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Robust and sustainable cultivation of microalgae: recycling of nitrogen from liquid digestate and control of biological contamination

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« La vie n’est pas un problème à résoudre mais une réalité dont il faut faire l’expérience. » Søren Kierkegaard

Acknowledgements

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Abstract

The present doctoral thesis aimed to achieve a robust cultivation of microalgae by recycling nitrogen from a liquid digestate. However, the use of non-sterile nutrient source increases the risks of contamination of undesired organisms such as bacteria, viruses, protozoa, and rotifers. Therefore, the thesis also aimed to develop a cheap and bio-compatible decontamination method, which can be applied to microalgae during their cultivation. To reach these objectives, three projects were carried out.

In the first project, microalgae (*Chlorella vulgaris*) were successfully cultivated at high-density with liquid digestate from anaerobic digestion as a nitrogen source. The cultivation was carried out at laboratory and pilot scales in glass-column and thin-layer photobioreactors, respectively. Biomass dry weight and productivity reached values up to 18.6 g L^{-1} and 0.93 g L^{-1} d^{-1}, respectively. To obtain these results, a cultivation method was developed to overcome inherent issues associated to the use of liquid digestate such as the acidification of the water, ammonium toxicity, turbidity, and nutrient imbalance. The nitrogen mass balance was carried out during the cultivation and it shows that only 40 to 60 % of the nitrogen supplied to the cultures were assimilated in the biomass. Surprisingly, accumulation of nitrogen in the supernatant accounted only for approximatively 3 %. Therefore, a large fraction of the nitrogen was lost to the atmosphere.

In the second project, a citrate-modified photo-Fenton (PF) process was used to treat microalgae cultures contaminated with the bacterium *Escherichia coli*. The aim was to set experimental conditions where physiological and morphological differences can be used to favor the inactivation of bacteria and to minimize the loss of microalgae. Results showed that the citrate-modified PF was more effective against bacteria than a solar light/H_2O_2 treatment, while being less effective against microalgae. If the treatment was applied at an early-stage contamination (10^4 cells mL^{-1} of *E. coli* versus 10^7 cells mL^{-1} of *C. vulgaris*), bacteria were fully inactivated, while the microalgae loss of viable cells was about three orders of magnitude.

In the third project, the citrate-modified PF process was used to treat microalgae cultures contaminated by the rotifer *Brachionus calyciflorus*, a predator of microalgae, which can lead cultures to failure due to its high consumption rate of algal cells. Then, treated cultures were cultivated up to 14 days to assess the efficacy of the treatment. Results show that the citrate-modified PF and solar light/H_2O_2 treatments had equivalent efficacy to inactivate rotifers. However, the PF process remained less harmful for microalgae. When the citrate-modified PF treatment was applied to an early-stage contamination (5 rotifers mL^{-1}), the decontamination was effective and no regrowth of rotifers was observed. However, the cultivation of contaminated cultures, which were not treated, failed due to an increasing rotifer population (>1000 individuals mL^{-1} after 14 days of cultivation).

As a whole, the present doctoral thesis contributes to the development of more robust and sustainable cultures of microalgae. Methods were developed to cultivate microalgae at high-density with liquid digestate and to control biological contaminants. Finally, challenges and opportunities were discussed as a perspective for further works.

Keywords

microalgae; high-density cultivation; liquid digestate; nutrient recycling; nitrogen mass balance; photo-Fenton; iron-citrate complex; bacterial inactivation; rotifer decontamination; near-neutral pH

Résumé

L'objectif de cette thèse de doctorat est d'obtenir une culture robuste de microalgues en recyclant l'azote contenu dans un digestat liquide. Cependant, l'utilisation d'une source non-stérile de nutriments augmente les risques de contamination par des organismes comme les bactéries, virus, protozoaires et rotifères. La thèse vise donc aussi le développement d'une méthode de décontamination, peu chère, biocompatible, qui peut être appliquée aux microalgues durant leur cultivation. Dans ce but, trois projets ont été menés.

Dans le premier projet, les microalgues (*Chlorella vulgaris*) ont été cultivées avec succès à haute densité avec le digestat liquide, provenant d'une digestion anaérobique, comme source d'azote. La culture a été menée en laboratoire et à échelle pilote respectivement dans des photobioréacteurs en tube de verre et à couche fine. Le poids sec de la biomasse et la productivité ont atteint respectivement 18.6 g L^{-1} et 0.93 g L^{-1} d^{-1}. Pour parvenir à ces résultats, une méthode de culture a été développée pour résoudre les défis liés à l'utilisation de digestat, comme par exemple, l'acidification de l'eau, la toxicité de l'ammonium, la turbidité, et le déséquilibre des nutriments. Le bilan massique d'azote a montré que seul 40 à 60 % de l'azote, fournis aux microalgues, ont été assimilés dans la biomasse. Etonnamment, l'accumulation d'azote dans le surnageant n'était que d'environ 3 %. Il a donc été conclu qu'une grande quantité d'azote a été perdue dans l'atmosphère.

Dans le second projet, un processus photo-Fenton (PF) modifié par l'ajout de citrate, a été utilisé pour traiter des cultures de microalgues contaminées par la bactérie *Escherichia coli*. Le but était d'utiliser les différences physiologiques et morphologiques pour favoriser l'inactivation des bactéries tout en minimisant la perte de microalgues. Les résultats montrent que le traitement PF était plus efficace contre les bactéries qu'un traitement lumière solaire/ H_2O_2, tout en étant moins efficace contre les microalgues. Si le traitement était appliqué à un stade précoce de la contamination (10^4 cellules mL^{-1} de *E. coli* pour 10^7 cellules mL^{-1} de *C.*

vulgaris), les bactéries étaient entièrement inactivées, alors que la perte de microalgues était d'environ trois ordres de grandeur.

Dans le troisième projet, le processus PF modifié par le citrate a été utilisé contre le rotifère *Brachionus calyciflorus*, un prédateur de microalgue, qui peut faire faillir la culture de microalgues. Les cultures traitées ont été recultivées jusqu'à 14 jours pour évaluer l'efficacité du traitement. Les traitements PF et lumière solaire/H_2O_2 ont démontré une efficacité équivalente pour inactiver les rotifères. Cependant, le traitement PF était moins néfaste pour les microalgues. Le traitement par le PF modifié des cultures de microalgues contaminées (5 rotifères mL^{-1}) a entièrement éradiqué les rotifères. En l'absence de traitement, les cultures contaminées ont été fortement impactées par la croissance des rotifères qui ont fait défaillir la culture (>1000 rotifères mL^{-1} après 14 jours de culture).

Dans son ensemble, cette thèse de doctorat contribue au développement de cultures de microalgues plus robustes et durables. Des méthodes ont été développées pour cultiver les microalgues à une haute densité avec du digestat liquide et pour maîtriser les contaminants biologiques. Finalement, les défis restants ainsi que les opportunités ont été discutés dans la perspective de futurs projets.

Mots-clés

Microalgues; culture à haute densité; digestat liquide; recyclage de nutriments; bilan massique d'azote; photo-Fenton; complexe fer-citrate; inactivation de bactéries; décontamination de rotifères; pH quasi-neutre

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Notation

Acronyms and symbols	meaning
BC	*Brachionus calyciflorus*
CFU	Colony-forming unit
Cit, CA	Citric acid
CHN	Carbon hydrogen and nitrogen
CV	*Chlorella vulgaris*
DOC	Dissolved organic carbon
EC	*Escherichia coli*
ECTS	European credit transfer and accumulation system
Ind.	Individuals
L	Ligand
LMCT	Ligand-to-metal charge transfer
logU	Logarithmic unit
LOQ	Limit of quantification
PBR	Photobioreactor
PF	Photo-Fenton
PLD	Pretreated liquid digestate
PLD-NaOH	Pretreated liquid digestate equimolarly mixed with NaOH
ROS	Reactive oxygen species
SAG	Culture collection of algae at Göttingen University
SEM	Standard error of the mean
TP	Total phosphorus

TN	Total nitrogen
w/o	Without
WWTP	Waste water treatment plant

Chapter 1. Introduction

The content of this chapter is partially published in:

- A. Pulgarin, S. Giannakis, C. Pulgarin, C. Ludwig, D. Refardt, A novel proposition for a citrate-modified photo-Fenton process against bacterial contamination of microalgae cultures, Applied Catalysis B: Environmental. 265 (2020) 118615. https://doi.org/10.1016/j.apcatb.2020.118615.

The content of this chapter is also partially included in:

- A. Pulgarin, A. Garcia Kapeller, M. Tarik, S. Egloff, M. Mariotto, C. Ludwig, D. Refardt, Cultivation of microalgae at high-density with pretreated liquid digestate as a nitrogen source: fate of nitrogen and improvements on growth limitations, Journal of Cleaner Production (under review).

- A. Pulgarin, J. Decker, J. Chen, S. Giannakis, C. Ludwig, D. Refardt, H. Pick, Effective decontamination of the rotifer *Brachionus calyciflorus* from a *Chlorella vulgaris* microalgal culture by homogeneous solar photo-Fenton at neutral pH: inactivation kinetics and successful recultivation of treated cultures, (manuscript to be published).

- A.Pulgarin, J. Decker, J. Chen, S. Giannakis, C. Ludwig, D. Refardt, H. Pick, A method for the continuous production of *Brachionus calyciflorus* rotifers in a 1-liter culture, (methodical protocol to be published).

1.1. Context

The scientific consensus about climate change became unequivocal in the last ten years: our world is warming and greenhouse gases emitted by human activities are the main contributor [1]. Climate change represent a direct threat for humankind as it impacts world ecosystems, which has deleterious effects on biodiversity, water availability and food production. The United Nations acknowledged in 2015 in the Paris agreement that climate change is a concern for humankind, and they organized a worldwide response to hold the increase of temperature below 2 °C above pre-industrial levels, and to purse efforts to limit the increase to 1.5 °C [2]. The world has to become climate neutral by 2050 to achieve this goal, which will require a massive decarbonization of the world economy and a shift towards green energy. As agriculture, forestry and other land use account for about one quarter of global greenhouse gas emissions [3], many European countries promoted the development of anaerobic digesters to produce biogas and mitigate greenhouse emissions from agriculture [4]. However, the anaerobic digestion produces digestate as a waste, which has to be handled properly to prevent environmental harm.

The development of renewable energy based on biomass has also raised attention on microalgae. Microalgae represent a very diverse group of marine and freshwater photosynthetic organisms with an estimated number of more than 70.000 species [5]. This diversity is reflected by the wide scope of their applications, including biofuels, human and animal nutrition, and high-value products (e.g. carotenoids) [6,7]. However, the high expectations regarding an upscaling of microalgae production to an industrial scale, especially for biofuels, were lowered over the last decades [8]. It became clear that several technological and operational limitations, such as energy and nutrient consumption, must be overcome to make microalgae-based products economically sustainable outside niche markets [9–11]. Therefore, in recent years, investigations were carried out to develop synergies between production of microalgae and recycling of waste streams such as liquid digestates from anaerobic digestion. These synergies aim to lower the production costs of algal biomass and to create new alternatives for the treatment of agricultural and industrial wastes.

In this context, the present study aimed to efficiently cultivate microalgae at high-density by recycling liquid digestate from a Swiss agricultural biogas plant. An important focus was also given to the control of biological contamination during the cultivation of microalgae, which is still an understudied research area. Indeed, if we assume that industrial production of microalgae will rely on the use of non-sterile waste streams, it is likely that the contamination of microalgae cultures by biological organisms such as viruses, bacteria, fungi, protozoa and rotifers, may become an important issue as these organisms may jeopardize the health of the cultures and the quality of the biomass.

1.2. Liquid digestate: a by-product of biogas production

In recent decades, the European Union became the world leader in biogas electricity production thanks to European policies of renewable energy, which stimulated the implementation of biogas plants to valorize organic matter such as manure, green waste and food waste via anaerobic digestion [12]. A biogas plant operates by gathering manure and organic wastes from agriculture, households and industry, which are then used to feed an anaerobic digester to produce CH_4 (Figure 1.1). The first step of the anaerobic digestion consists of the degradation of the carbohydrates, lipids and proteins into acetate, H_2 and CO_2 via biological activity of acidogenic, acetogenic and homoacetogenic bacteria [13]. In a second step, methanogenic bacteria convert acetate, H_2 and CO_2 into CH_4 via acetotrophic (Equation 1.1) or hydrogenotrophic methanogenesis (Equation 1.2) [14].These two processes account for about 70 % and 30 % of the CH_4 production, respectively [15].

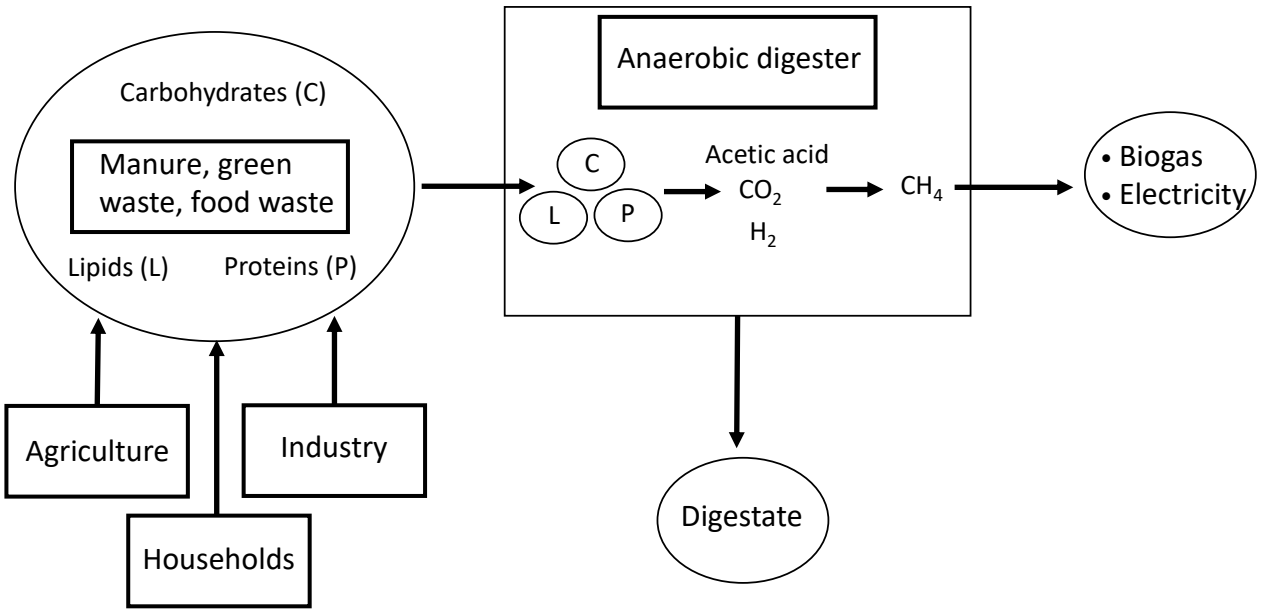


Figure 1.1. A simplified scheme of the anaerobic digestion inputs and outputs. Wastes from agriculture, households and industry are supplied to an anaerobic digester. Carbohydrates, lipids and proteins are then anaerobically converted into CH_4, which can be used as fuel or to generate electricity. The anaerobic digestion also releases a sludge named digestate, which contains salts and non-digestated (or non-digestible) organic matter.

Equation 1.1. Acetotrophic methanogenesis

$$CH_3COOH \rightarrow CH_4 + CO_2$$

Equation 1.2. Hydrogenotrophic methanogenesis

$$CO_2 + H_2 \rightarrow CH_4 + H_2O$$

The anaerobic digestion of organic matter may contribute to the provision of a sustainable source of bioenergy and, thus, to the reduction of greenhouse gas emissions [16,17]. However, the process produces a large amount of digestate as byproduct, which must be disposed of appropriately to prevent environmental harm. After digestion, the digestate is separated by a screw press, which separates the liquid and solid fraction of the digestate (Figure 1.2). The solid digestate is the minor fraction of the separation process and

is usually used as soil amendment. Recent studies also suggested to pyrolyze the solid digestate to produce fuels and charcoal [18,19].

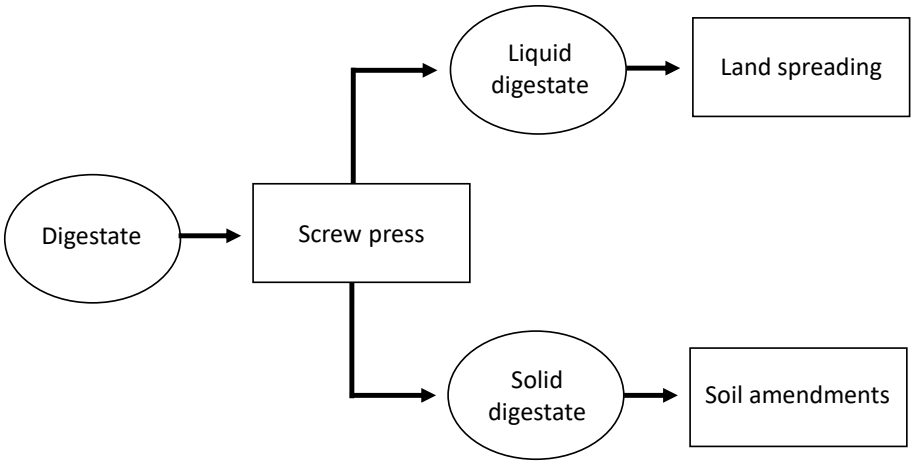


Figure 1.2. Agricultural liquid digestate is usually separated in solid and liquid phases with a screw press. Both solid and liquid digestates are mainly disposed of by land applications.

The liquid digestate is the main fraction of the digestate and its content (e.g., concentration of nutrients and elements) varies depending on the feedstock and treatments applied before or after digestion (Table 1.1). For example, feedstocks and processes shown in Fig. 1.1 and Fig. 1.2 are common for agricultural biogas plants, which produces a dark-brown turbid liquid digestate (Figure 1.3). Agricultural liquid digestate, usually contains high concentrations of ammonium (NH_4^+), phosphate (PO_4^{3+}), and dissolved carbon [20]. This type of digestate was used in this thesis to cultivate microalgae.

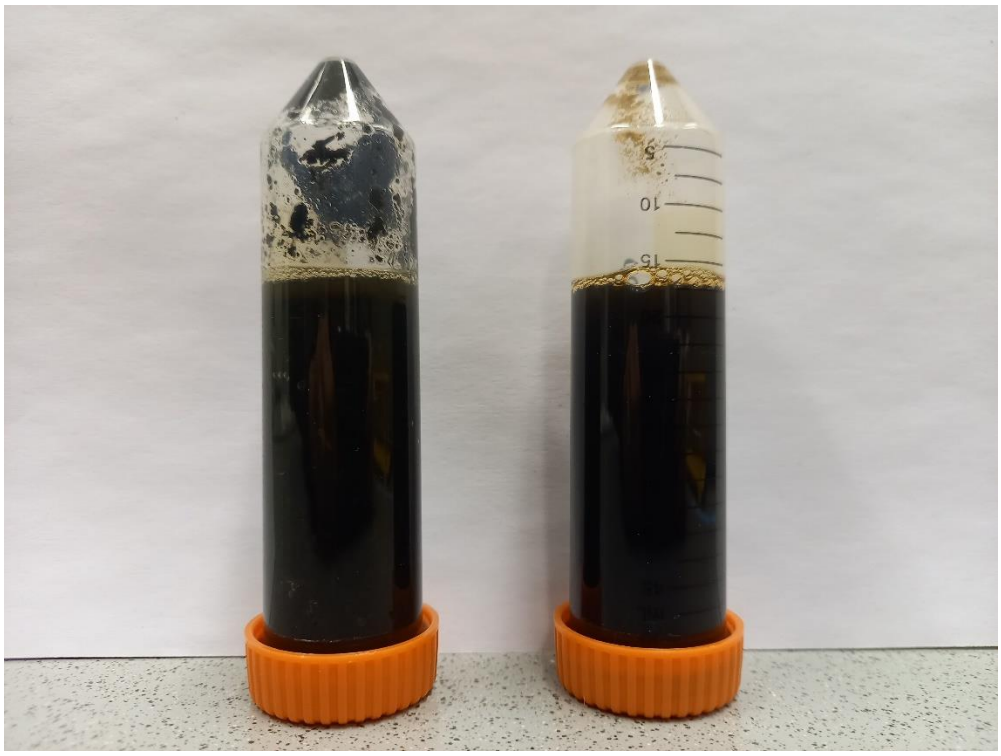


Figure 1.3. Liquid digestate from an agricultural anaerobic digester is characterized by its dark-brown coloration. Tubes contain unfiltered (left) and filtered (right) liquid digestate. Unfiltered liquid digestate is recognizable by the solid matter, which sticks to the tube's side.

Liquid digestate from biogas plants implemented in wastewater treatment plants (WWTP) is another type of liquid digestate. It has usually lower concentrations of carbon and nutrients than agricultural liquid digestate due to the WWTP treatment processes (e.g., settling, activated sludge and filtration), which aims to remove organic matter and solid particles. As WWTP treat human feces, the land spreading of this type of digestate is not always allowed. In this situation, the liquid digestate is treated by the WWTP until concentrations of nutrients, organic matter, pollutants and pathogens are sufficiently low to be discharged in the environment, accordingly to environmental policies. This type of liquid digestate was not used in this thesis.

Table 1.1. Range of value for the characteristics, and nutrient and element concentrations, of various liquid digestate. Adapted from [21].

Characteristic	**Range of value**	**Nutrient/element**	**Range of value (mg L^{-1})**
pH value	6.7-9.2	Cobalt	0.02-0.04
Percentage of NH_4^+ (NH_4^+-N/TN)	65-98 %	Copper	0.09-21.4
Percentage of PO_4^{-3} (PO_4^{-3}-P/TP)	82-90 %	Iron	0.9-65
Nutrient/element	**mg L^{-1}**	Lead	0.03-2.8
Dissolved organic carbon	210-6900	Magnesium	3-659
Total inorganic carbon	939-1353	Manganese	0.1-17
Total nitrogen (TN)	139-3456	Molybdenum	<1.8
Total phosphorus (TP)	7-381	Nickel	<1.4
Aluminum	0.1-34	Potassium	102-2707
Boron	0.9-4	Silicon	26-72
Cadmium	<1	Sodium	126-709
Calcium	65-1044	Sulphur	111-115
Chlorine	160-438	Zinc	0.9-13

The agricultural liquid digestate is mainly disposed of by land spreading to fertilize fields in the vicinity of the biogas plant. This practice is legally regulated in the European Union to limit the environmental impact associated with ammonia (NH_3) emissions and eutrophication [12,17,22], and, thus, limits the amount of disposable liquid digestate. The recent implementation of ultrafiltration and reverse osmosis technologies on biogas plants enabled the removal of a large water fraction from the liquid digestate (Figure 1.4), which improved transport cost-effectiveness [23]. This could also be coupled with stripping and scrubbing systems to produce aqueous ammonium sulfate [24]. Nevertheless, the development of alternative uses for liquid digestate is crucial to avoid a bottleneck due to an insufficient land availability in the vicinity of biogas plants

[25]. In this context, the cultivation of microalgae has been suggested as an alternative to simultaneously treat liquid digestate and produce algal biomass [26].

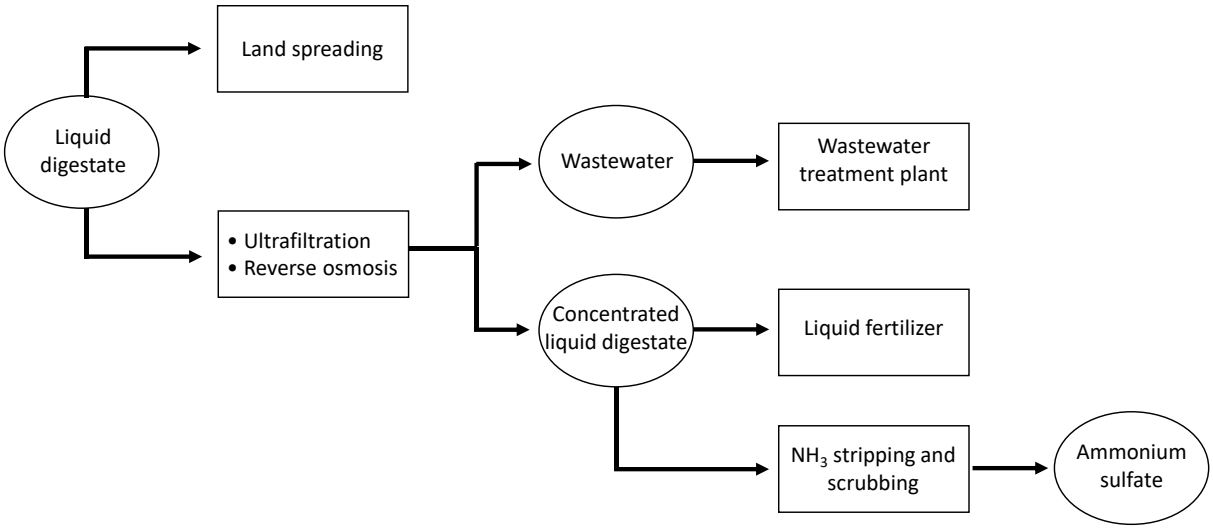


Figure 1.4. Liquid digestate is usually disposed of by land spreading. However, new processes were implemented in recent years to improve the cost-effectiveness of liquid digestate applications such as the concentration of the liquid digestate and recovery of NH_4^+.

1.3. Cultivation of microalgae and use of their biomass

Microalgae raised a lot of scientific interest in past decades thanks to their incredible diversity. However, the world market of microalgae remains modest with an estimated production of about 0.015 Mt per year in 2013 [27], which is marginal when compared to the 30.8 Mt of macroalgae produced worldwide in 2015 [28]. The production of microalgae is dominated by *Chlorella vulgaris* and *Arthrospira platensis,* which account for more than 90 % of the world production [27]. Microalgae-based products are mainly used in human and animal nutrition, cosmetics, and production of valuable molecules [6,7]. For example, *Dunaliella salina* and *Haematococcus pluvialis* are cultivated for their beta-carotene and astaxanthin content, respectively [10]. *Crypthecodinium cohnii* and *Schizochytrium* sp. are cultivated for the production of docosahexaenoic acid [29]. *Isochrysis* sp., *Chaetoceros* sp., *Tetraselmis* sp. and *Nannochloropsis* sp. are used in aquaculture to feed

zooplankton (e.g., *Brachionus* sp.), bivalve mollusks (clams, oyster and scallops), and larval stages of several marine gastropods, fish species and shrimps [30,31]. Recently, microalgae also raised interest for their potential application in biopolymer production [32].

Microalgae biomass has the advantage to not require arable land and microalgae are usually cultivated in open pond systems or closed photobioreactors (PBRs), which facilitates the management of nutrients and wastewater. The type of culture system has an important influence on the production costs and quality of the algal biomass (Table 1.2). Closed PBRs are more expensive than open ponds and the production of microalgae generally consume more energy [33]; however, they can reach higher biomass concentrations with a better control of the algal growth and biomass quality. Open ponds are usually much cheaper to build and consume less energy, but they also reach lower biomass concentrations and are more at risk for biological contaminations. Culture systems used for microalgae are diverse and are not restricted to closed PBRs and open ponds. For example, a greenhouse can be implemented above open pond systems to decrease the risk of contamination and to enable heating. Then, there are open PBRs, which can reach a high biomass concentration while having a better O_2 stripping and cooling ability than closed PBRs. However, the more complex the culture system is, the more difficult is the upscaling.

Table 1.2. Comparison of closed and open large-scale culture systems for the production of microalgae. Adapted from [9].

Culture systems	**Closed PBRs**	**Open ponds**
Contamination control	Easy	Difficult
Contamination risk	Reduced	High
Sterility	Achievable	None
Mixing	Uniform	Poor
Operation regime	Batch or semi-continuous	Batch or semi-continuous
Area/volume ratio	High (20-200 m^{-1})	Low (5-10 m^{-1})
Biomass concentration	High	Low
Investment	High	Low
Operations costs	High	Low
Light utilization efficiency	High	Poor
Temperature control	More uniform temperature	Difficult
Hydrodynamic stress on algae	Low-high	Very low
Evaporation of growth medium	Low	High
Gas transfer control	High	Low
O_2 inhibition	Greater problem in PBRs	PBRs > Ponds
Scale-up	Difficult	PBRs > Ponds

1.4. Microalgae and the hype for biodiesel

In the 2000s, a great hype emerged in the industry due a couple of overoptimistic studies about the potential of microalgae-based biofuels, and a substantial investment was made to achieve an economically sustainable algal biofuel production. In a study published in 2007 and cited more than 10 000 times, Chisti et al. wrote: "if microalgae are used to produce biodiesel. Between 1 and 3 % of the total U.S. cropping area would be sufficient for producing algal biomass that satisfied 50 % of the transport fuel needs", and concluded: "as demonstrated here, microalgal biodiesel is technically feasible. It is the only renewable biodiesel that can potentially completely displace liquid fuels derived from petroleum" [34]. Back then, the price of the algal biomass was estimated between 2.95 and 3.80 \$ kg^{-1} for an annual production of 100 t with a potential decrease to 0.47-0.60 \$ kg^{-1} thanks to economy of scale. However, the price of microalgae biodiesel was estimated around 2.8 \$ L^{-1} by using the lowest cost estimation for the algal biomass (i.e., 0.47-0.60 \$ kg^{-1}), and was already much higher than the average petrodiesel price (0.49 \$ L^{-1} in 2006). Therefore, the hype was based on the assumption that the most optimistic production cost for a liter of microalgae biodiesel can be decreased again by 80 % to compete with petrodiesel.

While a lot of progress was made in recent years to improve the cost-effectiveness of microalgae biomass and microalgae biodiesel, most industrial projects did not go beyond a demonstration facility [35]. Indeed, several techno-economic analyzes concluded that the cost of production remained between 0.5\$-14.9 \$ kg^{-1} for algal biomass and 1.66-5.42 \$ L^{-1} for microalgae biodiesel [36–38]. The industrialization of microalgae at large scale for biodiesel production also raised concerns about the environmental footprint. Indeed, it was estimated that 1 kg of microalgae biodiesel could require 3726 kg of water [39], and that the production of 19 billion liter of algal biofuels per year would use 32 to 49 % of the world surplus values of nitrogen and phosphorus [40]. This shows that microalgae still compete with food and feed for their needs in nutrients and water. Consequently, the upscaling of this industry remains unlikely as many techno-economic challenges have to be overcome yet. However, the hype around microalgae biodiesel had the benefit to stimulate research on energy applications for microalgae biomass [41].

1.5. Cultivation of microalgae in synergy with wastewater treatment

1.5.1. Cultivation of microalgae in wastewater

Some microalgae species from genera such as *Chlorella*, *Scenedesmus* and *Arthrospira* have the ability to grow in wastewater thanks to their robustness and versatility [42–44]. Wastewater may be used as a source of water and nutrients to produce microalgae [45,46], which can be converted into marketable products. However, the use of wastewater may prevent food and feed applications due to sanitary concerns [47]. Therefore, the production of microalgae with wastewater is often investigated for the production of biofuels such as biodiesel, bioethanol and biogas [47–51].

1.5.2. Cultivation of microalgae with anaerobic liquid digestate

In this context, it was suggested to cultivate microalgae close to a biogas plant to recycle liquid digestate nutrients (Figure 1.5), as well as CO_2-rich off-gas and heat produced during electricity generation [52]. Indeed, part of the synthetic fertilizers could be replaced by recycling CO_2 and nutrients, and the heat could be used to extend the cultivation period of microalgae cultures, for example, to spring or autumn. Therefore, this could decrease the operational cost of the cultivation, and, consequently, promote microalgae-derived products [33,35]. A number of studies used liquid digestate to cultivate microalgae and employed various cultivation methods (e.g. cylindrical glass PBR, Erlenmeyer flasks, plastic bags), experimental conditions, digestate types and pretreatment methods [21,53–55]. Most of them reported recurrent issues, which are inherent to the cultivation of microalgae with a liquid digestate.

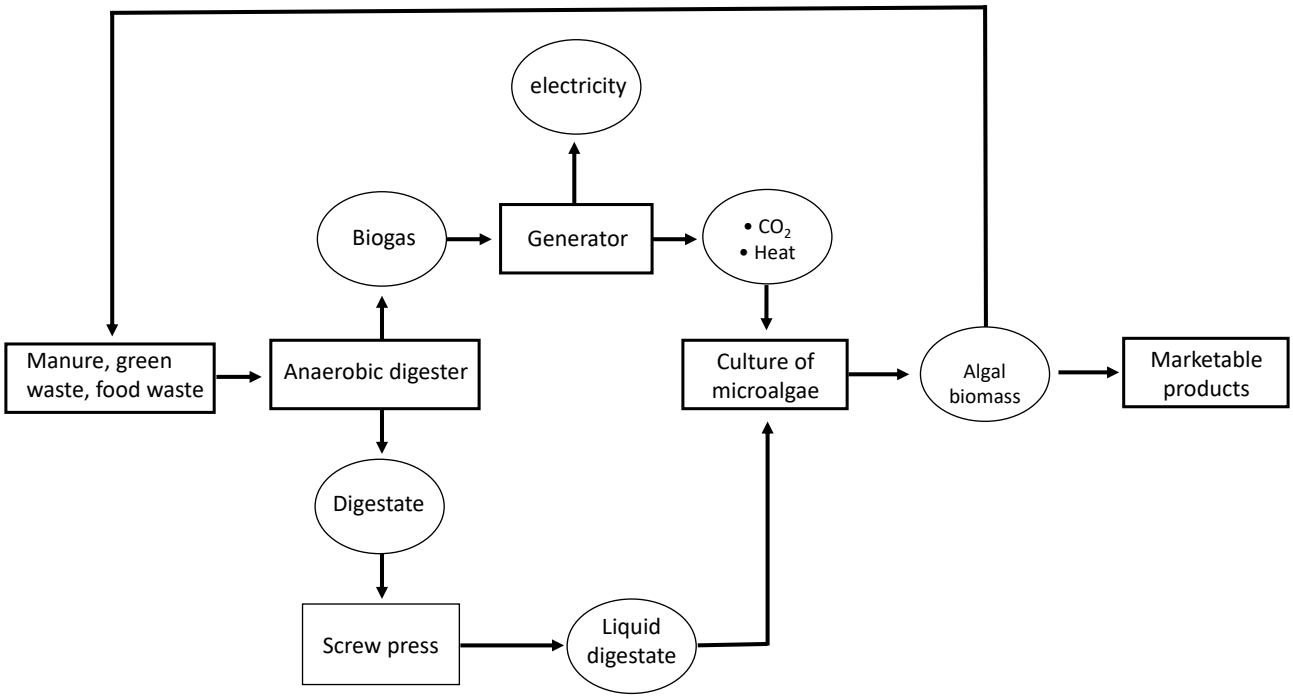


Figure 1.5. Scheme of the cultivation of microalgae with the recycling of effluents from an agricultural biogas plant.

First, liquid digestates are usually opaque and turbid, which decreases light transmission and contaminates the algal biomass with solid particles [56,57]. Second, liquid digestates often have an unbalanced composition of nutrients, which may limit algal growth [58,59]. Third, NH_4^+, usually the main source of nitrogen, can inhibit growth when supplied to microalgae due to its equilibrium with NH_3. A decrease in growth was reported for *Chlorella* species when free NH_3 concentration increased above 20 to 37 mg L^{-1} [60,61]. Fourth, the uptake of NH_4^+ by microalgae decreases the pH value due to an equimolar release of H^+ [62,63], which can inhibit growth [64]. Nevertheless, microalgae were successfully cultivated with liquid digestate when strategies were implemented to mitigate these issues (pretreatment of the digestate, tolerant algal strains, etc.), and densities of algal biomass from 2.0 to 4.8 g L^{-1} were achieved [65–67].

However, and to our knowledge, the aforementioned issues were not addressed together and systematically, and while previous studies reported promising results, the cultivation of microalgae was never carried out with liquid digestate in PBRs specifically designed for high density (biomass dry weight ≥ 10 g L^{-1}) [68,69]. The

cultivation of microalgae at high density has advantages compared to low density cultivation such as cheaper dewatering costs, better control of growth conditions, and lower risk of biological contamination [70]. Therefore, PBRs that achieve a high biomass density are promising candidates for a connection to a biogas plant [71].

1.6. Control of biological contamination in microalgae cultures

A serious threat that microalgae cultures face, without a proper countermeasure, is biological contamination by competitors, parasites or herbivorous predators. This considerably impacts the productivity of microalgae cultures, particularly if cultivation is conducted in outdoor open systems. Indeed, microalgae cultures are susceptible to a wide variety of organisms such as bacteria, fungi, viruses and rotifers, which can reduce the yield of biomass or even destroy a culture given the conditions [72,73]. Consequently, strategies have to be implemented throughout the cultivation process in order to maximize the probability of keeping the desired microalgae culture healthy and dominant; this includes the avoidance of bottlenecks due to an inoculation with low cell numbers, scaling nutrient feeding to the microalgae uptake rate and maintaining optimal growth conditions (i.e. light, temperature, pH) [74,75]. Nevertheless, these preventive strategies can only lower the probability of a biological contamination, and additional solutions are required to treat contaminated cultures.

At current state of knowledge, the available treatment methods are often specific to a kind of contaminant, increase operational costs and can have undesired effects [74]. Pesticides, antibiotics or antifungals can target specific biological contaminants, but they accumulate in the biomass and effluents, which in turn raises concerns about their impact on the culture, health and environment [76]. Filtration effectively removes larger organisms, such as zooplankton, but is ineffective if the biological contaminant has a smaller or similar size to the microalgae [77]. Information about viral infections is scarce and antiviral treatments remain mainly

unexplored [73]. Therefore, the development of efficient and environmentally friendly mitigation methods of biological contaminants is crucial for the industrial development of microalgae production [78].

The suggested methods are frequently adopted from other areas, such as sonication applied to ballast water [79] or low concentrations of hydrogen peroxide used as disinfectant in aquaculture [80]. However, their applicability to safely decontaminate microalgae cultures is yet to be proven. Surprisingly, studies investigating such alternative methods and their application to microalgae cultivation, e.g. the use of allelochemicals, non-persistent pesticides derived from plants or microalgae, predators, and pulsed electric fields [81–85], are rare.

1.6.1. Decontamination of rotifers in microalgae cultures

In aquatic ecosystems, microalgae are at the base of the food chain and they are predated by grazing microzooplankton. However, grazers have also been documented as occasional contaminants of industrial cultures of microalgae [86–88]. Grazers represent thus a threat for the production and quality of the algal biomass and there is a vital need for effective and easy applicable treatments for inactivating the growth of these contaminants [89]. Among grazers, rotifers, such as *Brachionus calyciflorus*, are small animals with a size between 100 and 200 µm [90] (Figure 1.6) , which are voracious predators of microalgae [91]. Rotifers thrive by producing diploid and haploid eggs, via amictic and mictic reproduction, respectively [92]. Diploid eggs from amictic females will develop into female, while haploid eggs from mictic females will develop into male, or if fertilized, into resting eggs (cysts).They can consume a large variety of microalgae at high rates and their predation rhythm may lead to the complete failure of microalgae cultures [93]. Moreover, microalgae are particularly vulnerable to rotifers when the cultivation is carried out at low cell densities [94], hence contamination has to be detected at an early stage after culture inoculation and corrective measures have to be taken to avoid the failure of the microalgal cultures. Rotifers are abundant in various aquatic or moist environments such as lakes and rivers but also marshes, moist soils, mosses, tree holes, etc. [95–97]. Therefore, the contamination of microalgae cultures by rotifers may increase if natural freshwater is used.

When the cultivation is carried out in open systems, wind and rain could also carry dirt into the cultures, which potentially contain rotifers or their eggs/cysts.

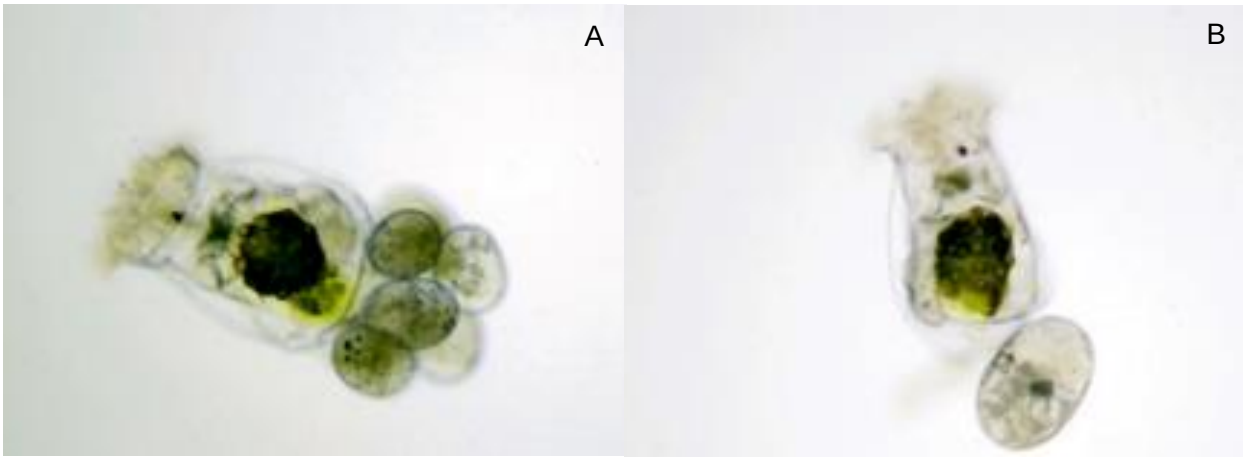


Figure 1.6. Mictic rotifer female with haploid eggs (A) and amictic female rotifer with a diploid egg (B).

Approaches for the removal of rotifers from microalgae cultures remain relatively unstudied, even if some innovative methods have been investigated and successfully applied so far, such as the use of hydrodynamic cavitation, surfactants, or botanical pesticides [82,98–101]. Sodium hypochlorite (0.45 to 0.6 mg Cl L^{-1}) was also successfully applied to inactivate rotifers in microalgae cultures, and to the best of our knowledge, this is the only study where an oxidative treatment was used for an *in-situ* inactivation of rotifers [102]. However, sodium hypochlorite and ozone treatments were used to produce bacteria-free cultures of rotifers, which shows that rotifers are equipped to face oxidative stress [103–105]. Hence, it would be interesting to develop new processes that may deal with the rotifers as the threat, considering their resistant nature.

1.6.2. Inactivation of biological contaminants in microalgae cultures with the photo-Fenton reaction

To our current knowledge, the advanced oxidation processes and more specifically, the photo-Fenton (PF) reaction at near-neutral pH (7-5 to 8.5), were never suggested as a disinfection method to disinfect biological contaminants in microalgae cultures and its feasibility was never assessed. However, the PF reaction was

successfully used at a laboratory scale to inactivate microalgae [106], or recently, TiO_2-mediated photo-catalysis [107]. The PF reaction has interesting applications for (waste)water treatment [108] and it has been used to inactivate a wide array of microorganisms, such as bacteria [109,110], viruses [111,112], fungi [113] , protozoa [114], even antibiotic resistant strains [115], leading to sterile effluents. The PF reaction consists in the production of hydroxyl radicals ($HO^{\bullet}$) through the simultaneous interaction between hydrogen peroxide (H_2O_2) and Fe^{2+}, with solar light catalyzing the reduction of Fe^{3+} to Fe^{2+} iron species. At near-neutral pH the reaction is described by the following equations (Eqs. 1.3-1.5) (for the complete mechanism, kinetics, interested readers are referred to, e.g., [116]):

Equation 1.3. Production of $HO^{\bullet}$ via the Fenton reaction

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + OH^{-}$$

Equation 1.4. Reduction of Fe^{3+} into Fe^{2+}

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2^{\bullet} + H^{+}$$

Equation 1.5 Photo-catalytic reduction of Fe^{3+} into Fe^{2+}

$$Fe^{3+} + H_2O \overset{h\nu}{\rightarrow} Fe^{2+} + HO^{\bullet} + H^{+}$$

Equation 1.6. Reduction of Fe^{3+} into Fe^{2+} via ligand-to-metal charge transfer

$$[Fe^{3+}L] + h\nu \rightarrow [Fe^{3+}L]^{*} \rightarrow Fe^{2+} + L_{ox}^{+}$$

Besides the classic (photo-)Fenton equations (Eqs. 1.3-1.5), the last reaction describes a Ligand-to-Metal Charge Transfer, with the ligand (L) acting as a sacrificial electron donor to Fe^{3+} resulting in Fe^{2+} (Eq. 1.6). This process is of high importance at neutral pH where iron presents low solubility and the photo-active iron complexes can assist in maintaining an effective photo-catalytic cycle.

Lately, oxidative processes involving H_2O_2, Fenton and UV-Fenton-like reactions were used as a cell disruption method to improve the lipid extraction from microalgae biomass for further biofuels conversion [117,118]. In this work, we propose the use of PF reaction at near-neutral pH for the disinfection of microalgae cultures, which entails some intrinsic advantages: 1) it can use the solar energy and widely available reagents which do not have toxic persistence and 2) the oxidative strength can be tuned by modifying the light exposition and reagents' concentration. However, the main challenge of this proposition is the non-selectivity of the generated oxidative species, which will target all biological species, including the microalgae.

It is our conviction though that there is a window of opportunity, since microalgae and other organisms (e.g., bacteria and rotifers) have different physiologies. Thus, these differences (e.g., size and cell wall thickness) may result in different inactivation mechanisms that influence their relative resistance. So far, it has been shown that several species of microalgae can overcome an initial H_2O_2 concentration in the range of 1 to 10 mg L^{-1} [119]. However, it remains largely unexplored whether the PF reaction can be adjusted to deliver an oxidative stress in a way that maximizes the biological contaminants inactivation while minimizing the inactivation of the microalgae.

1.7. Research objectives

The following questions were investigated in the framework of this thesis.

1.7.1. Can microalgae be cultivated at high-density with an agricultural liquid digestate as a nitrogen source?

The first novelty of this project was to use PBRs designed to reach a biomass dry weight above 10 g L^{-1} to cultivate microalgae with liquid digestate. Indeed, previous studies showed that microalgae can grow in liquid digestate, but data are scarce about the feasibility of reaching a high density. Therefore, the experimentation was first carried out in glass-column PBRs with 100 mL cultures in laboratory conditions, and then, upscaled

at 200 L in a thin-layer PBR located in a non-heated foil greenhouse. The second novelty was to address systematically the recurrent issues associated to the cultivation of microalgae with liquid digestate. For this purpose, issues such water acidification, NH_4^+ toxicity, turbidity and nutrients imbalance of the liquid digestate, were addressed in the protocol of cultivation. Finally, a nitrogen mass balance was carried out to have an overview of the nitrogen fate during the cultivation, which was important to determine if the liquid digestate was efficiently recycled.

1.7.2. Can a photo-Fenton reaction be used to treat microbial contaminants in microalgae cultures?

In this second project, microalgae and bacteria were mixed together and treated with H_2O_2 or photon-Fenton. The novelty was to employ experimental conditions, which would favor the inactivation of bacteria while minimizing the damage on microalgae. *Escherichia coli* was used as a model organism because its inactivation by oxidative processes was intensively studied over the past decades. Therefore, the results of the inactivation of bacteria in microalgae cultures were used as a basis to discuss potential mechanisms leading to the inactivation of microalgae, which remain relatively unstudied.

1.7.3. Does the photo-Fenton treatment work for more complex organisms such as rotifers? And do microalgae and rotifer populations regrow after the treatment?

The treatment of bacteria in microalgae cultures was used as proof of concept in the previous project. In this third project, the treatment was assessed on the rotifer *B. calyciflorus*, which is a voracious predator of microalgae. Rotifers are complex organisms and their inactivation by solar light/H_2O_2 and PF treatments in a microalgae culture has not been studied. Additionally, cultivation of microalgae cultures was continued after oxidative treatment to assess their resilience. The results were compared with control experiments to

determine if treated microalgae thrived better than contaminated microalgae which were not treated. These results are crucial to support further investigation of this process at a larger scale.

1.8. Structure of the thesis

The present doctoral thesis is organized in five chapters and four appendices as described below:

Chapter 1. Introduction

A presentation of the context and scope of the work carried out in the thesis.

Chapter 2. Cultivation of microalgae at high-density with liquid digestate

An investigation of the cultivation of microalgae at high-density, both at laboratory and pilot-scale, by using a liquid digestate from anaerobic digestion as a nitrogen source.

Chapter 3. A photo-Fenton process against bacterial contamination of microalgae cultures

A proof of concept of the use of a citrate-modified PF process to inactivate *E. coli* in microalgae cultures while minimizing the loss of algal cells.

Chapter 4. Effective decontamination of rotifers in microalgae cultures

An investigation of decontamination of *Brachionus calyciflorus* in microalgae cultures with oxidative treatments (solar light/H_2O_2 and citrate-modified PF) and the recultivation of the treated cultures.

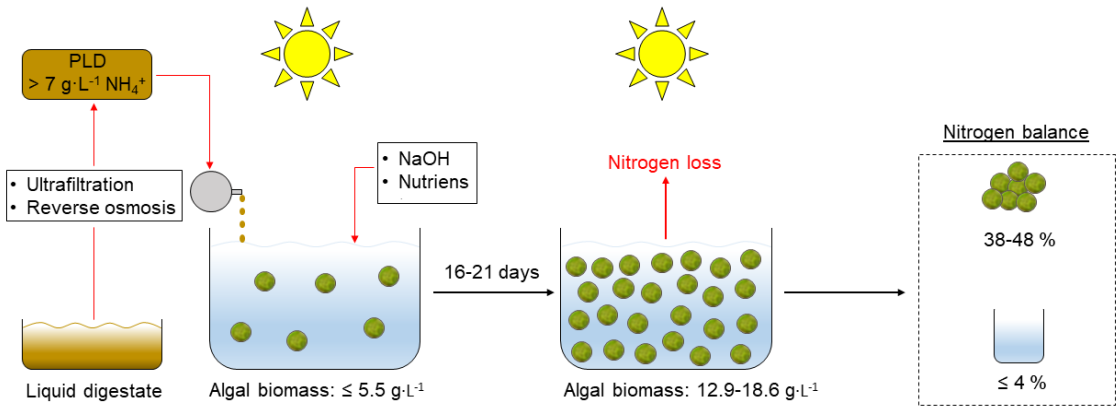
Chapter 5. Concluding remarks and outlook

Concluding remarks to the research questions investigated in the present work as well as recommendations and perspectives for further studies.

Appendices

Supplementary information used to support the content of Chapter 2 (Appendix A), Chapter 3 (Appendix B) and Chapter 4 (Appendix C), as well as additional activities carried out during the thesis (Appendix D).

Chapter 2. Cultivation of microalgae at high-density with liquid digestate



The content of this chapter is included in:

- A. Pulgarin, A. Garcia Kapeller, M. Tarik, S. Egloff, M. Mariotto, C. Ludwig, D. Refardt, Cultivation of microalgae at high-density with pretreated liquid digestate as a nitrogen source: fate of nitrogen and improvements on growth limitations, Journal of Cleaner Production (under review).

The author, Adrian Pulgarin, has carried out the conceptual and methodological work, data analysis and writing of the manuscript. The author has carried out the laboratory experiments jointly with Alexander Garcia Kapeller (Master's student) under the supervision of the author. The author was member of the team who performed the pilot-scale experiments. It is in the nature of such work that the author could not just do it alone or be present at all time. The close collaboration with the other members of the experimental team, Sophia Egloff, Marina Mariotto, and Dominik Refardt, is highly acknowledged.

Abstract

A liquid digestate rich in ammonium nitrogen (8.3 g L^{-1}) was collected from an agricultural biogas plant and supplied to microalgae cultures as the only nitrogen source. Cultivation of *Chlorella vulgaris* was carried out for up to 21 days both under controlled conditions in laboratory-scale glass-column photobioreactors as well as in an outdoor pilot-scale thin-layer photobioreactor. By systematically addressing issues associated with the use of liquid digestate (i.e., turbidity, nutrient imbalance, ammonium toxicity, and acidification), microalgae were robustly cultivated at a high density and cultures achieved a net biomass dry weight between 10 and 14 g L^{-1}, and a productivity up to 0.93 g L^{-1} d^{-1} (93 % of maximum expectation). Cultivation in the thin-layer photobioreactor achieved areal productivities between 7 and 10 g m^{-2} d^{-1}. A water acidification due to ammonium uptake by microalgae was prevented by the controlled addition of NaOH. A detailed mass balance showed that, despite high removal efficiencies (approximately 3 % of the supplied nitrogen remained in the medium), microalgae assimilated only 40 to 60 % of the supplied nitrogen and, consequently, a large amount of nitrogen was lost to the atmosphere.

2.1. Introduction

The aim of this study was to achieve stable high-density microalgae cultures with liquid digestate in glass-column (indoor) and thin-layer (outdoor) photobioreactors (PBRs) by using a systematic approach to address the issues mentioned in section 1.5.2 (i.e., turbidity, nutrient imbalance, ammonium (NH_4^+) toxicity and water acidification). First, the liquid digestate was pretreated by ultrafiltration to remove particles. Second, this pretreated liquid digestate (PLD) was used as a nitrogen source only and supplemented with missing nutrients. Third, the addition of PLD was distributed throughout the day to keep the NH_4^+ concentration in the culture low. Fourth, the pH was controlled by adding sodium hydroxide (NaOH) continuously or in batch to compensate H^+ release from the NH_4^+ uptake. Finally, growth performance and mass balance of nitrogen were calculated to assess if the cultivation of microalgae results in lower nitrogen loss than land spreading of liquid digestate, where losses are estimated between 20 and 60 % due to leaching, runoff, gas emission or volatilization [120,121].

2.2. Material and methods

2.2.1. Microalgae strain, and mineral medium

Chlorella vulgaris (strain SAG 211-11b) was acquired from the culture collection of algae at the Göttingen University (SAG) in Germany. The strain was cultivated in liquid mineral medium, which was prepared in ultrapure water with the following concentrations (mg L^{-1}): 1100 $CO(NH_2)_2$, 237 KH_2PO_4, 204 $MgSO_4 \cdot 7H_2O$, 40 EDTA-FeNa, 173.8 $CaCl_2 \cdot 6H_2O$, 0.83 H_3BO_3, 0.95 $CuSO_4 \cdot 5H_2O$, 3.3 $MnCl_2 \cdot 4H_2O$, 0.6 $CoSO_4 \cdot 7H_2O$, 2.7 $ZnSO_4 \cdot 7H_2O$, 0.17 $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.014 NH_4VO_3 [68]. A stock solution for each nutrient was also prepared for direct addition into the microalgae cultures. The recipe of the mineral medium is based on the elementary composition of the microalgae and sustains an algal biomass production of 6 g L^{-1}, which was confirmed empirically by years of experimentation with *C. vulgaris*.

2.2.2. Characteristics of the pretreated liquid digestate

PLD was obtained from the *Swiss Farmer Power Inwil* biogas plant located in Inwil, Switzerland. The biogas plant mainly processes waste from agriculture, gastronomy, and industry (e.g., manure, green waste, spoiled food, coffee grounds). The pretreatment of the digestate used in this study was carried out industrially with ultrafiltration and reverse osmosis to remove solid particles and to concentrate nutrients, respectively. While this removed solid particles, the dry weight remained high due to the concentration of salts and dissolved organic matter. At the plant, PLD is stored in an underground tank, from where it was sampled and stored at 4 °C. Due to the prolonged storage under non-sterile conditions at the plant, PLD was not considered sterile thereafter.

The characteristics of PLD such as physical properties, concentrations of nutrients and elements, are shown in Table 2.1. Electrodes were used to determine pH value (InLab semi-micro, Mettler Toledo, Switzerland), salinity and conductivity (InLab 738-ISM, Mettler Toledo). Dry weight was determined with a moisture analyzer (HC 103, Mettler Toledo). Photometric tests were used to determine the concentration of dissolved organic carbon (DOC) (LCK 385, Hach, Switzerland), NH_4^+-N (LCK 304), NO_3^--N (LCK 339), NO_2^--N (LCK 342), PO_4^{3-}-P (LCK 349), and total phenol content (LCK 345). An elemental analysis was carried out as follows: 2 mL from the PLD were mixed in the following order with 2 mL HCl (30 %), 4 ml HNO_3 (65 %) and 2 mL H_2O_2. A blank sample solution with the same acid content was prepared in the same way as PLD. The obtained solutions were diluted in three steps with ultrapure water and an acid-diluted solution (1 % HNO_3), respectively, giving a total dilution factor between 2400 and 9000. Calibration solutions were prepared by using multi-element commercial standards (Bernd Kraft, Germany) in the range of 5 to 500 µg L^{-1} for K and P and 1 to 100 µg L^{-1} for further 28 elements. Samples were measured by Inductively Coupled Plasma Optical Mass Spectrometry (ICP-MS, Agilent, 7000x, USA). Quantification was done by external calibration of each element.

Table 2.1. Characteristics of PLD. Analyses were carried out in triplicates and average values are shown with the standard error of the mean (SEM).

pH	7.85 ± 0.02	Conductivity (mS cm^{-1})	94.15 ± 1.05
Salinity (‰)	67.1 ± 0.9	Dry weight (g L^{-1})	72.95± 0.55
mg L^{-1}		**mg L^{-1}**	
DOC	5650 ± 50	Ca	392.6 ± 3.5
Phenols	305.0 ± 8.7	Si	82.00 ± 1.26
NH_4^+-N	8285 ± 30	Fe	5.20 ± 0.04
NO_3^--N	78.48± 1.86	Mn	5.69 ± 0.08
NO_2^--N	2.98 ± 0.40	B	1.96 ± 0.06
PO_4^{3-}-P	7.40 ± 1.97	Sr	1.50 ± 0.00
K	14485 ± 27	Se	< 1.4*
Na	5727 ± 17	Al	< 0.7*
Mg	705.5 ± 2.8		
µg L^{-1}		**µg L^{-1}**	
Zn	333.6 ± 13.1	Pb	14.47 ± 0.51
V	308.8 ± 14.9	Sn	4.11 ± 0.34
Ni	297.5 ± 13.9	Cd	< 24.8*
Li	162.0 ± 5.1	Cu	< 23.2*
Co	111.2 ± 1.1	Be	< 16.9*
Ba	107.4 ± 3.3	Hg	< 13.5*
As	98.81 ± 10.97	Sb	< 4.3*
Cr	82.44 ± 3.39	Ag	< 1.9*
Mo	27.56 ± 1.76	Tl	< 1.2*

*Below the limit of quantification (LOQ)

Nutrient concentrations in PLD were compared to the recipe of the mineral medium, both adjusted to a nitrogen concentration of 85.5 mg L^{-1}, which sustains the production of 1 g L^{-1} of algal biomass (Table 2.2). Higher concentrations in PLD or similar concentrations in both media were found for five nutrients (N, Mg, V, Ca and B), while they were lower in PLD for seven nutrients (P, Fe, Mn, Zn, Cu, Co, Mo). Consequently, PLD was used as a nitrogen source only and other nutrients were supplemented. Cultivations were carried out

with a daily supply of nitrogen (NH_4^+-N or urea-N) of 85.5 mg per liter of culture volume to allow a biomass productivity of 1 g L^{-1} d^{-1}.

Table 2.2. Nutrient concentrations of PLD and mineral medium. Concentrations are standardized to 85.5 mg L^{-1} of NH_4^+-N and urea-N for PLD and mineral medium, respectively.

	PLD	Mineral medium
mg L^{-1}		
NH_4^+-N	85.51	0
Urea-N	0	85.51
PO_4^{3-}-P	0.08	8.99
Ca	4.05	5.30
Mg	7.28	3.35
Fe	0.05	1.01
DOC	58.31	0
µg L^{-1}		
Mn	58.77	152.68
Zn	3.44	102.31
Cu	< 0.2*	40.30
B	20.29	24.19
Co	1.15	20.97
Mo	0.28	15.40
V	3.19	1.02

*Below LOQ

2.2.3. Design of glass-column photobioreactors and experimental plan

The setup of glass-column PBRs is described in a previous study [122]. Briefly, it consists of a set of glass-columns immersed into an aquarium kept at steady temperature and illuminated with a vertical panel of fluorescent tubes (Supplementary Figure A.1). Mass flow controllers (Vögtlin, Switzerland) were used to bubble a mixture of air and CO_2 into the PBRs to supply carbon and to keep microalgae in suspension. A peristaltic pump (REGLO digital MS-4/12, Ismatec, Germany) supplied PLD, PLD mixed with NaOH (equimolar to NH_4^+, PLD-NaOH), or urea to the microalgae cultures, respectively. Care was taken to supply identical amounts of nitrogen to all cultures. Ultrapure water was used for all cultivations.

Cultivations were carried out as follows: 100 mL of microalgae culture (dry weight: 4.1-4.9 g L^{-1}, pH 5.7-6.6) were added to each PBR and cultivated at 25 ± 0.5 °C and constant aeration (0.25 L_N min^{-1} 2 % (v/v) CO_2). A daytime of 12 hours per day was set with an incident photosynthetically active radiation of 800 µmol m^{-2} s^{-1} on the vertical surface of the aquarium. Nutrients were supplied to the cultures following a fed-batch strategy. One twelfth of a nitrogen solution prepared with PLD or urea was supplied to the microalgae cultures every hour during daytime for a cumulative nitrogen amount of 8.55 mg (i.e., 85.5 mg per liter of culture) per day, which sustained a productivity of 1 g L^{-1} d^{-1}. Other nutrients were supplied directly to the cultures every two to four days in amounts sufficient to prevent nutrient depletion. Evaporation loss was approximately 9 mL d^{-1} and was compensated by the nitrogen solution supplied.

Three different nitrogen solutions (9 mL each) were prepared daily as follows: 1) urea (8.55 mg urea-N) dissolved in ultrapure water, 2) PLD-NaOH, i.e. PLD (8.55 mg NH_4^+-N) diluted in ultrapure water and equimolarly mixed with 1 M NaOH (0.61 mmol), 3) PLD (8.55 mg NH_4^+-N) diluted in ultrapure water. Cultivations were carried out for up to 21 days in duplicates. The pH value of the cultures was controlled with an addition of 300 µL 1 M NaOH every day as long as the pH value remained below 6.5. This was not required for the microalgae cultures supplied with the PLD-NaOH solution.

Cultures were sampled periodically. To determine dry weight, a sample was washed two times with ultrapure water (centrifugation at 4000 rcf for 3 min, Z323K, Hermle, Germany) and re-suspended in its initial volume prior to analysis. To determine cell density, a sample of 10 µL was observed under a light microscope (400× magnification, Axiolab, Zeiss, Switzerland) and cells were counted in a counting chamber (Neubauer improved, Marienfeld, Germany). Photometric tests were used to determine the concentration of NH_4^+-N and total nitrogen (TN) (LCK 138) in the supernatant. The CHN content of dried algal biomass samples (100 µg) was determined by thermal conductivity and infrared spectroscopy (TruSpec Micro CHN, Leco Instruments Ltd., UK).

2.2.4. Design of the thin-layer photobioreactor and experimental plan

The cultivation of microalgae at pilot-scale was carried out in an open thin-layer PBR located in a non-heated foil greenhouse in August/September 2017 and in June/July 2020. The PBR consisted of an inclined (1.7 %) culture surface (18 m^2) made of glass sheets in a steel frame (Supplementary Figure A.2). Microalgae were pumped from a tank up to the top of the surface from where they flowed back to the tank as an 8 mm thick suspension layer. Sensors were used to monitor the conditions of cultivation such as the photosynthetically active radiation (SKL2620, Skye Instruments Ltd., UK), partial pressure of CO_2 (InPro 5000i, Mettler Toledo), pH value and temperature (InPro 3253i, Mettler Toledo). The thin-layer PBR has been described in detail elsewhere [69,123]. This thin-layer PBR differs in several aspects from the glass-column PBRs used in the first part of the study: it has an open design, its temperature fluctuates daily, and the illumination is not artificial. Key features that are comparable are evaporation (which allows the continued supply of PLD to the cultures) and high biomass density that can be reached [124].

Microalgae were cultivated in 200 L of freshwater for 16 to 19 days and supplied with PLD as a nitrogen source. The addition of nutrients followed a fed-batch strategy, like the one used for glass-column PBRs. PLD was supplied continuously 10 hours per day during daytime via a peristaltic pump for a cumulated amount of NH_4^+-N of 17.1 g d^{-1} (85.5 mg per liter of culture) to sustain a productivity of 1 g L^{-1} d^{-1}. All other nutrients were supplemented every three to six days directly to the culture to prevent nutrient depletion. Pure CO_2 was injected in the microalgae culture during daytime to maintain a partial pressure of 10 mbar. The CO_2 injection was switched off during the night. The pH value of the culture was kept above 7 with addition of NaOH pellets. Samples were collected periodically to determine the dry weight of the microalgae culture, CHN content of the algal biomass (in duplicate), and NH_4^+-N concentration in the supernatant (as well as TN for the cultivation of 2020) following the methods described in sections 2.2.2 and 2.2.3. The dominance of *C. vulgaris* in the microalgae culture was confirmed by visual observation under the light microscope.

2.3. Results and discussion

2.3.1. Cultivation of microalgae in glass-column photobioreactors

The cultivation of microalgae in glass-column PBRs at high-density with PLD-NaOH as a nitrogen source was successful and growth rates obtained were above those of cultures that were supplied with urea or PLD (Figure 2.1 and Supplementary Figure A.3). After 21 days of cultivation, cultures supplied with PLD-NaOH grew an additional 13.7 g L^{-1} (0.65 g L^{-1} d^{-1}) and reached a dry weight of 18.6 g L^{-1} ($3.72 \cdot 10^9$ cells mL^{-1}). Cultures supplied with urea grew an additional 3.4 g L^{-1} (0.16 g L^{-1} d^{-1}) and reached a dry weight of 8.4 g L^{-1} ($1.33 \cdot 10^9$ cells mL^{-1}). Thus, growth was two times and productivity four times higher for microalgae supplied with PLD-NaOH (Fig. 2.1A). Microalgae supplied with PLD without equimolar addition of NaOH did not grow and their cultivation was aborted after six days due to a pH value remaining below the tolerance level of *C.* vulgaris (pH value of 6) [64], despite a daily addition of 300 µL of 1 M NaOH (Fig. 2.1B). The use of PLD-NaOH stabilized the pH value around 7, which was like the cultivation of microalgae with urea.

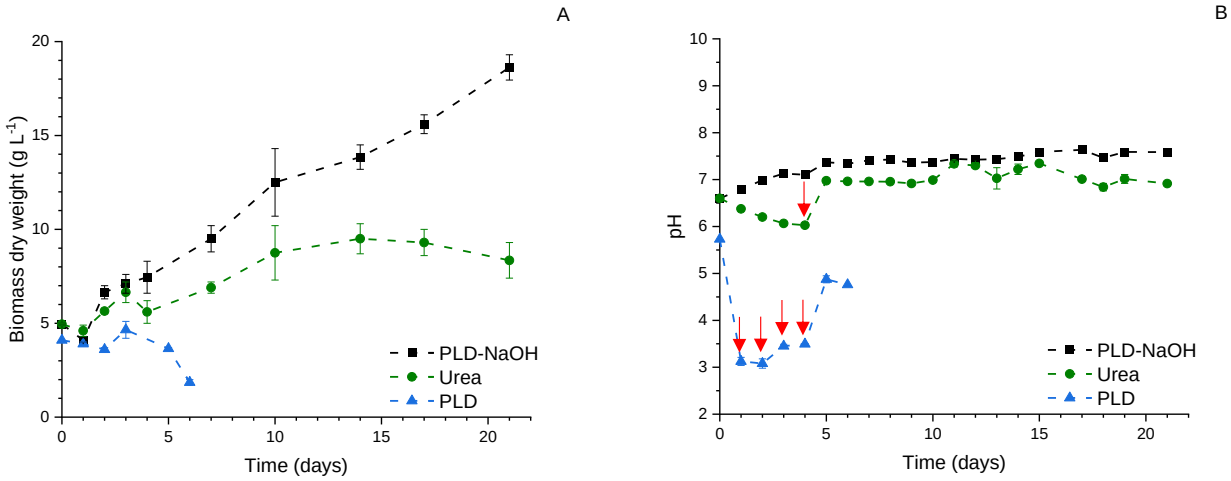


Figure 2.1. Evolution of the biomass dry weight (A) and pH value (B) for microalgae cultivated in glass-column PBRs; data points are means from duplicates and error bars are SEM. Microalgae cultures were supplied with three different sources of nitrogen: PLD-NaOH, PLD and urea. Arrows indicate the addition of 300 µL 1 M NaOH to the cultures to adjust the pH value.

The results show that microalgae can be robustly cultivated at high-density under laboratory conditions with PLD as a nitrogen source using the approach taken in this study. Indeed, the biomass dry weight (18.6 g L^{-1}) was much higher than numbers reported in other studies, which were usually below 4.8 g L^{-1} [65,125,126]. This shows that the cultivation potential of microalgae with liquid digestate is not limited to low-density PBRs. The consistent growth suggests that microalgae did not suffer from starvation nor NH_4^+-toxicity. The acidification of the growth medium was prevented, thanks to equimolar addition of NaOH, and the produced algal biomass did not contain solid particles. Surprisingly, growth of cultures supplied with PLD-NaOH did not only match but surpass growth in cultures supplied with urea. As culture were subjected to a day/night cycle, a light limitation is possible. Indeed, light intensity is usually set to a continuous 800 to 1000 µmol m^{-2} s^{-1} for glass-column PBRs, which enables a biomass dry weight up to 16 g L^{-1} [68]. This suggests that microalgae cultivated with PLD-NaOH were able to grow mixotrophically by using organic compounds contained in the liquid digestate [127–129]. As the trophic behavior of microalgae was not the focus of this study, this assumption remains to be verified.

In the following section, the cultivation of microalgae supplied with PLD as a nitrogen source was upscaled from 0.1 L to 200 L in a thin-layer PBR. The aim was to assess the feasibility of an upscaling at pilot-scale and under outdoor growth conditions.

2.3.2. Upscaling of the microalgae cultivation in a thin-layer photobioreactor

Microalgae cultures were supplied with PLD as a nitrogen source and successfully cultivated in a thin-layer PBR (Figure 2.2). The cultivation was carried out during 16 days in 2017 (Fig. 2.2A) and 19 days in 2020 (Fig. 2.2B). Microalgae grew an additional 10.1 and 12.6 g L^{-1}, and reached final biomass dry weights of 15.60 g L^{-1} and 13.0 g L^{-1}, respectively. The biomass productivity for the full cultivation period was similar between the two runs with a value of 0.63 g L^{-1} d^{-1} in 2017 and 0.66 g L^{-1} d^{-1} in 2020. These values were also like the productivity achieved with PLD-NaOH in glass-column PBRs (0.65 g L^{-1} d^{-1}). A closer inspection of biomass productivities revealed a change over time, with a decrease towards the end of the cultivation period.

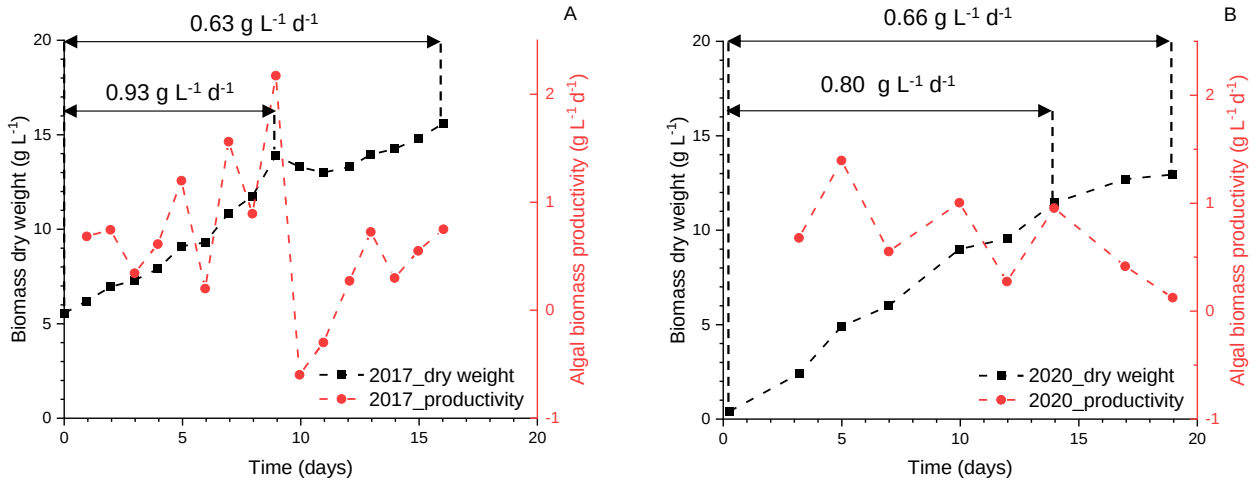


Figure 2.2. Evolution of the biomass dry weight and productivity (between sampling points) during the cultivation of microalgae at high-density in a thin-layer PBR that received PLD as a nitrogen source. Cultivation was carried out in August/September 2017 (A) and June/July 2020 (B). Arrows show average productivity for specific time intervals.

Microalgae had a robust growth during the first nine days of the cultivation carried out in 2017 with an average biomass productivity of 0.93 g L^{-1} d^{-1}. However, growth abruptly stopped, and loss of biomass occurred during two days before growth started again at a lower rate. The loss of productivity was associated to intermittent bad weather, which occurred during the cultivation (Supplementary Figure A.4). Indeed, average light intensity and temperature during daytime decreased from 448 µmol m^{-2} s^{-1} and 26.6 °C, for the first nine days of cultivation, to 121 µmol m^{-2} s^{-1} and 18.3 °C, for the three days of bad weather. In 2020, the average productivity was 0.80 g L^{-1} d^{-1} for the first 14 days. This was associated with good weather conditions that occurred during the full cultivation period with average light intensity and temperature during daytime of 461 µmol m^{-2} s^{-1} and 25.5 °C, respectively (Supplementary Figure A.5). However, productivity decreased during the last five days of the cultivation, which suggests that increased opacity and, thus, reduced light availability, caused by the continued addition of PLD (Supplementary Figure A.6) and the densification of the culture also contributed to the decrease. These results show that microalgae can be robustly cultivated both at high-density and pilot-scale under outdoor weather conditions with PLD as a nitrogen source. To our

knowledge, this is the first successful attempt of high-density cultivation. Previous studies at this scale conducted cultivation at a much lower density (i.e., dry weight below 1 g L^{-1}) [130–132]. The productivity of the cultures (0.8 and 0.93 g L^{-1} d^{-1}), approached a maximum expectations of 1 g L^{-1} d^{-1}, defined by the daily supply of PLD and nutrients. Possibly, cultivation could have been optimized by harvesting microalgae before they experienced bad weather or approached their stationary phase. The areal productivity can be derived from the volumetric productivities by assuming 200 L of circulating culture and 18 m^2 of illuminated surface and, thus, reached a maximum of 10.3 g m^{-2} d^{-1} (0.93 g L^{-1} d^{-1}).It has to be noted that the placement of the photobioreactor in a greenhouse results in a loss of sunlight of up to 50 % [69] and, thus, productivities are to some degree system specific.

The control of the pH value by addition of NaOH was a key factor leading to a successful growth (Figure 2.3). Indeed, the importance of the pH control was demonstrated again during the second run where insufficient NaOH addition caused the pH value to decrease to 2.5, during the first days of cultivation (Fig. 2.3B). The water acidification was due to the release of H^+ during the uptake of NH_4^+ by microalgae, and therefore, its intensity was related to the concentration of NH_4^+ supplied to the microalgae, which is higher for a high-density culture than for a low-density one [133].Despite the addition of NaOH, pH fluctuated daily, yet this was due to the CO_2 injection that was switched off during the night. *C. vulgaris* is tolerant to the basification of the growth medium [134], which has also the benefit of decreasing the risk of biological contamination [135]. While the PLD was not sterilized before its addition to the cultures, light-microscopic observations

indicated that *C. vulgaris* was the dominant species and no other organisms occurred at noticeable numbers.

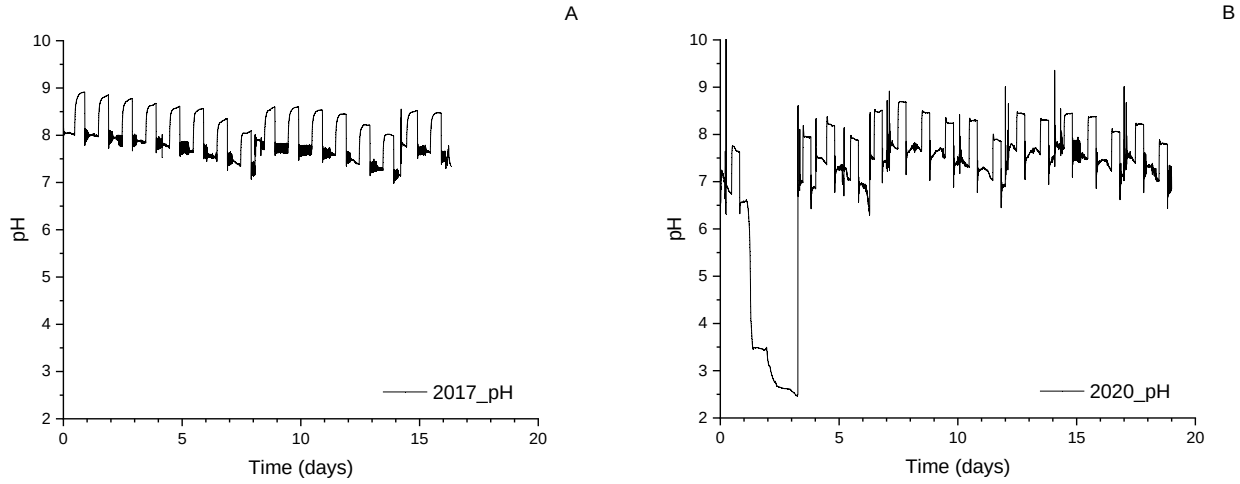


Figure 2.3. Monitoring of the pH value during the high-density cultivation of microalgae in a thin-layer PBR supplied with PLD as a nitrogen source. Cultivation was carried out in August/September 2017 (A) and June/July 2020 (B).

2.3.3. Nitrogen assimilation by microalgae cultivated at high-density

A nitrogen mass balance was carried out to determine whether microalgae efficiently assimilated nitrogen in their biomass or if accumulation occurred in the water. Results show that microalgae assimilated only 20.6 % and 38.1 % of the nitrogen supplied via addition of urea or PLD-NaOH, respectively, during the 21 days of cultivation in glass-column PBRs (Table 2.3). However, nitrogen did not accumulate in the water and the supernatant contained only 1 % and 3.7 % of the nitrogen supplied via addition of urea and PLD-NaOH, respectively. Concentration of NH_4^+ remained low in the supernatant during the cultivation period with values between 0.25 and 3.0 mg L^{-1} NH_4^+-N when microalgae were supplied with PLD-NaOH.

These results show that 78.4 % and 58.2 % of the nitrogen were removed from the culture system when microalgae were supplied with urea and PLD-NaOH, respectively. When cultures were supplied with PLD-NaOH, volatilization could occur due to the equilibrium of NH_4^+ with gaseous NH_3. Previous studies, which reported nitrogen assimilation between 20 and 35 % and nitrogen loss up to 80 %, suggested that NH_3

volatilization may be an important contributor [136,137]. In this study, this is unlikely the sole cause as nitrogen loss was not observed during a 2-day incubation of diluted PLD without microalgae (concentration of 90 mg L^{-1} NH_4^+-N and pH value adjusted to 7). Further, the phenomenon occurred with both NH_4^+ and urea. Cultures supplied with urea (which is not volatile) showed higher nitrogen loss and lower growth than microalgae supplied with PLD-NaOH. This suggests that the loss of nitrogen was at least aggravated by biological processes, which occurred in the presence of microalgae. The loss was possibly worsened by the constant feeding rate, which did not consider a possible reduction in demand over time, as well as suboptimal growth conditions, as there was a notable difference between cultures supplied with PLD-NaOH and urea.

Table 2.3. Nitrogen contained in the algal biomass and supernatant for microalgae cultivated with PLD-NaOH in glass-column PBRs. Concentrations were expressed per liter of microalgae culture and average values are shown with the SEM.

Time (days)	0	21	
Source of nitrogen		PLD-NaOH	Urea
Nitrogen in the biomass (mg L^{-1})	256 ± 2	941 ± 34	626 ± 71
Nitrogen in the supernatant (mg L^{-1})	6.5 ± 0	73 ± 2	24 ± 4
Nitrogen supplied to microalgae (mg L^{-1})	0	1795	1795

The nitrogen mass balance was also calculated for the cultivation in the thin-layer PBR (Figure 2.4). Microalgae assimilated 40.8 % and 48.3 % of the nitrogen supplied via the addition of PLD during the cultivation of 16 days in 2017 (Fig. 2.4A) and 19 days in 2020 (Fig. 2.4B), respectively. Like glass-column PBRs, nitrogen did not accumulate in the water during the cultivation in the thin-layer PBR. For example, the nitrogen in the supernatant accounted for only 2.4 % of the nitrogen supplied to the microalgae during the cultivation carried out in 2020, with a NH_4^+ concentration remaining between 0.1 and 2.2 mg L^{-1} (Supplementary Figure A.7).

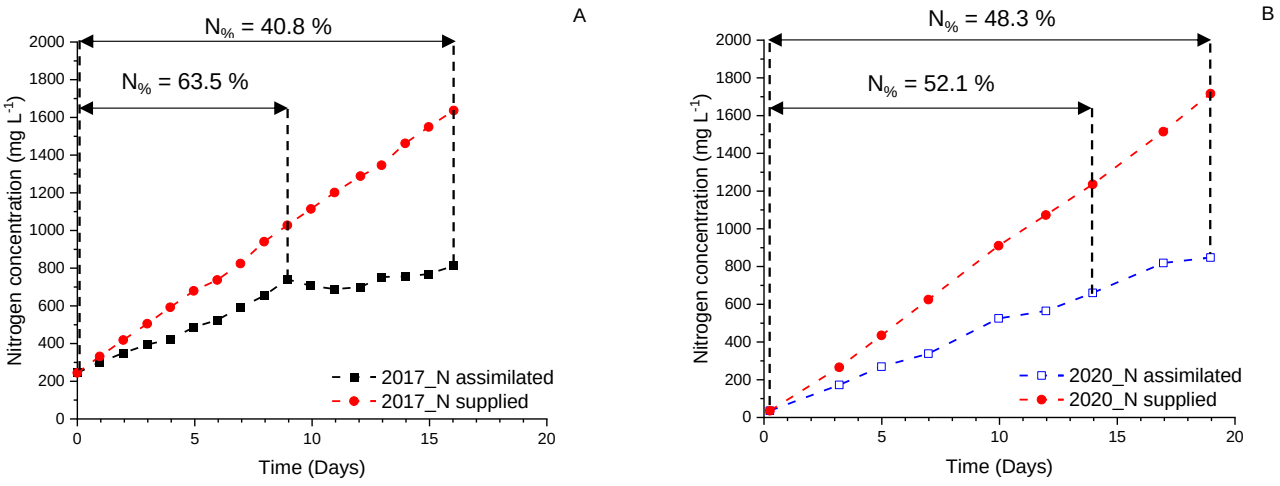


Figure 2.4 Nitrogen mass balance carried out during the cultivation of microalgae in the thin-layer PBR in 2017 (A) and 2020 (B), with PLD as a nitrogen source. The concentration of nitrogen assimilated by the algal biomass was compared to the concentration of nitrogen supplied to the culture. Concentrations of nitrogen are expressed per liter of microalgae culture. $N_{\%}$ shows the percentage of supplied nitrogen, which was assimilated by the algal biomass for specific time intervals represented by arrows.

These results show that a large fraction of nitrogen was lost to the atmosphere during the cultivation of microalgae in glass-column and thin-layer PBRs. Suboptimal growth conditions such as light-limitation or bad weather most likely contributed to the loss of nitrogen. However, the productivity was high during the first nine days of cultivation in 2017 and the first 14 days in 2020, and the assimilation of nitrogen for these specific time periods was 63.5 % and 52.1 %, respectively. While these percentages are higher than the ones calculated for the full cultivation period, they still show that microalgae assimilated only about half of the nitrogen supplied to the culture even at high productivity. The recipe of the mineral medium, to which the supply of PLD was scaled, may have contributed to the loss of nitrogen. Indeed, the nitrogen content of *C. vulgaris* may vary from about 4.5 % to 8.5 % but the average during the cultivation in the thin-layer PBRs was 5.5 ± 0.1 %. In comparison, the recipe of the mineral medium set the nitrogen content to 8.5 %, which may

result in an excessive fertilization. Therefore, tuning of the nitrogen supply may contribute to decrease the loss.

Loss due to the volatilization of NH_3 were not assessed in the thin-layer PBR, and therefore, they cannot be excluded. However, PLD was supplied gradually during daytime to prevent a high concentration of NH_4^+ that favors conversion to NH_3. Further, pH value was kept at 7.5 ± 0.5 during the feeding period, to favor an equilibrium towards NH_4^+. Additionally, results obtained in glass-column PBRs as well as other studies showed a relatively good stability of NH_4^+ for the pH and temperature range used in this study [138,139]. Therefore, it is assumed that biological processes were an important contributor to the loss of nitrogen. While the composition of these emissions remains unknown, this raises the question of the environmental impact of microalgae cultivation. For example, recent studies showed that *C. vulgaris* may emit nitrous oxide (N_2O), a greenhouse gas, via the oxidation of intracellular nitrite [140,141]. Until now, significant N_2O emissions were measured only when microalgae were supplied with nitrate or nitrite [142,143]. However, the large nitrogen loss reported in the present study during the cultivation of microalgae with NH_4^+-rich PLD points to the importance of a better understanding of the processes that cause these emissions and the different gas species involved.

2.4. Conclusions

Microalgae were successfully cultivated at high-density at laboratory and pilot scales with pretreated liquid digestate as a nitrogen source. Microalgae achieved biomass productivities up to 0.93 g L^{-1} d^{-1} under optimal weather conditions, which was close to expected values. The robust growth was made feasible by anticipating and resolving systematically the bottlenecks associated to the use of liquid digestate. The cultivation at high-density also mitigated the risk of contamination and cultures were not jeopardized using a non-sterile liquid digestate. The nitrogen mass balance showed that at least half of the nitrogen supplied to the culture was not assimilated in the algal biomass nor accumulated in the supernatant. It is noteworthy that this observation was true both at laboratory and pilot-scale despite distinct differences between the cultivation systems. Therefore, the cultivation of microalgae has nitrogen loss similar to that resulting from the land spreading of liquid digestate. Formation of volatile nitrogen compounds by microalgae is considered as a probable cause and must be investigated to better assess the environmental impact of microalgae cultures. Further optimization of the cultivation, e.g., via improved pH control and PLD dosage, will also allow to reduce the loss of nitrogen. Closed reactor systems may also be designed to recover nitrogen from gases.

Acknowledgments

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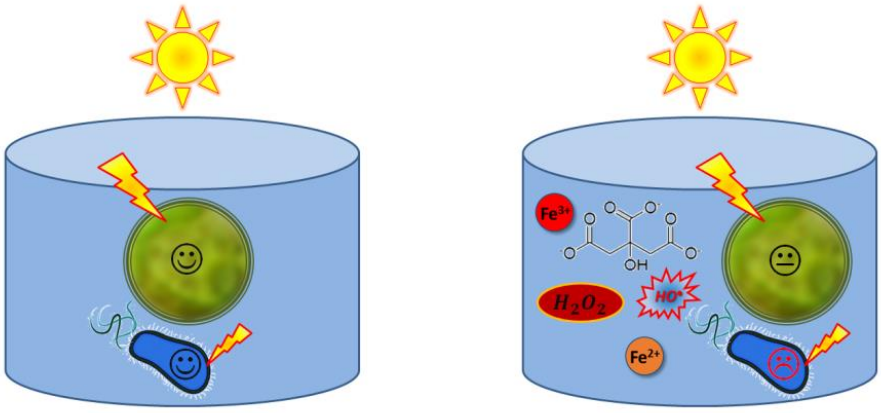
Chapter 3. A photo-Fenton process against bacterial contamination of microalgae cultures



Solar light (hv) | Citrate-modified photo-Fenton process

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The author, Adrian Pulgarin, has carried out the majority of the conceptual and methodological work, and writing of the manuscript. The author also carried out the experiments and data analysis.

Abstract

This study reports the first attempt of a citrate-modified photo-Fenton (PF) application at near-neutral pH (7.5 to 8.5, tap water) to disinfect *Escherichia coli* (as model bacterium), in *Chlorella vulgaris* cultures. The conditions are aimed towards bacterial inactivation, while minimizing the detrimental effect on microalgae viability. The presence of microalgae accelerates H_2O_2 consumption and may affect bacterial disinfection due to faster H_2O_2 depletion. Supplementation of citric acid before PF improved the inactivation efficacy by 1.34 to 1.96 logarithmic unit, alongside with a notably lower microalgae inactivation. Citric acid also considerably increased the lifetime of dissolved iron and prevented aggregation of microalgae, which was caused by Fe^{2+} addition. While these aggregates do not impact the already short lifetime of dissolved iron, they impede the citrate-favored homogeneous process that mainly inactivates *E. coli*. Finally, an integrated mechanism for *C. vulgaris* inactivation is suggested and compared with the one of *E. coli*.

3.1. Introduction

The PF reaction has been successfully used as a (waste)water treatment to inactivate microorganisms such as bacteria, viruses, fungi, and protozoa. The present study is the first attempt of a PF-mediated disinfection of a microalgae culture in tap water contaminated by bacteria. We simulate these conditions in a model system consisting of the microalgae species *Chlorella vulgaris* contaminated with the bacterium *Escherichia coli* and aim to improve the understanding of the PF process in this unexplored domain of application. More specifically, we tested its bactericidal capacity and compatibility with microalgae under near-neutral conditions. By assessing the contribution of each constituent of the composite PF process, we attempt to elucidate the mechanisms that lead to bacterial decontamination and adapt it to this aqueous matrix by addition of citric acid, which improves the homogeneous PF process thanks to its chelating ability that increases the lifetime of dissolved iron. We conclude with a proposal for the overall mechanism of the disinfection, in an effort to understand separately the different events that occur.

3.2. Material and methods

3.2.1. Chemicals and reagents

Iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) ≥ 99 %, hydrogen peroxide (H_2O_2) 30%, NaOH, ferrozine, hydroxylamine hydrochloride ≥ 99 %, acetate buffer (pH 4.66) and catalase from bovine liver (2000-5000 U mg^{-1}) were all provided by Sigma-Aldrich. The plate count agar and reagents for the preparation of the bacteriological media (NaCl, KCl, yeast extract) were also acquired from Sigma-Aldrich; tryptone was purchased from BD Biosciences. H_2SO_4 was provided by Merck and the citric acid 99.6 % by Acros Organics. Titanium (IV) oxysulfate 1.9-2.1 % ($TiOSO_4$) was provided by Fluka.

3.2.2. Bacterial methods

3.2.2.1. Preparation of bacterial cultures

Escherichia coli K12 is used here as a model organism to contaminate microalgae cultures because, even if it is not known as pathogenic for microalgae, it has been studied in detail under oxidative processes, allowing us to focus on the unknown variables of the experimental plan. Furthermore, the specific strain is a wildtype isolate, hence no concerns are raised on a possible over sensitivity to minor changes and effects during the treatment. The culture was originally obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, strain no. 498) and stored in 20 % glycerol at -20 °C. To prepare a working stock, frozen bacteria were streaked on plate count agar and incubated at 37 °C for 24 h. Single colonies were inoculated in 5 mL lysogeny broth (10 g L^{-1} NaCl, 5 g L^{-1} yeast extract, 10 L^{-1} g tryptone, autoclaved) and incubated at 37 °C for 8 h under agitation, after which 2.5 mL of the bacterial suspension was diluted 100-fold with lysogeny broth in a sterile bottle and incubated at 37 °C for 16 h to reach stationary phase (optical density 3 to 5.5 at 600 nm). The culture was then centrifuged (Z323K, Hermle) for 15 min at 4000 rcf and 4 °C and washed three times with saline solution (8 g L^{-1} NaCl, 0.8 g L^{-1} KCl, pH adjusted to 7-7.5 with 0.1 M NaOH, autoclaved). Pellets were then re-suspended in saline solution to reach a cell density of approximatively 10^9 colony-forming units (CFU) per mL and stored at 4 °C for up to 10 days. This suspension was then diluted in tap water or in the microalgae culture to reach the desired concentration used for the experiments. The characteristics and preparation of the tap water is available in the supplementary information (Table B.1).

3.2.2.2. Determination of the concentration of cultivable bacteria by plate counting

30 µL of a catalase suspension (3 mg mL^{-1}) was added to 1 mL of sample to remove residual H_2O_2. The concentration of cultivable bacteria was then determined by spreading 0.1 mL of sample on plate count agar. Plates were incubated at 37 °C for 24 h and colonies were manually counted. All experiments were performed at least in duplicate, in two separate plating series (statistical replicates), in minimum two consecutive dilutions (technical replicates), in order to obtain countable colony numbers.

3.2.3. Microalgae methods

3.2.3.1. Preparation of microalgae stocks and mineral medium

Chlorella vulgaris is used here as a model organism due to its widespread use both in scientific studies as well as commercial applications. Strain SAG 211-11b was acquired from the Culture Collection of Algae at the Göttingen University (SAG), Germany, and cultured in mineral medium that was designed for microalgae. The liquid medium was prepared in ultrapure water with the following concentrations (mg L^{-1}) : 550 $CO(NH_2)_2$, 118.5 KH_2PO_4, 102 $MgSO_4 \cdot 7H_2O$, 20 EDTA-FeNa, 86.9 $CaCl_2 \cdot 6H_2O$, 0.415 H_3BO_3, 0.475 $CuSO_4 \cdot 5H_2O$, 1.65 $MnCl_2 \cdot 4H_2O$, 0.3 $CoSO_4 \cdot 7H_2O$, 1.35 $ZnSO_4 \cdot 7H_2O$, 0.085 $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.007 NH_4VO_3 [68]. The reagents were provided by Sigma-Aldrich and the minimum purity was ≥ 98 %. The solid medium was prepared with agar (Sigma-Aldrich) and half the aforementioned concentrations. Stocks of microalgae were prepared by streaking microalgae on agar plates, which were incubated for two weeks at room temperature and constant illumination of 60 µmol $m^{-2} \cdot s^{-1}$. Plates were then stored at 20 °C and low illumination for up to one month.

3.2.3.2. Photobioreactors for the microalgae culture

A set of glass-columns (height: 50 cm, internal diameter 3.0 cm) was immersed into an aquarium, whose temperature was maintained with a chiller (TK 1000, TECO) coupled to a pump (compact 1000, EHEIM). Columns were illuminated with a vertical panel of eight fluorescent tubes (55 W DULUX cool white, OSRAM). The photosynthetically active radiation was measured at the surface of the aquarium with a quantum sensor (from Li-COR). Mixing and injection of air and CO_2 were carried out with mass flow controllers (Vögtlin) to provide a steady flow rate and CO_2 percentage.

3.2.3.3. Microalgae culture procedure

Single colonies were picked from stocks on agar plates and inoculated in 100 mL mineral medium in the photobioreactor. Cultivation was carried out under constant aeration (0.15 L_N min^{-1} 2 % (v/v) CO_2) at 25 ± 0.5 °C for six days. Illumination was gradually increased from 100 to 500 µmol $m^{-2} \cdot s^{-1}$ (24 h at 100 µmol $m^{-2} \cdot s^{-1}$,

72 h at 250 µmol $m^{-2}·s^{-1}$ and 48 h at 500 µmol $m^{-2}·s^{-1}$). pH was kept between 6.5 and 7.5 with 1 M NaOH. After six days, microalgae were washed two times (4000 rcf for 3 min) with tap water and re-suspended in 100 mL tap water. To determine cell count and dry weight, a sample was washed three times with ultrapure water and cells were counted under a light microscope (magnification 400, Axiolab, Zeiss) with a counting chamber (Neubauer improved, Marienfeld) and dry weight was determined with a moisture analyzer (HC 103, Mettler Toledo). The microalgae culture was then adjusted with tap water to a concentration of 100 mg dry weight L^{-1} (approximately 10^7 CFU mL^{-1}). The microalgae cultures were always fresh and used the same day of the preparation.

3.2.3.4. Determination of the concentration of cultivable microalgae by plate counting

30 µL of a catalase suspension (3 mg mL^{-1}) was added to 1 mL of sample to remove residual H_2O_2. The concentration of cultivable microalgae was then determined by spreading 0.1 mL of sample on mineral medium agar plates. Plates were incubated at room temperature and constant illumination of 60 µmol $m^{-2}·s^{-1}$ for about nine days and colonies were counted. Two separate plating series and consecutive dilutions were performed to obtain countable colony numbers.

3.2.4. Experimental procedure and analytical methods

3.2.4.1. Experimental conditions

Experiments were carried out in 100-mL Pyrex reactors previously acid-washed with H_2SO_4 and autoclaved. The reactors were placed in a water batch on a multi-position magnetic stirrer (MIX 15 eco, 2 mag) set at 350 rpm into a solar simulator (Suntest CPS, Atlas) and irradiated at 700 W m^{-2} with a Xenon lamp (wavelengths: 290-800 nm; approx. 0.5 % UVB, 5 % UVA; more information about the spectral distribution can be found in the supplementary material Fig. B.3). Global irradiance was measured with a pyranometer (CM6b, Kipp & Zonen). The aforementioned chiller and pump were used to keep the temperature at 25 ± 0.5 °C during experimentation. 100 mL of a bacterial suspension (in tap water or in a microalgae culture) were poured into each reactor and a sample to assess bacterial and microalgal cultivability at time zero was taken. Then,

reagents were added where required by the treatment in the following order (two-minute intervals between every addition): 1) citric acid, 2) Fe^{2+}, 3) H_2O_2. Samples for the analyses of dissolved Fe^{2+}/Fe^{3+} and H_2O_2 were taken immediately (<30 s) after the addition of the respective reagent. The lamp then was switched on and the experiment was carried out for two hours. Samples were taken periodically to determine the concentration of cultivable bacteria and microalgae, dissolved Fe^{2+}/Fe^{3+} and/or H_2O_2, and pH (InLab semi-micro electrode, Mettler Toledo).

3.2.4.2. Experimental plan

Three different set of experiments were carried out in tap water and/or microalgae cultures with variations of the treatments and bacterial concentrations but always with a constant simulated solar light intensity of 700 W m^{-2}. All experiments were repeated at least twice. The first set compared the effect of the presence of simulated solar light alone (henceforth solar light) and the PF (1 mg L^{-1} Fe^{2+} and 10 mg L^{-1} H_2O_2) treatment both in tap water and microalgae culture with bacterial concentrations of 10^6 CFU mL^{-1} each. The second set compared the effects of solar light, PF (1 mg L^{-1} Fe^{2+} and 25 mg L^{-1} H_2O_2) and citrate-modified PF (1 mg L^{-1} Fe^{2+},25 mg L^{-1} H_2O_2 and 17.5 mg L^{-1} of citric acid) treatments in tap water with a bacterial concentration of 10^6 CFU mL^{-1}. For the third set of experiments, solar light, PF (1 mg L^{-1} Fe^{2+} and 25 mg L^{-1} H_2O_2), citrate-modified PF (1 mg L^{-1} Fe^{2+},25 mg L^{-1} H_2O_2 and 17.5 mg L^{-1} of citric acid), and solar light/H_2O_2 (25 mg L^{-1} H_2O_2) treatments were carried out in microalgae cultures with 10^6 CFU mL^{-1} and 10^4 CFU mL^{-1} respectively. For all experiments, average pH measurements at time zero (before addition of chemicals) were 8.15±0.01 and 7.91±0.02 for tap water and microalgae cultures, respectively. While the addition of Fe^{2+} slightly decreased the pH, the addition of citric acid lowered the pH to around 7.2 for both tap water and microalgae culture. However, it was noticed that the pH recovered during experiments to values close to these measured at time zero. Also, separate control tests and existing literature showed that citric acid at a concentration of 0.09 mM is not harmful for *E. coli* or *C. vulgaris* [144–147].

3.2.4.3. H_2O_2 and dissolved iron measurements

The evolution of H_2O_2 concentration was measured by colorimetry (method: DIN 38.402 H15). Briefly, 10-20 µL of titanium (IV) oxysulfate were mixed with 1 mL of a filtered sample and subsequently measured at 410 nm with a spectrophotometer (UV-1800, Shimadzu). A calibration curve of seven points between 0 and 25 mg L^{-1} (R^2 > 0.99) was used to determine the H_2O_2 concentration. Dissolved Fe^{2+} and Fe^{3+} were measured spectrophotometrically at 562 nm using the Ferrozine method [148]. 1.6 mL of the sample was filtered and mixed with 0.2 mL of a 4.9 mM ferrozine solution, 0.2 mL of a hydroxylamine hydrochloride solution (10% w/w), and 0.5 mL of an acetate buffer (pH 4.66). The calibration curve was prepared by measuring pre-defined iron standards and the limit of quantification (LOQ) was 0.01 mg L^{-1}. Filtrations were performed with syringe filters (0.45 µm). Error bars represent the standard error of the mean.

3.2.4.4. Data treatment and presentation of cell inactivation kinetics

Data on cell cultivability were standardized to the initial concentration and are given as the inactivation of cells in logarithmic unit (logU) per mL (logCFU mL^{-1}). Error bars represent the standard error of the mean of said data. The limit of detection for both cultivable bacterial and microalgal cells is 10 CFU mL^{-1}, which corresponds to a single colony on an agar plate if 0.1 mL is spread.

3.3. Results and discussion

3.3.1. Effect of photo-Fenton and solar light on bacteria in presence and absence of microalgae

Figure 3.1 showcases the inactivation of bacteria (10^6 CFU mL^{-1}) during the solar light and PF treatments in tap water and in a microalgae culture (10^7 CFU mL^{-1}). In tap water (Fig. 3.1A), the PF reaction (adding 1 mg L^{-1} Fe^{2+} and 10 mg L^{-1} H_2O_2) inactivated all bacteria within 90 min, while solar light only yielded a measurable

but limited inactivation of bacterial cells after two hours (only 1.36 logU). When bacterial inactivation within a microalgae culture was assessed, inactivation was markedly less effective (Fig 3.1B): solar light and PF treatments reduced the cultivable bacteria concentration by 0.51 and 1.00 logU, respectively.

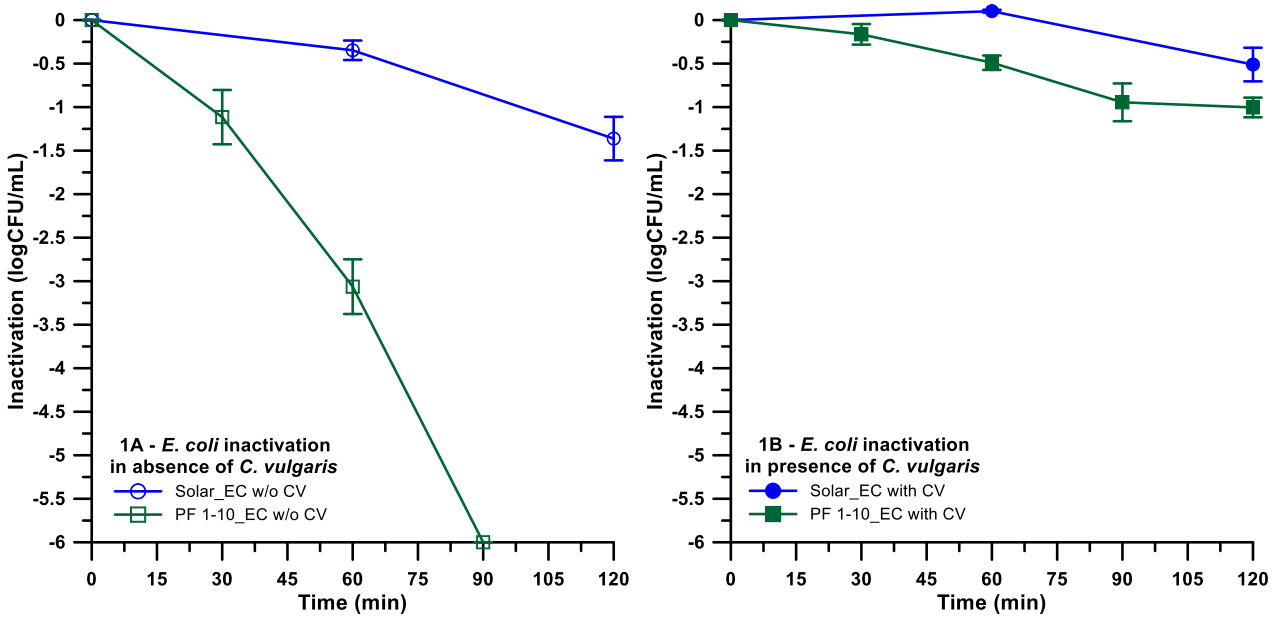

Figure 3.1. Inactivation of bacteria (10^6 CFU mL^{-1}) during solar light and PF (1 mg L^{-1} Fe^{2+} and 10 mg L^{-1} H_2O_2) treatments, in tap water (A) and in a microalgae culture (10^7 CFU mL^{-1}) (B).

Figure 3.1A confirms that even in near-neutral tap water, the PF reaction can significantly improve the inactivation of bacteria compared to solar light alone. Although the inactivation mechanisms have been recently summarized [111], updated and enriched [149–151], we will mention here the main events that lead to bacterial inactivation, in order to use this as a basis for the upcoming modifications introduced in this work, without their repetition each time. The action of solar light (UVB, UVA and visible light) affects the generation of intracellular reactive oxygen species (ROS). This takes place by the initiation of an intracellular Fenton process [116] by the accumulation of H_2O_2 [150] and its reaction with Fe^{2+}. H_2O_2 accumulates because scavenging enzymes are inactivated [149], leaving the bacterial cell defenseless. If one considers the addition of H_2O_2 and Fe, a further ROS imbalance takes place. H_2O_2 and iron can be transferred into the cell, where they further fuel the internal PF process [151]. Also, their simultaneous presence in the bulk can be described

by Eqs. 1.3-1.5 which show the general mechanism of the PF reaction. The Fe^{2+} produces highly bactericidal hydroxyl radicals ($HO^{\bullet}$) by the oxidative action of H_2O_2, while Fe^{2+} is regenerated from Fe^{3+} under light exposure, via a ligand-to-metal charge transfer (LMCT) (Eq. 1.6) [111]. In near-neutral and aerated conditions, the Fe^{2+} is oxidized into Fe^{3+} oxides, which precipitate in a few minutes [152]. Nevertheless, the duration is long enough to allow a diffusion of the iron cations and H_2O_2 into the bacteria, inducing major internal cell damages under light exposure [151]. The solid iron oxides generated from iron salts during treatment are also intrinsically reactive (photo)catalysts by different ways [153] such as: a) the iron photo-leaching which assures a steady state concentration of soluble iron (required for homogeneous PF) [154], b) the iron complexation and transportation into the bacterial cell by siderophoric proteins excreted by bacteria [155], c) the (photo)catalytic action for heterogeneous Fenton and PF reactions [156], d) the semiconductor action mode with O_2 and specially H_2O_2 as electron acceptors, which generate bactericidal species [153] and e) the possible LMCT with the bacterial membrane playing the role of the sacrificial ligand [157].

However, when bacteria in a microalgae culture are exposed to the PF process, a notable decrease of the bacterial inactivation kinetics occurs (Fig. 3.1B). This reduction has to be associated with the presence of microalgae and affects both the solar light and PF treatments. For the solar light treatment, the lower efficacy is likely caused by microalgae that shade bacteria from photons. Indeed, for the same photonic flux the targets are now ten times more, since ~10^7 CFU mL^{-1} of microalgae cells are present. For the PF treatment, the reduced germicidal capacity is most likely caused by the competition for the Fenton reagents, the multiplication of targets for the ROS generated by the PF process, and probably also to the intrinsic consumption of H_2O_2 by the scavenging mechanisms of the microorganisms. In order to assess these last points, further experiments were performed.

3.3.2. Impact of solar light and photo-Fenton process on microalgae cultivability during bacterial disinfection

Figure 3.2A presents the course of inactivation of microalgae (10^7 CFU mL^{-1}) during solar light and PF treatments of bacteria (10^6 CFU mL^{-1}) in a microalgae culture. It shows that an exposure of two hours under solar light had no detrimental effects on the microalgae cultivability, while the PF treatment reduced cultivability by only 0.29 logU. The weak effect of solar light was expected since microalgae are photosynthetic organisms and an irradiance of 700 W m^{-2} is well within the range of ambient solar radiation. It is also noteworthy to point out that while the observed effect of PF against microalgae appears to be lower than against bacteria (Fig. 3.1B), it is reversed when absolute cell numbers are considered, because a higher concentration of microalgae (10^7 CFU mL^{-1}) than bacteria (10^6 CFU mL^{-1}) was treated (we remind: PF inactivated $4.9 \cdot 10^6$ mL^{-1} microalgal cells and $9.0 \cdot 10^5$ mL^{-1} bacterial cells).

Consumption of H_2O_2 and dissolved iron during the PF treatment of bacteria in both tap water and microalgae culture are shown in Fig. 3.2B. While in tap water the concentration of H_2O_2 remained above 7 mg L^{-1} during the two hours of treatment, the presence of microalgae caused the H_2O_2 concentration to drop to less than 1 mg L^{-1} during the same time. At time zero, the concentration of dissolved iron in tap water and microalgae culture was 0.42 and 0.65 mg L^{-1} respectively, which is below the 1 mg L^{-1} of Fe^{2+} that were added. The concentration of dissolved iron at 15 min was below the LOQ of 0.01 mg L^{-1}. This indicates that the lifetime of dissolved iron is only a few minutes at these conditions.

If we assume that all H_2O_2 was effectively consumed (i.e. to generate $HO^{\bullet}$ and in a possible intracellular transfer), the number of H_2O_2 molecules required to inactivate each microalgae cell remains lower than for the bacteria. Nevertheless, due to the different starting concentrations, the experimental design does not allow to conclude which organism is more resistant to oxidation. It has to be kept in mind, though, that microalgae are not immune against oxidative stress. Therefore, tuning of oxidative stress levels, as well as

the relative concentrations between the targeted biological contaminant and the microalgae are key to a successful application.

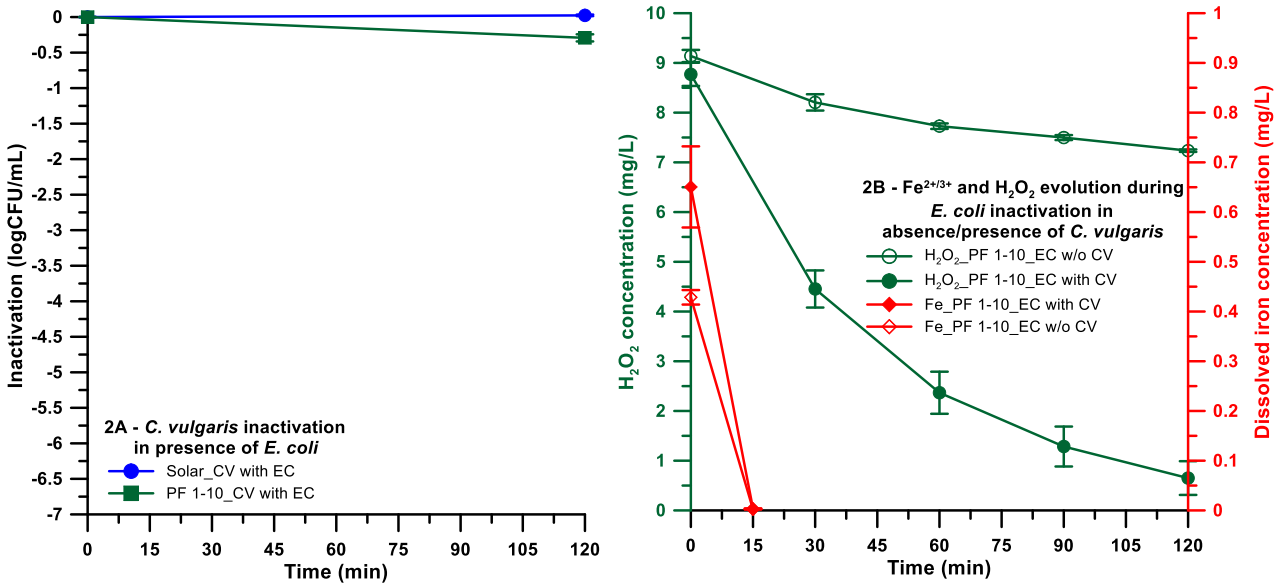

Figure 3.2. Inactivation of microalgae during solar light and PF (1 mg L^{-1} Fe^{2+} and 10 mg L^{-1} H_2O_2) treatments of bacteria in a microalgae culture (A). Consumption of H_2O_2 and dissolved iron concentration during the PF treatment for bacteria alone in tap water, and bacteria in a microalgae culture (B). Initial concentrations of bacteria and microalgae were 10^6 and 10^7 CFU mL^{-1}, respectively.

It can also be concluded that bacteria and microalgae compete for H_2O_2, as its concentration sharply drops from the beginning of the experiment in the presence of microalgae. Bacteria are known to have a complex system of H_2O_2 and superoxide radical scavenging [158], and it is likely that a similar system exists for microalgae. In fact, microalgae exhibit mechanisms to address oxidative stress from their metabolism (e.g. photosynthesis) and from external factors such as excessive irradiance or nutrient depletion [159,160]. Additionally, extracts from microalgae have been shown to have antioxidant properties [161,162] and their release from disrupted cells during the oxidative treatment may increase H_2O_2 consumption further. Therefore, the biggest complication from this matrix is that in the studied conditions, microalgae and bacteria are very quickly exposed to a much lower concentration of oxidant than in the situation where bacteria are

alone. The measurements of dissolved iron show that, in slightly basic conditions, the dissolved Fe^{2+} precipitates in a matter of minutes in both tap water and microalgae culture. Indeed, the concentrations measured at time zero were already much below the targeted initial concentration of 1 mg L^{-1}.

The presence of microalgae did not decrease the availability of the cationic iron forms. Therefore, the lower efficiency is likely due to the increase of oxidative targets. It was also noticed that microalgae cells aggregated after the addition of iron during the first minutes of PF experiments (Fig. B.1). Indeed, addition of $FeSO_4$ to microalgae cultures (tap water, near-neutral pH) triggers the formation and precipitation of iron hydroxides, which results in the coagulation of microalgae, most likely by destabilizing cell surface charges and also by favoring the aggregation via the anchoring sites of iron precipitates [163]. Consequently, the potential action in a heterogeneous PF process predominantly impacts microalgae, as they are the closest target.

In conclusion, in a mixed culture of microalgae and bacteria, we observed a decline in cultivability of both organisms that indicates that both are susceptible to the oxidative stress. However, only a small proportion of the organisms (in logU terms) was inactivated in the studied conditions, which is not the desired effect for the bacteria. For this reason, citric acid, a biocompatible and non-persistent natural organic complex, was added to limit precipitation or aggregation of iron cations, and thus, to extend the duration of a homogeneous PF process [164,165]. The H_2O_2 concentration was increased from 10 to 25 mg L^{-1} in order to prevent a depletion of the oxidative species required to maintain the effective production of $HO^{\bullet}$ from the photocatalytic iron recycling, as well as to compensate the possible consumption of H_2O_2 by 1) anti-oxidant and scavenging mechanisms of the studied organisms and 2) the oxidation of the citric acid itself.

3.3.3. Citrate-modified photo-Fenton process: towards the enhancement of the homogeneous Fenton events

3.3.3.1. Effect of citric acid addition and H_2O_2 concentration on bacterial inactivation in absence of microalgae

Figure 3.3A depicts the inactivation of bacteria (10^6 CFU mL^{-1}) in tap water under solar light only, PF (1 mg L^{-1} Fe^{2+} and 25 mg L^{-1} H_2O_2) and citrate-modified PF (1 mg L^{-1} Fe^{2+}, 25 mg L^{-1} H_2O_2, and 17.5 mg L^{-1} citric acid). Figure 3.3B shows the corresponding H_2O_2 consumption and dissolved Fe^{2+}/Fe^{3+} concentration. In tap water, the disinfection effect is partially attenuated by the presence of competitive anions, namely NO_3^-, HCO_3^-, Cl^-, and SO_4^{2-} (3, 110, 11 and 47 mg L^{-1}, respectively, see Table B.1 with the detailed tap water analysis), which compete with the $HO^\bullet$ generated by the PF process [166,167]. Citric acid is a known Fe^{2+}/Fe^{3+} chelator, and has been previously reported to enhance the action of the Fenton process at neutral pH [165,168]. In this range of pH, the citric acid added to water is deprotonated, and therefore encountered as citrate.

Figure 3.3A shows that the PF and citrate-modified PF reactions effectively inactivated bacteria at a concentration of 10^6 CFU mL^{-1} in tap water. However, PF inactivation rates of bacteria were neither impacted by the addition of citric acid nor by the increase of H_2O_2 concentration from 10 to 25 mg L^{-1}; most probably because an optimal efficacy was already reached with 1 mg L^{-1} of Fe^{2+} and 10 mg L^{-1} of H_2O_2. As a matter of fact, the H_2O_2 consumption is rate-controlled by the gradient of its transfer into the cell; a higher amount of H_2O_2 will rapidly enter the healthy cell, which will scavenge H_2O_2 as long as it is viable, i.e. it produces catalases; however, at lower H_2O_2 concentrations this effect is lower [169,170].

Furthermore, H_2O_2 is consumed at a higher rate when citric acid is added (Fig. 3.3B), that is not translated to a higher inactivation rate of bacteria. Additionally to the possibility of an already reached optimal efficacy, we can safely assume that a) the H_2O_2 concomitantly reacted with the citric acid (oxidation) [171] and b) citric acid is also a target of the $HO^\bullet$ (as all organic molecules), hence represents a non-effective $HO^\bullet$ consumption

[164]. Nevertheless, in both cases the H_2O_2 concentration remained high enough to keep the PF at its maximum efficacy and ensure that the reaction is not limited by the absence of the reagent.

The citrate-modified PF treatment keeps the iron in solution for more than 30 min while no dissolved iron was detected after 15 min for the PF treatment (Fig. 3.3B). Additionally, a solar light/Fe^{2+} treatment with citric acid (1 mg L^{-1} of Fe^{2+} and 17.5 mg L^{-1} of citric acid) revealed that iron remained in solution for at least 90 min in absence of H_2O_2. Treatments with citric acid (citrate-modified PF and solar light/Fe^{2+}) also showed an initial concentration of dissolved iron close to the expected 1 mg L^{-1} of Fe^{2+} (0.95 and 0.96 mg L^{-1}, respectively). The lifetime of the dissolved iron in tap water was therefore considerably improved by the addition of citric acid and enabled its consumption by reacting with the H_2O_2 to produce $HO^{\bullet}$.

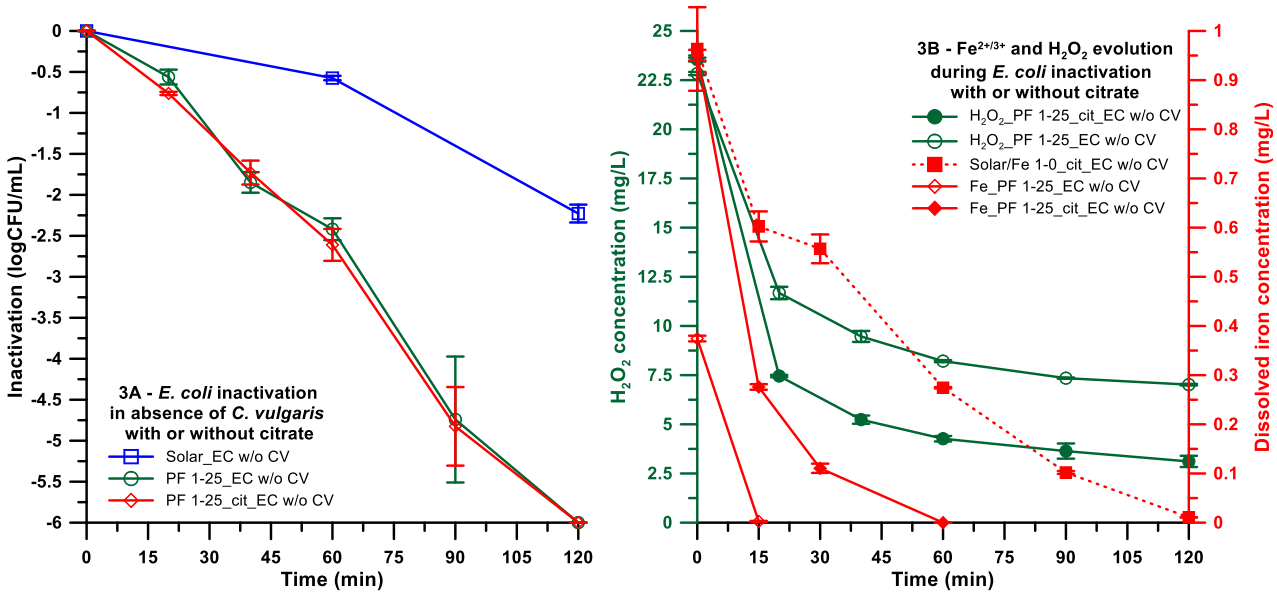

Figure 3.3. Inactivation of bacteria (10^6 CFU mL^{-1}) in tap water during solar light, PF (1 mg L^{-1} Fe^{2+} and 25 mg L^{-1} H_2O_2), and citrate-modified PF (1 mg L^{-1} Fe^{2+}, 25 mg L^{-1} H_2O_2, and 17.5 mg L^{-1} citric acid) treatments (A). Consumption of H_2O_2 and dissolved iron concentration during the PF and citrate-modified PF treatments, as well as during the solar light/Fe^{2+} treatment with citric acid (1 mg L^{-1} Fe^{2+}, 17.5 mg L^{-1} citric acid) (B).

The experimental modifications of the PF process explored in this section are therefore not crucial for the inactivation of bacteria. Neither the increase of H_2O_2 concentration nor the complexation of iron cations by

citric acid enhanced the inactivation rate of bacteria. On the contrary, the H_2O_2 increase resulted in a seemingly unnecessary consumption of reagents. However, citric acid efficiently maintained iron in solution and allowed an efficient reaction with H_2O_2. These modifications could be beneficial for the inactivation of bacteria in presence of microalgae where competing targets are numerous.

3.3.3.2. Effect of bacterial concentration during citrate-modified photo-Fenton inactivation in presence of microalgae

A bacterial concentration of 10^6 CFU mL^{-1} is commonly used for kinetic studies [108,116,149], yet it may be above the level of a realistic contamination in a microalgae culture, depending on the conditions. To address the role of bacterial concentration, inactivation of bacteria was studied in treatments with different initial concentrations of bacteria (10^6 and10^4 CFU mL^{-1}), always in presence of microalgae (10^7 CFU mL^{-1}) (Figure 3.4).

In a microalgae culture that contained 10^6 CFU mL^{-1} of bacteria (Fig. 3.4A), the highest inactivation efficacy was reached with the citrate-modified PF treatment (1 mg L^{-1} Fe^{2+}, 25 mg L^{-1} H_2O_2, and 17.5 mg L^{-1} citric acid). Around 3.04 logU of bacterial cells were inactivated in two hours. Without citric acid, the inactivation kinetics of the PF treatment (1 mg L^{-1} Fe^{2+} and 25 mg L^{-1} H_2O_2) was comparable to the solar light/H_2O_2 treatment (25 mg L^{-1} H_2O_2), while the concentration of bacteria was reduced by 1.70 and 1.88 logU, respectively. Within two hours, none of the treatments achieved a complete inactivation of all bacteria (i.e. a reduction of 6 logU).

In a microalgae culture that contained 10^4 CFU mL^{-1} of bacteria, all treatments (solar light/H_2O_2, PF and citrate-modified PF), except exposure to solar light only, reduced the concentration of cultivable bacteria by 4 logU within two hours (Figure 3.4B). Citrate-modified PF inactivated all bacteria in less than 90 min, PF and solar light/H_2O_2 had similar inactivation rates and required 120 min. In both sets of experiments, with 10^4 CFU mL^{-1} and 10^6 CFU mL^{-1} of bacteria, the order of the efficacy of the individual treatments remained the same. Likewise, inactivation efficacies of PF and solar light/H_2O_2 were comparable in both sets. This supports the aforementioned mechanistic interpretation of the results.

Despite the 3-logU inactivation (efficacy of 99.9 %) that was achieved in a microalgae culture with the citrate-modified PF treatment, with an initial concentration of 10^6 CFU mL^{-1} of bacteria (Fig. 3.4A), the remaining bacterial concentration of 10^3 CFU mL^{-1} may be a potential threat to the quality and further growth of the microalgae culture [172]. However, the use of higher concentrations of PF reagents at this stage is not advised because the possible increase of the inactivation of bacteria would concomitantly be accompanied by an enhanced detrimental impact on the microalgae culture viability. Ideally, the PF treatment has to be effective during the earliest step of contamination (when the antagonist population is still low), in order to increase the probability of their "total" inactivation with a limited detrimental impact on the comparatively higher population of microalgae.

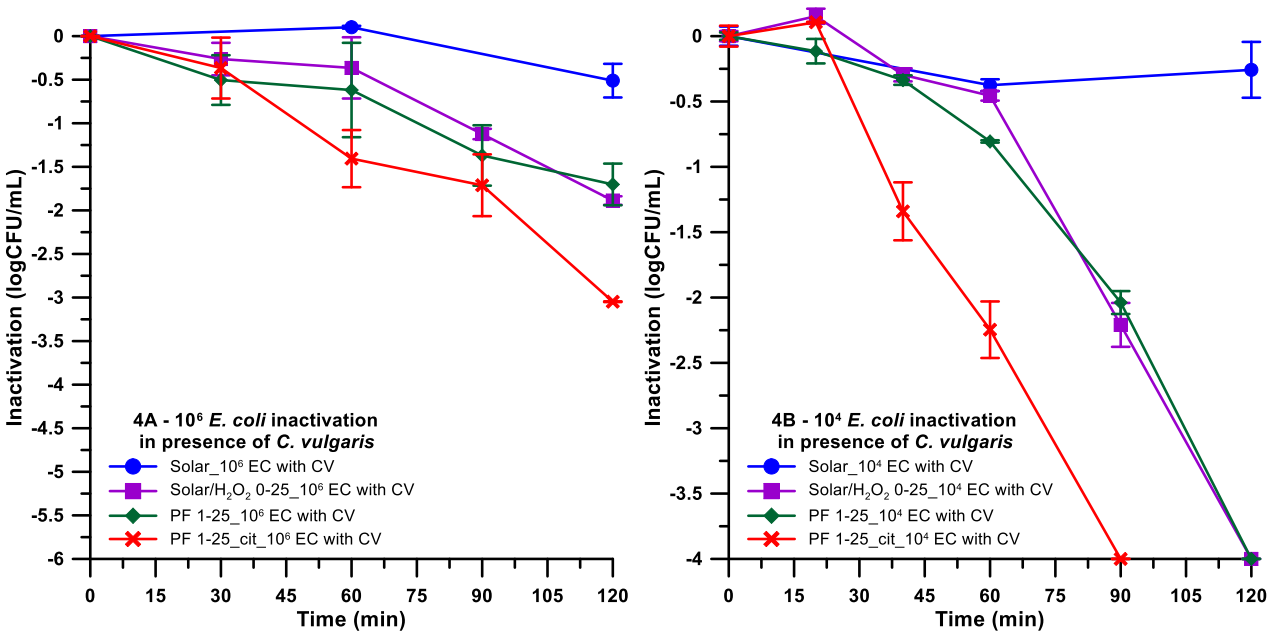

Figure 3.4. Inactivation of bacteria in a microalgae culture (10^7 CFU mL^{-1}) during solar light, solar light/H_2O_2 (25 mg L^{-1} H_2O), PF (1 mg L^{-1} Fe^{2+} and 25 mg L^{-1} H_2O_2) and citrate-modified PF (1 mg L^{-1} Fe^{2+}, 25 mg L^{-1} H_2O_2, and 17.5 mg L^{-1} citric acid) treatments. The initial concentration of bacteria was 10^6 (A) and 10^4 (B) CFU mL^{-1}.

Considering the solar light treatment, it could be expected that an initial bacterial concentration of 10^4 CFU mL^{-1} would results in a faster inactivation than for 10^6 CFU mL^{-1}. However, in both cases bacterial concentrations were modestly changed by solar light, which indicates that the limiting factor is the shading from light by microalgae, which does not depend on the concentration of bacteria. For a bacterial concentration of 10^4 CFU mL^{-1}, the 4-logU inactivation of bacteria with the solar light/H_2O_2, PF and citrate-modified PF treatments shows that microalgae do not prevent the oxidative species to reach the bacterial targets. Therefore, interactions between microalgae and bacteria do not hinder the inactivation processes.

These results support our previous findings that the lifetime of the dissolved iron, at near-neutral pH and in absence of citric acid, is too short to react efficiently with the H_2O_2 and to produce enough $HO^{\bullet}$, considering the higher number of oxidative targets when microalgae are present. If the iron had been available, the PF reaction without citric acid would have inactivated bacteria in a larger extent than the solar light/H_2O_2, which was not observed (Fig 3.4A and Fig. 3.4B). The addition of citric acid keeps the iron in solution, and enables the PF cycle to effectively take place. Without addition of citric acid, the PF reaction under these conditions is only able to inactivate bacteria via the less effective solar light/H_2O_2 process.

Measurement of dissolved iron was performed during treatments for both concentrations of bacteria (10^6 and 10^4 CFU mL^{-1}) in microalgae cultures (Figure 3.5). During the PF treatment, a decrease was already observed at time zero and no dissolved iron was detected after 15 min. However, during the citrate-modified PF treatment, dissolved iron was still present after two hours (0.058 and 0.043 mg L^{-1} of dissolved iron for 10^6 and 10^4 CFU mL^{-1} of bacteria, respectively). Additionally, a solar light/Fe^{2+} treatment with citric acid was performed as a reference for the bacterial concentration of 10^6 CFU mL^{-1}, and 0.143 mg L^{-1} of dissolved iron was detected after two hours of treatment. It was also observed that the addition of citric acid prevented an aggregation of microalgae during the experiments (Fig. B.1). These results are in accordance with the previous assumptions. Indeed, the similar results between the solar light/H_2O_2 and PF treatments may be associated to the low iron availability in comparison to the number of oxidative targets. The addition of citric acid does not only improve the solubility of the iron but also enables its reaction with H_2O_2, which results in

a higher bacterial inactivation during the citrate-modified PF treatment. Additionally, the prevention of microalgae aggregates during the citrate-modified PF treatment may also improve the homogeneous PF reaction with bacteria by not being in the aggregates of microalgae.

The H_2O_2 consumption during both experiments is also shown in Fig. 3.5. It reveals that decreasing the bacterial concentration from 10^6 to 10^4 CFU mL^{-1} reduces also the H_2O_2 consumption rate. Indeed, a bacterial concentration of 10^4 CFU mL^{-1} not only decreases the number of cell targets for light absorption and oxidative species that are generated, but also metabolites and cell fractions generated by their oxidative lysis and disruption. Also, as long as bacteria are healthy, there is a 2-logU difference in the number of cells, which decreases their H_2O_2 scavenging capacity. Furthermore, we noticed the same H_2O_2 consumption between the PF and solar light/H_2O_2 treatments without citric acid. We also report that the addition of citric acid increased the H_2O_2 consumption for both tested bacterial concentrations, which is partially associated to the increased availability of the dissolved iron.

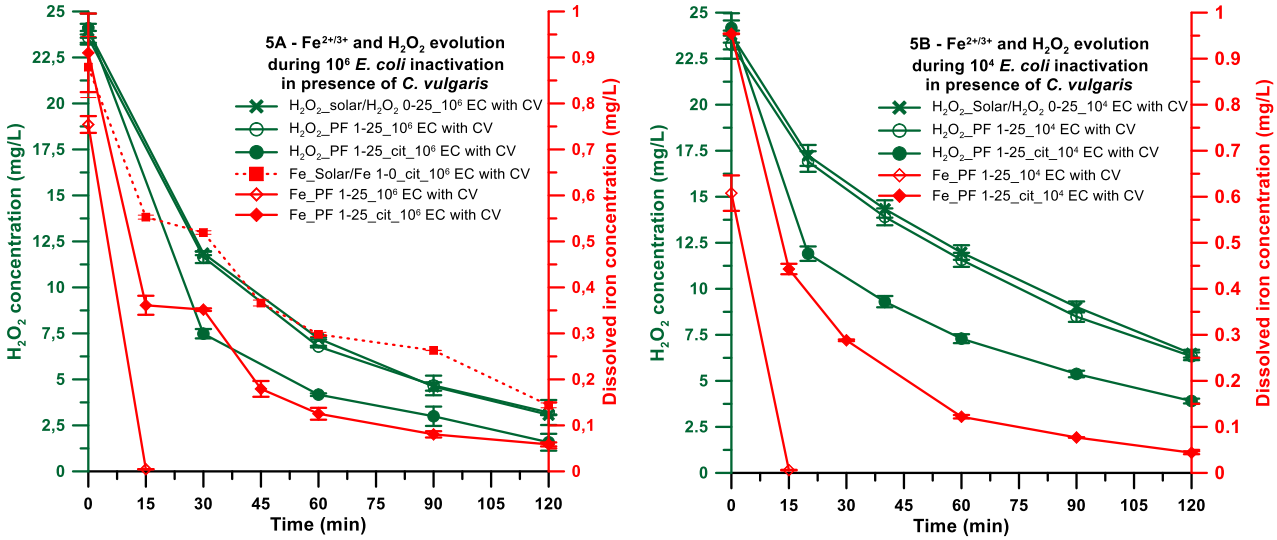

Figure 3.5. Consumption of H_2O_2 and concentration of dissolved iron during the solar light/H_2O_2 (25 mg L^{-1} H_2O_2), PF (1 mg L^{-1} Fe^{2+}, and 25 mg L^{-1} H_2O_2) and citrate-modified PF (1 mg L^{-1} Fe^{2+}, 25 mg L^{-1} H_2O_2, and 17.5 mg L^{-1} citric acid) treatments in microalgae cultures (10^7 CFU mL^{-1}) with a bacterial concentration of 10^6 (A), and 10^4 (B) CFU mL^{-1}, respectively Additionally, a solar light/Fe^{2+} treatment with citric acid (1 mg L^{-1} Fe^{2+}, 17.5 mg L^{-1} citric acid) was performed for a concentration of 10^6 CFU mL^{-1} of bacteria in a microalgae culture.

3.3.3.3. Impact of bacterial concentration on microalgae cultivability during the photo-Fenton process: competition for the generated ROS.

The cultivability of the microalgae was assessed while they experienced the treatments with bacterial concentrations of 10^6 (Figure 3.6A) and 10^4 CFU mL^{-1} (Figure 3.6B), respectively. When the microalgae culture is treated in presence of 10^4 instead of 10^6 CFU mL^{-1} of bacteria an increase of the microalgae inactivation rate is observed for all the oxidative treatments even if the order of efficacy for the microalgae inactivation is similar to that observed with a bacterial concentration of 10^6 CFU mL^{-1}. The solar light/H_2O_2 and the PF treatments without citric acid showed the highest damaging effect on the microalgae after two hours: a mean reduction of 1.82 and 4.16 logU of microalgae was observed for bacterial concentrations of 10^6 and 10^4 CFU mL^{-1}, respectively. The addition of citric acid resulted in a lower damage than the previous treatments exhibiting a microalgae inactivation, after two hours, of only 1.30 and 3.32 logU in presence of 10^6 CFU mL^{-1} and 10^4 CFU mL^{-1} of bacteria, respectively.

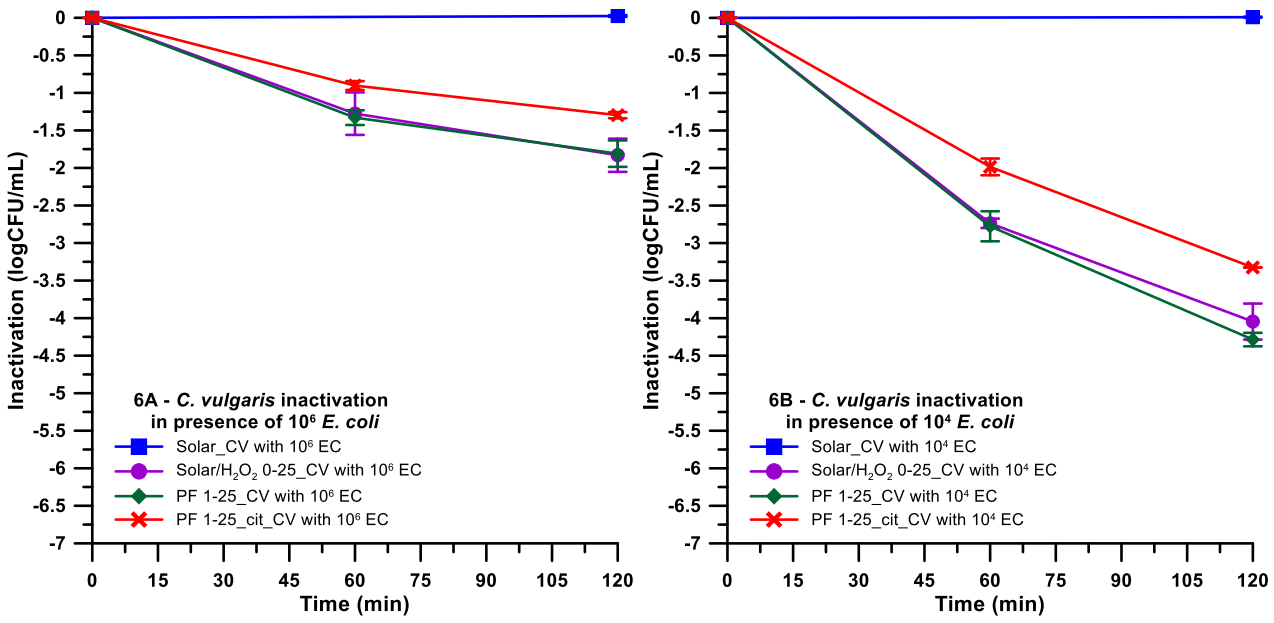

Figure 3.6. Inactivation of microalgae (10^7 CFU mL^{-1}) during solar light, solar light/H_2O_2 (25 mg L^{-1} H_2O_2), PF (1 mg L^{-1} Fe^{2+}, and 25 mg L^{-1} H_2O_2) and citrate-modified PF (1 mg L^{-1} Fe^{2+}, 25 mg L^{-1} H_2O_2, and 17.5 mg L^{-1} citric acid) treatments in microalgae cultures with a bacterial concentration of 10^6 (A), and 10^4 (B) CFU mL^{-1}, respectively.

It could be expected that this more effective citrate-modified PF process would be also more germicidal for microalgae. Interestingly enough, the citrate-modified PF inactivates microalgae in a lower extent than during the solar light/H_2O_2 and PF treatments, while it showed the best bacterial inactivation rate (Fig. 3.4A and Fig. 3.4B). This result is apparently in contradiction with the general behavior of bacterial, viral or yeast systems which normally show positive correlation between the oxidative stress force and the inactivation capability of the applied oxidative process [154,156,165,173]. However, we report that the addition of citric acid and the shift to a longer homogeneous PF duration enhances the ROS production in the bulk. If we assume that bulk ROS are the driving force of the inactivation, then *C. vulgaris* with its thicker cell wall is more protected than *E. coli* against this extracellular oxidative stress. In order to further evidence this unexpected behavior of the microalgae inactivation, the cellular volume, wall thickness and iron content of *C. vulgaris* were compared with *E. coli* (Table 3.1). With these data, we will try to assess the likeliness that the driving force for the inactivation of *C. vulgaris* is an intracellular PF process.

The higher cellular volume of the microalgae results in a lower surface/volume ratio, which combined with a thicker cell wall, probably slows down the speed of diffusion of H_2O_2 and Fe^{2+}, compared to bacteria. Therefore, we hypothesize that during the citrate-modified PF reaction, the iron and H_2O_2 permeate faster inside bacteria than in microalgae. Under initiation of the light exposure, the bacteria immediately suffer a strong internal and external oxidation, while the respective intracellular effect is attenuated for the microalgae. Most likely, this happens because the reagents need more time to permeate or creates holes in the cell wall and reach the cytoplasm [174]. Therefore, the permeation into the microalgae is weakened when the citric acid is added due to the faster decrease of the H_2O_2 concentration, thanks to its PF reaction with iron in the bulk. At this point, we need to consider that microalgae have approximatively ten times more iron than bacteria, although most of it is used by the photosynthetic apparatus [175]. Consequently, microalgae are likely more sensitive to a possible intracellular PF, which makes the H_2O_2 concentration more critical than the availability of the added iron; this might explain the higher efficacy of the solar light/H_2O_2 process compared to the homogeneous PF.

The prevention of microalgae aggregates by the citric acid is another factor that may reduce the inactivation of microalgae during the citrate-modified PF treatment. Indeed, if the iron precipitates are not mainly concentrated close to the microalgae (as it is expected with the aggregates of microalgae), their inactivation by a heterogeneous PF reaction is expected to be weaker. However, the contribution of the heterogeneous PF reaction is expected to be small under present experimental conditions as no noticeable adverse effect was observed on the cultivability of microalgae.

Table 3.1. Typical values for the cellular volume, wall thickness, dry weight and iron content of C. vulgaris and E. coli.

	C. vulgaris	*E. coli*
Volume (μm^3 $cell^{-1}$)	30[a)] [176]	0.42-1.79 [177]
Wall thickness (nm $cell^{-1}$)	100[a)]	6.35 [178]
Dry weight (pg $cell^{-1}$)	6.28 ± 0.51[b)]	0.44 [179]
Iron content (% dry weight)	0.2-0.68 [180]	0.022 [181]

a) Values were estimated from Fig. B.2, b) The average was calculated from six runs of microalgae cultures

3.3.4. Integrated inactivation mechanism during the simultaneous presence of *E. coli* and *C. vulgaris* by the citrate-modified photo-Fenton process

In order to present an overview of the photo-catalytic disinfection mechanisms taking place in the proposed intervention for decontamination of microalgae cultures, we summarize our findings and contextualize our propositions alongside the existing literature propositions for the pathways to inactivation (for simplicity, references will not be repeated anew).

Concerning the *E. coli* inactivation in a *C. vulgaris* culture, the following mechanisms summarized in Figure 3.7A can be considered, in an inside-out approach (see pathways 1-6):

1) The addition of H_2O_2 and Fe^{2+} in the bulk initiates a transport of the reagents in the bacterial intracellular domain. Considering that the Fenton process is naturally occurring at low, non-bactericidal levels inside the cell, the presence of additional H_2O_2 and/or Fe^{2+}/Fe^{3+} replenishes the Fenton reagents. However, we note here that this process is attempted to be controlled by the H_2O_2 scavenging enzymes (catalases, peroxidases) and Fe^{2+}/Fe^{3+}-sequestering mechanisms.
2) Illumination induces the inactivation of the H_2O_2 scavenging enzymes inside the cell, which in turn incapacitates the cell against the intracellular PF process. Hence, the production of H_2O_2 in the cell increases, and an important occurrence of intracellular oxidative stress ensues.
3) The H_2O_2 moderately affects cell viability, due to its low oxidation potential when reacting with the bacterial membrane, but its reaction with Fe^{2+} in the bulk results to $HO^{\bullet}$ generation and Fe^{3+} (homogeneous Fenton process). In presence of O_2 (and H_2O_2) iron oxidation leads to the generation of iron oxides (denoted Fe_xO_y), mostly Goethite and Lepidocrocite for the $FeSO_4$ salt. These oxides can play the role of the heterogeneous photo-catalyst and sustain a heterogeneous PF.
4) Solar light can reduce Fe^{3+} back to Fe^{2+} via a LMCT, if a ligand is found. The Fe^{3+} in water can form complexes with water or with surface functional groups of the bacterial membrane (e.g. carboxyl, hydroxyl or amine groups). Upon light irradiation, these can play the role of the sacrificial ligand and

electron donor, facilitating the Fe^{3+}/ Fe^{2+} turnover. This Fe^{2+} can either react with bulk H_2O_2 or enter the cell (pathway 1). Fe^{3+} can also get chelated by siderophores produced by *E. coli*, for as long as the cell is healthy and produces these proteins.

5) The presence of iron oxides can play the role of a semiconductor photo-catalyst in the following ways: i) By attachment to the cell wall, which oxidizes it by the (low potential) generated holes, or ii) by the electron excitation, which can lead to a reaction with O_2 and generation of superoxide radical anion. $O_2^{\bullet-}$ can either reduce components in the bacterial cell wall or Fe^{3+} to Fe^{2+}, further enhancing the PF process. A two-electron transfer would lead to H_2O_2 generation, further fueling the $HO^{\bullet}$production by the PF process.
6) Finally, solar light increases the leaching of Fe^{2+} into the bulk, via Fe^{2+}/Fe^{3+}-oxide surface reactions that lead to photo-dissolution. The Fe^{2+}/Fe^{3+} released can participate in the aforementioned path-ways, enhancing the oxidative action of the process.

Concerning *C. vulgaris*, there are some strong indications for existing pathways similar to *E. coli*, but some new ones proposed in our work are related to the presence of iron in the bulk. More specifically, the proposed mechanism is as follows (Figure 3.7B, pathways 1-6):

1) Solar light is not largely affecting *C. vulgaris*, since it is the driving force for its photo-synthetic activity and is well-equipped to handle these types of stress. However, the addition of H_2O_2 or iron in the bulk during illumination (without citric acid) entails the possibility of having a similar pathway to *E. coli*, as *C. vulgaris* needs iron for photo-synthetic purposes and holds significantly higher amounts in its cell. Also, H_2O_2 can pass through lipids but *C. vulgaris* can scavenge intracellular amounts, for as long as its enzymes are active. Hence there is a possibility of an enhanced intracellular PF process, similarly to how it was discussed before.
2) The presence of H_2O_2 in the bulk can lead to reactions with the microalgal cell wall, as seen by the high solar light/H_2O_2 germicidal effect, but the kinetics and the oxidative potential are unknown, so we cannot yet pinpoint the contribution of this process. However, it was made evident that the

addition of iron has enhanced the aggregation of *C. vulgaris* cells, forming large aggregates that settle rapidly.

3) The simultaneous addition of Fe^{2+} and H_2O_2 surely induces the Fenton process, and under solar light, the PF process affects *C. vulgaris* in a similar way as it affects *E. coli*. However, the aggregates formed (in absence of citric acid) may increase the effect of heterogeneous PF on the microalgae.
4) The LMCT reported for bacteria is very likely to happen in algae as well. The high negative charges of *C. vulgaris* cell wall assures its affinity to Fe^{3+}, and under solar light, the LMCT is possibly taking place. However, it is unknown if the detrimental effect may be attenuated by the thicker cellular formation.
5) Similarly to 4), pathway 5), i.e. the semiconductor action mode surely takes place, but its efficacy is yet to be elucidated. It is important to determine the reductive potential of the *C. vulgaris* cell wall to assess the possibility of direct oxidation by the holes, or its reduction by the $O_2^{\bullet-}$ generated by the excited electrons. Also, the superoxide's dismutation to H_2O_2 may enhance the $HO^{\bullet}$ generation in the bulk.
6) Finally, the enhanced photo-dissolution of iron oxides will fuel the Fe^{2+}-mediated processes mentioned above. From our data, we can assert that this process will affect the aggregation or the LMCT on the cell wall.

Finally, a special mention has to be made to the most effective process among the studied ones in this research, i.e. the modification of the PF process by citric acid. By the addition of this low-molecular weight acid, the homogeneous aspects of the process enhanced bacterial inactivation and reduced the respective microalgae one. Figure 3.7C presents a schematic overview, which depicts the possible routes to inactivation induced by the citrate-modified PF process (pathways 1-6):

1) Upon irradiation, the Fe^{3+}-citrate ligand is absorbing energy which facilitates the LMCT from citrate to iron.
2) The LMCT process is reducing Fe^{3+} to Fe^{2+}, leading to a citrate radical and Fe^{2+}.
3) The oxidized citrate radical reacts with molecular oxygen and generate superoxide radical anions.

4) The superoxide radical anions $O_2^{\bullet-}$ generated can reduce Fe^{3+} to Fe^{2+}, which in turn will re-participate in the PF process taking place in the bulk. Also, it can reduce components in the bacterial cell wall, and most likely in the respective microalgal counterpart, or generate H_2O_2 (via dismutation).
5) The generation (and/or presence) of Fe^{2+} in the bulk is critical; compared to the process without citric acid, the complexation and regeneration process is very important to the proposed disinfection process. However, according to the mechanisms discussed for *E. coli* and *C. vulgaris*, respectively, it is yet to be determined if these Fe^{2+} amounts can affect the intracellular domain of *C. vulgaris* with the same severity as *E. coli*.
6) The newly produced Fe^{2+} will react with the H_2O_2, resulting to a PF process with higher disinfection capacity, since it offers more readily available iron to the Fenton process. The fact that through this pathway the enhancement of the homogeneous process is more germicidal for *E. coli* rather than *C. vulgaris* is noteworthy. As such, we propose that either the intracellular process is not effectively inactivating *C. vulgaris*, or that the generation of $HO^{\bullet}$ in the bulk is naturally more effective to disrupt cell wall of *E. coli*, and hence, lead to faster inactivation. Further testing has to be performed in order to assess the two aforementioned possibilities, as we have strong indications for the validity of both pathways.

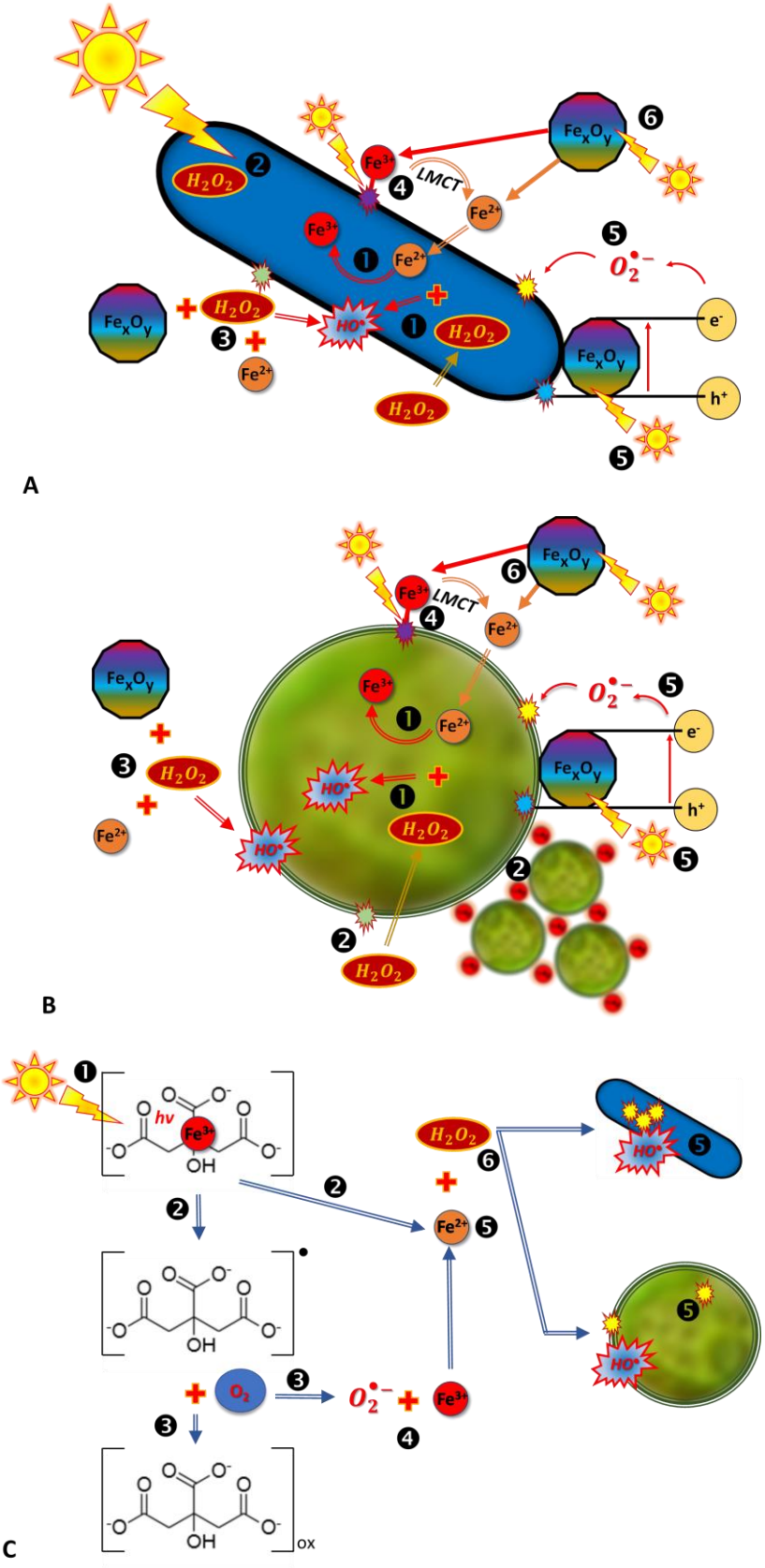

Figure 3.7. Integrated proposal for the inactivation mechanisms of E. coli (A), C. vulgaris (B), and the modifications of E. coli and C. vulgaris inactivation by the citrate-modified PF process (C). The numbers (1 to 6) refer to the text.

3.4. Conclusions

In this work, we used the photo-Fenton process to eradicate bacterial contamination in a microalgae culture. While the results indicate that both bacteria and microalgae are harmed by oxidative stress, the mechanism and kinetics of inactivation differ between them and are further altered by their simultaneous presence. Firstly, shading by microalgae protects bacteria from the oxidative stress induced by radiation. Secondly, the presence of microalgae increases the oxidative targets and increases H_2O_2 consumption, both of which reduce the bacterial inactivation. Also, the addition of citric acid results in a homogenous catalysis, which is shown to preferentially harm bacteria. This feature of the citrate-modified photo-Fenton process, namely the differential effect on bacteria and microalgae, has a potential applicability for large scale processes, in which early signs of contamination should be handled by addition of the Fenton reagents in presence of citric acid. The high microalgae survival rates found here indicate that the culture will continue to thrive after disinfection of the bacterial contaminant.

The present study laid the groundwork for a new disinfection method and further studies are now required to determine the optimal operating space and boundary conditions. The appropriate concentration of the Fenton reagents is a critical factor and varies as a function of the species involved and their respective cell concentration. Furthermore, a continuous feed of reagents as well as a homogenous distribution of photons would allow steady conditions and thus, operation at overall lower levels of reagents without risk of shortage. This would also reduce the impact of the initial oxidative stress, which is experienced by the microalgae under a batch regime. Also, the efficacy of the method relies on the application of an appropriate oxidative stress when the concentration of the biological contaminant is much smaller than the population of microalgae, thus the culture of microalgae has an increased probability of recovery. Once the operating range for an effective decontamination has been described for a series of biological contaminants and cultivation units, this could define the potential applications in the commercial production of microalgae. Then, cost assessments should be done and compared with current biocidal agents to determine the economic feasibility of the process.

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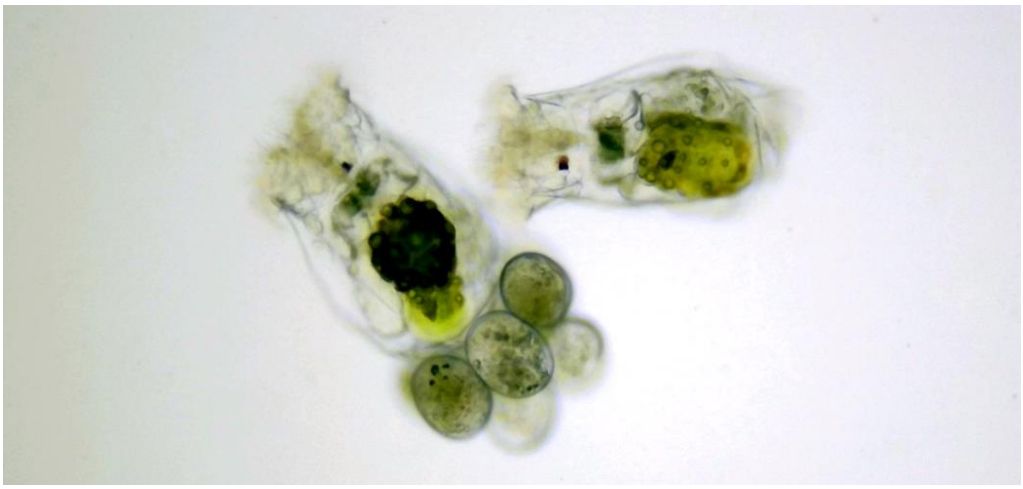
Chapter 4. Effective decontamination of rotifers in microalgae cultures



The content of this chapter is included in:

- A. Pulgarin, J. Decker, J. Chen, S. Giannakis, C. Ludwig, D. Refardt, H. Pick, Effective decontamination of the rotifer *Brachionus calyciflorus* from a *Chlorella vulgaris* microalgal culture by homogeneous solar photo-Fenton at neutral pH: inactivation kinetics and successful recultivation of treated cultures, (manuscript to be published).

- A. Pulgarin, J. Decker, J. Chen, S. Giannakis, C. Ludwig, D. Refardt, H. Pick, A method for the continuous production of *Brachionus calyciflorus* rotifers in a 1-liter culture, (methodical protocol to be published).

The author, Adrian Pulgarin, has carried out the majority of the conceptual and methodological work, data analysis and writing of the manuscript. The author has carried out the experiments jointly with Jérémie Decker (Master's student) and developed the rotifer methods jointly with Jiahua Chen (trainee), both under the supervision of the author.

Abstract

In this study, a citrate-modified photo-Fenton process was successfully applied to decontaminate a microalgae culture spiked with 5 rotifers mL^{-1} from the species *Brachionus calyciflorus*. The treatment did not jeopardize the regrowth of the microalgae and the cultures were successfully cultivated during 14 days post-treatment. The decontamination was effective as no regrowth of rotifers was observed during the cultivation of the microalgae. The impact of the citrate-modified photo-Fenton treatment was also studied with a starting concentration of 20 rotifers mL^{-1} and compared with a solar light/H_2O_2 treatment. Results show that both treatments had similar efficacies on the rotifers but that the citrate-modified photo-Fenton treatment had a lower impact on the cultivability of microalgae than the solar light/H_2O_2. However, while rotifers were impacted by the treatment, their inactivation was not complete and post-treatment regrowth occurred, which highlights the importance to apply the treatment at an early stage of the contamination. Moreover, an initial contamination level of 5 rotifers mL^{-1} is already a significant threat as the rotifer population reached more than 1000 rotifers mL^{-1} after 14 days and resulted in the failure of the microalgae culture.

4.1. Introduction

In the previous chapter, employing the photo-Fenton (PF) process under (simulated) solar light and with the help of a natural chelator (citrate) has been proven effective in eliminating bacteria from a microalgae culture. This application bears the advantage over existing oxidative decontamination processes of performing well at a neutral-basic pH value by virtue of citrate [182], and utilizing solar irradiation, a key component in algal cultures [183]. Furthermore, despite the non-selective nature of the hydroxyl radicals [184] and its documented capability of disrupting algae cells [106,185], a selectivity over bacteria has been noted, leaving the microalgal culture relatively unharmed, with a potential to regrowth [122]. As such, it would make an excellent case if a similar effect was attained in eliminating rotifers from a microalgal culture, without significantly hampering microalgae growth.

Here, solar based treatment methods, namely solar light/H_2O_2 and PF treatments were applied on microalgae cultures contaminated by rotifers. Light-driven autotrophic processes are the main culturing mode for producing large quantities of microalgae biomass [186]. Thus, the decontamination process presented here is compatible with this mode of cultivation. The ultimate objective is to determine if the homogeneous, citrate-modified PF method developed against bacterial contaminations (Chapter 3) could also be used to remove a microalgae predator, which is a bigger and more complex organism than bacteria. In order to attain this goal, we studied i) the effects the solar-based treatments exert on microalgae (*Chlorella vulgaris*) and rotifers (*Brachionus calyciflorus*) and ii) the recovery of both organisms after treatment. For this purpose, the effects on microalgae and rotifers were assessed immediately after treatments as well as up to two weeks later to assess the long-term efficacy of the treatments. Ultimately, the study aims to contribute to the development of a cheap, sustainable, and bio-compatible in-situ oxidative treatments for the protection against biological contaminants in microalgae cultures.

4.2. Material and methods

4.2.1. Preparation of synthetic freshwater

A moderately hard synthetic freshwater was prepared with the following concentrations (mg L^{-1}): 122.85 $MgSO_4 \cdot 7H_2O$, 60 $CaSO_4 \cdot 2H_2O$, 4 KCl, 96 $NaHCO_3$ in ultrapure water (milli-Q, Merck) [187]. This solution was stored in the dark at ambient temperature and used as liquid medium for the cultivation of rotifers and preparation of microalgae suspensions.

4.2.2. Microalgae methods

4.2.2.1. Cultivation of microalgae

Chlorella vulgaris (SAG 211-11b, Göttingen University, Germany) was cultivated in order to feed rotifers and to prepare standardized microalgae cultures, i.e., cultures with a dry weight of 100 mg L^{-1} and a cell concentration of ~10^7 cells mL^{-1}; these were then used to carry out inactivation experiments. Microalgae methods and the setup of glass-column photobioreactors (PBRs) were identical to what was described in section 3.2.3, with a series of modifications:

i) Tap water was replaced by synthetic freshwater.

ii) If microalgae were mixed with rotifers, samples collected to determine the cultivability of microalgae were filtered with a 40-µm mesh to remove rotifers.

iii) The culture procedure in glass-column PBRs was slightly altered: a) the nutrient concentration of the liquid mineral medium was doubled, b) instead of a gradually increasing light intensity, a constant intensity of 250 µmol m^2 s^{-1} of photosynthetically active radiation was used, c) the cultivation period was increased from 6 to 7 days, d) liquid mineral medium was added to the culture after 3 days to increase the volume from 100 to 200 mL.

iv) Microalgae used to carry out inactivation experiments were always harvested after 7 days of growth and used fresh the same day. About 100 mL of the microalgae culture was required to

prepare the experiment and the stock to feed rotifers. Therefore, and if needed, the remaining 100 mL were adjusted to 200 mL with liquid mineral medium and the cultivation in a glass-column PBR was extended to 14 days. However, microalgae harvested during this extended period were only used to prepare stocks to feed rotifers.

4.2.2.2. Preparation of microalgae stocks to feed rotifers

Synthetic freshwater was used for every step of the following procedure: microalgae were harvested, washed two times (4000 rcf for 3 min), and re-suspended in their initial volume. The cell count was determined, and microalgae were diluted to ~$2 \cdot 10^8$ cell mL^{-1}. This stock was stored up to 4 days at 4 °C and used daily to feed rotifers.

4.2.3. Rotifer methods

4.2.3.1. Counting method to monitor rotifers

Counting of *Brachionus calyciflorus* rotifers (AB-R10F, Florida Aqua Farms Inc., USA) was carried out in a watch glass or in petri dishes under a binocular (SZ51, Olympus) with a 10 to 20× magnification, which allowed counting of female and male rotifers as well as free and attached eggs. Female rotifers are recognizable by their large size, while male rotifers are much smaller and swim faster. Female rotifers were categorized as follows: swimming, motile, or inactive. Swimming rotifers (male and female) were counted with a 10× magnification. Motile and inactive female rotifers, as well as eggs were counted with a 20× magnification. Non-swimming female rotifers were observed for 5 s; if life signs were noticed (e.g., movement, activity of the mastax or ciliary corona, etc.), the rotifer was categorized as a motile rotifer, if not, it was categorized as an inactive rotifer (i.e., most likely dead). Healthy female rotifers were noticeable because they usually swim, predate and eat microalgae. Swimming female rotifers were the focus of this study, and therefore, male rotifers and motile/inactive female rotifers are not shown in the results.

To determine the concentration of rotifers in cultures, a 1-mL sample was counted in a watch glass. Above a concentration of 40 individuals per mL, counting is difficult, and therefore, the sample was subdivided by pouring it first in a 1.5-mL tube and counting rotifers successively on smaller volumes (e.g., 200 µL). Then, the empty tube was rinsed with synthetic freshwater and the number of rotifers contained in the rinsing water was added to the count.

Rotifer viability tests were carried out during the experiments, which required to incubate 1-mL samples in petri dishes and to monitor the growth. To do the counting, the petri dish was first gently shaken to homogenize the content and a grid was placed below as a reference. Rotifers were then counted by screening the petri dish following the pattern of the grid. Each petri dish was screened twice with a 10 and 20× magnification.

4.2.3.2. Cysts hatching and cultivation in petri dishes

A 40-µm sieve (40 µm nylon cell strainers, WVR) was placed in a petri dish (diameter: 9 cm) filled with 30 mL of synthetic freshwater. Rotifer cysts were added to the sieve and incubated at 25°C under low light intensity (~3 $\mu mol \cdot m^{-2} \cdot s^{-1}$) (Supplementary Figure C.1). The hatching of rotifers was first checked after 24 h and then twice per day. Microalgae were added to the petri dish to reach a concentration of 10^7 cells mL^{-1} when the first swimming rotifers were observed. Ten rotifers were transferred to a new petri dish filled with freshwater and microalgae, and rotifers produced in these petri dishes were also used to create new petri dish with ten rotifers. This step was carried out repeatedly until 10 to 20 petri dishes were obtained. Then, rotifers were incubated until their population reached 50 to 100 rotifers per petri dish, and they were transferred in a 0.5-L reactor. It was found necessary to maintain the cultivation of rotifers in petri dishes until the upscaling of the culture was successful.

4.2.3.3. Upscaling of the culture volume

A 0.5-L semi-opaque plastic bottle was used as a reactor to grow rotifers. It was sterilized with 80 % ethanol and washed with ultrapure water to remove all alcohol traces prior to use. The reactor was then filled with

100 mL of synthetic freshwater and microalgae at a final concentration of 10^7 cells mL^{-1}. Rotifers from petri dishes were sieved with a 40-µm mesh and transferred into the reactor to reach a concentration of at least 10 rotifers mL^{-1}. The reactor was covered with a lid, but not closed hermetically in order to allow gas exchange, and placed in a water bath at 25 ± 1.5°C, under an incident light intensity of 100 µmol m^{-2} s^{-1} at the surface of the bottle. The bottle was gently shaken manually once a day. The culture volume was adjusted to 400 mL with synthetic freshwater when the concentration reached 40 rotifers mL^{-1}. The concentration of microalgae was adjusted to 10^7 cells·mL^{-1} and an air pump (air 100, Eheim), paired with a 0.45-µm filter (Vacusart, Sartorius), was implemented to provide a constant air flow of 0.25 L_N min^{-1} at the bottom of the reactor via a tube. The culture volume was adjusted to 1 L when the concentration reached 40 rotifers mL^{-1}. To do so, the 0.5-L reactor was replaced by a clean 1.2-L reactor (i.e., semi-opaque plastic bottle), which was filled with 1 L of synthetic freshwater and 10^7 cells·mL^{-1} of microalgae. The 400-mL culture was sieved into a beaker with a 0.04-mm^2 mesh to remove large aggregates of dead rotifers and microalgae. Then, rotifers were harvested with a 40-µm sieve and transferred into the new reactor. Incubation was at the same light intensity and temperature conditions but with an increased air flow of 0.5 L_N min^{-1}. The setup is shown in Supplementary Figure C.2.

4.2.3.4. Maintenance of culture and harvest of rotifers

Rotifers approximately double their concentration daily and consume all microalgae. To maintain stable conditions, concentrations of rotifers and microalgae were measured daily and adjusted to 60 rotifers mL^{-1} by replacing a part of the culture volume with synthetic freshwater and 10^7 microalgae cells mL^{-1} by addition of microalgae culture. Adjusted cultures were controlled again by measuring both concentrations. Replacement of the culture volume also allowed to remove waste produced by rotifers, which may impact the health of the culture. Once per week, in addition to the daily maintenance, the culture was filtered with a 0.04-mm^2 mesh, and rotifers were then harvested with a 40-µm sieve and transferred into a new clean reactor filled with 1 L of synthetic freshwater and microalgae. In this study, the culture was stably maintained

for 60 days and it provided a consistent source of rotifers for the experiments. The monitoring of the concentration of rotifers and microalgae is shown in Supplementary Figure C.3.

On the day of the experiment, the concentration of rotifers was determined, and the volume of culture required for the experiment was poured into a beaker. From there, rotifers were harvested with a 40-µm sieve and transferred into the experimental reactor, which was filled beforehand with synthetic freshwater with or without microalgae, as determined by the experimental plan. The final concentration of rotifers in the experimental reactor was checked, and if necessary, adjustments were made by addition or removal of rotifers with the sieve.

4.2.3.5. Assessment of the rotifer viability during the experiments

The viability of rotifers was determined at regular time points during the experiments by assessing the growth of duplicate 1-mL samples, which were incubated up to 7 days (at 25 °C and under a low light intensity) in petri dishes filled with 30 mL of synthetic water and microalgae (10^7 cells·mL^{-1}). Additionally, 30 µL of a 3 mg mL^{-1} bovine catalase solution was added to neutralize the residual H_2O_2. Rotifers were counted every 24 h and the monitoring ended one day after their concentration surpassed 100 rotifers per petri dish (appearing as a red line in our manuscript graphs, *vide infra)*.

4.2.4. Experimental procedure and analytical methods

4.2.4.1. Experimental conditions

Reactors consisted of 250-mL beakers with floating magnetic stirring bars (DS6630, Thermo Scientific), both of which were acid-washed and autoclaved before carrying out inactivation experiments. The setup of the solar simulator, light intensity and temperature were the same as described in section 3.2.4.1, but the stirring was decreased to 150 rpm. 200 mL of a microalgae and/or rotifers suspension was poured into each reactor and samples were taken to assess the cultivability of microalgae and growth of rotifers at time zero. Reagents were added into reactors, according to the treatment, in the following order: 1) citric acid, 2) Fe^{2+} 3) H_2O_2,

with a two-minutes interval between each addition. The lamp was switched on and the experiment was carried out for 2 h. The first sample for the analyses of dissolved Fe^{2+}/Fe^{3+} and H_2O_2 was taken 30 s after the addition of reagents.

Samples were taken regularly to determine the viability of rotifers and microalgae (i.e., the growth and cultivability, respectively), dissolved Fe^{2+}/Fe^{3+}, H_2O_2, and pH value. For all the inactivation experiments, the average pH value at t=0 was 8.0 ± 0.1 for synthetic freshwater alone and 7.6 ± 0.1 when microalgae were added. It dropped to 6.9 ± 0.1 in both cases when Fe^{2+} was added. However, the average pH value increased during experiments to reach 7.4 ± 0.1 in synthetic freshwater and 7.7 ± 0.2 in microalgae cultures at the end of the 2-h treatment.

If a treated culture was cultivated after the 2-h treatment, H_2O_2 was neutralized in the reactor by addition of 1.4 mL of a bovine catalase solution (3 mg mL^{-1}). Then, the stirring bar was removed and nutrients were added to reach one fourth the concentration of the liquid mineral medium. The reactor was placed under a light intensity of 100 µmol m^{-2} s^{-1} in a water bath at 25 ± 1.5 °C. A mass flow controller provided to the culture a constant air flow of 0.1 L_N min^{-1} (2 % CO_2 (v/v)), and 200 µL of a 1 M NaOH solution were added after 30 min to stabilize the pH value around 7. The algal cell and rotifer concentrations were determined daily by counting under light microscope (in duplicate) or binocular (in triplicate), respectively. Water evaporation was compensated daily by addition of synthetic freshwater. The stability of the pH value was verified every three days.

4.2.4.2. Experimental plan

Four different sets of experiments were carried out in microalgae cultures or synthetic freshwater with different treatments and concentrations of rotifers, always at a simulated solar light intensity of 700 W/m^2. The first set of experiments focused on microalgae cultures (dry weight: 100 mg L^{-1}, cell concentration: ~10^7 cells mL^{-1}) without rotifers and aimed to assess the effect of i) simulated solar light alone, ii) solar light and Fe^{2+} with citric acid (1 mg L^{-1} Fe^{2+}, 17.5 mg L^{-1} citric acid), iii) solar light and H_2O_2 (20 mg L^{-1} H_2O_2), and iv) the

homogeneous citrate-modified PF (1 mg L^{-1} Fe^{2+}, 20 mg L^{-1} H_2O_2, 17.5 mg L^{-1} citric acid). Further sets of experiments consisted of the same treatments but focused on rotifers (20 rotifers mL^{-1}), in synthetic freshwater without microalgae (second sets) and in a microalgae culture (third set), respectively. The fourth set of experiments consisted of 5 rotifers mL^{-1} in a microalgae culture to which a citrate-modified PF treatment (1 mg L^{-1} Fe^{2+}, 20 mg L^{-1} H_2O_2, 17.5 mg L^{-1} citric acid) was applied. Two biological replicates were carried out for every treatment in each set of experiments.

Microalgae cultures with 20 rotifers mL^{-1} were recultivated after their treatment with solar light/H_2O_2 and citrate-modified PF. Microalgae cultures with 5 rotifers mL^{-1} were also recultivated after the treatment with citrate-modified PF. Additionally, microalgae cultures with and without rotifers, which were not treated, were cultivated under the same conditions and their data were used as a reference.

4.2.4.3. H_2O_2 and dissolved iron measurements

The determination of H_2O_2 and dissolved Fe^{2+}/Fe^{3+} was carried out as described in section 3.2.4.3.

4.2.4.4. Data treatment

Data on algal cell cultivability were standardized to the initial concentration and are given as colony forming unit (CFU), which was expressed graphically as the inactivation of cells in logarithmic units (logU) per mL (logCFU/mL). Data on algal cell count were also standardized to the initial concentration and are given as the concentration of cells per mL in logU. Data on rotifers are given as the number of swimming female rotifers per petri dish or per mL. Errors bars represent the standard error of the mean of these data. The limit of detection for the microalgae cultivability on agar plates and cell counting under the light microscope is 10 CFU mL^{-1} and 10^4 cells mL^{-1}, respectively. The limit of detection for the counting of rotifers is 1 per petri dish for viability tests carried out during the treatments, and 1 mL^{-1} for the monitoring carried out during the cultivation of treated cultures.

4.3. Results and discussion

4.3.1. Effect of solar light alone, solar light/Fe^{2+} with citric acid, solar light/H_2O_2, and homogeneous (citrate-modified) photo-Fenton on microalgae cultures without rotifers

Experiments were carried out in microalgae cultures to assess the effects of the four different solar-based processes on their cultivability and to determine the associated consumption of reagents (Figure 4.1). Inactivation curves show that the process with solar light/Fe^{2+} and citric acid was similar to an exposure to solar light alone and had no detrimental effects on the overall microalgae cultivability. Indeed, iron plays a key role in many metabolic processes of microalgae, such as photosynthesis, respiration and nitrogen uptake [188]. Moreover, the concentration of iron used in this study was in the range, or below, the concentration usually used to cultivate *C. vulgaris* [68,189]. Therefore, a solar light intensity of 700 W m^{-2}, which is in the range of ambient irradiation, and the presence of a small concentration of iron, was unlikely to have a detrimental effect. However, the solar light/H_2O_2 and citrate-modified PF treatments inactivated 2.46 ± 0.13 and 1.83 ± 0.31 logU of microalgae per mL of culture after 2 h, respectively (Fig. 4.1A). These results corroborate observations made in our previous study [122], i.e., that the solar light/H_2O_2 treatment is more efficient than the citrate-modified PF to inactivate microalgae. However, in the present study, the loss of microalgae was moderate for both treatments due to an adjusted H_2O_2 concentration, which was decreased to 20 mg L^{-1} compared to our previous work (Chapter 3). Indeed, a concentration of viable microalgae in the range of 10^5 cells mL^{-1} is expected to be high enough to enable successful regrowth of the culture [190].

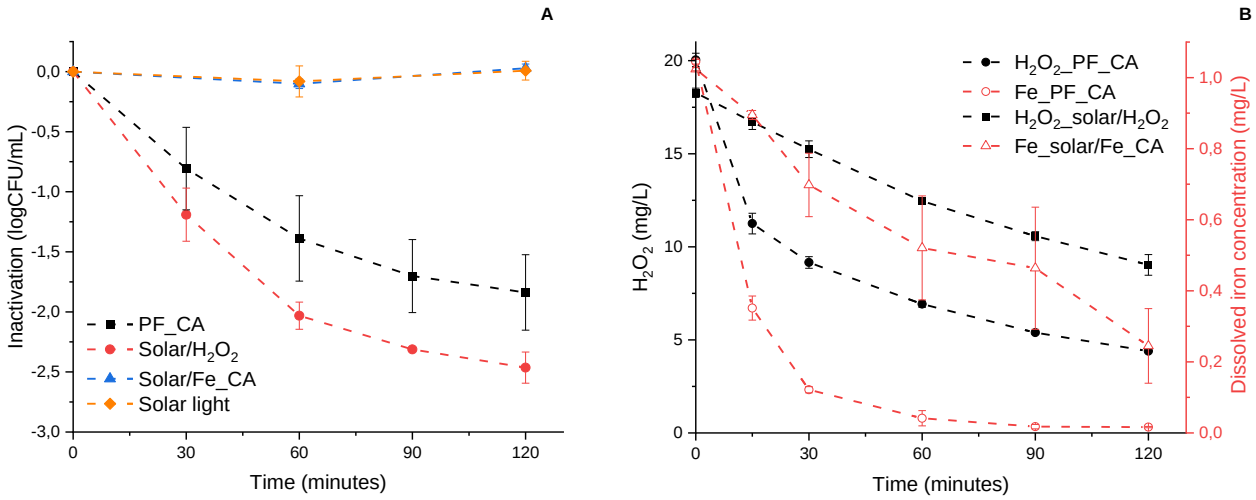


Figure 4.1. Inactivation of microalgae during the solar light, solar light/Fe^{2+} with citric acid, solar light/ H_2O_2, and citrate-modified PF processes/treatments in a microalgae culture (A). The initial concentration of microalgae was 10^7 CFU mL^{-1}. Consumption of H_2O_2 and dissolved iron concentration during the solar light/Fe^{2+} with citric acid, solar light/ H_2O_2 and citrate-modified PF processes/treatments in a microalgae culture (B).

The consumption curves of reagents during treatment of microalgae (Fig. 4.1B) were compared with curves of the same treatments carried out without microalgae in ultrapure water and synthetic freshwater (Supplementary Figure C.4). The results show that microalgae induce the high consumption of H_2O_2 during solar light/ H_2O_2 and citrate-modified PF treatments (i.e., this is not a matrix effect). It is also likely that a fraction of the dissolved Fe^{2+}/Fe^{3+} was consumed by interaction with microalgae (binding or uptake). However, the iron concentration of the culture medium was also impacted by the use of freshwater, which is most likely due to the near-neutral pH value that favors its precipitation. The results also show that the citrate-modified PF reaction occurred mainly during the first 30 min. The consumption of H_2O_2 associated to the PF reaction was 6 or 8 mg L^{-1} when the reaction was carried out in microalgae culture or synthetic water, respectively.

4.3.2. Effect of solar light, solar light/Fe^{2+} with citric acid, H_2O_2/light and citrated-modified photo-Fenton processes on rotifer alone without microalgae

The previous solar-based processes were then applied to a rotifer dispersion, with a concentration of 20 rotifers mL^{-1} in synthetic freshwater, to assess the effects on their growth and to determine the consumption of reagents during the treatment period (Figure 4.2). Rotifers were neither adversely impacted by solar light (in the range of ambient irradiation), nor by a concentration of 1 mg L^{-1} of Fe^{2+} and 17.5 mg L^{-1} of citric acid (Fig. 4.2A and Supplementary Figure C.5). Indeed, adverse effects of iron on the rotifer growth were only reported for a much higher concentration [191]. Therefore, the rotifer inactivation observed during the solar light/H_2O_2 and citrate-modified PF treatments resulted from oxidation of rotifers by H_2O_2 or the generated radicals, such as $HO^{\bullet}$, produced during the reaction.

Rotifers were fully inactivated by the solar light/H_2O_2 treatment in 90 min. Moreover, the growth was strongly impacted after 60 min of treatment with an average value of only 11 ± 11 rotifers per petri dish after 7 days of incubation (Fig. 4.2B). As far as the efficacy of the PF process is concerned, rotifers were fully inactivated by the citrate-modified PF treatment in 120 min and regrowth was severely impacted after 90 min of treatment, as an average of only 42 ± 42 rotifers were counted in petri dishes after 7 days of incubation (Fig. 4.2C). Comparatively, the growth of untreated rotifers was much higher, and the incubation was stopped after 3 days as the average value reached 196 ± 12 and 277 ± 95 rotifers per petri dish for samples taken at time zero (i.e., before the addition of chemicals) of the solar light/ H_2O_2 and citrate-modified PF treatments, respectively.

These results show that rotifers can be fully inactivated, and their subsequent regrowth prevented by applying the oxidative treatments (i.e., H_2O_2 and citrate modified PF). Similarly to the treatment of microalgae, the solar light/H_2O_2 treatment appeared to be more efficient than the citrate-modified PF. Rotifers are even bigger in size than microalgae, and therefore, it is possible that they are also more resistant to a strong, but short, external oxidative stress induced by the citrate-modified PF reaction [122]. However,

rotifers are much more complex organisms than microalgae and their reproduction relies on their ability to produce eggs and cysts. A single viable egg in a petri dish is enough to induce regrowth and replenish the population of rotifers. Therefore, the efficacy difference of the two treatments, which is relatively narrow, may also be due to the variability amongst the population of rotifers (e.g., size of rotifers, number of mictic and amictic females, etc.).

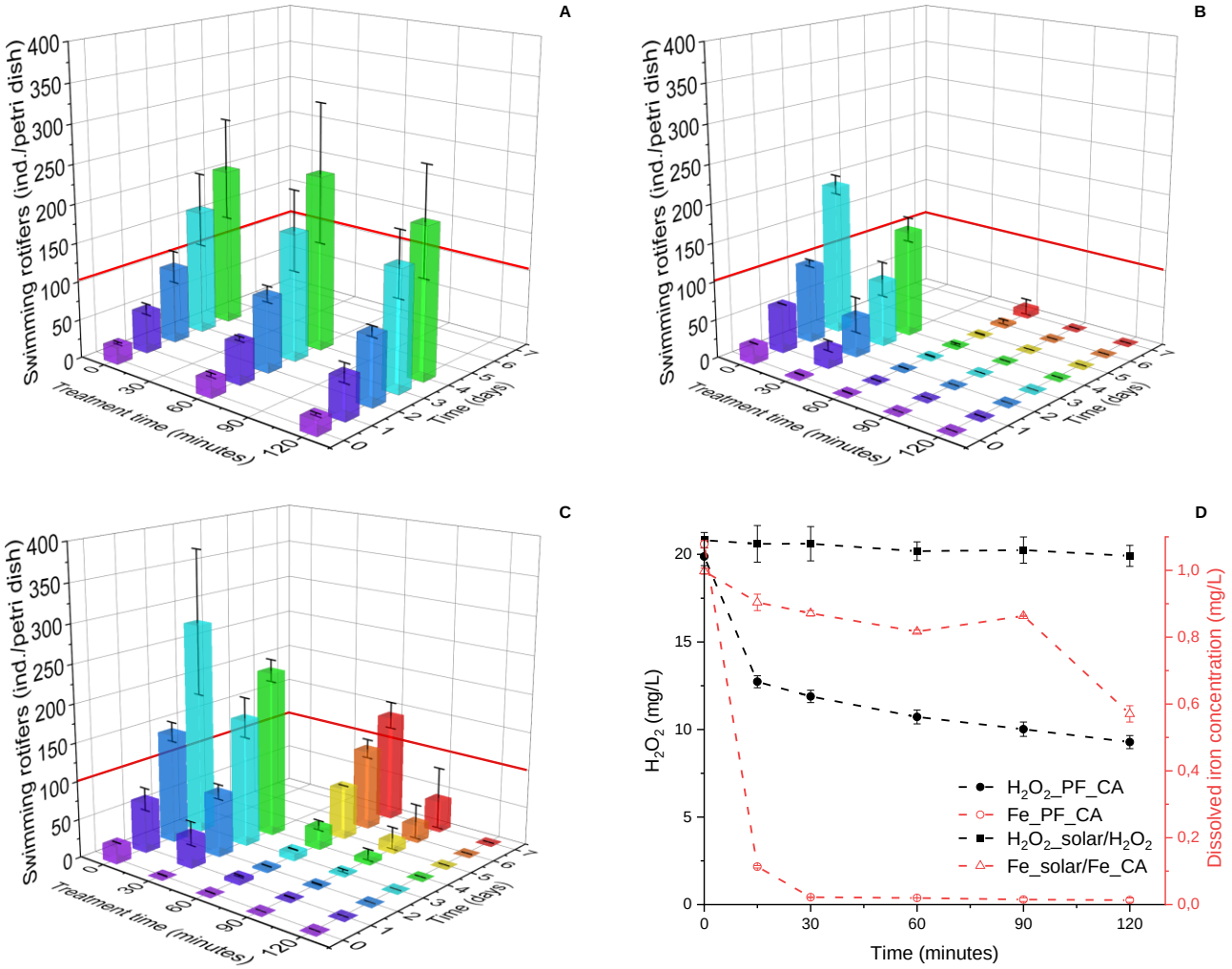


Figure 4.2. Growth of rotifers during their incubation in petri dishes for regular time points of the solar light process (A), solar light/ H_2O_2 (B), and citrate-modified PF (C) treatments in synthetic freshwater. Each petri dish was incubated with a 1-mL sample and the incubation was stopped after 7 days or one day after the concentration of rotifers exceeded 100 rotifers per petri dish (red line). Consumption of H_2O_2 and concentration of dissolved iron during the solar light/Fe^{2+} process with citric acid, solar light/H_2O_2 and citrate-modified PF treatments in synthetic water with rotifers (D). The initial concentration of rotifers was 20 individuals mL^{-1}.

Concentrations of H_2O_2 and dissolved Fe^{2+}/Fe^{3+} show that a population of 20 rotifers mL^{-1} did not consume much of the reagents, when compared to the corresponding curves in microalgae (Fig. 4.2D). This is likely due to the lower dry weight and contact surface, resulting from the rotifer concentration used in this study. Indeed, the dry weight of a single rotifer is estimated between 0.11 and 0.47 µg, which results in 2.2 to 9.4 mg L^{-1} for a concentration of 20 rotifers mL^{-1} [192]. Comparatively, the dry weight of microalgae was 100 mg L^{-1} for a higher number of cells (~ 10^7 cells mL^{-1}), hence the specific surface was higher. In any case, these results are a good indication that the inactivation of rotifers may be feasible within a microalgae culture, without leading to a faster reagent consumption than for microalgae alone.

4.3.3. Treatment of a microalgae culture highly contaminated (20 rotifers mL^{-1}) with solar light/H_2O_2 and citrate-modified photo-Fenton processes, and subsequent cultivation of treated cultures

Microalgae cultures were contaminated with a starting concentration of 20 rotifers mL^{-1}, and then treated with solar light/H_2O_2 or citrate-modified PF treatments in an attempt to decontaminate the cultures. Solar light and solar light/Fe^{2+} (with citric acid) processes were also carried out and their data were used as a reference. Cultivability of microalgae, growth of rotifers, and concentrations of H_2O_2 and dissolved Fe^{2+}/Fe^{3+} were monitored (Figure 4.3 and Supplementary Figure C.6). Microalgae were inactivated by 1.8 ± 0.13 and 2.00 ± 0.01 logU per mL of culture by the citrate-modified PF and solar light/H_2O_2 treatments, respectively (Fig. 4.3A), which was similar to the experiments with microalgae alone (in absence of rotifers). However, rotifers were not fully inactivated during the treatment with citrate-modified PF (Fig. 4.3B) and solar light/ H_2O_2 (Fig. 4.3C) contrary to their experiments without presence of microalgae. As a result, rotifer regrowth occurred during the 7-days incubation, even if cultures were treated for 2 h. This shows that the efficacy of the treatments was mainly impacted by the presence of microalgae, which dominated the consumption of H_2O_2 (Fig. 4.3D), and therefore, decreased its oxidative effect on rotifers. However, we must note here that the growth of rotifers was carried out under ideal conditions (for rotifers) and it is not known if their weakened population would recover in conditions ideal for microalgae growth, i.e., in liquid mineral medium,

illuminated and bubbled with an air/CO_2 flow. Thus, the conditions used here constitute a worst-case scenario for a field-application.

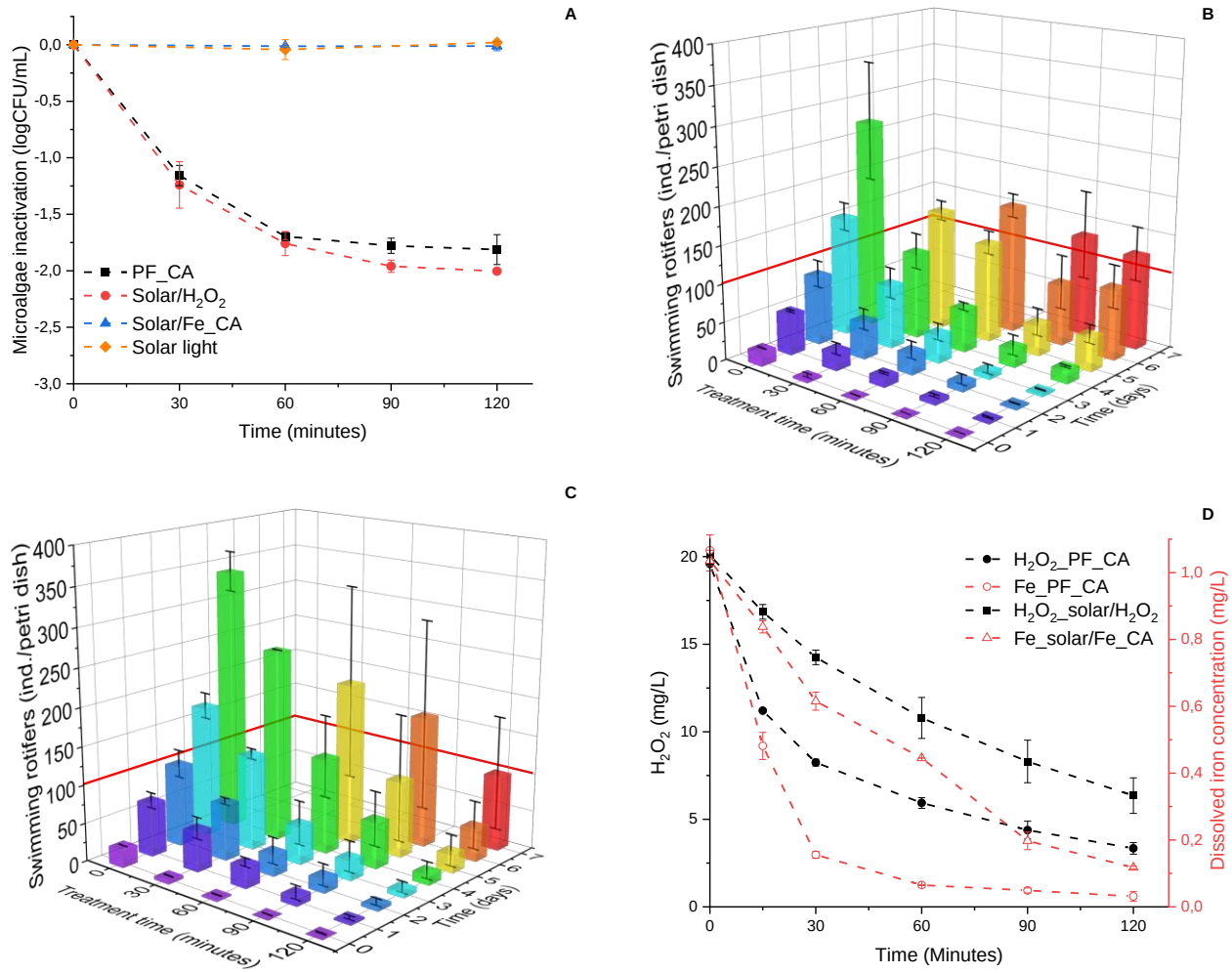


Figure 4.3. Inactivation of microalgae during the solar light process, solar light/Fe^{2+} process with citric acid, solar light/ H_2O_2, and citrate-modified PF treatments in a microalgae culture contaminated with 20 rotifers mL^{-1} (A). The initial concentration of microalgae was 10^7 CFU mL^{-1}. Growth of rotifers during their incubation in petri dishes for regular time points of the citrate-modified PF (B) and solar light/ H_2O_2 (C) treatments. Each petri dish was incubated with a 1-mL sample and the incubation was stopped after 7 days or one day after the concentration of rotifers exceeded 100 rotifers per petri dish (red line). Consumption of H_2O_2 and dissolved iron concentration for the aforementioned treatments as well as for the solar light/Fe^{2+} process with citric acid (D).

Following the oxidative solar processes application, the treated cultures were cultivated for 7 days and the ability of microalgae and rotifers to recover was assessed by the monitoring of algal cell and rotifer concentrations (Figure 4.4). The growth was compared with a baseline, of untreated microalgae cultures, and untreated microalgae but contaminated with 20 rotifers mL^{-1}. The growth of rotifers was, on average, not prevented by the cultivation conditions, but only delayed. Indeed, rotifers reached a concentration of 9 ± 8 and 16 ± 16 rotifers mL^{-1} after 7 days of cultivation in cultures treated with solar light/H_2O_2 and citrate-modified PF, respectively. However, these concentrations were much lower than the concentration of rotifers of untreated contaminated microalgae cultures, where rotifers reached an average concentration of 145 ± 26 individuals mL^{-1}.

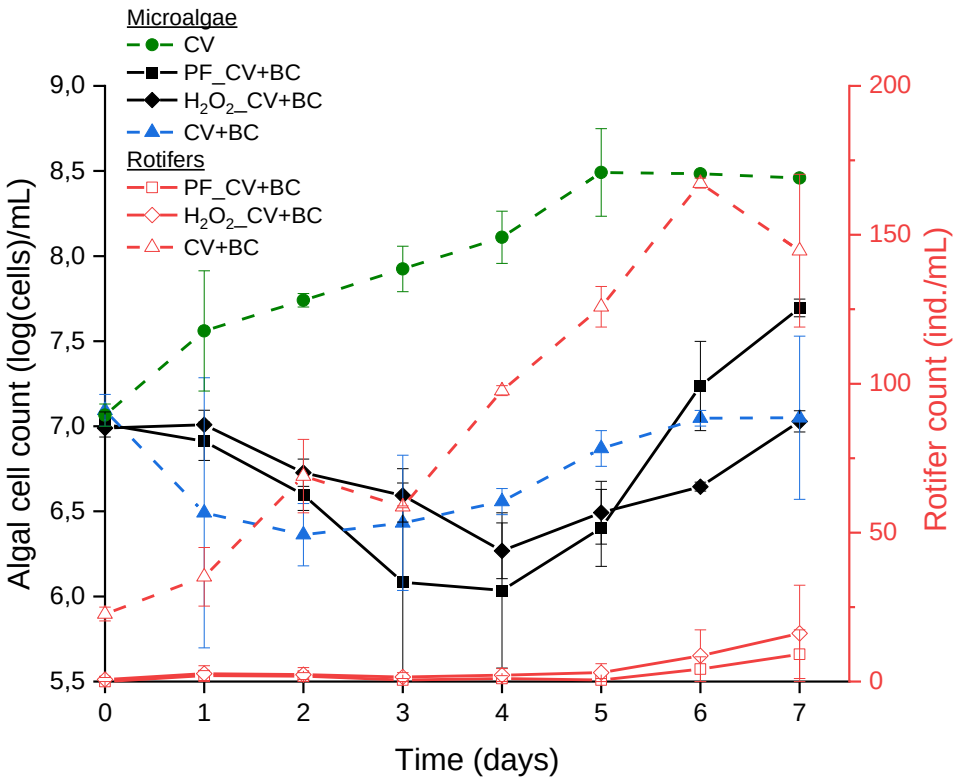


Figure 4.4. Evolution of the concentration of algal cells and rotifers during the cultivation of microalgae contaminated with 20 rotifers mL^{-1}, which were treated during two hours with solar light/H_2O_2 and citrate-modified PF. A baseline was carried out with the cultivation of untreated cultures with microalgae alone or microalgae with rotifers. The initial cell concentration of microalgae was 10^7 cells mL^{-1}. Continuous and dash lines between points are only used as a visual help.

The two treatments also had effects on the growth of microalgae. The cultures reached cell concentrations of 7.03 ± 0.06 and 7.70 ± 0.05 logU mL^{-1} for the solar light/H_2O_2 and citrate-modified PF treatments after seven days, respectively, which was lower than the 8.46 ± 0.02 logU mL^{-1} reached by untreated cultures without rotifers, in five days. However, the reported growth curves indicate that microalgae recovered from the treatments. Indeed, their cell concentration decreased during the first four days, most probably due to the residual effect of the inflicted oxidative damages, and then increased until the end of the cultivation. Microalgae cultures showed better growth when they were treated with citrate-modified PF instead of solar light/H_2O_2, which is consistent with results of their cultivability on agar plates.

The comparison between untreated microalgae cultures, with and without rotifers, demonstrated the considerable impact that rotifers have on the algal growth. Indeed, the cell density fluctuated between 6.36 and 7.09 logU mL^{-1} and did not approach the concentration of microalgae, which were not contaminated with rotifers (i.e., 8.46 LogU mL^{-1}). Additionally, the increasing population of rotifers (145 rotifers mL^{-1} after 7 days) indicated that they were adapting themselves to the cultivation conditions of microalgae, and that microalgae were most probably destined to be consumed.

4.3.4. Treatment of contaminated microalgae cultures with citrate-modified photo-Fenton at an early-stage contamination (5 rotifers mL^{-1})

In the previous section it was shown that the intensity of the oxidative treatments was appropriate to preserve the viability of the microalgae cultures. However, the oxidative stress was not sufficient to fully inactivate 20 rotifers per mL^{-1}, in presence of microalgae. Therefore, the citrate-modified PF reaction was applied to a lower concentration of 5 rotifers mL^{-1}, which could correspond to a contamination at an early stage in a field application [87]. The inactivation of the microalgae and rotifers, the subsequent growth of rotifers, and the concentration of reagents were monitored in order to assess the impact of the PF treatment (Figure 4.5). The intensity of the treatment is modulated by the biomass of microalgae: their inactivation, as well as the consumption of reagents was similar to the previous experiments of microalgae alone and

microalgae contaminated by 20 rotifers mL^{-1}. This led to moderate microalgae inactivation and most of the reagents were consumed at the end of the 2-h treatment (Fig. 4.5A). However, in these tests with 5 rotifers mL^{-1}, rotifers were fully inactivated by the citrate-modified PF treatment in 90 min, which suggests that the treatment is effective and can address contamination issues if treatment occurs at an early stage.

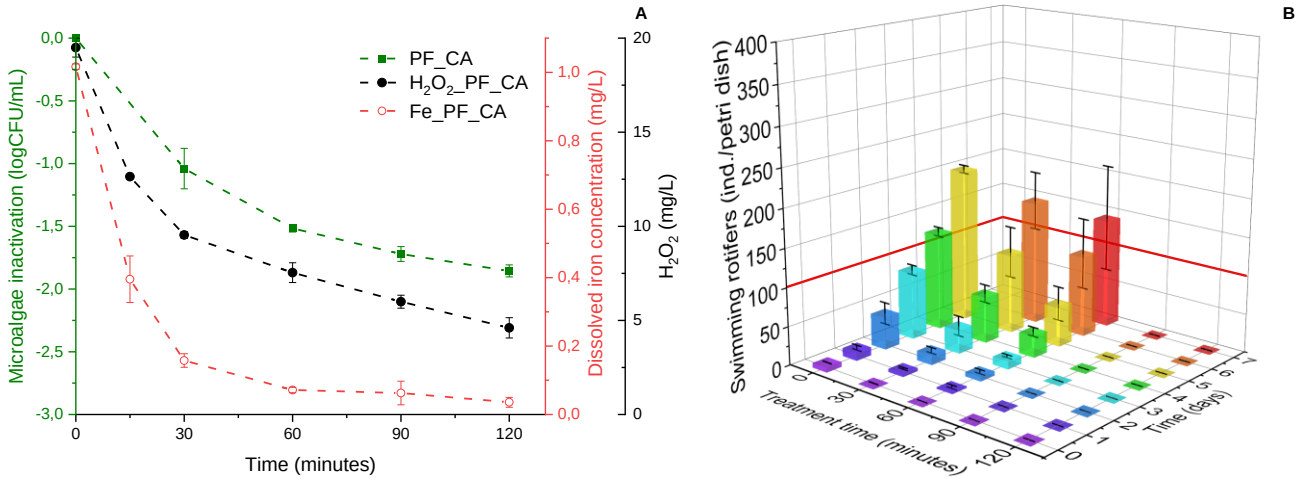


Figure 4.5. Inactivation of microalgae and associated consumption of reagents during the citrate-modified PF treatment of microalgae cultures contaminated with 5 rotifers mL^{-1}. (A). The initial concentration of microalgae was 10^7 CFU mL^{-1}. Growth of rotifers during their incubation in petri dishes for regular time points of the treatment (B). Each petri dish was incubated with a 1-mL sample and the incubation was stopped after 7 days or one day after the concentration of rotifers exceeded 100 rotifers per petri dish (red line).

The treated cultures were also cultivated for 14 days to confirm that the inactivation of rotifers was effective over time, and to assess the regrowth levels of microalgae (Figure 4.6). Baseline experiments were also done by the cultivation of untreated microalgae cultures, with and without a concentration of 5 rotifers mL^{-1}. The successful inactivation of rotifers by the treatment was confirmed, as no regrowth was observed during the 14 days of post-treatment cultivation. Moreover, microalgae of treated cultures were able to reach their stationary phase after only 9 days of cultivation, enumerating a 8.23 ± 0.06 logU mL^{-1}. In comparison, the

non-contaminated microalgae cultures reached their stationary phase (8.27 ± 0.03 logU mL^{-1}) in 7 days. Therefore, the treatment delayed the growth of microalgae by two days only.

This delay in growth is a modest drawback compared to the fate of microalgae, which were not treated. As a matter of fact, the threat of rotifers, even at a low concentration of 5 individuals per mL, was demonstrated in untreated, but contaminated, microalgae cultures. Microalgae were first moderately impacted by rotifers during the first 8 days and their cell concentration approached the value of the uncontaminated culture by reaching up to 8.07 ± 0.1 logU mL^{-1}. However, during the second week of cultivation, the culture failed due to the exponential growth of rotifers, reaching up to 1521 ± 412 rotifers mL^{-1}. These results demonstrate the efficacy of the citrate-modified PF treatment to stop rotifer contamination in microalgae culture, but also the dramatic decay of microalgae and growth of rotifers, if no measures were applied to control the contamination.

The citrate-modified PF treatment (1 mg L^{-1} Fe^{2+}, 20 mg L^{-1} H_2O_2, 17.5 mg L^{-1} citric acid) achieved a full decontamination of rotifers while microalgae were able to regrow. It was as effective as other known methods used to stop the growth of biological contaminants, namely, botanical pesticides, surfactants, or disinfectants. A complete inactivation of 5 rotifers mL^{-1} was achieved with 3 µM of cetyltrimethylammonium bromide in *C. vulgaris* cultures (biomass dry weight: 160 mg L^{-1}) [101], and with 1.8 µg L^{-1} of toosendanin in *Chlorella* sp. cultures (cell density: $2 \cdot 10^{-7}$ cells mL^{-1}) [82]. However, the citrate-modified PF process has the advantage of not being persistent in the water after the treatment. Sodium hypochlorite (0.45 to 0.6 mg Cl L^{-1}) supplied every two hours during one to three days inactivated a concentration of 7 rotifer mL^{-1} in *Chlorella kessleri* cultures (biomass dry weight: 60 to 90 mg L^{-1}) [102]. However, this treatment time was much longer than for the citrate-modified PF process, which required 90 min.

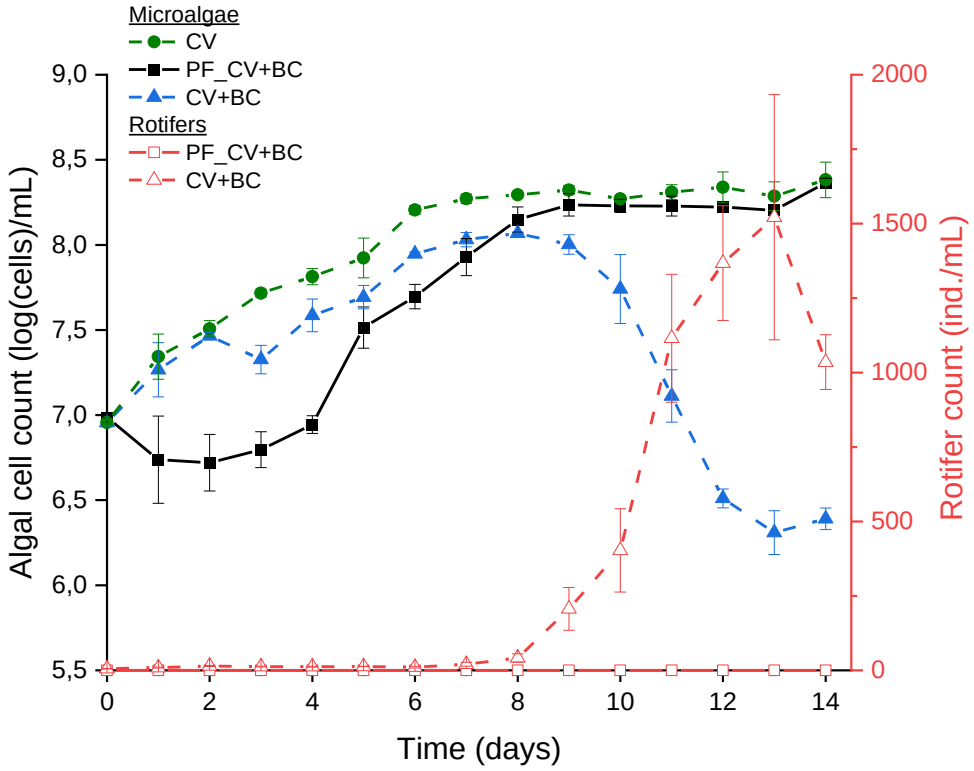


Figure 4.6. Evolution of the concentration of algal cells and rotifers during the cultivation of microalgae contaminated with 5 rotifers mL^{-1}, which were treated during two hours with citrate-modified PF. A baseline was carried out with the cultivation of untreated cultures with microalgae alone or microalgae with rotifers. The initial cell concentration of microalgae was 10^{7} cells mL^{-1}. Continuous and dash lines between points are only used as a visual help.

4.4. Conclusions

A citrate-modified photo-Fenton was used to decontaminate rotifers in a microalgae culture. The treatment aimed to reach a full inactivation of rotifers and their eggs while minimizing the loss of viable algal cells thus cultures can be cultivated after the treatment. The treatment was efficiently applied to a concentration of 5 rotifers mL^{-1} in a microalgae culture. Rotifers were fully inactivated after a short (90 min) treatment and no re-growth was observed during the 14-days cultivation post-treatment. Treated microalgae recovered from the oxidative stress and reached, only slightly delayed by 2 days, the stationary phase with similar cell concentration than non-contaminated microalgae. Citrate-modified photo-Fenton and solar light/H_2O_2 seemed to have similar efficacy on the inactivation of rotifers in contaminated microalgae cultures. Nevertheless, the citrate-modified photo-Fenton tends to inflict less damage on microalgae and offers a better control on the reagent removal as most of the H_2O_2 and dissolved Fe^{2+}/Fe^{3+} was consumed at the end of the two-hour treatment. The present study also shows the importance of applying the treatment at an early stage of the contamination. Indeed, citrate-modified photo-Fenton and solar light/ H_2O_2 treatments failed to fully inactivate rotifers in microalgae cultures when their concentration was 20 rotifers mL^{-1}. The results of the present study demonstrate the successful application of an oxidate treatment for in-situ inactivation of microalgae predators. The next step of the project would be to apply the treatment at larger scale in a commercial PBR designed to produce microalgae.

Acknowledgements

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Chapter 5. Concluding remarks and outlook

5.1. Concluding remarks

While microalgae have untapped potential for many applications, overly optimistic projections heralded them as superorganisms. This prevented a critical discussion of the techno-economic challenges that this industry must overcome. It is proven that microalgae can be robust and grow quickly, and that they can reach high content of lipids, proteins, or expensive molecules such as astaxanthin. Nevertheless, it is unrealistic to think that one algal strain has all these properties. There are still numerous challenges about the costs of production where technological breakthroughs are needed to allow the upscaling of the production. Recent studies aim to develop synergies between the production of microalgae and the treatment of wastewater to lower the production costs of microalgae-based products. While they have potential to make microalgae biomass more affordable, they also raise new challenges such as the control of biological contaminations during the cultivation.

The present doctoral thesis investigated the cultivation of microalgae at high-density with an agricultural anaerobic liquid digestate as a nitrogen source and the treatment of contaminated microalgae cultures with a photo-Fenton (PF) process. The following sections aim to answer the research questions by summarizing the main results obtained in this work. The perspectives for further work are also discussed based on the results obtained in this thesis.

5.1.1. Cultivation of microalgae with liquid digestate from anaerobic digestion

Microalgae were successfully cultivated with a pretreated anaerobic liquid digestate both at laboratory and pilot scales. The microalgae reached high densities during the cultivation (12.95 to 18.6 g L^{-1}), which shows a great potential to produce microalgae while recycling liquid digestate. However, the cultivation with liquid

digestate required strategies to control pH and to prevent excessive concentration of NH_4^+, which may become toxic for microalgae. The pretreatment of the liquid digestate to remove solid particles was also crucial to avoid accumulation of solid particles, which could have clogged the pipes of the photobioreactor (PBR) and contaminated the algal biomass. Therefore, it is not recommended to use raw liquid digestate for the cultivation of microalgae. Filtration of the liquid digestate has a cost but the implementation of ultrafiltration and reverse osmosis system is economically viable because they substantially decrease transportation costs. For example, the liquid digestate used in this thesis was collected at a biogas plant, which filtrates and concentrates large volumes. This creates an opportunity to implement the cultivation of microalgae close to the biogas plant to recycle the liquid digestate, heat and CO_2.

The determination of the nitrogen mass balance showed that about half or more of the nitrogen supplied to the cultures was not assimilated in the algal biomass nor in the water, which means that it was lost to the atmosphere. This raises questions about the environmental footprint of microalgae until gas species contained in these emissions are determined. Indeed, the use of microalgae to treat liquid digestate, may lose attractiveness if NH_3 or N_2O emissions are detected because this would mean that the pollution is shifted from the water to the atmosphere. However, the use of liquid digestate is still recommended if the goal is to produce microalgae as nitrogen losses were also noticed with a synthetic source of nitrogen such as urea.

5.1.2. Inactivation of bacteria in microalgae cultures

Oxidative processes are not selective and both microalgae and bacteria where inactivated when a contaminated microalgae culture was treated. However, in this project, a kind of selectivity was created by having both organisms mixed together thanks to their different physiology. The citrate-modified PF treatment was more efficient than solar light/H_2O_2 treatment to inactivate bacteria but less efficient for microalgae. This is counter-intuitive because advanced oxidative treatments such as the PF reaction aim to improve the formation of highly reactive $HO^{\bullet}$, and they are considered as more effective to inactivate any biological contaminant. These results showed that two different organisms may have a different sensitivity towards the pathways leading to their inactivation. Therefore, an advanced oxidative process such as the

citrate-modified PF can be less effective than a solar light/H_2O_2 treatment because it optimizes an oxidative pathway, to which microalgae are much more resistant than bacteria thanks to their physiology.

This differential sensitivity was used to favor the survivability of microalgae over bacteria, and it was shown that bacteria can be fully inactivated if the treatment is applied to a relatively low concentration of bacteria (i.e., 10^4 cells mL^{-1} of bacteria versus 10^7 cells mL^{-1} of microalgae), which corresponds to a contamination at an early stage. Nevertheless, this concentration of bacteria could already be concerning for food applications, which warrants the treatment [193,194]. Therefore, these results successfully laid the basis for the development of oxidative treatments to inactivate biological contaminants in microalgae cultures.

5.1.3. Decontamination of rotifers in microalgae cultures and regrowth of treated cultures.

In this project, the treatment method was extended to rotifers, which are predators of microalgae. It was shown that the citrate-modified PF process fully inactivated a contamination of 5 rotifers mL^{-1}. The treated microalgae cultures were successfully cultivated and reached biomass concentrations similar to reference cultures, which were not contaminated nor treated. However, the use of citrate-modified PF and solar light/H_2O_2 treatments to decontaminate a higher concentration of 20 rotifers mL^{-1} in microalgae cultures did not fully remove rotifers. Indeed, regrowth of rotifers was observed, which showed the importance of applying the treatment to an early-stage contamination. While the two treatments had a similar efficacy on rotifers, the solar light/H_2O_2 treatment damaged more microalgae. However, this higher loss of algal cells did not prevent microalgae to regrow during their cultivation post-treatment. Therefore, it could be considered to use H_2O_2 instead of a PF process to decrease the cost of the treatment. However, the citrate-modified PF offers a better control on the reagents as the H_2O_2 residual was much lower than for H_2O_2 alone, after the two-hour treatment. If the residual is not neutralized, it may extend the inactivation of microalgae over the desired period, and thus, potentially jeopardize the regrowth of the culture.

The main undesired impact of the treatment on the culture was to delay the regrowth by about two days. However, this is a modest drawback compared the fate of the cultures if microalgae were not treated. Indeed, cultures contaminated with 5 rotifers mL^{-1} were decimated by rotifers, which reached a concentration above 1000 rotifers mL^{-1} in less than two weeks of cultivation. Therefore, the productivity of the microalgae and the quality of their biomass was heavily impacted. These results show the efficacy of the treatments to inactivate rotifers in a microalgae culture, and they encourage the development of this method for applications in real conditions. While the main advantages of the citrate-modified PF treatment are the ability to target any biological contaminant and the use of non-persistent chemicals, it creates also an opportunity to use the infrastructure, implemented to carry out the treatment, to sterilize the photobioreactors and clean the wastewater recovered during the harvesting process. However, several aspects still have to be investigated such as the feasibility of the upscaling and the tolerance of commercial PBRs towards the treatment (e.g., the resistance against oxidation). In comparison, other chemical treatments such as pesticides, antibiotics and surfactant may remain easier to use and cheaper for pest control. However, their persistence in the water and biomass could decrease the acceptance by consumers. Moreover, their selectivity could also require the use of chemical cocktails to face the variety of biological contaminants.

5.2. Outlook

5.2.1. Demonstration at a biogas plant and optimization of the cultivation

The cultivation of microalgae with liquid digestate was carried out at high-density and at pilot-scale. Therefore, the next step of the project would be to implement a thin-layer PBR close to an agricultural biogas plant, which would enable the recycling CO_2 and heat, in addition to the liquid digestate. Techno-economic studies should be carried out to assess by how much the recycling of waste streams decrease the cost of production of microalgae. The optimization of the cultivation should also be an important focus. For example, the pretreated liquid digestate used in this study had the potential to replace also the magnesium, calcium,

boron and vanadium. Then, the supply of nitrogen and the duration of the cultivation could be adjusted to decrease the nitrogen losses during suboptimal growth conditions. Customization of the mineral medium recipe could also be investigated such as decreasing the concentration of nitrogen from 8.5 to 6 % to better fit the percentage of nitrogen, which was measured in the algal biomass. Finally, the nitrogen gas emissions by microalgae cultures should be investigated to determine if greenhouse or toxic gases are emitted during the cultivation. To this end, a closed PBR can be designed to recover and analyze gases emitted during the cultivation. Gas samples can be analyzed by gas chromatography to determine species such as N_2O. Emission of NH_3 can also be determined by bubbling the gas flow through an acidic bath to convert NH_3 into NH_4^+, which can then be quantify spectrophotometrically.

5.2.2. Treatment of biological contaminants in microalgae cultivated at pilot-scale

It was shown in this project that solar light/H_2O_2 or citrate-modified PF treatments can fully inactivate biological contaminants such as bacteria or rotifers, in microalgae cultures. The next step would be to apply the treatment in more realistic conditions, for example, in a PBR designed to cultivate microalgae. It is crucial to investigate the adaptability of this method to different PBRs and cultivation conditions. Indeed, the efficacy of the treatment rely on an equilibrium between several parameters such as the concentrations of contaminant, microalgae and reagents, as well as the light exposure and intensity. Therefore, it should be determined if the treatment can be applied at different stages of the cultivation and with different types of illumination (i.e., natural and artificial lights). To this end, it is strongly recommended to automate the supply of the reagents and the measurement of their concentration. This would enable the development of more elaborate treatment methods with a better control of the growth conditions, and therefore, higher efficacy and shorter duration of the treatment. Finally, the selection of algal strains may also be used to decrease the damages of the oxidative stress. For example, microalgae could be repetitively exposed to H_2O_2 and recultivated on agar plates to select the most resistant strains.

Appendices

Appendix A. Supportive information for Chapter 2

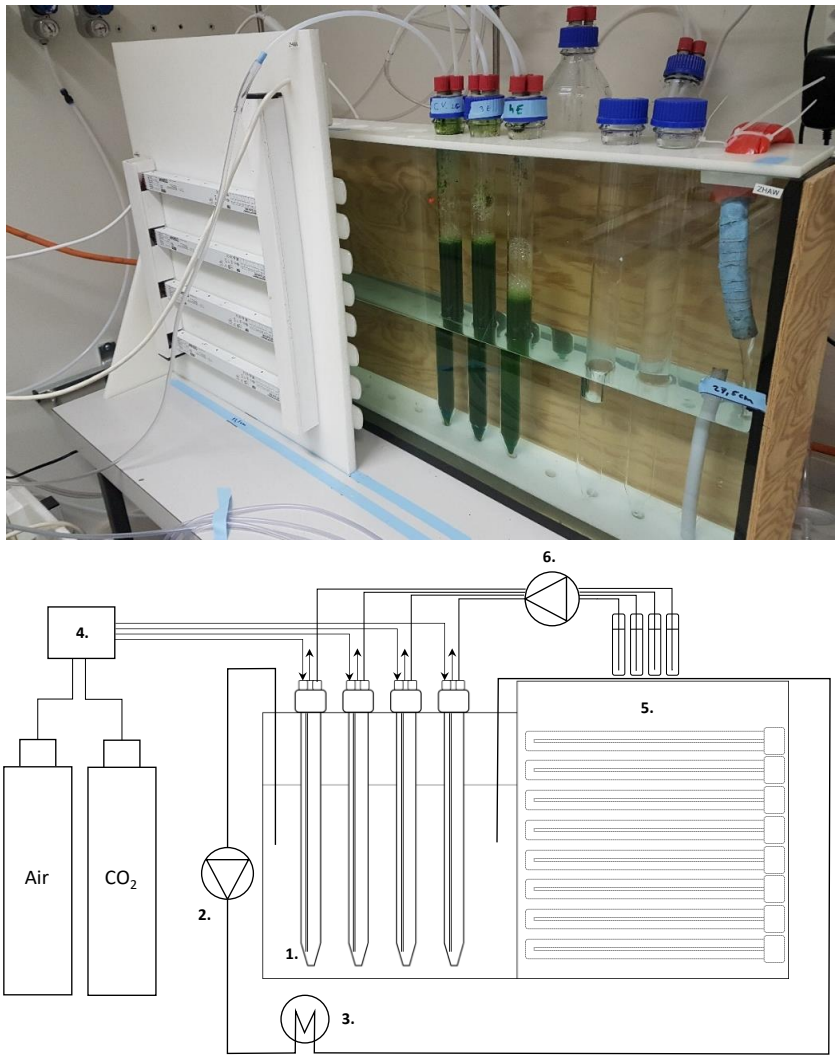

Figure A.1. Picture and drawing of the glass column PBRs setup, which consists of glass columns immersed in an aquarium (1), a water pump (2), a chiller (3), a gas mixing and injection unit (4), a light panel (5), and a peristaltic pump (6). The drawing was created by Alexander Garcia Kapeller.

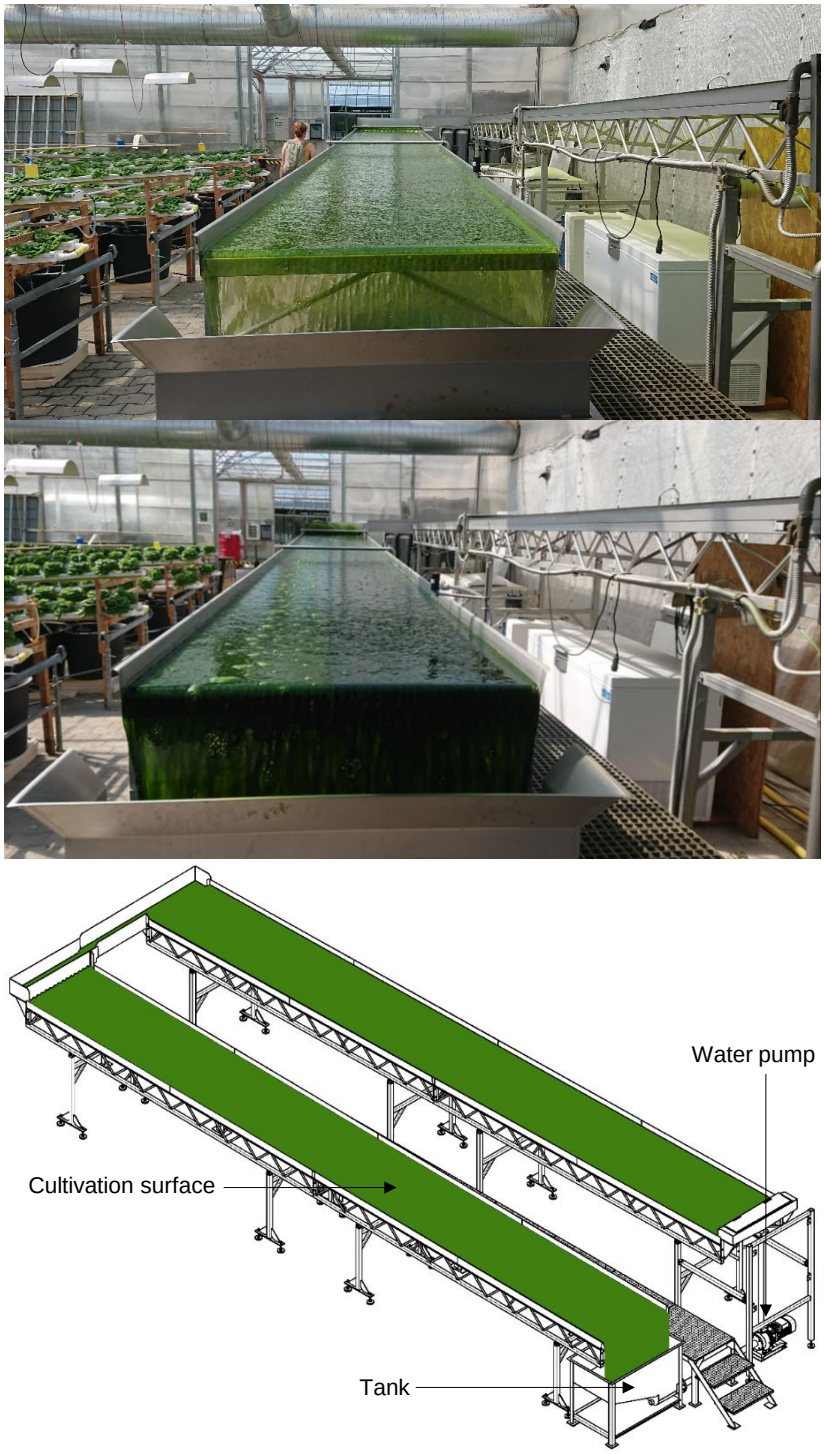

Figure A.2. Cultivation of microalgae in a thin-layer PBR with PLD as a nitrogen source. The PBR is located in a non-heated foil greenhouse. Pictures were taken at the start (A) and after 11 days of cultivation (B), and the successful growth is noticeable by the dark green color. The drawing shows the design of the thin-layer PBR with the cultivation surface of 18 m^2, 200 L tank and water pump (C) [69].

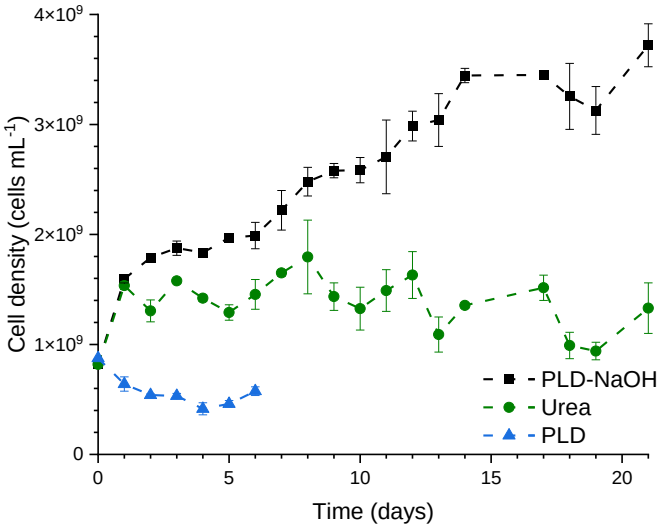

Figure A.3. Cell density of microalgae cultivated in glass-column PBRs with PLD-NaOH, PLD, and urea as a nitrogen source.

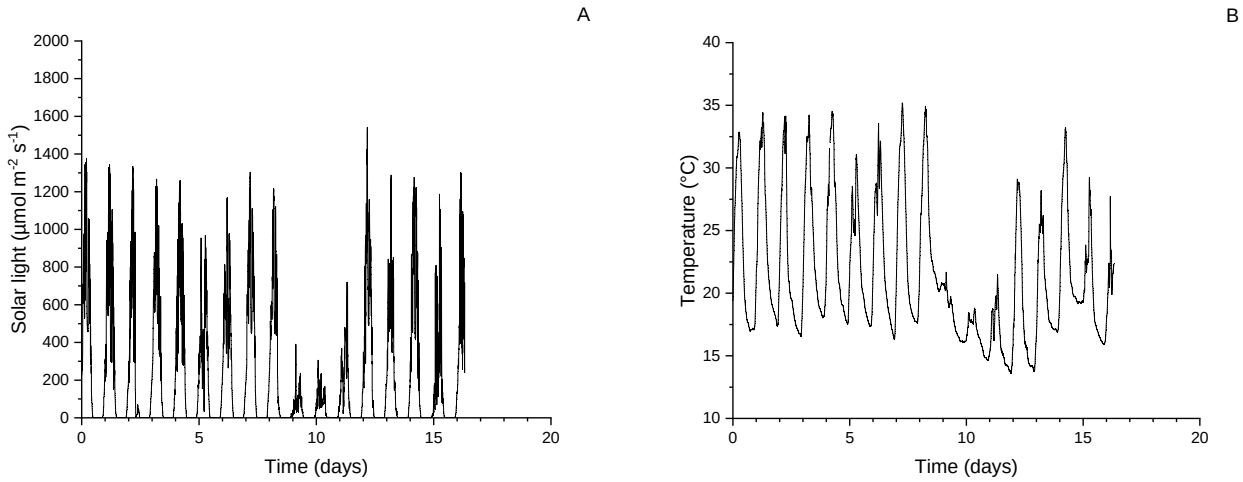

Figure A.4. Sun intensity at the surface of the culture (A) and temperature in the culture (B) during the first cultivation of microalgae at high-density in a thin-layer PBR supplied with PLD-NaOH as a nitrogen source. The cultivation was carried out during 16 days in August/September 2017.

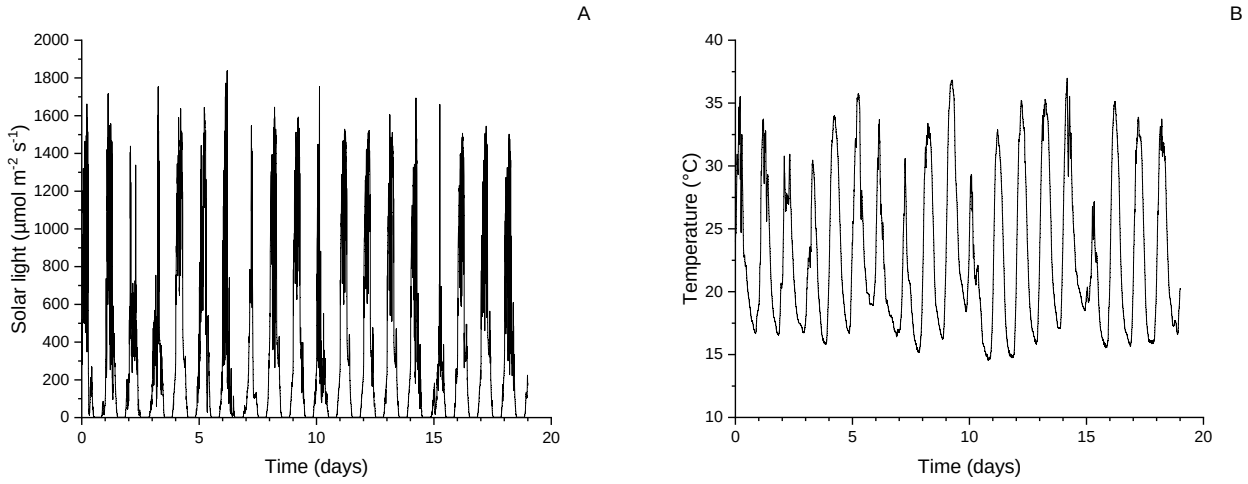

Figure A.5. Sun intensity at the surface of the culture (A) and temperature in the culture (B) during the second cultivation of microalgae at high-density in a thin-layer PBR supplied with PLD-NaOH as a nitrogen source. The cultivation was carried out during 19 days in June/July 2020.

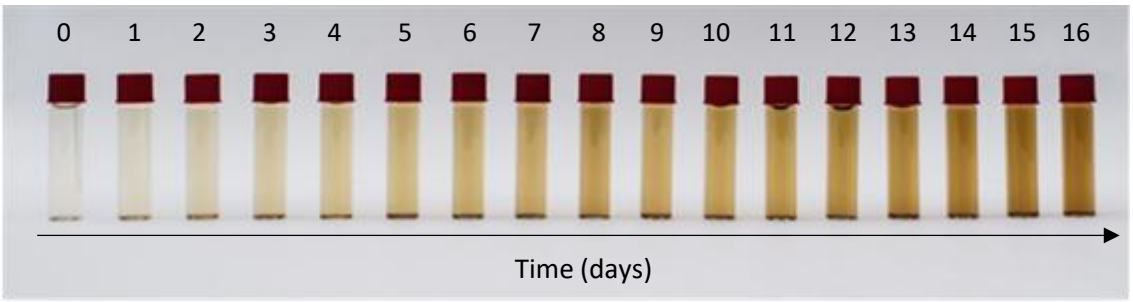

Figure A.6. Samples of supernatant taken during the cultivation of microalgae carried out in August/September 2017. The supernatant became darker over time due to the accumulation of organic carbon coming from the addition of PLD.

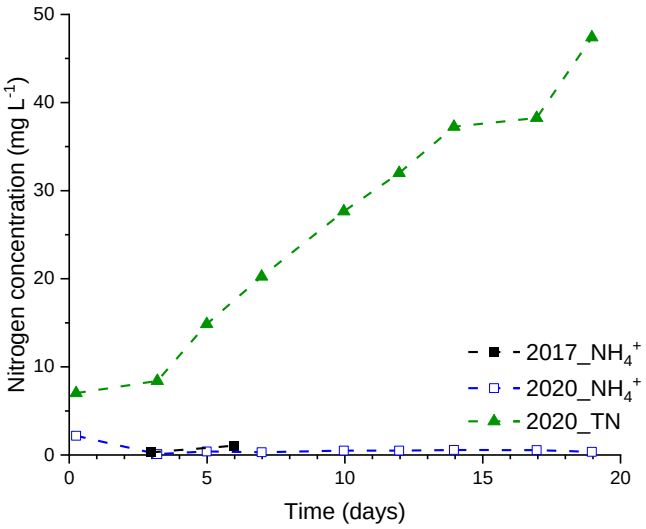

Figure A.7. Accumulation of NH_4^+ and TN in the supernatant during the cultivation in the thin-layer PBR with PLD as a nitrogen source.

Appendix B. Supportive information for Chapter 3

The tap was opened two minutes before pouring the water into a sterile glass bottle. The tap water was stirred two hours and then kept with the lid open (but covered) overnight to evaporate potential chlorine gas residues. The pH of the tap water is around 8 and its characteristics are shown in Table B.1:

Table B.1. Characteristic of the tap water used during the experiments: own measurements by ion chromatography and inductively coupled plasma optical emission spectrometry (ICP-OES), and data provided by the Water Services of the city of Lausanne*.

Conductivity	µS/cm at 25°C	287
Hardness	°f	13
Calcium (Ca^{2+})	mg/L	44
Magnesium (Mg^{2+})	mg/L	6
Sodium (Na^{+})	mg/L	6.9
Potassium (K^{+})	mg/L	1.8
Bicarbonate (HCO_3^{-})	mg/L	110
Chloride (Cl^{-})	mg/L	11
Sulfate (SO_4^{2-})	mg/L	47
Nitrate (NO_3^{-})	mg/L	3
Total iron (Fe)	mg/L	0.092
Phosphate (PO_4^{3-})	mg/L	< 0.05
Nitrite (NO_2^{-})	mg/L	< 0.05
Total inorganic carbon	mg/L	18.3
Total organic carbon	mg/L	0

*https://www.lausanne.ch/vie-pratique/energies-et-eau/eau/qualite/reservoir-recherche/reservoir-resultat?adresse=EPFL%20ECUBLENS

***Figure B.1**. Reactors containing microalgae cultures (100 mg L^{-1}) mixed with bacteria (initial concentration 10^6 CFU mL^{-1}) after two hours of illumination with solar light and the following treatments: 1) 1 mg L^{-1} Fe^{2+}, 25 mg L^{-1} H_2O_2 and 17.5 mg L^{-1} citric acid (i.e. citrate-modified PF reaction), 2) 1 mg L^{-1} $Fe^{2+}$and 25 mg L^{-1} H_2O_2, 3) 25 mg L^{-1} H_2O_2, 4) no reagents added. Aggregates of microalgae were observed only with treatment 2).*

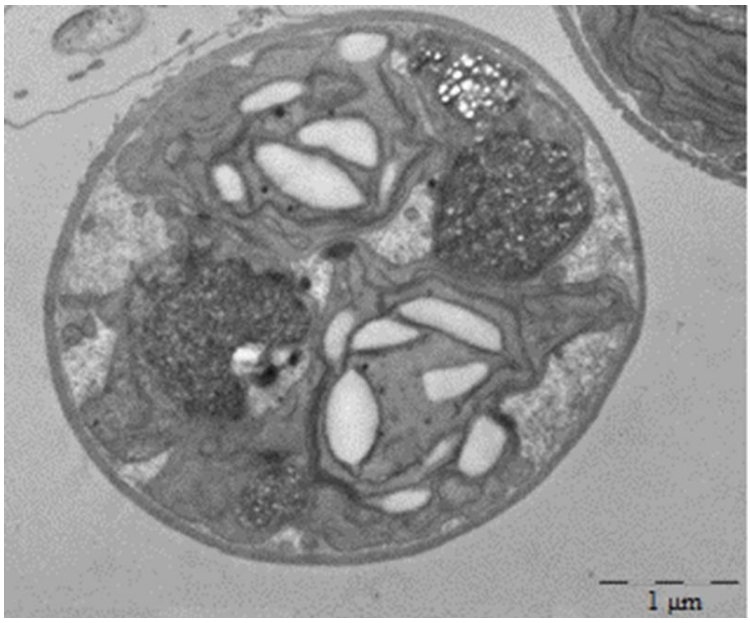

Figure B.2. Picture of typical C. vulgaris cell (SAG 211-11b) acquired by transmission electron microscopy (TEM). The picture was used to support estimations made in Table 2.

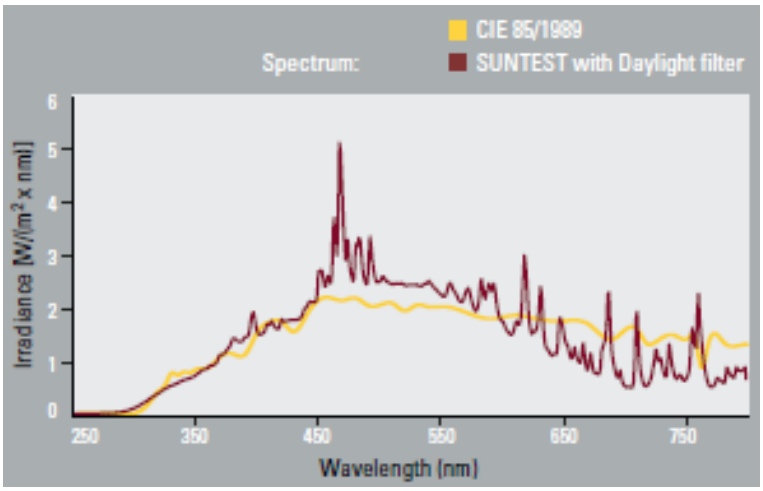

Figure B.3. Suntest solar simulator light wavelength emission spectrum (Manufacturer: Suntest Xenon Test-Instruments Brochure).

Appendix C. Supportive information for Chapter 4

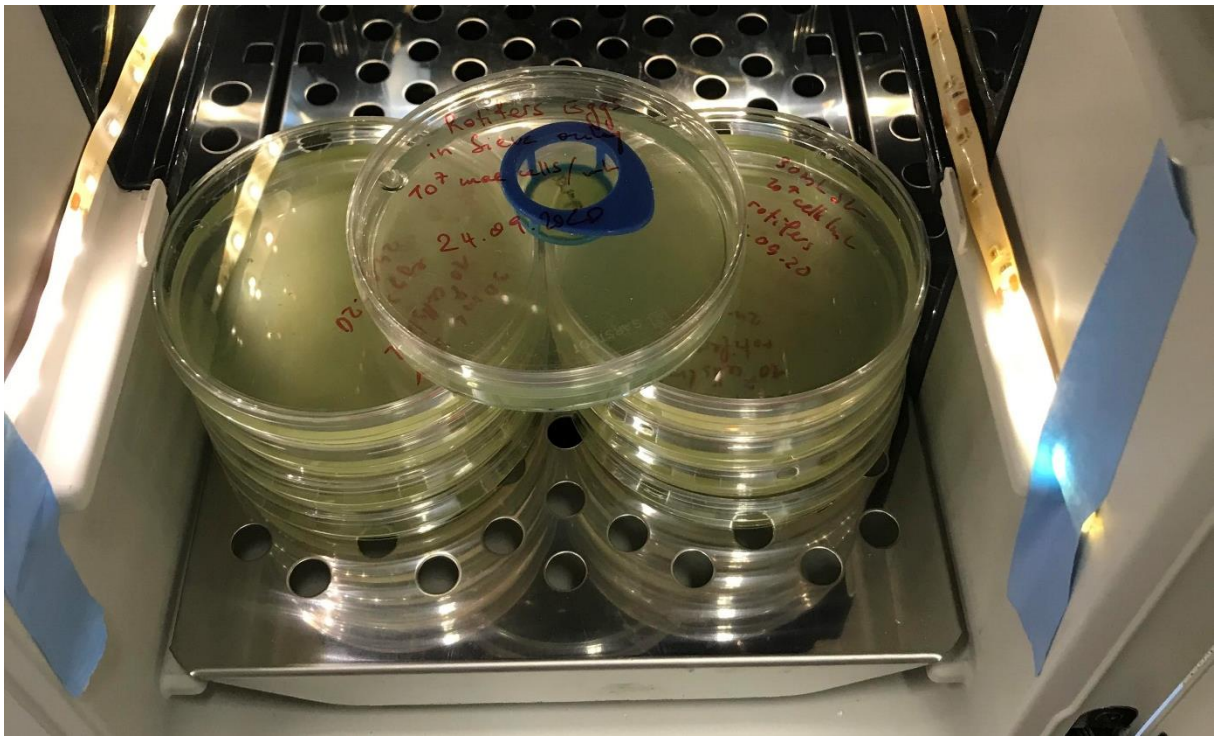

Figure C.1. Incubation of rotifers in petri dishes filled with synthetic water and a concentration of 10^7 cells mL^{-1} of microalgae. Cysts are hatched in the blue 40-µm sieve.

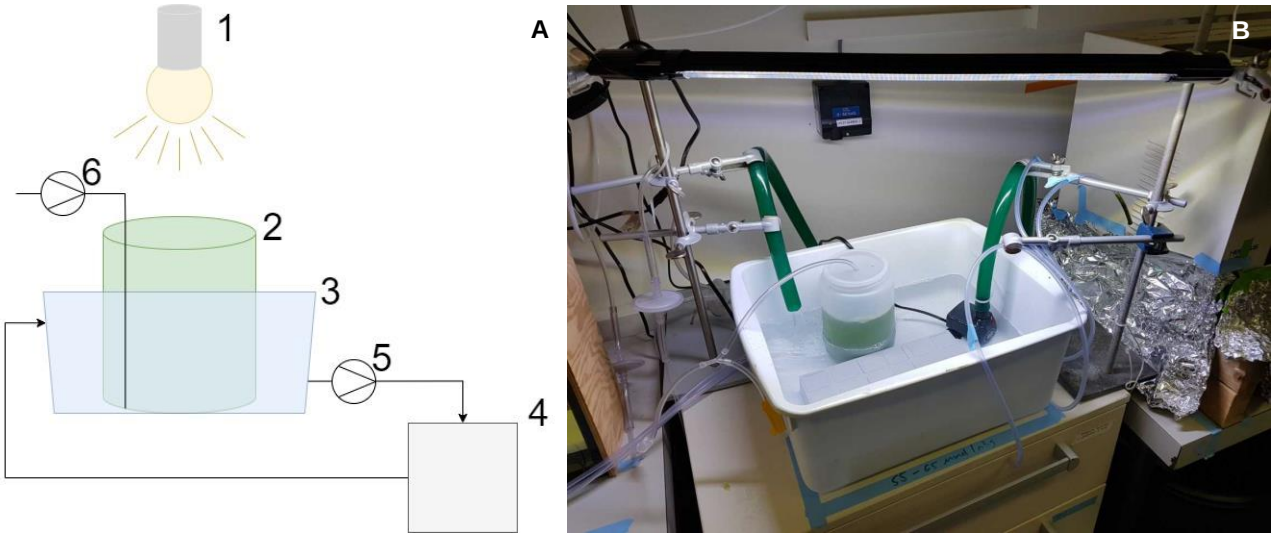

Figure C.2. A) Scheme of the setup used to cultivate rotifers in a reactor made of semi-opaque plastic. It consisted of a LED lamp, under which the reactor (2) was placed in a a water bath (3). A chiller (4) and a water pump (5) were used to keep the temperature at 25 ± 1.5 °C. The culture was bubbled with an air flow provided by a pump paired with a 0.45 µm filter (6). B) Picture of the setup during the cultivation of rotifers.

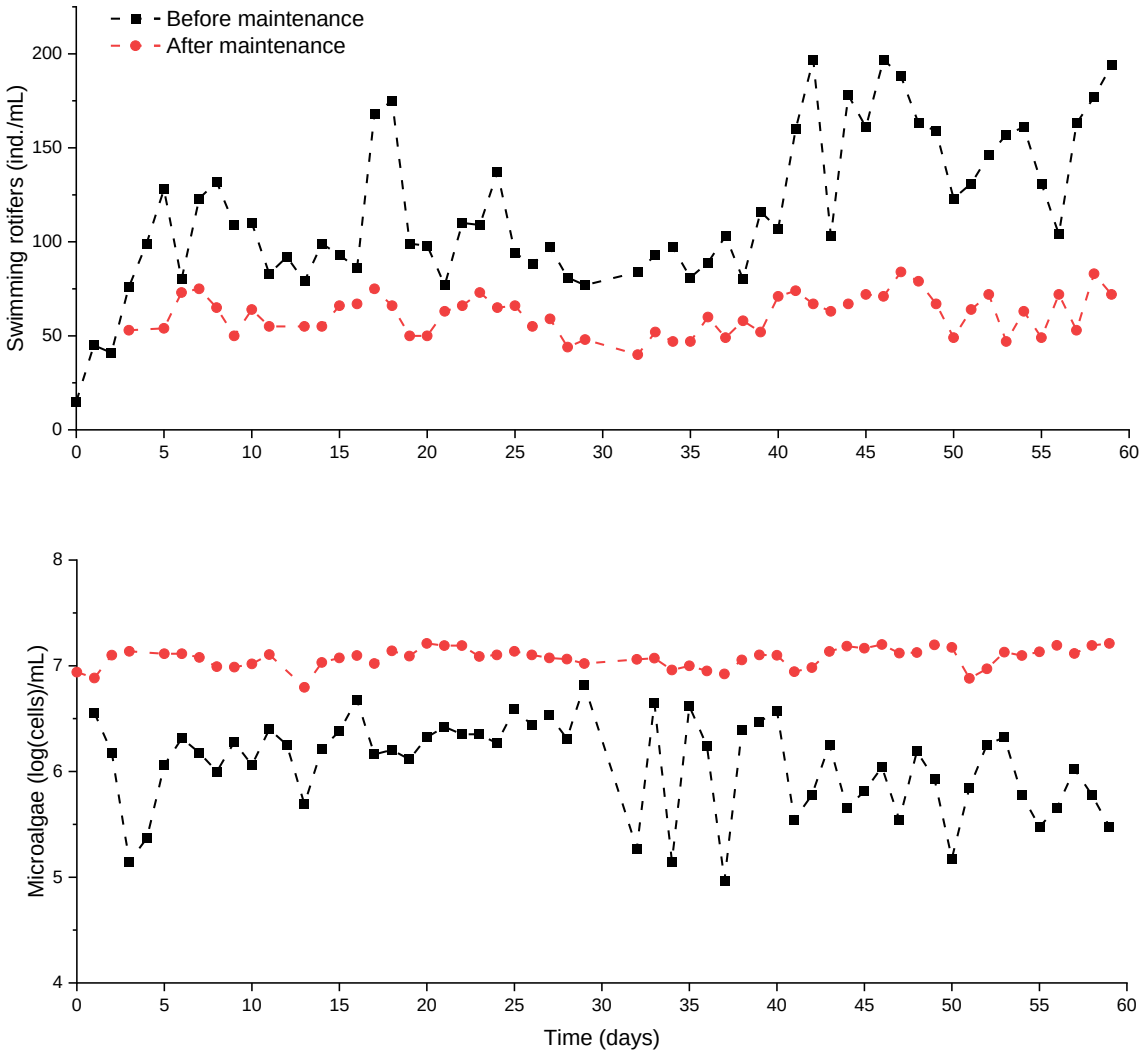

Figure C.3. Evolution of the concentration of rotifers (A) and microalgae (B) in the rotifer culture carried out during 60 days. Counts were performed before and after the maintenance of the culture.

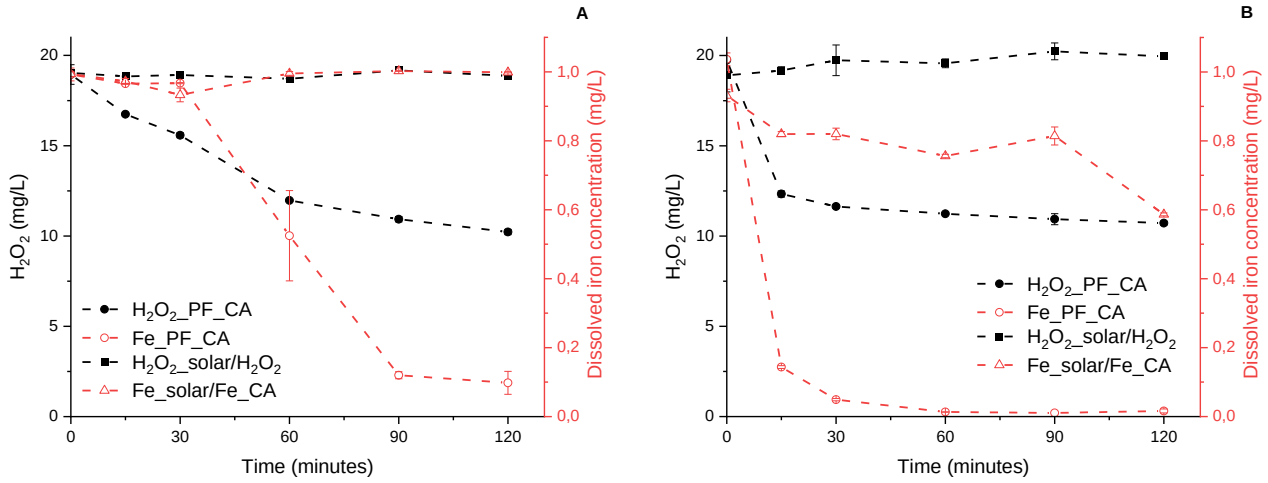

Figure C.4. Consumption of H_2O_2 and concentration of dissolved iron during the solar light/Fe^{2+} process with citric acid, solar light/ H_2O_2 and citrate-modified PF treatments in ultrapure water (A) and synthetic freshwater (B) without microalgae and rotifers.

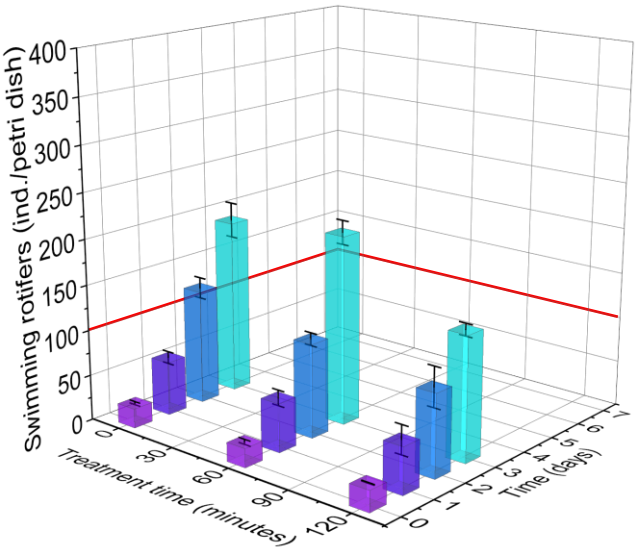

Figure C.5. Growth of a concentration of 20 rotifers mL^{-1} during their incubation in petri dishes for regular time points of the solar light/Fe^{2+} process with citric acid in synthetic freshwater. Petri dishes were incubated with 1-mL samples and the incubation was stopped after 7 days or one day after the concentration of rotifers exceeded 100 rotifers per petri dish (red line).

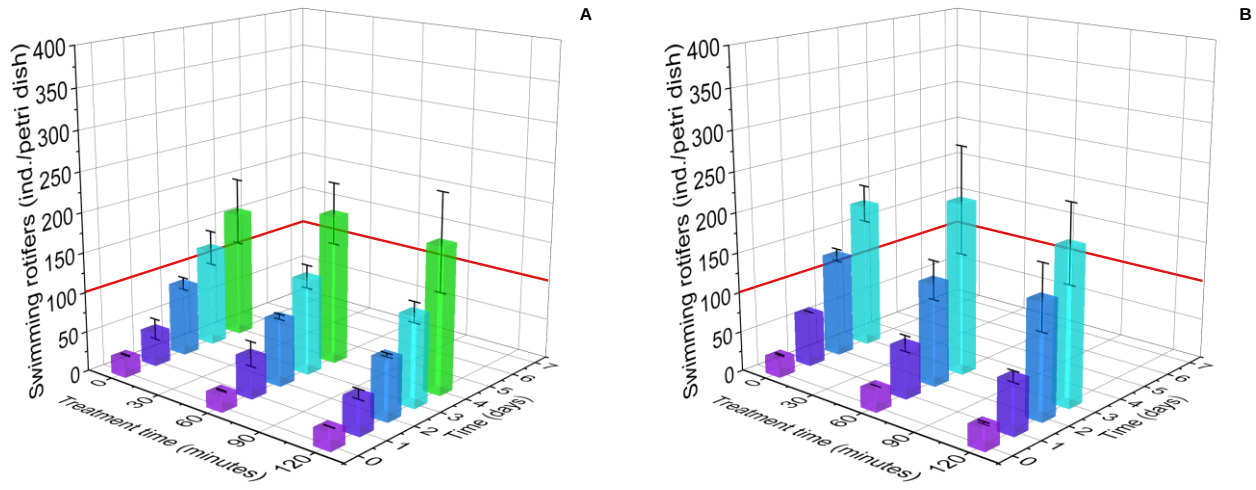

Figure C.6. Growth of a concentration of 20 rotifers mL^{-1} during their incubation in petri dishes for regular time points of the solar light process (A) and solar light/Fe^{2+}process with citric acid (B) in microalgae cultures. Petri dishes were incubated with 1-mL samples and the incubation was stopped after 7 days or one day after the concentration of rotifers exceeded 100 rotifers per petri dish (red line).

Appendix D. Additional activities

Co-authorship

- E. Zanchetta, E. Damergi, B. Patel, T. Borgmeyer, H. Pick, A. Pulgarin, C. Ludwig, Algal cellulose, production and potential use in plastics: Challenges and opportunities, Algal Research. 56 (2021) 102288. https://doi.org/10.1016/j.algal.2021.102288.

Supervision and teaching

Supervision

- Yasser Baddour, Photo-Fenton mediated decontamination of algal cultures, Master's thesis (2018).
- Alexander Garcia Kapeller, Cultivation of green microalgae *Chlorella vulgaris* using an effluent of the anaerobic digestion as nitrogen source, Master's thesis (2018).
- Jiahua Chen, Continuous cultivation of *Brachionus calyciflorus* for laboratory experiments, internship's project (2019).
- Jérémie Decker, Inactivation of rotifer contamination in microalgae cultures via photo-Fenton process, Master's thesis (2021, nominated for a prize).

Teaching

- Pollutants analysis in the environment, practical work, 2019-2020.

Other activities

- Safety Coordinator, first contact and coordinator for the safety and health of the collaborators as a part of the research activities of the group, 2017-2021.
- Person in charge of the implementation and development of a laboratory for chemical, microbiological and analytical work, 2016-2021.
- Promotion of scientific activities, Scientistic Valais, Sion, 29-30 Apr. 2017.

Scientific workshops and training

Courses

- Understanding statistics and experimental design, 4 ECTS, 2017.
- Biomass conversion, 2 ECTS, 2017.
- Academic writing for doctoral students, 2 ECTS, 2017.
- Climate economics for engineers, 2 ECTS, 2018.
- Environmental economics for engineers, 2 ECTS, 2018.

Training

- Obligatory basic safety training (FOBS 1 + 2), 28 Sept. 2017.
- Working in a P2 and P3 environment (FOBS 3), 26 Sept. 2017.
- Connaissances de base en sécurité au travail et protection de la santé pour préposé à la sécurité, 13-14 Nov. 2017.
- Training for the Flow Cytometry Platform,22-23 June 2017.

Workshops and presentations

- Workshop at Justus Liebig University Giessen, research group of Prof. Dr. Stefan Gäth, 13-15 June 2016.
- Workshop at the Laboratory of Cells Cycle of Algae at the institute of Microbiology in Trebon, Czech Republic, 8-14 May 2017.
- Scientific presentation: “A novel proposition for a citrate-modified photo-Fenton process against bacterial contamination of microalgae cultures”, ZHAW Wädenswil :5 Dec. 2019, Paul Scherrer Institute: 30 Mar. 2020.
- Submission of an abstract: “A new approach to inactivate bacteria in microalgae cultures”, 11th European Conference on Solar Chemistry and Photocatalysis Environmental Applications (SPEA 11), 2020, conference cancelled due to the Covid-19 sanitary crisis.

Photographic credits

Pictures included in the present doctoral thesis were taken without distinction by the author or team members involved in the research projects.

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[193] Département fédéral de l'intérieur, Ordonnance du DFI sur l'eau potable et l'eau des installations de baignade et de douche accessibles au public, (2017).
[194] Département fédéral de l'intérieur, Ordonnance du DFI sur l'hygiène dans les activités liées aux denrées alimentaires, (2020).

Curriculum Vitae

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Work experience

Apr. 2016 – Present — Doctoral assistant

École polytechnique fédérale de Lausanne – EPFL

Ludwig Group, Lausanne. In collaboration with the Zurich University of Applied Science (ZHAW), Wädenswil.

Nov. 2015 – Mar. 2016 — Research assistant

Swiss Federal Institute of Aquatic Science and Technology – Eawag

Department of water and resources & drinking water, Dübendorf.

Sept. 2014 – Aug. 2015 — Compulsory civilian service

Swiss Federal Institute for Forest, Snow and Landscape Research – WSL

Laboratory of ecological systems, Lausanne.

Sept.2013 – June 2014 — Master's student and trainee

École polytechnique fédérale de Lausanne – EPFL

Group for photochemical dynamics, Lausanne.

Technical skills

Development and management of a scientific project	Strong experience of project development and coordination in a scientific work environment, that is to review the state-of the art, to develop novel ideas, to coordinate and supervise the work between team members, to obtain high-quality scientific outputs in a defined timeframe, and to communicate the results via the publication in peer reviewed journals.
Laboratory work	Strong experience of working in chemical, microbiological and analytical research laboratories, carrying out scientific experiments, and developing laboratory infrastructure and activities.
Safety coordinator	Strong experience of keeping safe the work environment of a laboratory via the implementation of safety measures and promotion of good practices.
Software	Word, Excel, PowerPoint, Origin, Visual Studio.

Academic projects

2021 — Doctoral thesis

Robust and sustainable cultivation of microalgae: recycling of nitrogen from liquid digestate and control of biological contamination.

2014 — Master's project

Kinetics of charge separation and recombination in hybrid perovskite solid-state solar cells.

Education

2014 — Master of Science in Molecular and Biological Chemistry

École polytechnique fédérale de Lausanne – EPFL

Focus on physical chemistry, biological chemistry and biophysics, inorganic chemistry.

2012 — Bachelor of Science in Chemistry and Chemical Engineering

École polytechnique fédérale de Lausanne – EPFL

Focus on synthetic and analytical chemistry, biological and biophysical chemistry, computational chemistry.

Languages

French – Mother tongue

English – Advanced, C1 level

Spanish – Intermediate, B1 level

German – Elementary, A2 level

Programming language C# – Beginner

Peer reviewed journal publications

- A. Pulgarin, S. Giannakis, C. Pulgarin, C. Ludwig, D. Refardt, A novel proposition for a citrate-modified photo-Fenton process against bacterial contamination of microalgae cultures, Applied Catalysis B: Environmental. 265 (2020) 118615. https://doi.org/10.1016/j.apcatb.2020.118615.
- E. Zanchetta, E. Damergi, B. Patel, T. Borgmeyer, H. Pick, A. Pulgarin, C. Ludwig, Algal cellulose, production and potential use in plastics: Challenges and opportunities, Algal Research. 56 (2021) 102288. https://doi.org/10.1016/j.algal.2021.102288.
- B.J.M. Robroek, R.J.H. Albrecht, S. Hamard, A. Pulgarin, L. Bragazza, A. Buttler, V.E. Jassey, Peatland vascular plant functional types affect dissolved organic matter chemistry, Plant Soil. 407 (2016) 135–143. https://doi.org/10.1007/s11104-015-2710-3.

Interests

Piano, hiking, yoga, software and video game development.

Individual situation

Born on 14 February 1989, Swiss citizen, single, one child.