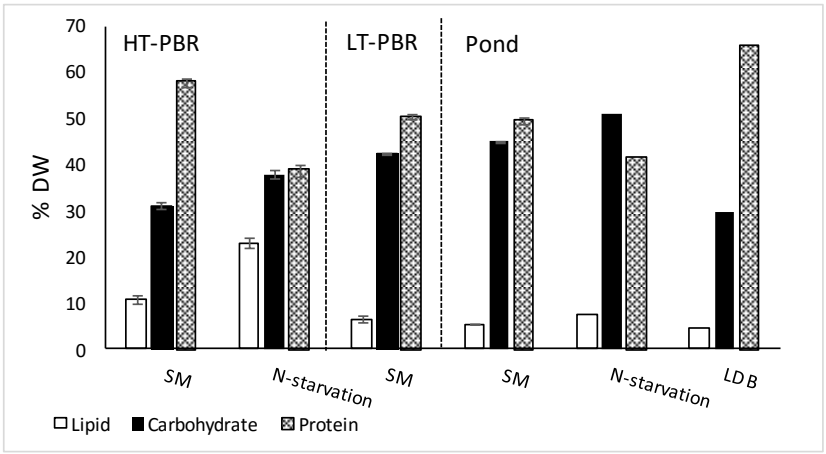

Fig. 2 Lipid, carbohydrate and protein content (% ash free DW) (mean ±SD) of *P. tricornutum* biomass grown in open ponds and PBRs on standard medium (SM), N starvation and LD B.

Miron et al. (2003) evaluated in *P. tricornutum* biomass produced in PBR outdoor in Almeria, Spain, a mean protein content of about 50% DW and observed that protein contents tend to be low in older cells than in the rapidly growing younger cells; Chrismadha and Borowitzka (1994) reported a protein content range of 30–70% DW, values in line with our results. Lower protein content was always observed in N starvation condition both in HT-PBR both in open ponds as expected. Interestingly, the highest value of protein concentration was recorded in open ponds fertilized with LD B, probably due to the high concentration of ammonium Nitrogen (see Table 1) which was highly available to microalgae (Veronesi et al. 2015; Wang et al. 2014).

As far as the lipid content it was observed that in indoor HT-PBR was reached respect the others technologies the highest value of lipid productivity, that on SM was 2.3±0.3 $gm^{-2}d^{-1}$, where the biomass productivity was also high. In outdoor systems on SM the lipid productivity was 0.6±0.1 $gm^{-2}d^{-1}$ in open pond and 0.3±0.1 $gm^{-2}d^{-1}$ in LT-PBR.

It is known that stress conditions due to N deprivation induced a reduction in protein content and promote the conversion of carbon dioxide fixed into energy-rich compounds such as lipids and carbohydrates (Ho et al. 2013). This influenced negatively the cell growth decreasing the biomass productivity and resulting in lower lipid productivity as yet observed by Procházková et al. (2014). Using NS regime in HT-PBR it was observed that the protein content and productivity were lower respect to SM (38.8±0.9 % and 6.2±0.8 $gm^{-2}d^{-1}$ espectively), while the carbohydrate fraction (37.9 %) was comparable with that observed in the SM (Fig. 2).

Nitrogen starvation increased the lipid content of *P. tricornutum* during the cultivation in indoor HT-PBR: a gradually increase was observed reaching the maximum lipid content of 31.9 % after 14 cultivation days (Fig. 3), however a decrease was observed if the cultivation was prolonged more than two weeks.

In particular, as reported in Figure 3 an increase of non-polar lipids respect to the polar lipids was observed. Similar results was observed by Bondioli et al. (2012) with a different algal strain (*Nannochloropsis* sp), where the major lipids were represented by TAGs (non-polar lipids) under nitrogen starvation. The results obtained are in agreement with the assumption that under nutrient limitation algae may react by storing surplus energy as neutral lipid, without an increase of structural lipids (polar lipid) (Rodolfi et al. 2009; Sharma et al. 2012). Due to reduced cell division rate a decrease of biomass productivity and CO_2 assimilation were observed compared with SM, showing data of 16.0±2.0 $gm^{-2}d^{-1}$ and 27.1±3.1 $gm^{-2}d^{-1}$ respectively, while the lipid productivity average was 3.7±0.5 $gm^{-2}d^{-1}$ about 1.18 $gm^{-2}d^{-1}$ higher than the observed value in SM (2.3±0.3 $gm^{-2}d^{-1}$).

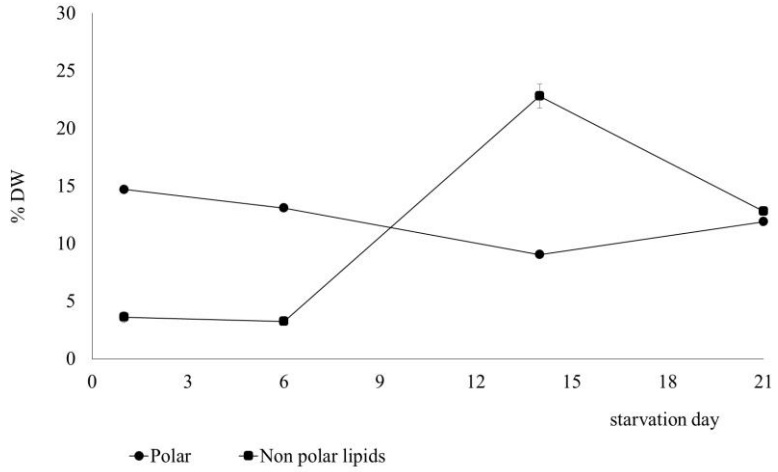

Fig. 3 Lipid content trend in *P. tricornutum* under nitrogen starvation in indoor HT-PBR

A lipid content around 10-25% DW was reported by different authors (Chauton et al. 2013; Yao et al. 2014; Torzillo et al. 2012; Wawrik et al. 2010). Griffiths et al. (2009) reported from a review of literature data a lipid content of 21% in complete medium and 26% in N starvation, while we observed in HT-PBR a variation from 10.8 % DW in SM to 23.1 % DW in NS.

N Starvation in open pond did not affect significantly the lipid content but only the biomass productivity and CO_2 assimilation, which were 4.5±2.0 $gm^{-2}d^{-1}$ and 7.5±1.8 $gm^{-2}d^{-1}$ (Table 2) respectively, and lower than that observed in SM and in indoor HT-PBR in NS condition. *P. tricornutum* biomass obtained in LD B showed a protein fraction of 65.6±0.7 %, while carbohydrate and lipid fraction were 29.9±0.1 % and 4.4±0.2 % (Fig. 2). The most abundant fatty acids extracted from *P. tricornutum* were: saturated C16:0 (palmitic acid), monounsaturated C16:1 (palmitoleic acid) and poly-unsaturated C20:5 (eicosapentaenoic acid, EPA) (Table 4).

Table 4 Fatty acid composition of *P. tricornutum* cultivated in PBRs and open pond under different growth medium (SM= standard medium; NS= nitrogen starvation; LD B= liquid digestate B).									
Production technology/ growth medium	**C14:0 (%)**	**C16:0 (%)**	**C16:1 (%)**	**C16:2 (%)**	**C18:0 (%)**	**C18:1 (%)**	**C18: 2 (%)**	**C18:3 (%)**	**C20:5 (%)**
HT-PBR SM	5.8±0.3	19.3±0.8	41.5±1.6	-	1.5±0.2	4.6±0.2	1.1±0.1	2.9±0.2	12.6±0.9
HT-PBR NS	4.9±0.3	43.5±1.7	33.9±1.7	-	1. 8±0.2	7.1±0.5	0.6±0.1	1.1±0.1	10.1±0.1
LT-PBR SM	7.8±0.42	25.5±0.8	24.8±0.9	-	1.4±0.1	14.1±0.9	-	6.4±0.3	19.6±1.2
Open pond MS	13.2±0.9	23.4±1.1	21.9±1.3	5.2±0.1	2.7±0.2	5.3±0.2	1.2±0.2	-	19.7±0.9
Open pond NS	9.5±0.5	20.2±1.0	20.6±0.8	6.3±0.4	3.4±0.3	12.5±0.9	2.9±0.2	-	17.5±0.7
Open pond LD B	6.7±0.4	15.2±0.9	24.2±0. 6	7.9±0.3	1.5±0.1	6.5±0.9	2.8±0.2	-	28.7±0.9

Low concentrations of C18:1 (oleic acid), C16- and C18- polyunsaturated fatty acids were observed. EPA, one of the omega-3 fatty acids used in the human and animal diet, was produced during cultivation in SM at a concentration of 13 - 20 %. This value is lower respect to the data reported by Torzillo et al. (2012) who reported a 35 % of EPA in both using PBRs and open pond. When microalgae was cultivated on NS condition in HT-PBR, the relative abundance of EPA decreased slightly as described also by Breuer et al. (2012) that reported a reduction of about 11 % (from 24 to 13 %): a significantly reduction was observed from 13 to 10 % in HT-PBR and from 20 to 18 % in open ponds, differences of about 2-3%, lower than reported by Breuer that worked indoor using small volumes. The decreasing of C20:5 acid, was compensated by an increase of the concentration of C16:0 and C18:1 fatty acids, data in line with Breuer et al. (2012) for C16:0 but not for C18:1. During the cultivation in open pond using NS condition, the decrease of C20:5 was not observed; this was justified by the absence of the lipid increase, as before described. During the cultivation in open ponds on LD B, respect to the SM was observed a decrease of SFAs (saturated fatty acids) (from 39.3 to 23.4 %) and an increase of unsaturated fatty acids (from 53.3 to 70.1 %). This is also a remarkable features of the use of this type of wastewater as in many case the unsaturated fatty acid are useful as building block for bioplastic or for nutritional supplements. Microalgae cultivated in outdoors achieved high content of PUFAs (polyunsaturated fatty acids) (in the range between 26-39 %) and lower MUFAs (monounsaturated fatty acids) (in the range between 27-39 %) compared to those obtained in indoor HT-PBRs (PUFA 12-16 %; MUFA 40-46 %). Moreover, a higher SFAs was observed in microalgae cultivated outdoor in NS (50.18 %). Looking at the biochemical composition of *S. obliquus* in indoor HT-PBR grown in SM the biomass showed the lowest lipid content (6.9±0.1 % DW) (Fig. 4) but, respect to the outdoor systems, reached the highest protein and carbohydrate productivity (4.2±0.4 g m-2d-1 and 4.3±0.4 g m-2d-1 respectively).

Biomass produced in HT-PBR in NS conditions presented respect to the cultivation in SM, a reduction in protein content from 46.3±1.4 to 33.4±0.4 % DW, whereas the lipid and carbohydrate contents increased from 6.9±0.1 to 10.2±0.3 % DW and from 46.8±1.1 to 56.4±0.8 % DW (Fig. 4) respectively. This value are in line with the results by Ho et al. (2012), during the cultivation in batch system under nitrogen starvation. During the cultivation in open pond in NS, the decreasing of the biomass productivity

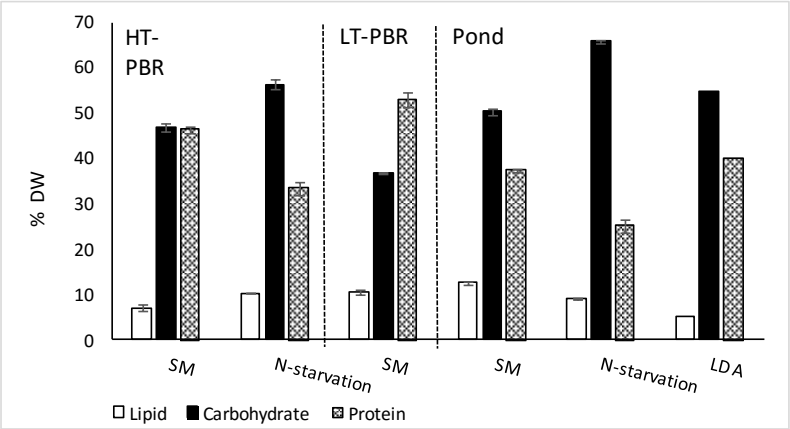

Fig. 4 Lipid, carbohydrate and protein content (% ash free DW) (mean ±SD) of *S. obliquus* biomass grown in open ponds and PBRs on standard medium (SM), N starvation (NS) and LD B.

(5.9±1.9 $gm^{-2}d^{-1}$), CO_2 assimilation (10.8±1.3 $gm^{-2}d^{-1}$) and protein fraction (25.0±0.28 % DW) was compensate by an increase of the carbohydrate fraction (65.9±0.7 % DW), without observing an increase of the lipid fraction (Fig. 4). Interestingly, the composition of microalgae grown in open ponds with LD-A did not change significantly compared to SM: the protein was 40.0±1.3 % (vs 37.3±1.6 % DW) the carbohydrate content was 54.9±0.3 (vs 50.4±0.3 % DW); while the lipid content decreases from 12.6±0.6 to 5.1±0.1 % (Fig. 4). Abou-Shanab et al. (2013) reported a 31 % lipid content after 20 days of batch-mode cultivation of *S. obliquus* in sterilized piggery wastewater. These results are higher than those obtained in the present study. However, that work was conducted in batch mode where the medium probably became nutrient-depleted favoring stress

condition and thus increased lipid content. Numerous studies have demonstrated that microalgae are able to accumulate high lipid content under stressful conditions (Xin et al. 2010), however in our experiments a significant increase in lipid content under NS conditions was observed only in HTPBR. The proximate biochemical composition of *S. obliquus* in complete medium reported in literature (Becker 2007) was 50-56 % DW protein, 10-17 % DW carbohydrates and 12-14 % DW lipid, data in line with our data only for lipid content. The main fatty acids of *S. obliquus* were saturated C16:0 (palmitic acid), mono-unsaturated 18:1 (oleic acid), and poly-unsaturated 18:2 (linoleic acid) and 18:3 (linolenic acid) (Table 5). During the NS cultivation in indoor HT-PBR was observed a decrease of SFAs (from 30 to 23 %) and MUFAs (from 44 to 31 %) counterbalanced by an increase of PUFAs (from 26 to 45 %), respect to the standard medium (Table 5).

Table 5 Fatty acid composition of *S. obliquus* cultivated in PBRs and open pond under different growth medium (SM= standard medium; NS= nitrogen starvation; LD B= liquid digestate A).								
Production technology/ growth medium	**C16:0 (%)**	**C16:1 (%)**	**C16:2 (%)**	**C16:3 (%)**	**C18:0 (%)**	**C18:1 (%)**	**C18:2 (%)**	**C18:3 (%)**
HT-PBR SM	27.8±0.1	8.4±0.2	2.7±0.1	4.7±0.1	2.3±0.1	35.7±1.1	7.5±0.1	10.7±0.3
HT-PBR NS	21.0±0.1	6.1 ±0.1	2.4±0.1	9.7±0.5	2.4±0.1	25.4±1.0	18.5±0.4	14.6±0.3
LT-PBR SM	27.1±0.2	4.1±1.1	3.3±0.1	11.2±0.2	3.7±0.1	16.3±0.9	10.5±0.2	21.6±0.4
Open pond SM	20.6±0.1	2.7±0.1	3.1±0.1	11.3±0.7	2.1±0.1	19.3±0.8	8.5±0.1	31.0±0.9
Open pond NS	19.6±0.1	2.0±0.1	1.4±0.1	7.9±0.2	2.5±0.1	39.1±1.3	9.1±0.4	16.1±0.3
Open pond LD A	20.7±0.2	3.6±0.1	2.7±0.1	6.8±0.2	2.5±0.1	31.4±1.1	11.9±0.8	14.8±0.1

Outdoor the biomass showed an increase in C18:1 when a NS regime was applied, as described by Breuer (2012); in HT-PBR this trend was not observed. The highest level of C18:3 was assessed in biomass cultivated in SM in LT-PBR (21.6±0.4 %) and in ponds (31±0.9 %). In open pond with LD A, the content of MUFAs (34.9 %) was higher than the content in the biomass cultivated in SM (22 %), where PUFAs were abundant (53.9 %). Like *P. tricornutum*, also in *S. obliquus* cultivated outdoor was achieved a higher content of PUFAs and SFAs and a lower content of MUFAs, compared to those obtained under indoor conditions (table 5). As described by Breuer et al. (2012) in the oleaginous strains in N starvation condition it is possible to observe an accumulation of TAGs, with a different fatty acids composition than that of functional and structural lipids. In the non-oleaginous strains this could indicate a shift in lipid class composition, for example a reduction in thylakoid membrane content. Strains that accumulated the largest amounts of fatty acids also showed the largest change in fatty acid composition. The biochemical composition data have been evaluated to assess their potential use for the production of biofuels, chemicals and omega-3, and as animal feed and human food (Dibenedetto et al submitted).

4. Conclusions

P. tricornutum and *S. obliquus* can be successfully grown in both open ponds under greenhouse in South of Italy and HT-PBRs, when a semi-continuous regime was applied. However the use of HT-PBR imply high costs of investment and management. Our data showed that satisfactory productivity can be obtained in open ponds under greenhouse with much less investment. Looking at the cultivation with the use as culture medium of liquid digestates from anaerobic digestion they provide good biomass and CO_2 fixation performance suggesting that the optimization of the cultivation parameters in low technology production plant is the optimal way to achieve profitable tradeoff between production costs, environmental sustainability and value of the microalgae biomasses. Even if the microalgae showed at lab scale a flexibility of their metabolism, which could be adapted to produce specific molecules, it's regulation in big volumes seems to be more difficult probably due to the complex biological systems that come into play in large scale productions. Finally, the biomasses produced outdoor showed for both strains the higher PUFA content respect the indoor cultivations, while the cultivation in NS conditions showed the lower PUFA content respect to standard medium.

The EPA production by *P. tricornutum* in the range from 20 to 28 % also using wastewater as medium, suggests its application in nutraceutical, food and feed sectors, while *S. obliquus* for its ability to grow on wastewater and its resistance to contamination and changes in temperature can be grow very profitable for both wastewaters depuration and animal feeding purpose.

In conclusion, it was found that the microalgae productivity in standard medium were about doubled in HTPBR respect to open ponds for *P. tricornutum* and about 20% higher for *S. obliquus*. However, the performance obtained using ponds under greenhouse and wastewater as growth medium are satisfactory and guarantee at our latitudes, the best trade-off between the production yield and the investment, maintenance and management costs.

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Appendix 1 Duplicate of participation in conferences, posters and abstracts
Appendix 2 List of publications and participation in conferences

UNIVERSITY OF PERUGIA

Department of Agricultural, Food and Environmental Sciences

XX Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology

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Sustainable production of microalgae for feed purposes: the cases of *A.maxima and S.obliquus*

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The aim of the present study was to evaluate the potential of different microalgae species to produce renewable biomass and at the same time removing and recycling nutrients from Liquid Digestates (LD). *Arthrospira maxima* and *Scenedesmus obliquus* were choosen for this study as possible candidates for biomass production with feed purpose. Two Liquid Digestates (LD) collected from industrial Anaerobic Digesters, fed with different organic substrates (vegetable biomass and urban organic waste),were analyzed.
Finally, the removal efficiency (RE) in terms of P and N and the biochemical composition of microalgae was assessed.

Produzione sostenibile di microalghe per la mangimistica: *A.maxima* e *S.obliquus*

Lo scopo del presente lavoroè stato quello di valutare la crescita di diverse specie microalgalisu Digestati Liquidi, valutandone l'efficienza di rimozionedei nutrienti, la produttività e la composizione biochimica al fine di ottenere biomasse utilizzabili nel settore della mangimistica.
Arthrospira maxima e *Scenedesmus obliquus* sono state selezionate come possibili candidateper il raggiungimento di tali obiettivi.Sono stati analizzati due differenti LDs raccolti da digestori anaerobici industriali, alimentati con substrati organici diversi (biomasse vegetali e rifiuti organici urbani).
Infine, è stata valutata sia l'efficienza di tali microalghe nella rimozione dei nutrienti(*phytoremediation*), sia la loro composizione biochimica.

Key words: Liquid digestate, microalgae, biochemical composition, removal efficiency, phytoremediation.

1. Introduction

This oral communication reports the main results of the following three activities directed to:
A1) determine productivity of *A.maxima* and *S.obliquus* growth on Liquid Digestates from vegetable biomass (LD1) and urban organic waste (LD2);
A2) phytoremediation capacity and the chemical nutritional characterization of microalgal biomass;
A3) ecotoxicity tests with *D. magna* and *P. Subcapitata* on liquid digestates before and after microalgae growth.

2. Materials and methods

2.1 Organism and cultivation

LDs were filtered grossly and analyzed for: pH, salinity, dry matter, Total Organic Carbon (TOC), Total Solid (TS) (according to APHA, Standard Methods, 1992); the data were shown in Table 1.
A.maxima was grown and maintained routinely in standard media Zarrouk (Zarrouk,1966),under continuous artificial illumination of 200 µmol m^{-2} s^{-1}(Light Emitting Diode) and the culture mixing was provided with an orbital shaker (150 rpm rotation speed). The pH was set at 10.5 and monitored daily, while the temperature was maintained at 24 ± 2 °C.

Table.1 *Chemical and physic chemical parameter of liquid digestate from vegetable biomass and urban solid waste*

Parameter	LD1	LD2
pH	7.68	4.1
EC (mS/cm)	5.4	1.2
Salinity (g/L)	1.9	0.64
Tds (g/L)	2.4	0.77
Total solids g/L	0.46	52.8
N-NH4 (mg/L)*	5000	5300
N-NO3 (mg/L)*	890	720
N-NO2 (mg/L)*	0.47	0.36
PO4 (mg/L)*	200	73
BOD5 (mgO_2/L)	6410	5000

S.obliquus was grown and maintained routinely in standard media BG11 (Rippka *et al.*, 1979, modificated by Rippka and Herdman, 1993), under the same conditions described for *A. maxima*. The temperature was maintained at 22 ± 2 °C.

In a preliminary test the strains were inoculated at a ratio of 1:4 in 250 mL erlenmeyer flasks containing 150 mL of liquid digestates (different dilution factors between 0.1 and 50 % v/v). The strains were cultivated in batch for 15 days, in order to define the growth-curves. The culture growth was estimated experimentally by measuring the optical density (OD) at species-specific wavelength (λ560 nm), using a spectrophotometer (HACH –Milano- DR 2400): a linear relationship between OD and dry weight (DW) was determined.

After the preliminary test, *A.maxima* and *S.obliquus* were cultivated in semi-continuous in tubular photobioreactors bubble column (PBRs) (diameter 14 cm, height 24 cm and a working volume of 10 L) and incubated at 23±2C°. The PBRs were continuously illuminated by 8000–10,000 lux LED lights (WD-TM-D35W, Widen Photodiode Technology Co.,China) and the culture mixing was provided through air bubbling.

On the basis of growth curves defined in the preliminary test, when the microalgae culture arrived near the end of exponential phase, the harvest of 1/3 of culture was carried out (about three times a week) with a continuous centrifuge (10.000 g). Each supernatant was collected for chemical analysis and ecotoxicology tests, while the wet microalgae biomass was frozen and dried in lyofilizer to evaluate the productivity (mg of dried biomass/L/day).Mean productivity P (g DW/l/d) for cultivation cycle lasting n days was calculated as (1):

$$P = \frac{\Sigma(X/Lt)}{n} \qquad (1)$$

where X is the sum of dried biomass harvested, Lt the total liters centrifuged and n the number of production days.

All the experiments were carried out in triplicate and average values with standard deviation were reported in the results. Analysis of variance (ANOVA) was applied, using raw data, to evaluate significant differences in growth among LDs and control (significance level was always set at $p = 0.05$).

2.2 Chemical biomass composition

Before and after the microalgae growth, the LD samples were analyzed for total nitrogen, ammonium ($N\text{-}NH_4$), nitrate ($N\text{-}NO_3$), nitrite ($N\text{-}NO_2$) and phosphate ($P\text{-}PO_4$) content, using a spectrophotometer (HACH –Milano- DR 2400) to define the efficiency of microalgae phytoremediation. Microalgae biomass was analyzed for lipid content (Bligh and Dyer, 1959) and protein concentration (AOAC, 1995). Carbohydrate determination was performed on 1 g of freeze dried samples treated with hydrochloric acid (0.2 M) at 85°C for 1 h. After neutralization by sodium hydroxide, reducing sugars were determined using the Fehling test(AOAC, 1995).

2.3 Ecotoxicity assays

The ecotoxicity was evaluated by performing a battery of tests with organisms belonging to different trophic levels and using ISO standards methods: *D. magna* (UNI EN ISO 6341:2011), *P. subcapitata* (UNI EN ISO 8692: 2005). The results were expressed in terms of EC50, EC20 and EC1 estimated with the EPA ICp procedure (US EPA 1993).

3. Results and discussion

3.1 Ecotoxicology study

The ecotoxicological effects of LDs were reported in Table 2 in terms of EC50, EC20 and EC1.

The most toxic sample for both organisms utilized was the LD2 that induced an EC 50 at a concentration below to 0.003% (v/v).

Table.2 *Ecotoxicological effects of the liquid digestates (LD1 and LD2) from anaerobic digestion expressed in terms of EC50, EC20 and EC1. EC50s, EC20s and EC1s were estimated with the EPA ICp procedure (US EPA 1993). Values are the upper and lower limits of 95 % confidence interval*

Ecotoxicological test LD1	*EC50 (% v/v)*	*EC20 (%v/v)*	*EC1 (%v/v)*
Daphnia magna 24h	*2.9375 [2.6250-3.6364]*	*2.3750 [2.2500-3.0000]*	*2.0188 [2.0100-2.0375]*
P. subcapitata	*2.6818 [2.5000-2.9375]*	*2.2727 [2.2000 -2.3750]*	*2,0136 [2.0100-2.0250]*
Ecotoxicological test LD2	*EC50 (% v/v)*	*EC20 (%v/v)*	*EC1 (%v/v)*
Daphnia magna 24h	*1.5000 [1.5000-1.5000]*	*1.2000 [1.2000-1.2000]*	*1.0100 [1.0100-1.0100]*
P. subcapitata	*0.2355 [0.0665-0.3997]*	*0.0073 [0.0054-0.0311]*	*0.0004 [0.0003-0.0006]*

The most toxic effects observed for the sample from anaerobic digestor feed with urban organic fraction (LD 2)was probably due to the differentiation level of organic waste input, that for municipal plant can present a grade of impurity. The removal efficiencies (% RE) of *A.maxima*, after fourteen-days of culture, were 97.85% for N-$NH_3$and 98.23% for phosphates while, *S.obliquus* showed a RE of 98.2% for N-$NH_3$and 96.9% for phosphates. Values in line with the literature data (Martinez *et al.*, 2000).

3.2 Algal growth in the LDs

In Fig.1 (A and B)growth curves of *A.maxima* grown on1% and 2% v/v liquid digestates were shown.
At the 1% LDs dilution, productivity control of *A. maxima* was 98 mg/L/d, in according to the literature data (Fatma *et al.*, 1994; Xin *et al.*, 2011). In particular, the productivity data of the sample LD2 (98mg/L/d) was not statistically different from the control. The productivity data of the sample LD1 (65 mg/L/d) was statistically different from the control.
At the 2% LDs dilution, productivity control was 190 mg/L/d not in line with literature reports (Fatma *et al.*, 1994; Xin *et al.*, 2011). The productivity data of the sample LD1 (120mg/L/d) and of the sample LD2 (101mg/L/d) were statistically different from the control. *A. maxima* showed a significant biomass productivity at 2% diluition (v/v).
In Fig.2 growth curves of *S.obliquus* grown on1% v/v liquid digestates were shown.

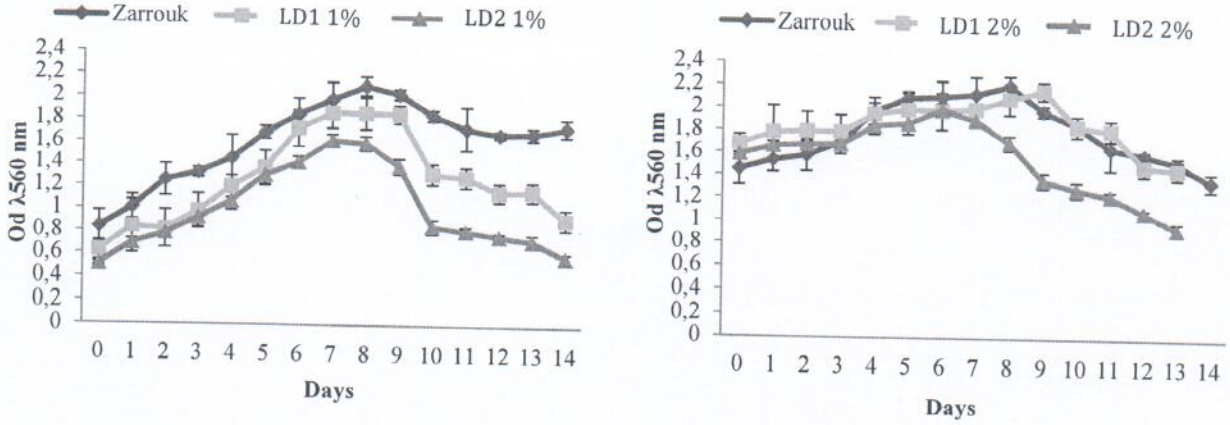

Figure1 *A.maxima growth curve(A)on LDs at 1% (v/v) dilution and(B) on LDs at 2% (v/v) dilution for 14 days. Zarrouk was the control.*

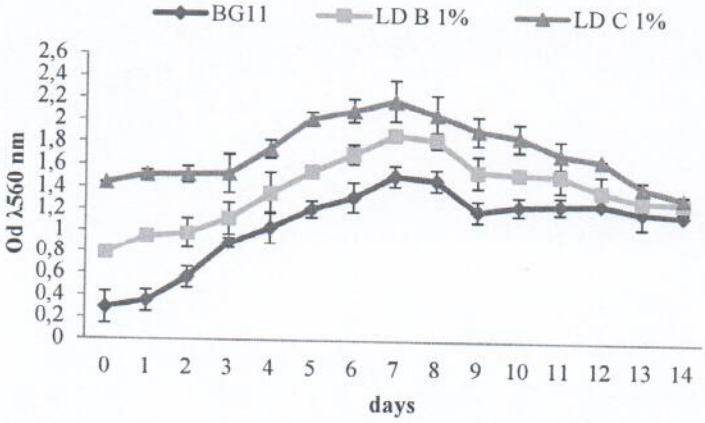

Figure 2 *S.obliquus growth curve on LDs at 1% v/v dilution for 14 days. BG11 was the control.*

The *S.obliquus* productivity, at 1% v/v dilution, were 10 and 9 mg/L/din LD 1 and LD 2, respectively. The control productivity (BG11 medium) was 10 mg/L/d, not statistically different than the productivity of samples. Kim *et al.*(2007) reported a maximum productivity of 6 mg/L/d for *S. obliquus*, on fermented swine urine.

3.3 Biochemical composition

Proteins are the main macronutrients present in *A. maxima* and the highest content was 531±12.3mg/g observed for the LD2 at 1% (v/v) and 492±12.3 mg/g for the LD2 at 2% (v/v). The results were in accordance with literature data (Jimènez *et al.*, 2003) except for the values reported for the LD1 at 1% and 2% (v/v).

Maximum carbohydrates concentration was evaluated for the LD2 at 2% (v/v)(226±1.3mg/g) and it was statistically different from the control (190±1.5mg/g). Lipid content for all sample analyzed was high and statistically different from the control (108±6.1mg/g). Challem *et al.* (1981)reported values of about 7% (lower than our data) (Table 3).
The control phycocyanin concentration was (11.4±0.05 mg/g),statistically different than the values reported for LD1 and LD2 (table 3). Chaiklahan *et al.* (2011) were reported a phycocyanin content of 6.17 mg/g, value statistically different than the our experimental data.
Chlorophyll content of the dried biomass from *A. maxima* cultivated under standard conditions was 13.6±0.14mg/g. The chlorophyll contents of the LD1 and LD2 (at 1% and 2%) were not statistically different from the control (table 3). The results were higher than literature data; Jiménez *et al.*(2003) showed a chlorophyll content between 6 and 9 mg/g.
Lipid content of *S.obliquus* for the LD1 and LD2 was 261±5mg/g and 254±11.5mg/g, respectively. These values were in line with the control (279±7.3 mg/g) and the literature data (Mata *et al.*, 2013). The lowerprotein content was reported for LD2 (245±7.3mg/g) and it was statistically different from the control (324±9.2 mg/g) (table 4). According to Martinez *et al.*,(2000) the protein content was roughly 30% in standard medium.
Carbohydrates concentration were188±11.1mg/g in standard conditions, 169±12.3mg/g for LD1 and 174±8.3 for LD2. The data were in accordance with literature (Soeder and Hegewald, 1988;Martinez *et al.*, 2000).The our chlorophyll contents were within the margin of chlorophyll percentages reported for different green microalgae of 0.1 - 6.0% (Senger and Fleischhacker, 1978;Martinez *et al.*, 2000).

Table 3 *Biochemical composition of dried biomass of A.maxima on LD from vegetable biomass and urban solid waste*

Nutrients (mg/g)	*Standard medium*	*LD1 (1%v/v)*	*LD2 (1%v/v)*	*LD1 (2%v/v)*	*LD2 (2%v/v)*
Proteins	*508±15.3*	*329±9.1*	*531±12.3*	*337±6.2*	*492.5±12.3*
Lipids	*108±6.1*	*126±9.4*	*129±10.8*	*160±10.6*	*157±6*
Carboydrates	*190±1.5*	*125±0.02*	*139±0.3*	*163±1.6*	*226±1.3*
Phycocyanin	*11.4±0.05*	*8.2±0.2*	*7.3±0.02*	*10.5±0.01*	*9±0.1*
Chlorophyll	*13.6±0.14*	*12.2±0.3*	*13.5±0.4*	*12.4±0.02*	*12.1±0.02*

Table 4 *Biochemical composition of dried biomass of S.obliquus on LD from vegetable biomass and urban solid waste*

Nutrients (mg/g)	*Standard medium*	*LD1 (1% v/v)*	*LD2 (1% v/v)*
Proteins	*324±9.2*	*340±10.3*	*245±7.3*
Lipids	*279±7.3*	*261±5*	*254±11.5*
Carbohydrates	*188±11.1*	*169±12.3*	*174±8.3*
Chlorophyll	*6.6±0.14*	*7.2±0.3*	*8.5±0.4*

4. Conclusions and Future Perspectives

From this paper it is clear that the cultivation cost of algal biomass may be reduced (about 20%) by coupling the algal biomass production with wastewater treatment. This integration will solve two major problems, wastewater management and energy crisis, in a single stroke. Moreover, use of wet biomass for anaerobic digestion further minimizes the harvesting cost of algal biomass. The toxicity of LDs (as assessed by ecotoxicological tests) decreased by approximately 50%. The high biomass productivity of liquid digestate-grown microalgae suggests that these cultivation method offer real potential as the production of sustainable and renewable energy, for cattle feed industry and high added value molecules production.

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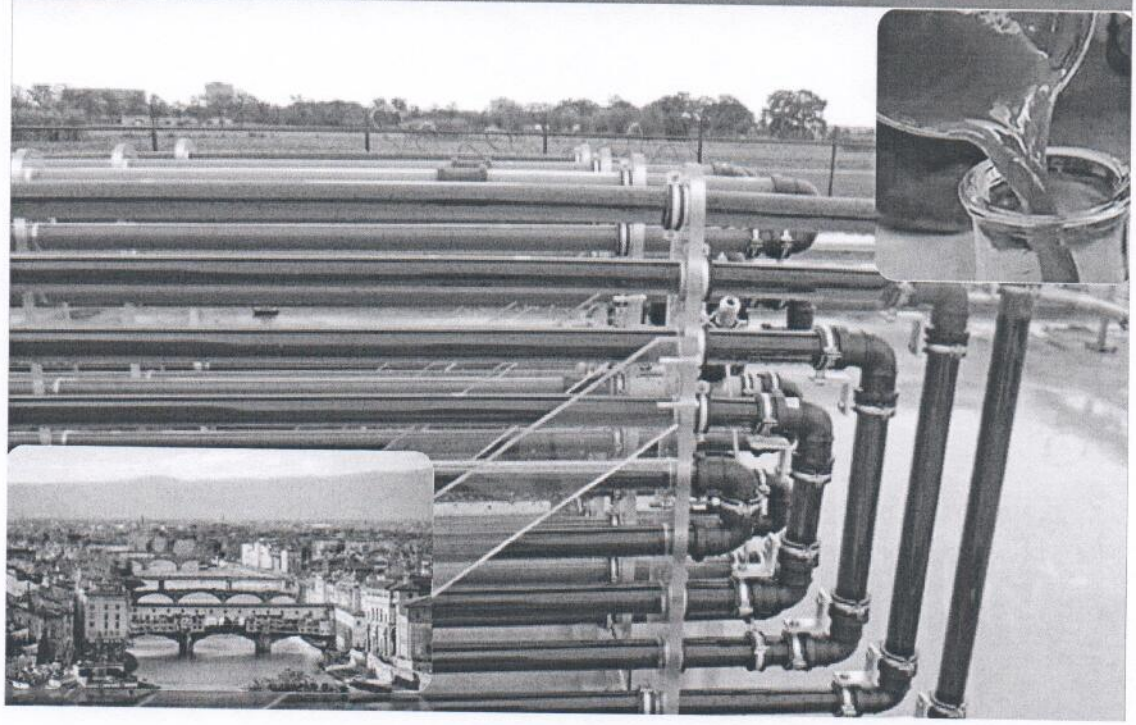

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List of poster presentations

Aquaculture and wastewater

1. *Amir Neori1, Sofie Van Den Hende2* - Greenwater algal ponds, combined with microalgal bacterial flocculation (MaB-floc): the cheapest source for aquatic biomass
2. *Soren Laurentius Nielsen* - Optimization of *Rhodomonas salina* as feedstock for the production of live feed for aquaculture, based on copepods
3. *Ajijo Muyiwa Reuben* - Production of microalgae for shrimp larviculture
4. *A. Moro* - Microalgae for flue gases and wastewater treatment: a challenge for the future
5. *S. Buono1, A. L. Langellotti1, A. Martello1, M.Massa1, V. Fogliano2* - Sustainable fertilizers for microalgae culture

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10. *Silvia Helena Govoni Brondi1*, Dalva Aparecida de Souza1, Eny Maria Vieira2, Ana Teresa Lombardi3* - Cultivation of microalgae *Chlorella vulgaris* for the lutein production aiming to supply the pharmaceutical, food, cosmetic and fine chemicals industry
11. *Kalpesh Sharma* - Have you got your anti-oxidans from microalgae?
12. *Faruq Ahmed, Wenxu Zhou, Peer M Schenk* - Microalga Pavlova lutheri as a new source of phytosterols : compounds with many health benefit
13. *A. L. Langellotti1, S. Buono1, A. Martello1, G. Mariano1, V. Fogliano2* - Extraction and drying of Blu Aqueous Extract of Arthrospira Maxima

Value Chains for Commercialising the Technology – the EU projects

14. *L. Sijtsma et al - SPLASH: sustainable polymers from algae sugars and hydrocarbons*

Limits to cultivation and new technologies for biomass production

15. *Ana Barros1, Tiago Guerra1, Edgar Santos1, Joana Silva1, Luis Costa1, Manuel Simões2 and João Navalho1,* - Mass balance analysis of carbon and nitrogen in mixotrophic microalgae cultures*
16. *Arnaud Muller-Feuga, Eric Abellan, Rémi Pradelles - Development of an artificial light preparative photobioreactor*
17. *Diana Fonseca et al. - Evaluation of the shear stress effect*
18. *Arnaud Muller-Feuga - Acess to microalgae biodiversity passes through cell fragility*

SUSTAINABLE FERTILIZERS FOR MICROALGAE CULTURE

S. Buono[1], A. L. Langellotti[1], A. Martello[1], M. Massa[1], V. Fogliano[2]
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The recent hike in fertilizers price may increase the production costof microalgal biomass substantially, inducing another negative point to Life Cycle Assessment.
The use of nutrient rich wastewaters is an attractive option to reduce the production cost.While the term'waste' is commonly used for these materials, it is however an unfortunatelabel, as it suggests that the materials have no further use and aremerely a nuisance by-product to be treated to meet environmental issues.
The microalgae selection was a critical point; they should be able to grown ina wide range of environmental parameters and medium compositionand mustbe very robust and preferably mixotrophic, able to consume organic and mineral nutrients present in wastewaters.
Our experimental data on growth of *Galdieriasulphuraria* and *Arthrospira maxima* on different kinds of wastewaters as liquid digestate from anaerobic digesters and plus wastes from candied fruit process were showed. The produced microalgae biomass was analyzedin terms of chemical composition and some biological activities.
The analyzed wastewaters showed to be a good and cheap alternative fertilizer for *G. sulphuraria* and *A. maxima* growth. The produced biomass presented a relatively lower productivity respect the control but macronutrients composition and biological activitieswere in line with the control and sometimes higher.

Sustainable fertilizer for microalgae culture

S. Buono[1], M. Massa [1], A. L. Langellotti [1] A. Martello [1], V. Fogliano [2]
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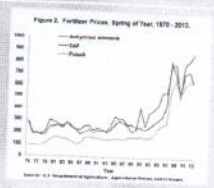
Introduction

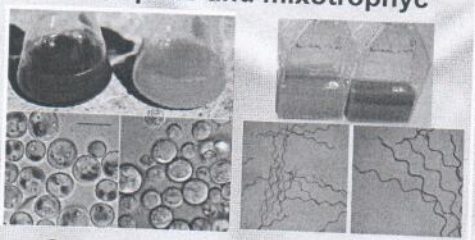
One of Critical Energetic Burdens in Algal Cultivation is represented by Fertilizers consumption. The recent hike in fertilizer prices may increase the production cost of microalgal biomass substantially, inducing another negative point to Life Cycle Assessment. The use of nutrient rich wastewaters is an attractive option to reduce the production cost.



Different kinds of wastewaters as liquid digestate from Anaerobic Digesters and plus waste sugars from candied process were analyzed as alternative microalgae growth medium.

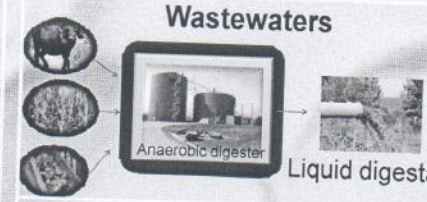
Materials & Methods

Microalgae: extremophile and mixotrophyc



G.sulphuraria *A.maxima*

Wastewaters



Cherry-blanching water

Chemical analysis: pH , Total solid, salinity, $N\text{-}NH_4$, PO_4, $N\text{-}NO_3$, $N\text{-}NO_2$, TOC ;

Wastewater treatments: sedimentation, filtration, dilution

The culture growth was monitored experimentally measuring Optical Density and Dry Weight.

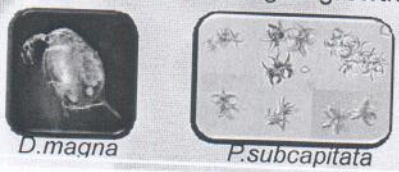
Productivity estimation: photobioreactors 10L

Macronutrient composition of microalgae biomass

Lipid (Bligh and Dyer, 1959); Carbohydrate (Fehling, 1848); Protein (AOAC, 1995); Polyphenols (Folin-Ciocalteu,1927); Antioxidant activity (Gallati, 1979); Phycocyanin (Yoshikawa and Belay, 2008).

Ecotoxicology study

Before and after *microalgae* growth



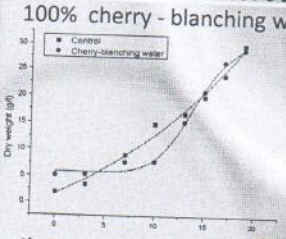
D.magna *P.subcapitata*

D. magna (UNI EN ISO 6341:2011)
P. subcapitata (UNI EN ISO 8692: 2005)

Results

***Galdieria sulphuraria* on cherry –blanching water Heterotrophic growth**

100% cherry - blanching water



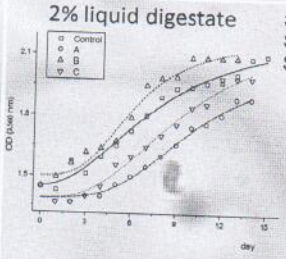
In the control the productivity was 0,79±0,03 g/L/day, resulting not statistically different from the value observed for cherry wastewater (0,73±0,04 g/L/day, red symbol).

Antioxidant activity
(mmol TE/kg)

Control 290 Cherry-blanching 422

***Arthrospira maxima* on liquid digestate**

2% liquid digestate



Sample A : digestor feed with zootechnical biomass;
Sample B : digestor feed with vegetable biomass;
Sample C : digestor feed with urban organic fraction.

Productivity

Zarrouk : 0.190 g/L/day
A: 0.126 g/L/day
B: 0.12 g/L/day
C: 0.10 g/L/day

Biomass nutrient composition		
Nutrients (mg/g)	Standard medium	Cherry wastewater
Proteins	245±21	215±21
Lipids	77±1,4	75±3,5
Carbohydrates	684±9	625±49
Polyphenols	1.6	5.3

Biomass nutrient composition				
Nutrients (mg/g)	Standard medium	A	B	C
Proteins	521±18.3	424±8	341±12.3	507±12.3
Lipids	118±6	182±6	204±9	177±3.4
Phycocyanin	14.5±0.3	13±0.2	12.5±0.01	9±0.1

Conclusions

The wastewaters showed to be a good and cheap alternative fertilizer for *G. sulphuraria* and *A. maxima growth.* The produced biomass presented a relatively lower productivity respect to the control but nutrients composition and biological activities were in line with the control and sometimes higher.

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UNIVERSITÀ DEGLI STUDI DI BARI ALDO MORO

Production of *Galdieria sulphuraria* on cherry-blanching wastewater

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Tutor: Prof. Vincenzo Fogliano.

The first two activities of the PhD thesis project are described below. Firstly, Wastewaters, obtained from an industrial cherry-blanching, were used as growth media for *Galdieria sulphuraria* (SAG 107.79), an acidophile and thermophile microalgae, that typically grows in heterotrophic and/or autotrophic conditions in solfatara areas and shows a very interesting biochemical composition. Secondly, its nutrient removal efficiency (phytoremediation capacity) and its chemical composition were assessed.

Produzione di *Galdieria sulphuraria* su refluo di ciliegie solforate

Le prime due attività del progetto di tesi di dottorato sono di seguito descritte. In primo luogo, le acque reflue di lavorazione delle ciliegie solforate sono state utilizzate come mezzo di crescita per *Galdieria sulphuraria* (SAG 107.79), una microalga acidofila e termofila, che tipicamente cresce in autotrofia e /o in eterotrofia in zone vulcaniche sulfuree e che mostra un'interessante composizione biochimica. In un secondo tempo si è proceduto a valutare la sua efficienza nella rimozione dei nutrienti (*phytoremediation*) di tale tipo di acque reflue e la sua composizione chimico nutrizionale.

Key words: wastewater, microalgae, biochemical composition, cherry.

1. Introduction

This poster reports the main results of the first two activities concerning: 1) productivity of *G. sulphuraria* growth on cherry-blanching wastewaters; 2) phytoremediation capacity and the chemical nutritional characterization of microalgal biomass; 3) Ecotoxicity tests with *D. magna* and *P. subcapitata* on cherry wastewater before and after Galdieria growth.

2. Materials and methods

2.1 Organism and cultivation

G. sulphuraria was grown and maintained routinely in Allen medium in heterotrophy conditions, where organic carbon was supplemented with glucose (30 g/L). The pH was set at 1.5, whereas temperature was maintained at 39 ± 2 °C. In a preliminary tests the selected strains were inoculated at a ratio of 1:4 in 250 mL erlenmeyer flasks containing 150 mL of cherry wastewater. The strain was cultivated in batch for 19 days, in order to define the growth-curves. The culture growth was estimated experimentally by measuring the PCV (packet cell volume), numbers of cells for mL (with Burker chamber) and dry weight. On the basis of growth curves defined in the preliminary test, when the microalgae culture arrived near the end of exponential phase, the harvest of 1/3 of culture was carried out three times a week with a continuous centrifuge. Each supernatant was collected for chemical analysis and ecotoxicology tests, while the wet microalgae biomass was frozen and dried in lyofilizer to evaluate the productivity (mg of dried biomass/L/day). The wastewater was filtered grossly and analyzed for: pH, salinity, dry matter, Total Organic Carbon (TOC), Total Solid (TS).

2.2 Chemical biomass composition and ecotoxicity assays

Before and after the microalgae growth, the wastewater samples were analyzed for total nitrogen, ammonium ($N\text{-}NH_4$), nitrate ($N\text{-}NO_3$), nitrite ($N\text{-}NO_2$) and phosphate ($P\text{-}PO_4$) content, using a spectrophotometer (HACH – Milano- DR 2400) to define the efficiency of microalgae phytoremediation. Microalgae biomass was analyzed for lipid content with Bligh and Dyer method and protein concentration with AOAC method. Carbohydrate were determined using the Fehling test. Total antioxidant activity was determined using a direct measurement of ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6- sulfonic acid). Polyphenols were determined using a Folin method with the Folin-Ciocalteu reagent. The ecotoxicity was evaluated by performing a battery of tests with organisms belonging to different trophic levels and using ISO standards methods: *D. magna* (UNI EN ISO 6341:2011), *P. subcapitata* (UNI EN ISO 8692: 2005).

3. Results and discussion

3.1 Ecotoxicology study

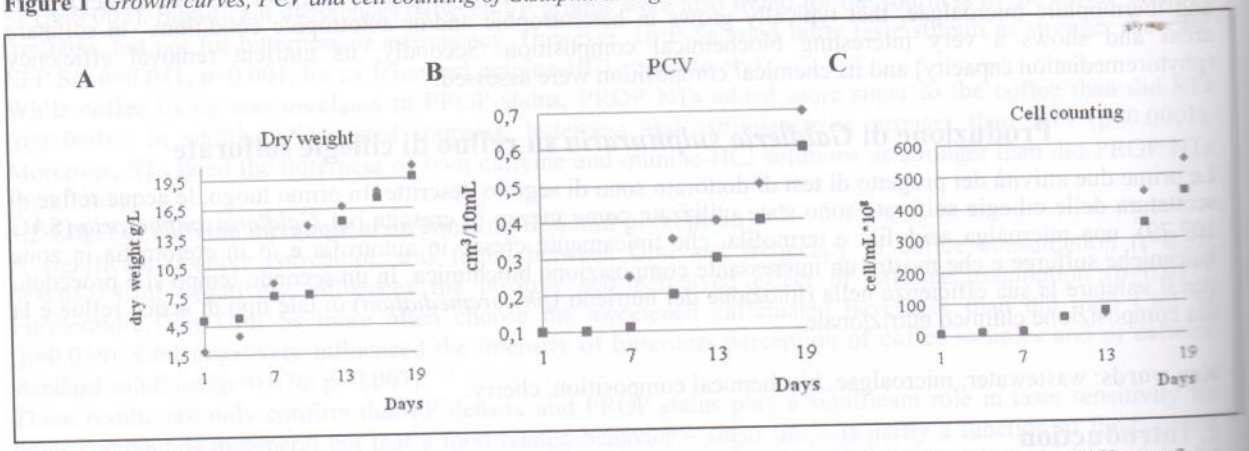
The ecotoxicological effects of the cherry-blanching wastewater were reported in table 2 in terms of EC50, EC20 and EC1. The most sensible organism was *P. subcapitata* with an EC50 value ranging from 5.49 to 5.56%. Whereas, the removal efficiencies of *G.sulphuraria* were 97.85% for ammoniacal-nitrogen and 98.23% for phosphates.

Table 2 *Ecotoxicological effects of the cherry wastewaster expressed in terms of EC50, EC20 and EC1. EC50s, EC20s and EC1s were estimated with the EPA ICp procedure (US EPA 1993). Values are the upper and lower limits of 95 % confidence interval.*

Ecotoxicological test	EC50 (% v/v)	EC20 (%v/v)	EC1 (%v/v)
***Daphnia magna* 24h**	18.86 [17.5 - 19.37]	12.72 [11.5 –13.75]	1.5 [0.5 -10.18]
P. subcapitata	5.5301 [5.49 - 5.56]	2.21 [2.19 -2.22]	0.11 [0.10 - 0.11]

3.2 Algal growth in the wastewater

Figure 1 *Growth curves, PCV and cell counting of G.sulphuraria grown on 100% cherry wastewater were shown.*



In the control (Allen medium), the productivity was 0,79±0,03 g/L/day, resulting not statistically different from the value observed for cherry wastewater (0,73±0,04 g/L/day). According to Selvaratnam *et al.*, (2014), the productivity of *G.sulphuraria* grown on urban wastewater was 0.165 g/L/d. The results of PCV (**Figure 1B**) and of cell counting (**Figure 1 C**) showed the same behavior despite the heterogeneity of cellular dimension of this strain. The sample showed a performance comparable to the control.

3.3 Macronutrient composition

The lipid content was very low in both culture conditions: lipids concentration was 10,5 ±1,4 mg/g in heterotrophic conditions and 17,5±3,5 mg/g on cherry wastewater condition. The protein content in heterotrophic and in cherry wastewater biomass was 215±21 mg/g and 195±21 mg/g, respectively. These values are mainly due to the high amounts of phycobiliproteins. Carbohydrates are the main macronutrients present in *G. sulphuraria*: carbohydrates concentration was 684±9 mg/g in heterotrophic conditions and 625±49 mg/g on cherry wastewater conditions. The data refer to carbohydrates content were in accordance with literature (Graziani *et al.*, 2013).

3.4 Antioxidant capacity

Antioxidant capacity (TEAC) of the dried biomass from *G. sulphuraria* cultivated under heterotrophic conditions and on wastewater was of 290 mmol/kg and of 422 mmol/kg dry weight, respectively. The high value of biomass on wastewater was parallel to the higher concentrations of antocyanin and pigments, molecules well known in the literature for their antioxidant activity. Graziani *et al.*, (2013) reported that the antioxidant activity absolute value of the dried biomass from *G. sulphuraria* was 5.6 mmol/kg. This value was statistically different from that obtained by our results. Another parameter that denote the antioxidant activity is the total polyphenol content that is higher than the control, 1,06 mg/ml gallic acid and 0.33 mg/ml gallic acid, respectively

4. Conclusions

According to data of this paper, fertilizer cost production is approximately 30%, the toxicity of sulfur containing water (as assessed by ecotoxicological tests) decreased by approximately 50%, with the ability in phytoremediation of *G.sulphuraria*. The cherry- blanching wastewater was proved as good growth substrate for *G. sulphuraria*.

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UNI EN ISO 8692 (2005) Water quality - Freshwater algal growth inhibition test with unicellular green algae.

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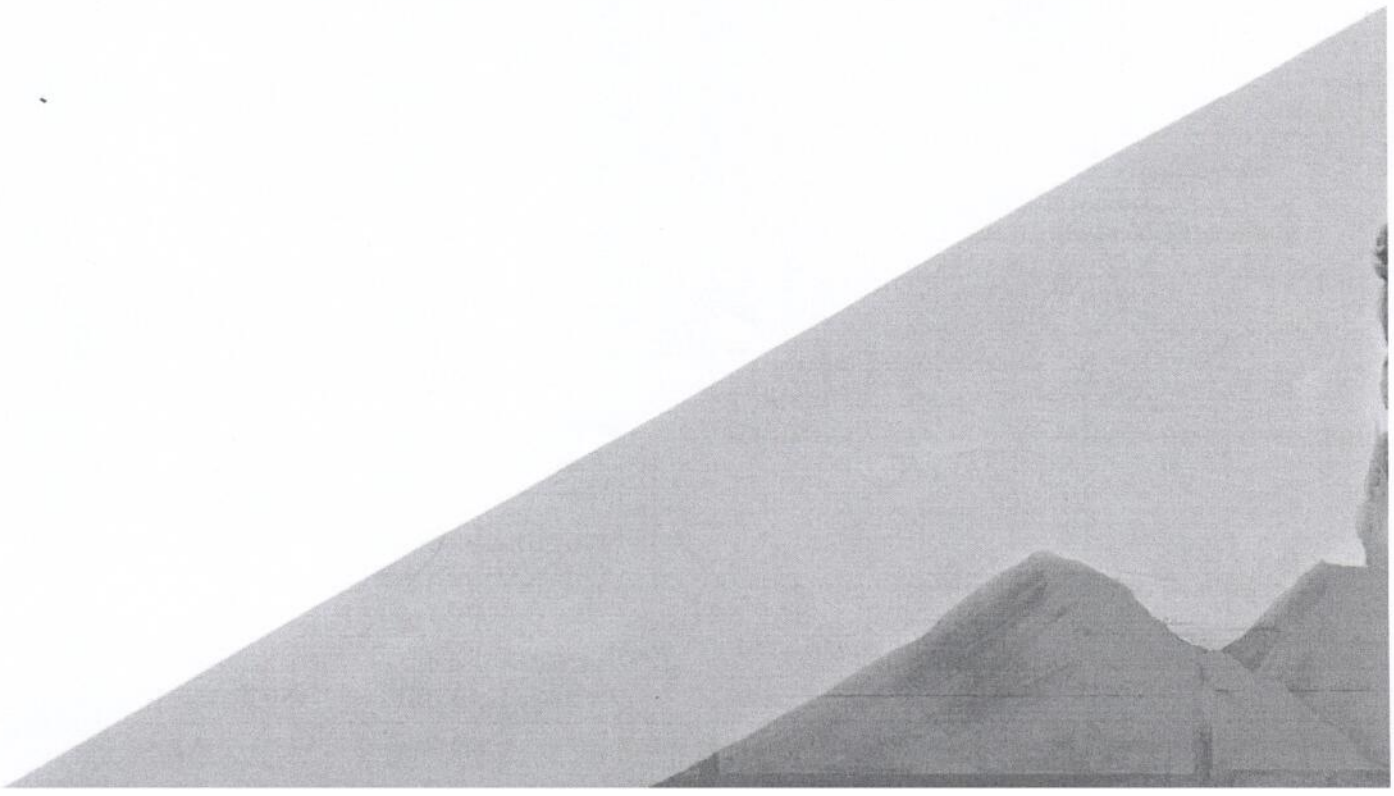
2014

PROGRAMMA



TITANIA MESOPOROUS CLEANING UP IN THE MARINE ENVIRONMENT: ECOTOXICOLOGICAL STUDY FOR THE DEFINITION OF ECO-FRIENDLINESS

E. Bocci[1], S. Buono[2], C. Gambardella[3], V. Vitiello[4], M.L. Vannuccini[1], F.S. Freyria[5,6], M. Massa[2], B. Bonelli[5], M. Faimali[3], I. Buttino[4], I. Corsi[1]

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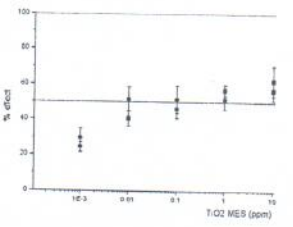
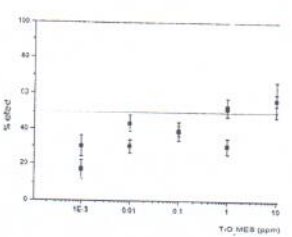
Aim

The aim of the present study done in the framework of the NanoRemEco research project is to validate and define ecotoxicological tools to plan and realize *ecosafe* remediation techniques in the mar environment based on the use of *eco-friendly* NMs. The study has been organized using ecotoxicological bioassays in selected marine organisms belonging to different trophic levels (from algae benthic grazer) in order to assess any potential risk related to trophic transfer as well as toxicity. In the present study we have investigated the *ecosafety* of newly synthesized pure *mesoporous tita* (MT) as potential NM for seawater remediation on algae (*Rhodomonas baltica*,*Rhinomonas reticolata)*, zooplankton (*Artemia franciscana, Amphibalanus amphitrite, Acartia tonsa, Brachionus plicat* and on primary consumer (*Paracentrotus lividus*). Algal growth inhibition, zooplankton mortality, crustaceans and rotifers swimming behaviour alteration and developmental abnormalities of sea urc embryos were evaluated as end-points.

R. baltica



R. reticolata

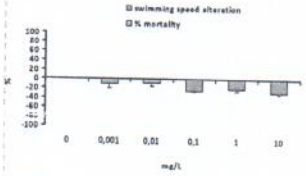


R. baltica	MT (ppm)
NOEL[a]	NV
EC1	NV
EC20	0.0007 [0.0006-0.0009]
EC50	0.325 [0.04-0.62]

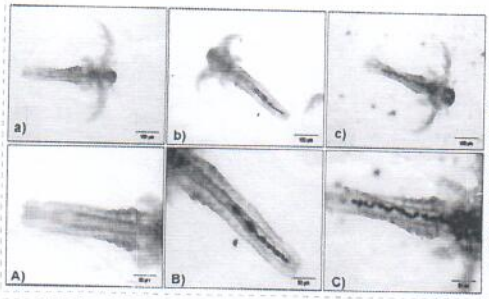
R. reticulata	MT (ppm)
NOEL[a]	0.001
EC1	NV
EC20	0.0008 [0.0006-0.0028]
EC50	6.1429 [0.7-8.2]

In the algal growth inhibition test the most sensible microalgae *R. baltica*.

A. franciscana

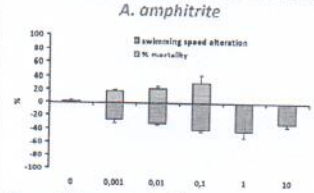


In all MT concentration tested there were low percentages of swimming alteration in larvae exposed to 0,1 until 10 mg/L, it was also possible to calculate the EC_{20}=1,47 mg/L (confidence limits 0.73-1.73). The mortality rate is not affected in the larvae exposed all MT concentration.



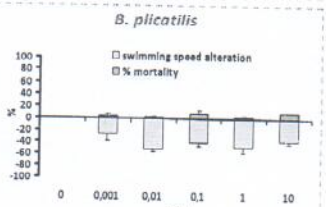
The microscopy observation showed that aggregation of MT nanoparticles occurred after 48h of exposure in all suspensions in the filtered natural seawater. The larvae accumulate aggregates of MT and once ingested these seem to be compressed within the larval gut. a-c) magnification 10X; A-C) magnification 20X; a-A) Ctrl; b-B) MT 1 mg/L; c-C) MT 10 mg/L.

A. amphitrite



The mortality rate is affected in the larvae exposed at MT concentration until 0,1 mg/L, but concentrations of 1 and 10 mg/L do not induce mortality. In all MT concentration tested there were swimming alteration in larvae exposed all MT concentration. The swimming alteration were not dose-response.

B. plicatilis



The mortality rate is not affected in the la. rae exposed all MT concentration.
In all MT concentration tested there were swimming alterations, but not related to dose-response.

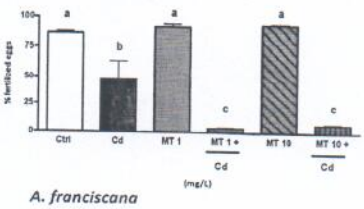
P. lividus: embryotoxicity

The embryo development bioassay showed moderate developmental defects MT exposure in the range of 0.01-10 mg/L. Although both treatments shown slight but not significant embryos developmental abnormalities were observed but still consider in the range of normality. The slight alteration shown are A) normal development; B) separated tips; C) Fused arms; D) Crossed tips. The imaging are 40 X.

Species	Endpoint	Test	MT	
Rhodomonas baltica	**Sub-lethal** (Inibition growth 72 h)	IRSA-CNR (IRSA-CNR, 1978)	EC_{20} 0.0007 mg/L (0.0006-0.0009) EC_{50} 0.325 mg/L (0.04-0.62)	
Rhinomonas reticolata	**Sub-lethal** (Inibition growth 72 h)	IRSA-CNR (IRSA-CNR, 1978)	EC_{20} 0.0008 (0.0006-0.0028) EC_{50} 6.1429 mg/L (0.7-8.2)	
Artemia franciscana	**Lethal** (Mortality 48h)	ASTM E1440-91(1998) APAT/IRSA-CNR 8060 ISO 14669:1999 Unichim M.U.2244:12	No toxicity <10mg/L	
	Sub-lethal (Swimming speed alteration 48h)	Garaventa *et al.*, 2010	EC_{20} 1,47 mg/L (0,73-1,73)	
Amphibalanus amphitrite	**Lethal** (Mortality 48h)	Unichim M.U. 2245:12	No toxicity <10mg/L	
	Sub-lethal (Swimming speed alteration 48h)	Garaventa *et al.*, 2010 Piazza *et al.*, 2012	Swimming alteration no dose-response	
Brachionus plicatilis	**Lethal** (Mortality 48h)	ASTM E1440-91 (2012)	No toxicity <10mg/L	
	Sub-lethal (Swimming speed alteration 48h)	Garaventa *et al.*, 2010	Swimming alteration no dose-response	
Acartia tonsa	**Lethal** (Mortality 48h)	ISO 14669:1999 Unichim M.U. 365:12	No toxicity <10mg/L	
Paracentrotus lividus	**Sub-lethal** (Spermiotoxicity 1h20')	Dinnel et al., 1987 USEPA 1991 Giuliani *et al.* (2002) ICRAM D.D. 3.12.2002	No toxicity <10mg/L	
	Sub-lethal (Embriotoxicity 48h)	Giuliani *et al.* (2002)	No toxicity <10mg/L	

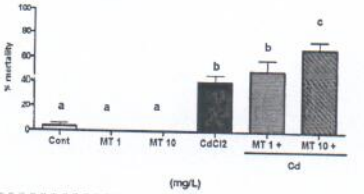
Co-exposure: MT + EC50 $CdCl_2$

P. lividus: spermiotoxicity



The treatment with EC50 Cd (1.5 mg/L) significantly reduces the fertilization compared with the control and MT (1 and 10 mg/L).
The co-exposure MT with EC50 Cd reduces significantly the fertilization.

A. franciscana



The treatment with EC50 Cd (400 mg/L) significantly increases the mortality compared with the control and MT (1 and 10 mg/L).
The co-exposure MT (1 and 10 mg/L) with EC50 Cd increases significantly the mortality

Concluding remarks

Based on this first data, MT resulted *ecosafe* since any lethal and significant sub-lethal effects were produced in marine organisms belonging to various trophic levels. Nevertheless, future investigations will evaluate the interaction of MT with toxic contaminants to be remediated in sea water and their effects to marine biota.

Progetto finanziato nell'ambito del POR CRO FSE 2007-2013 Asse IV – Capitale Umano: **NanoRemEco (Testing Eco-friendly Nanoparticles for sediment and marine waters Remediation).**

TITANIA MESOPOROSA PER LA BONIFICA IN AMBIENTE MARINO: STUDIO ECOTOSSICOLOGICO PER LA DEFINIZIONE DI ECO-FRIENDLINESS

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[1]Dipartimento di Scienze Fisiche, della Terra e dell'Ambiente, Università degli Studi di Siena;
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[3]Istituto di Scienze Marine, Consiglio Nazionale delle Ricerche, Via de Marini 6, 16149 Genova;
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[6]Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Ave, 02139 Cambridge, (MA, USA).

L'uso di nanomateriali (NMs) per la bonifica *in situ* è detta nanoremediation, questa tecnica rappresenta una soluzione promettente e all'avanguardia, garantendo una rapida ed efficiente rimozione degli agenti inquinanti. Tuttavia, l'uso di tali NMs non deve comportare rischi aggiuntivi per l'ambiente marino e più importante non deve indurre tossicità agli organismi marini. I fattori ed i processi che influenzano l'ecotossicità dei MNs, nonché l'efficacia degli interventi di bonifica sono molto complesse. Inoltre, soprattutto negli ecosistemi marini, la conoscenza dei potenziali impatti associati con il rilascio NMs è ancora limitata. Pertanto, è necessario una corretta valutazione ecotossicologica dei NMs utilizzati per la bonifica in acqua di mare, rendendo quindi necessario comprendere a fondo le interazioni di tali sostanze con il biota marino, al fine di evitare un potenziale effetto negativo dovuto alla loro applicazione *in situ*. Lo scopo del presente studio è svolto nell'ambito del progetto di ricerca NanoRemEco ed è quello di validare e definire strumenti ecotossicologici per pianificare e realizzare tecniche di bonifica ecosostenibili nell'ambiente marino basate sull'uso di NMs eco- friendly. Lo studio è stato organizzato utilizzando test biologici ecotossicologici negli organismi marini selezionati appartenenti a diversi livelli trofici, al fine di valutare il rischio potenziale legato all'eventuale trasferimento trofico, nonché valutarne la tossicità. Nel presente studio abbiamo studiato la tossicità di una nuova nanoparticella di sintesi, la titania mesoporosa (MT) come potenziale NM per la bonifica delle acque di mare. Lo studio è stato svolto su alghe (*Rhodomonas Baltica* e *Rhinomonas reticolata*), zooplancton, quali crostacei e rotiferi (*Artemia franciscana*, *Amphibalanus anfitrite*, *Acartia tonsa*, *Brachionus plicatilis*) ed un principale consumatore il riccio di mare (*Paracentrotus lividus*). Sono stati valutati end-point letali e sub-letali quali: l'inibizione della crescita delle alghe, la mortalità e l'alterazione della capacità natante dello zooplancton, alterazioni e anomalie dello sviluppo di embrioni di riccio di mare.
L'inibizione della crescita algale (72h) ha mostrato per la *R. baltica* e *R. reticolata*, rispettivamente, EC_{20} pari a 0,0007 mg/L [0,0006-0,0009], EC_{50} 0,325 mg/L [0,14-0,62] e EC_{20} 0,0008 mg/L [0,006-,0028], EC_{50} 6,1429 mg/L [4,2-8,2] rispettivamente. Per quanto riguarda lo zooplancton, nessuna mortalità è stata osservata fino a 48 ore di esposizione, ma sono state osservate delle lievi alterazioni, come ad esempio l'inibizione del nuoto. Nell'echinoderma *P. lividus* sono stati osservate solo lievi ma non significative malformazioni durante lo sviluppo embrionale, ma e considerate nel range di normalità . Sulla base di questi primi dati, il MT sembra rappresentare che non determina effetti sub-letali e letali significativi negli organismi marini testati appartenenti a diversi livelli trofici. Tuttavia, le indagini future saranno volte a valutare l'interazione di MT con contaminanti tossici da bonificare in acqua di mare e i loro effetti sul biota marino.

NANOMATERIALI ECOFRIENDLY PER LA BONIFICA DI ACQUE MARINE: IL PROGETTO DI RICERCA *NANOREMECO*

E. Bocci[1], S. Buono[2], C. Gambardella[3], V. Vitiello[4], M. Vannuccini[1], F.S. Freyria[5,6], M. Massa[2], B. Bonelli[5], M. Faimali[3], I. Buttino[4], I. Corsi[1]

[1]Dipartimento di Scienze Fisiche, della Terra e dell'Ambiente, Università degli Studi di Siena; [2]CAISIAL, Università degli studi di Napoli Federico II; [3]Istituto di Scienze Marine, Consiglio Nazionale delle Ricerche, Via de Marini 6, 16149 Genova; [4]ISPRA Istituto Superiore per la Ricerca e la Protezione Ambientale-STS Livorno. [5]Dipartimento di Scienza Applicata e Tecnologia, Politecnico di Torino, Corso Duca degli Abruzzi 24, I-10129, Torino, IT. [6]Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Ave, 02139 Cambridge, (MA, USA).

L'uso di nanomateriali (NMs) per la bonifica *in situ* è detta *nanoremediation* e rappresenta una soluzione promettente e all'avanguardia, garantendo una rapida ed efficiente rimozione degli agenti inquinanti. La *nanoremediation* è in grado non solo di ridurre i costi totali di bonifica, in particolare per i grandi siti, ma anche di ridurre rapidamente *in situ* alcune concentrazioni di agenti contaminanti. Sebbene questa nuova tecnica migliora le attuali tecniche di bonifica, anche in modo significativo in termini di tempo e costi, è necessario prevedere che l'applicazione delle specifiche NMs ingegnerizzate non rappresenti un rischio aggiuntivo per l'ambiente e più importante per il biota. I fattori ed i processi che influenzano l'ecotossicità dei NMs, nonché l'efficacia degli interventi di bonifica sono molto complessi. Inoltre, soprattutto negli ecosistemi marini, la conoscenza dei potenziali impatti associati con il rilascio nell'ambiente di NMs è ancora limitata. Pertanto, è necessario una corretta valutazione ecotossicologica dei NMs utilizzati per la bonifica di acque e sedimenti marini, rendendo quindi necessario comprende le interazioni di tali sostanze con la matrice acquosa ed il biota marino, al fine di evitare un potenziale effetto negativo dovuto alla loro applicazione *in situ*. Il progetto di ricerca NanoRemEco (*Testing Eco-friendly Nanoparticles for sediment and marine waters Remediation- POR CRO FSE*) rappresenta il primo studio che affronta tale argomento, testando la sicurezza ambientale dei NMs per la bonifica di acque marine utilizzando un approccio ecotossicologico. Lo scopo di questo progetto è quello di validare e definire strumenti ecotossicologici per pianificare e realizzare tecniche di bonifica ecosostenibili nell'ambiente marino basate sull'uso di NMs eco-friendly. Lo studio si articola in varie fasi basate su strumenti ecotossicologici standardizzati e innovativi negli organismi marini appartenenti a diversi livelli trofici al fine di valutare il rischio potenziale legato all'eventuale trasferimento trofico, nonché valutarne la tossicità. Il NMs oggetti di studio sono stati selezionati tra quelli disponibili in commercio per la bonifica in acqua di mare e nuovi NM sintetizzati per questo scopo. Il progetto NanoRemEco ha permesso di identificare le caratteristiche fisico-chimiche e comportamentali specifiche degli NMs in acqua di mare, che potrebbero comportare rischi per l'ambiente marino in modo che possano essere usati per progettare nuove NMs ecosostenibili basati sul concetto di sicurezza. In particolare, sono stati indagati l'interazione tra NM ed il biota marino nonché con il contaminante da bonificare. Un caso studio verrà presentato con riguardo al biossido di titanio (TiO2 Aeroxide © - P25) come uno dei NM più diffusi in commercio. Il P25 è stata valutato utilizzando test ecotossicologici su alghe (*Rhodomonas Baltica* e *Rhinomonas reticolata*), zooplancton, come ad esempio crostacei (*Artemia franciscana*, *Amphibalanus anfitrite*, *Acartia tonsa*), rotiferi *(Brachionus plicatilis*) e consumatori primari, come l'echinoderma *Paracentrotus lividus*. Le alghe risultato essere l'organismo più sensibile all'esposizione al P25 mentre nello zooplancton abbiamo osservato solo risposte comportamentali, come l'alterazione della velocità del nuoto. Nel test di spermio tossicità del riccio di mare non è osservato alcun effetto di P25. I risultati preliminari di co-esposizione con cadmio (Cd), come inquinante marino da bonificare mediante P25, hanno mostrato un aumento della tossicità del Cd sia nel riccio di mare *P. lividus* che nel crostaceo *A. franciscana*. I meccanismi di tossicità negli scenari di co-esposizione, nonché l'interazione di P25 con il Cd in acqua di mare sono ancora in studio. Questi primi dati suggeriscono tuttavia l'importanza di condurre studi ecotossicologici al fine di promuovere NM ecofriendly per la bonifica di acque marine contaminate.

Appendix 2 List of publications and participation in conferences:

Publications

1. Silvia Buono, Antonella Colucci, Antonella Angelini, Antonio Luca Langelotti, Marina Massa, Anna Martello, Vincenzo Fogliano, Angela Dibenedetto. (2015). Productivity and biochemical composition of *Scenedesmus obliquus* and *Phaeodactylum_tricornutum*: effects of different cultivation approaches. Journal of Applied Phycology. (Accepted with major revision)

2. Marina Massa, Silvia Buono, Antonio Luca Langellotti, Anna Martello, Lina Madau, Giovanna Mariano, Luigi Castaldo, Dario Troise, Vincenzo Fogliano. Production of *Galdieria sulphuraria* on spent cherry-blanching liquid. (Submitted)

3. Marina Massa, Silvia Buono, Antonio Luca Langellotti, Anna Martello, Vincenzo Fogliano. Biological treatment of liquid digestates from anaerobic digestions: the microalgae and cyanobacteria potential. (Submitted)

Comunication at congress

1. Massa M., Buono S., Langellotti A.L., Martello A., Fogliano V. (2015). Sustainable production of microalgae for feed purposes: the cases of *A. maxima and S. obliquus.*

2. Massa M., Buono S., Langellotti A.L., Martello A., Fogliano V. (2014). Production of Galdieria sulphuraria on cherry-blanching wastewater. In: Proceedings book XIX Workshop on Devolopments in the italian PhD Research on Food Science, Technology and Biotechnology. p. 173-174, ISBN: 978-88-88793-48-1, Bari, 24-26 Settembre, 2014

3. S. Buono, Massa M., A. L. Langellotti, A. Martello, V. Fogliano (2014). Sustainable fertilizer for microalgae culture. In: EABA . Firenze, 1-3 dicembre 2014

4. L. Langellotti, S. Buono, A. Martello**,** G. Mariano, Massa M., V. Fogliano (2014). Extraction and drying of blu aqueous extract of *Arthrospira maxima*. In: EABA . Firenze, 1-3 dicembre 2014

5. E. Bocci, S. Buono, C. Gambardella, V. Vitiello, M. Vannuccini, F.S. Freyria, Massa M., B. Bonelli, M. Faimali, I. Buttino, I. Corsi. (2014). *Titania mesoporous* cleaning up in the marine environment: ecotoxicological study for the definition of eco-friendliness. In: I congresso nazionale della società italiana di nanotossicologia. Napoli, 27-28 giugno 2014

6. E. Bocci, S. Buono, C. Gambardella, V. Vitiello, M. Vannuccini, F.S. Freyria, Massa M., B. Bonelli, M. Faimali, I. Buttino, I. Corsi. (2014). Nanomateriali ecofriendly per la bonifica di acque marine: il progetto di ricerca nanoremeco. . In: I congresso Nazionale della società italiana di nanotossicologia. vol. I, p. 20, Napoli, 27-28 giugno 2014

7. E. Bocci, S. Buono, C. Gambardella, V. Vitiello, M. Vannuccini, F.S. Freyria, Massa M., B. Bonelli, M. Faimali, I. Buttino, I. Corsi. (2014). *Titania mesoporosa* per la bonifica in ambiente marino: studio ecotossicologico per la definizione di eco-friendliness. In: I congresso nazionale della società italiana di nanotossicologia. p. 22, Napoli, 27-28 giugno 2014