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Chloride Salt Inhibition on Lipid Production in Wastewater-Grown Algae for Biofuel Production

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Abstract

Algae are increasingly being recognized as useful organisms for many applications in today's world. Their ability to remove nitrogen, phosphorus, and trace metals from water while adding oxygen to water makes them an attractive tertiary treatment technology in municipal wastewater treatment facilities. At the same time, algae produce lipids and carbohydrates that are useful for biofuel production, and they are not a human food crop unlike many biofuel feedstocks. In this study the effect of increased chloride concentrations in wastewater was assessed on the ability of two species of algae, *Chlorella vulgaris* and *Scenedesmus dimorphus*, to function as a biofuel feedstock. In the first phase of the experiment, algae was cultivated in synthetic wastewater with varying chloride concentration; in the second phase secondary effluent samples from the Westside Wastewater Treatment Plant in Fayetteville, AR, were used to cultivate algae, and the chloride concentration was measured. The capacity of *C. vulgaris* to produce biomass and lipids was not heavily affected by increased chloride concentrations in the synthetic wastewater, but that of *S. dimorphus* was diminished at increased chloride concentrations. The secondary effluent had a chloride concentration ranging from six to nine times that of the synthetic wastewater recipe. *S. dimorphus* produced more biomass and chlorophyll than *C. vulgaris* in these trials, but neither species was effective in in producing lipids. Overall, the results from both phases of the experiment require replication for validation, and there are many opportunities to further this work.

1. Introduction

1.1 Importance of Algae

Algae are photoautotrophic organisms that thrive in a wide variety of environments around the world. These organisms are increasingly being cultivated due to their potential as a feedstock for biofuel production. In the face of climate change in an energy-intensive global community, algae offer many benefits. Production and consumption of algae-based biofuels is a carbon recycling process in which carbon dioxide is taken up by algae during production and released during consumption of fuels. This presents a significant opportunity for mitigating carbon dioxide emissions from fossil fuel usage in the transportation industry, as combustion of fossil fuels increases the amount of carbon dioxide in the atmosphere. Algae are not a human source of food and can grow in non-arable, high stress environments, so they do not present a challenge to food sources like other plant-based biofuels (Kraan, 2010). Thus, algae-based biofuels present a potential solution to several problems without generating additional challenges with respect to the water-food-energy nexus.

1.2 Algae in Wastewater Treatment

In addition to their promise as a biofuel feedstock, algae can also be used to treat wastewater. Wastewater is typically treated in several stages, each of which removes contaminants of different sizes and types. Solids are removed in preliminary and primary treatment, and dissolved organic matter is removed in secondary treatment. Depending on the regulations for a particular facility, effluent from secondary treatment can contain moderate levels of nutrients such as nitrogen and phosphorus that could lead to eutrophication if discharged into some environments (Auer et al., 2010). Algae are not a typical component of modern wastewater treatment systems, but their use as a biological component of the wastewater treatment process has been well established. Algae can play a vital role in the process because of their success in removing nutrients from water and adding dissolved oxygen to water

(Oswald, 2003). Incorporating algae after secondary treatment could potentially lower the level of nutrients discharged to the environment.

The contaminants that algae remove from wastewater serve as a nutrient source for algal biomass production. Two important groups of compounds for biofuel synthesis contained in algal biomass are lipids and carbohydrates. Lipids can be converted into biodiesel, and carbohydrates can be used for ethanol production. Research in algae cultivation has already shown that limiting the availability of nitrogen significantly increases lipid production within algae, and nutrient starvation has already become a common component in the procedures of studies (Zhou et al., 2014). The use of algae in the wastewater treatment process may simultaneously improve water quality and generate a biofuel feedstock.

It is important to note here that the benefits that algae provide in the wastewater treatment process are accompanied by some disadvantages. One of these is harvesting the algae. Algae are harvested from wastewater first through flocculation which can be expensive if chemically-induced flocculation methods are used. Further harvesting can proceed through gravity or centrifugal sedimentation, but the small size of microalgae often results in a settling rate that is too low for gravity sedimentation methods to be effective. Centrifugal sedimentation is an energy-intensive process, and this would increase the cost of implementing algae in wastewater treatment facilities (Pittman et al., 2011). To satisfy algae's energy requirements, implementation of algae in the wastewater treatment process might require clarifiers with larger surface area and more exposure to the sun. Increased process footprint increases the capital cost of the process.

1.3 Chloride in Wastewater

Chloride salts make up a portion of the major inorganic constituents that are found in water used for many applications in today's world. Freshwater typically contains much lower concentrations of these salts than ocean water, but chloride is still found in freshwater due to contributions from surface water and groundwater (Auer, et al., 2010). Chloride is eventually discharged into the environment after being used by people in food, beverages, and other products (Mullaney, et al., 2009). There are no threats to human health when chloride is taken in along with appropriate amounts of fresh water, so chloride removal has not been a concern of wastewater treatment in the past. However, chloride could pose a challenge to other living organisms in water, such as algae. The effect of salinity has been studied on marine algae, but only for several freshwater algae species such as *B. braunii* (Rao et al., 2007) and *Botryococcus spp.* (Yeesang and Cheirsilp, 2011). However, both of these works examine algae strictly in growth media, not wastewater.

1.4 Objectives

The main objective of this study was to assess how chloride salts in wastewater impact freshwater algae's performance as a biofuel feedstock. This study focused on lipids and biomass production of algae as they grew in both synthetic wastewater and secondary wastewater effluent from the Westside Wastewater Treatment plant (Westside), located in Fayetteville, AR. Chloride concentration was manipulated in the synthetic wastewater experiments, and chloride concentration was measured in the secondary effluent experiments. The synthetic wastewater experiments were an attempt to establish a relationship between algae's performance and chloride concentration present, whereas the secondary effluent experiments served to gauge the algae's performance in an environment that more closely represents a real world scenario.

2. Methods and Materials

The species of algae selected for this study were *Chlorella vulgaris* and *Scenedesmus dimorphus*; these species were selected based on previous studies that demonstrated their potential to produce compounds useful for biofuel production. *C. vulgaris* has been shown to produce lipids up to 50% of its dry weight (Huang & Su, 2013), and *S. dimorphus* has been shown to produce lipids up to 30% of its dry weight (Gour et al., 2016). Pure cultures of these species were purchased from the University of Texas

UTEX Algae Collection (UTEX 2714 *Chlorella vulgaris* and UTEX 1237 *Scenedesmus dimorphus*) and were maintained throughout the trials. Figure 1 shows images of both the species used in this study.

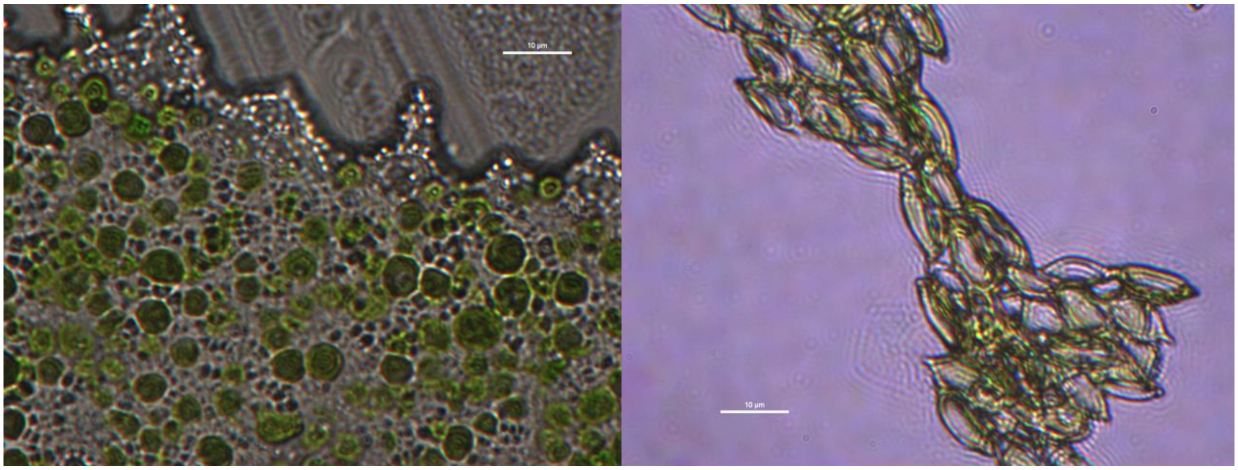


Figure 1. *C. vulgaris* (left) and *S. dimorphus* (right) at 100x magnification. Note 10 µm scale bar in both photos.

Each of these trials consisted of a two-week period; in the first week, each species of algae was inoculated in a 500 mL flask of liquid proteose media (see Table 1). Prior to inoculation, the pH of the proteose media was adjusted to 8 to optimize algal growth (Lavens & Sorgeloos, 1996). Additionally, the media were autoclaved to prevent any potential contamination from bacteria or other microorganisms.

Table 1. Recipe for proteose media.

Compound		M.W	Final Concentrations	Final Weights Required
		g/mol	g/L	g
NaNO3	Sodium Nitrate	84.99	0.025	0.025
CaCl2.2H2O	Calcium Chloride dihydrate	146.98	0.0025	0.0025
MgSO4.7H2O	Magnesium Sulfate heptahydrate	246.366	0.0075	0.0075
K2HPO4	Potassium Phosphate dibasic	174.2	0.0075	0.0075
KH2PO4	Potassium Phosphate monobasic	136.086	0.0175	0.0175
NaCl	Sodium Chloride	58.44	0.0025	0.0025
	Peptone		1	1

After a week of growth in the proteose media, the algae were separated from the media using a centrifuge, and they were then inoculated into flasks of wastewater for the experiments (see Table 2). The synthetic wastewater was prepared for inoculation in a similar manner to the proteose media. Flasks of the synthetic wastewater were adjusted to a pH of 8 using concentrated sodium hydroxide and hydrochloric acid and then autoclaved. However, in these trials, the chloride concentration was varied by adding sodium chloride to the synthetic wastewater. Over the course of the project, trials were conducted at two times (12.8 mg/L), three times (19.2 mg/L), five times (32 mg/L), and ten times (64 mg/L) the chloride concentration of the synthetic wastewater recipe. These concentrations are presented in Table 3 and are based on common characteristics of wastewater outlined by Pescod (1992) in a wastewater treatment and usage document by the Food and Agriculture Organization of the United Nations.

Table 2. Synthetic wastewater recipe

Formula	Name	mg Compound	Calculated
CH3COONa	Sodium Acetate		
CH3COONH3	Ammonium Acetate	240.88	240.88
KH2PO4	monopotassium phosphate	43.94	43.94
NaHCO3	Sodium Bicarbonate	125	125
CaCl2	Calcium chloride	10	10
FeCl3 6H2O		0.804	0.804
MnSO4	Manganese sulfate	0.038	0.038
ZnSO4	Zinc sulfate	0.035	0.035
MgSO4	Magnesium sulfate	25	25
Yeast extract		50	50
FeCl2	Iron chloride	0.375	0.375

Table 3. Chloride Concentrations in synthetic wastewater trials

Trial	Cl^- Concentration (mg/L)
1x	6.4
2x	12.8
3x	19.2
5x	32
10x	64

In the trials conducted with secondary effluent samples from Westside, pH was also adjusted to 8, and only half of the samples were autoclaved prior to inoculation with algae. The practice of autoclaving only half of the flasks in these trials is an attempt to bring this study closer to realistic conditions because secondary effluent in treatment plants will likely contain some amount of other organisms. Throughout the trials, all the flasks of algae were continuously stirred by using a magnetic stir bar and a stir plate; the stir plate was kept under LED lights with a timer cycling the lights on and off every 12 hours. All trials included control flasks of wastewater at the same chloride concentration that were not inoculated with either algal species.

During the week of growth in wastewater, samples were collected daily to measure the algae's productivity and the chloride concentration of the wastewater. Measurements of the algae's productivity were collected by removing a 100 μL sample from each flask of synthetic wastewater for analysis using a Biotek Synergy H1 Microplate Reader. The microplate reader first measured optical density and chlorophyll. Optical density was detected via absorbance of light at 680 nm, and chlorophyll was detected via fluorescence with excitation at 440 nm and emission at 685 nm. Optical density was used as an indicator for the intensity of the algae's growth, and chlorophyll was an indicator of the carbohydrate production in the algae. After collecting these measurements, 100 μL of Nile red solution was added to each of the wells in the microplate to measure lipids (Chen et al., 2009). Lipid content was detected via fluorescence with excitation at 530 nm and emission at 570 nm. All microplate-based measurements are dimensionless as a result of these absorbance and fluorescence methods.

Ion chromatography (IC) was used to measure the chloride concentration present in the wastewater throughout the trials. 1.5 mL samples were collected daily, filtered with a 0.1-micron filter, and diluted with deionized water prior to IC measurement. These samples were analyzed using a Metrohm 850 Professional ion chromatograph, and the data was processed in Metrohm's MagIC software. Additionally at the end of each trial, pH of the wastewater was measured with a pH meter, and total solids produced by the algae were measured using Standard Method 2540B (Eaton, 2005). Microsoft Excel and Matlab were used for processing and visualizing all data from the experiments. All procedures were approved by the University of Arkansas's Institutional Biosafety Committee prior to execution of the experiments (IBC protocol #17029 approved on 2/15/17).

3. Results and Discussion

3.1 Synthetic Wastewater Trials

In these trials, increased levels of chloride appeared to improve the overall productivity of *C. vulgaris*. As seen in figure 2, *C. vulgaris* reached its peak biomass production during the 10x trial, while its lowest biomass production occurred during the 1x trial. *S. dimorphus* had optimal biomass production at the lowest chloride concentration, although it behaved similarly at the highest chloride concentration. Its performance at the middle of the range was fairly consistent across the trials. With the exception of the 1x trial, *C. vulgaris* produced more biomass than *S. dimorphus*.

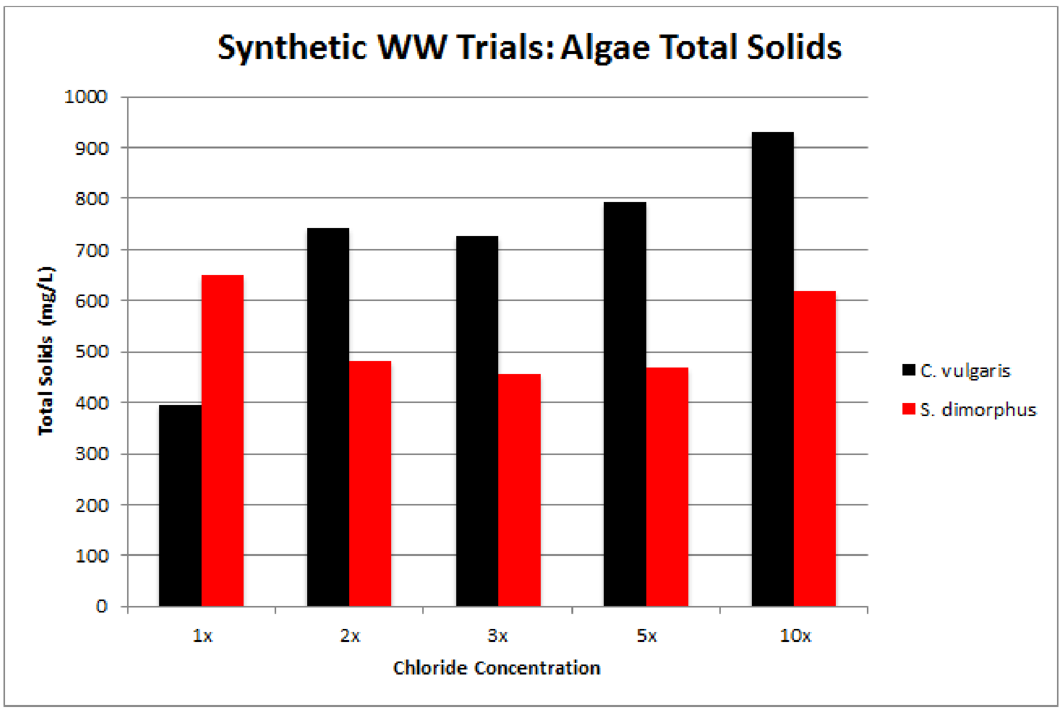


Figure 2. Total solids production by both algal species in the synthetic wastewater trials. 1x trials refer to a chloride concentration of 6.4 mg/L, 2x trials refer to a concentration of 12.8 mg/L, etc. (See Table 3 for a full listing of concentrations).

The data for chlorophyll production in *C. vulgaris* reflect similar results to the total solids measurements; at increased chloride concentrations, this species of algae produced more chlorophyll. The top panel of figure 3 shows the change in chlorophyll over the course of all of these trials.

Chlorophyll production was at its lowest for this phase of the experiment during the 1x trial. Chloride concentration had only a small effect on chlorophyll production, as evidenced by the measurements from the 2x, 3x, 5x, and 10x trials. The trend of chlorophyll levels reaching a maximum toward the beginning of the growth period supports findings by Zhou et al. (2014) for polysaccharide production in pure *Chlorella* cultures used to treat wastewater.

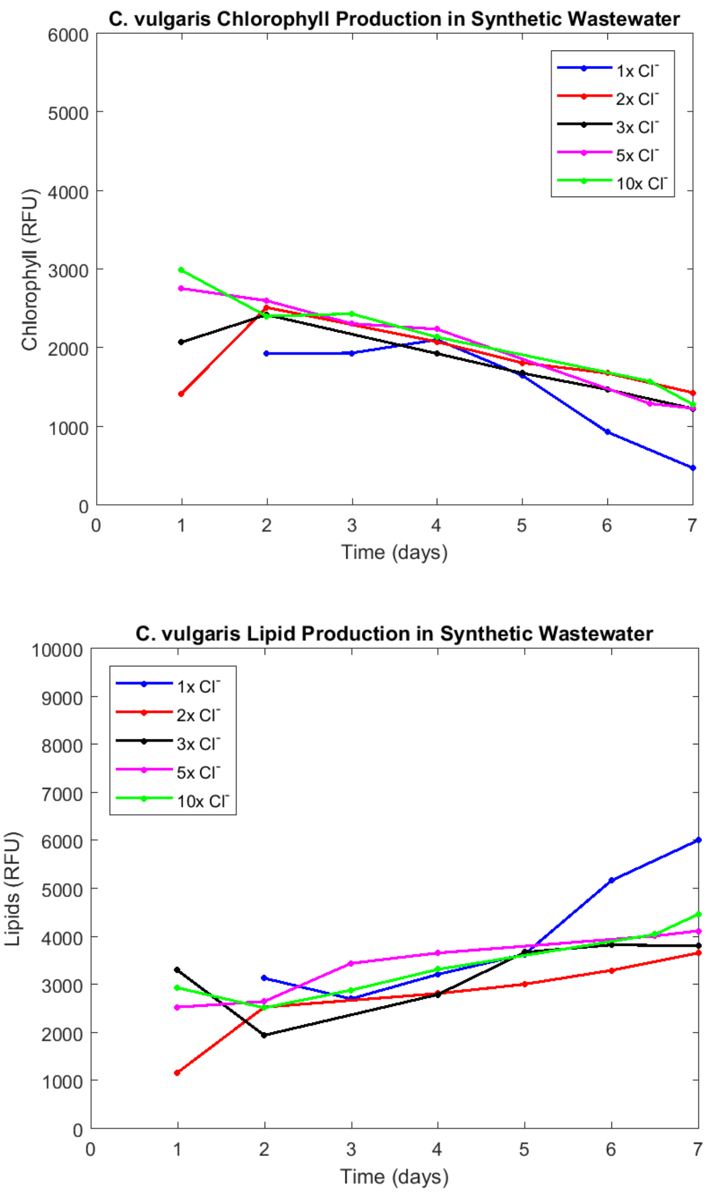
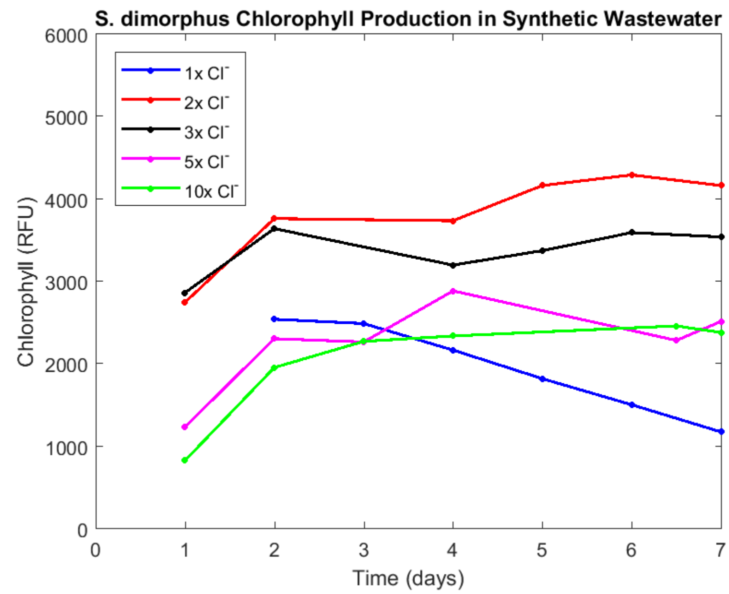


Figure 3. *C. vulgaris* chlorophyll and lipid production over the course of synthetic wastewater trials. 1x trials refer to a chloride concentration of 6.4 mg/L, 2x trials refer to a concentration of 12.8 mg/L, etc. (See Table 3 for a full listing of concentrations).

While biomass and chlorophyll production increased in *C. vulgaris* at higher chloride concentrations, the bottom panel of figure 3 indicates that lipid production showed the opposite trend. Lipid production was greatest during the 1x trial, and the other four trials showed lower lipid production. The general trend of increased lipid production toward the end of trials again agrees with the results of Zhou et al. (2014). The algae use up a greater portion of nutrients in the water over the course of the trial, and this nutrient starvation induces greater lipid production.

Overall, it appears that increased chloride concentration does not negatively impact *C. vulgaris*, instead providing some small benefit for the species. Total biomass and chlorophyll production improved under conditions with elevated chloride. While lipid production was negatively impacted at higher chloride concentrations, this impact was not large, and lipid production at the highest chloride concentrations (5x and 10x) was actually higher than that at the 2x and 3x concentrations. Lipid measurements are also an indirect indicator of how well the algae remove nutrients from wastewater, which is algae's main function in the wastewater treatment process. These results suggest that *C. vulgaris*'s ability to treat wastewater is not greatly compromised by increased chloride concentrations.

Figure 4 shows chlorophyll and lipid production by *S. dimorphus* during the synthetic wastewater trials. Chlorophyll production reached a maximum during the 2x and 3x trials and a minimum during the 1x trial. This indicates that *S. dimorphus* was most successful in producing carbohydrates when exposed to chloride concentrations between 12.8 and 19.2 mg/L and was moderately impacted at higher concentrations. In stark contrast to chlorophyll production, lipid production in *S. dimorphus* was heavily impacted when chloride concentration was increased. As indicated by the bottom panel of figure 4, this species was only able to produce lipids at the lowest of chloride concentrations.



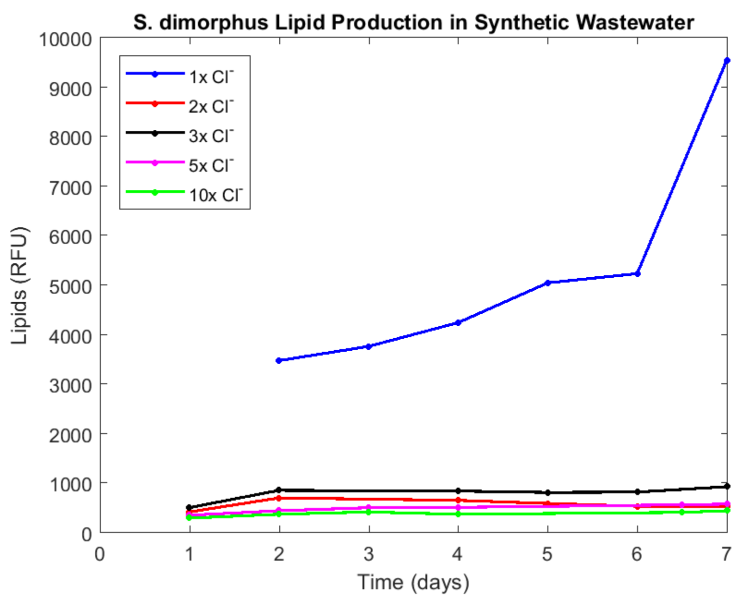


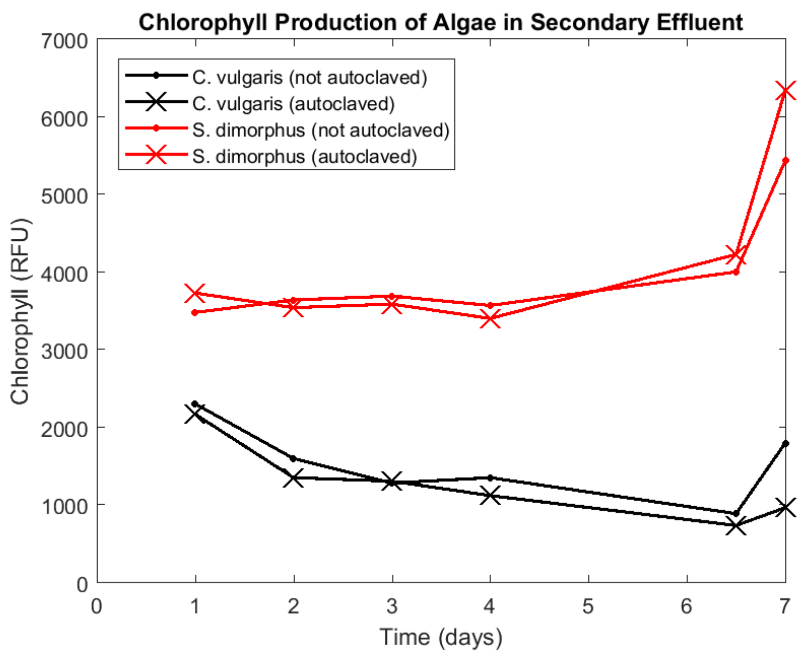
Figure 4. *S. dimorphus* chlorophyll and lipid production over the course of synthetic wastewater trials. 1x trials refer to a chloride concentration of 6.4 mg/L, 2x trials refer to a concentration of 12.8 mg/L, etc. (See Table 3 for a full listing of concentrations).

The data indicate that *S. dimorphus* was heavily affected as a biofuel feedstock by chloride salt stress. While chlorophyll production was lowest during the 1x trial, total biomass and lipid production were at a maximum during this trial. Lack of biomass production in the 2x, 3x, and 5x trials in conjunction with moderate chlorophyll production and poor lipid production make *S. dimorphus* a poor feedstock for energy at increased chloride concentrations. Poor lipid production at all chloride concentrations greater than 6.4 mg/L also suggests that *S. dimorphus*'s capacity to remove nutrients from wastewater was severely diminished as chloride concentration increases. Additionally, in the 2x, 3x, 5x, and 10x trials with *C. vulgaris* and *S. dimorphus*, pH at the end of the trials had increased substantially, occasionally being greater than 10.5. This change in pH could have affected the growth of the algae and impacted results from the study.

3.2 Secondary Effluent Trials

The IC measurements of chloride in the secondary effluent samples ranged from 39 to 56 mg/L. In the context of this study, these measurements put the trials in between the chloride concentrations of the 5x and 10x synthetic wastewater trials. *C. vulgaris* produced total biomass of 694 mg/L when the effluent was not autoclaved prior to inoculation and 710 mg/L when the effluent was autoclaved prior to inoculation. This is similar to the results of the 2x and 3x synthetic wastewater trials. *S. dimorphus* produced total biomass of 847 mg/L when the effluent was not autoclaved prior to inoculation and 804 mg/L when the effluent was autoclaved prior to inoculation. This is particularly interesting because it indicates that *S. dimorphus* might be more successful in secondary effluent from a real world treatment plant than it was in synthetic wastewater.

S. dimorphus also produced chlorophyll well when compared to its trials in synthetic wastewater and to *C. vulgaris* in the secondary effluent trials. This is evident in the top panel of figure 5 for both the autoclaved and non-autoclaved *S. dimorphus* trials. In the bottom panel of figure 5, it is evident that neither species of algae was effective in producing lipids during the secondary effluent trials. This indicates that neither species of algae would be effective in removing nutrients from wastewater in a treatment plant at these chloride concentrations. Another point of interest in these trials is the manner in which chlorophyll and lipid production changed over the course of the trials. Both species showed increased chlorophyll production and decreased lipid production at the end of the trials, which is contrary to the findings of the synthetic wastewater trials and other studies like Zhou et al. (2014). It could be possible that the algae experienced a greater lag time in their growth cycle while in the secondary effluent or that the difference in nutrient content between the synthetic wastewater and the secondary effluent caused this behavior.



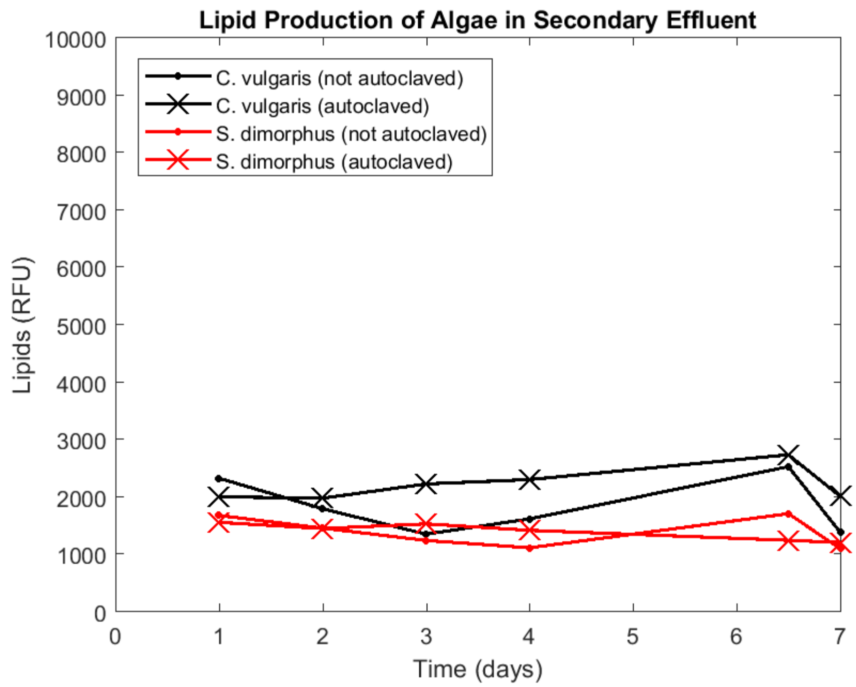


Figure 5. Chlorophyll and lipid production in both species of algae during the secondary effluent trials.

4.0 Conclusion and Future Opportunities

In the synthetic wastewater trials *C. vulgaris* was more resistant to chloride stress than *S. dimorphus*, producing greater total biomass and lipids during the majority of the trials. While *C. vulgaris* was less successful at removing nutrients when exposed to higher chloride concentrations, it was still able to produce lipids, and thus was still removing nutrients from the wastewater. In contrast, *S. dimorphus* showed no ability to remove nutrients when exposed to chloride concentrations greater than that in the original synthetic wastewater recipe. *S. dimorphus* was still able to produce chlorophyll at higher chloride concentrations, but its lack of biomass and lipid production indicate that chloride stress diminishes its potential as a biofuel feedstock and a component of the wastewater treatment process. In the secondary wastewater trials, *S. dimorphus* produced more biomass and chlorophyll than *C. vulgaris*. It appears that neither species was able to remove nutrients well in these trials. Autoclaving the secondary effluent samples prior to inoculation did not have an effect on the performance of either species.

Moving forward, there are still many opportunities related to this study that need to be explored. A weakness of this study is its lack of replication; the results presented here would have greater certainty if the trials were carried out in duplicates or triplicates. A greater understanding of the algae's ability to remove nutrients from wastewater would also benefit this experiment; this could be achieved by measuring total nitrogen and total phosphorus over the course of the trials. Additionally, it is still unclear why the growth of both species showed such great differences between the synthetic wastewater and secondary effluent trials. This question could be answered by a more thorough chemical characterization of the secondary effluent samples. Control of pH through the course of trials also has the potential to refine the results of this experiment. It could be that the increase in pH during the trials was a greater driver of algal activity than ambient chloride concentration. Other characteristics of wastewater could also be manipulated to get a more detailed picture of the conditions in which algae could be a viable wastewater treatment technology and biofuel feedstock.

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References

Auer, M. T., Mihelcic, J. R., & Zimmerman, J. B. (2010). *Environmental engineering: Fundamentals, sustainability, design*. Hoboken, NJ: John Wiley & Sons/Wiley.

Chen, W., Zhang, C., Song, L., Sommerfeld, M., & Hu, Q. (2009). A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *Journal of Microbiological Methods,77*(1), 41-47. doi:10.1016/j.mimet.2009.01.001

Eaton, A. D. (2005). *Standard methods for the examination of water and wastewater*(21st ed.). New York: American Public Health Association.

Gour, R. S., Chawla, A., Singh, H., Chauhan, R. S., & Kant, A. (2016, May 19). Characterization and Screening of Native Scenedesmus sp. Isolates Suitable for Biofuel Feedstock. *PLOS ONE PLoS ONE, 11*(5). doi:10.1371/journal.pone.0155321

Huang, Y., & Su, C. (2013, March 20). High lipid content and productivity of microalgae cultivating under elevated carbon dioxide. *International Journal of Environmental Science and Technology, 11*(3), 703-710. doi:10.1007/s13762-013-0251-y

Kraan, S. (2010, December 15). Mass-cultivation of carbohydrate rich macroalgae, a possible solution for sustainable biofuel production. *Mitigation and Adaptation Strategies for Global Change, 18*(1), 27-46. doi:10.1007/s11027-010-9275-5

Lavens, P., & Sorgeloos, P. (1996). *Manual on the production and use of live food for aquaculture*(Food and Agriculture Organization of the United Nations). Rome: Food and Agriculture Organization of the United Nations.

Mullaney, J. R., Lorenz, D. L., & Arntson, A. D. (2009). *Chloride in groundwater and surface water in areas underlain by the glacial aquifer system, northern United States* (United States of America, U.S. Geological Survey). Reston, VA: U.S. Geological Survey.

Oswald, W. J. (2003). My sixty years in applied algology. *Journal of Applied Phycology, 15*(2/3), 99-106. doi:10.1023/a:1023871903434

Pescod, M. B. (1992). *Wastewater treatment and use in agriculture*. Rome: Food and Agriculture Organization of the United Nations.

Pittman, J. K., Dean, A. P., & Osundeko, O. (2011). The potential of sustainable algal biofuel production using wastewater resources. *Bioresource Technology,102*(1), 17-25. doi:10.1016/j.biortech.2010.06.035Rao, A. R., Dayananda, C., Sarada, R., Shamala, T., & Ravishankar, G. (2007). Effect of salinity on growth of green alga Botryococcus braunii and its constituents. *Bioresource Technology,98*(3), 560-564. doi:10.1016/j.biortech.2006.02.007

Yeesang, C., & Cheirsilp, B. (2011). Effect of nitrogen, salt, and iron content in the growth medium and light intensity on lipid production by microalgae isolated from freshwater sources in Thailand. *Bioresource Technology,102*(3), 3034-3040. doi:10.1016/j.biortech.2010.10.013

Zhou, D., Li, Y., Yang, Y., Wang, Y., Zhang, C., & Wang, D. (2014, December 18). Granulation, control of bacterial contamination, and enhanced lipid accumulation by driving nutrient starvation in coupled wastewater treatment and Chlorella regularis cultivation. *Applied Microbiology and Biotechnology, 99*(3), 1531-1541. doi:10.1007/s00253-014-6288-0