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Assessing Auto-flocculation of Microalgae in Wastewater Treatment

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Abstract

Biofuels produced from algae have the prospect to provide a cheap, carbon neutral alternative to fossil fuels. However, the high cost for harvesting algae limits its wide application, as the preferred algae strains for biofuel production are typically unicellular microalgae that do not settle in water very well. Recently, researchers have been focusing on developing a biological method to achieve the sedimentation of algae through flocculation. A recent study has concluded that introducing microalgae that self-flocculates increases the recovery of the desired microalgae, similar to the effect of using coagulant to flocculate the algae. This option can potentially be more eco-friendly without added cost of extra chemicals.

This project explored the effect of auto-flocculation in microalgae when wastewater is used as nutrient source. This project showed that growing the self-flocculating algae *Scenedesmus dimorphus* with *Chlorella vulgaris* did not result in an increase of recovery efficiency from the wastewater after nine hours of sedimentation. When grown together, there was a slight shift to larger particle sizes than that of *C. vulgaris* alone, but it did not correlate into an increase of recovery. The growth of the two species together produced a lower amount of lipids than that of *C. vulgaris* alone, but approximately the same amount of biomass concentration. Further study is recommended to determine if other species of self-flocculating microalgae can produce the desired flocculation effect.

Background:

1. Algae's Use in Biofuels

As the population of the earth continues to grow so will the demand for fossil fuels. However, as the supply of these fuels begin to diminish, scarcity of the fuel and rising prices will create a need for alternative fuel sources. Green unicellular microalgae have been identified as a possible source of fuel due to their ability to provide substantial amounts of biomass and lipids that can be transformed into biodiesel. Another important motive for algal biofuels is it is estimated to have greater biomass productivity per area used for cultivation, as well as lower cost per yield than plant crops (Pittman et al., 2011).

The two main pathways for algal biofuel production are through conversions from biomass and/or lipids in algal cells. The lipids in algal cell are best used for producing biodiesel, while the biomass portion can be used to produce a variety of fuels such as bioethanol, biogas, bio hydrogen as well as burning just biomass (Singh and Olsen, 2011). Different types of algae produce differing amount of lipids by volume, so it is important to select suitable species for cultivation.

2. Algae Harvesting

Currently, one of the greatest challenges with producing algae for biofuels occurs at the algae harvesting step. Due to the planktonic growth mode of many microalgae, the cells must be concentrated for subsequent biofuel production. The most common harvesting methods for concentrating algae are gravity sedimentation, centrifugation, and filtration, which are typically proceeded by a flocculating step (Pittman et al, 2011). Currently, commercial algae harvesting is done by centrifugation, with just this one step contributing to 30% of the cost of algae production

(Salim, et al., 2011). In order for algal biofuel production to be competitive, the harvesting step needs to be completed more economically.

Flocculation is the formation of algae aggregates which can be achieved by a variety of strategies. They include chemical induced flocculation, extreme changes in pH, nutrient depletion, temperature, and level of dissolved oxygen (Salim, et al., 2011). However, a major problem with all of these methods is the cost of adjusting the properties of the water to induce flocculation as well as making it suitable for release into the water system. Recently, studies have begun to research the possibility of introducing bacteria or self-flocculating algae into the system in order to form flocs with desirable planktonic microalgae species.

3. **Algae production in Wastewater Systems**

A major criticism of algal biofuel production is whether the costs of input resources such as nutrients needed for algae growth lead to an overall carbon/energy neutral or positive process. Domestic wastewater can serve as the nutrient source for cultivating microalgae, which can remove excess nutrients such as nitrogen and phosphorus. Both *Scenedesmus dimorphus* and *Chlorella vulgaris* have been shown to achieve 90% removal of ammonia (Gonzalez, et al., 1997). *S. dimorphus* has been shown to be extremely tolerant of adverse conditions, including extremes in pH, CO_2, and salinity (Vidyashankar, et al., 2013). In addition, *S. dimorphus* tends to grow in clusters/aggregates, which could potentially serve as the auto-flocculation species that aids the sedimentation of other planktonic microalgae such as *C. vulgaris*.

This study focuses on the performance of *S. dimorphus* as an auto-flocculation algae species when incorporated into the growth of *C. vulgaris* in wastewater. Recovery efficiency of this

consortium was compared to individual species, in addition to the production of biomass and lipid content.

Materials and Methods

Preparation of Algae

Pure cultures of the microalgae *C. vulgaris* (UTEX 2714) and *S. dimorphus* (UTEX 1237) were obtained from the University of Texas Culture Collection Of Algae and maintained as pure cultures on plates. Each species was inoculated into proteose media [$NaNO_3$ (25 mg L^{-1}), $CaCl_2 \cdot H2O$ (2.5 mg L^{-1}), $MgSO_4 \cdot 7H_2O$ (7.5 mg L^{-1}), K_2HPO_4 (7.5 mg L^{-1}), KH_2PO_4, (17.5 mg L^{-1}), NaCl (2.5 mg L^{-1}), Peptone (1 g L^{-1})] at 26°C and allowed to grow for several days to reach exponential growth. To extract the algae for inoculation into synthetic wastewater, a centrifuge operating at 4000 rpm for 5 minutes was used. The supernatant was removed from the centrifuge tube and the algae were rinsed thoroughly with distilled deionized water to minimize nutrient intrusion into the synthetic wastewater media. For each trial in this experiment, four 120 mL flasks of synthetic wastewater were prepared and autoclaved using the formula included in Table 1.The chemical composition described in Table 1 is for the original strength of wastewater (1x concentration). For subsequent trials, 2x, 3x, and 4x concentrations were prepared using multiples of the formulation described.

Table 1: The chemical composition of the 1x synthetic wastewater.

Component	Concentration (mg Compound/L)
CH_3COONH_3	240.88
KH_2PO_4	43.94
$NaHCO_3$	125
$CaCl_2$	10
$FeCl_3$	0.804
$MnSO_4$	0.038
$ZnSO_4$	0.035
$MgS0_4$	25
Yeast extract	50

The synthetic wastewater was adjusted to pH 8 before each trial using NaOH. Each trial consisted of a control flask with no algae inoculum, a flask inoculated with both species of algae, and two flasks inoculated with one of each species of algae. All flasks were constantly stirred in a warm room at 26°C, with on and off LED lights above the flasks mimicking diurnal pattern. Each day the optical density (OD) was measured using a spectrophotometer to record the growth of the algae. Once the OD value stabilized for two days in a row the following quantification procedure was performed.

Quantification of Algae

To determine recovery efficiency, 1 mL of each flask of algae was placed into a plastic cuvette and OD was measured at the 680 nm wavelength every 15 minutes for 9 hours. The OD

values were used to calculate recovery using the following equation where OD_i:is the OD at time 0, and OD_t is the OD at the time of recovery:

$$\%\ Recovery = \frac{OD_i - OD_t}{OD_i} \times 100$$

The lipid and chlorophyll content were determined using a microplate reader (Synergy H1 Multi-Mode Microplate Reader, Biotek Instruments, Inc., Winooski, VT). For this measurement, 100 µL of sample were put into black-sided clear bottomed 96-well plates (Corning 3603, Corning, Tewksbury, MA). To find the chlorophyll content, fluorescence was measured using excitation at 440 nm and emission at 685 nm wavelengths. Immediately following the chlorophyll measurement, 100 µL of 2X Nile Red were added into each well and allowed to incubate for 10 minutes. The lipid content was recorded by fluorescence intensity at 530 nm of excitation wavelength and 570 nm of emission wavelength (Chen, Zhang, Song, Sommerfield &Hu, 2009).

To help determine if flocculation occurred in the flasks with both algae species, a Multisizer 4 Coulter Counter (Beckman Coulter, Brea, CA) was used to determine particle size and distribution in each flask. A 100 µL sample was diluted into 10 mL of Isoton solution and measured by the Coulter Counter. The size range of particles counted were from 2 to 20 µm.

Total solids concentration were measured following the standard procedure. 50 mL of sample were dried in an oven at 100°C until all of the liquid had evaporated. The dried biomass was weighed, and total solids calculated.

Replicates of measurements were performed where possible. Three samples were taken from each flask when computing lipid and chlorophyll values, and two 50 mL samples were taken from each flask when computing total solids. Average values and standard deviations were

computed for all measurements with replicates. Two-tailed student t-test was performed on different treatments.

Results

Sedimentation

As shown in Figure 1, sedimentation occurs rapidly in the first 4 hours and the rate of sedimentation is minimal after that. In order to determine how well sedimentation separated algae from the water, the recovery efficiency is included in Table 2. The 30-minute and 1-hr recovery efficiencies for all trials were low, with the 4x synthetic wastewater having a much lower recovery then both the 2x and 3x trials. For 9-hr recovery, the 2x, 3x, and 4x trials all yield similar percentages showing a possible max recovery efficiency of approximately 70%. There is no significant difference in recovery percentages between the different species of algae for all sedimentation time as shown in table 3. This offered evidence that growing *S. dimorphus* did not increase the sedimentation efficiency of *C. vulgaris*.

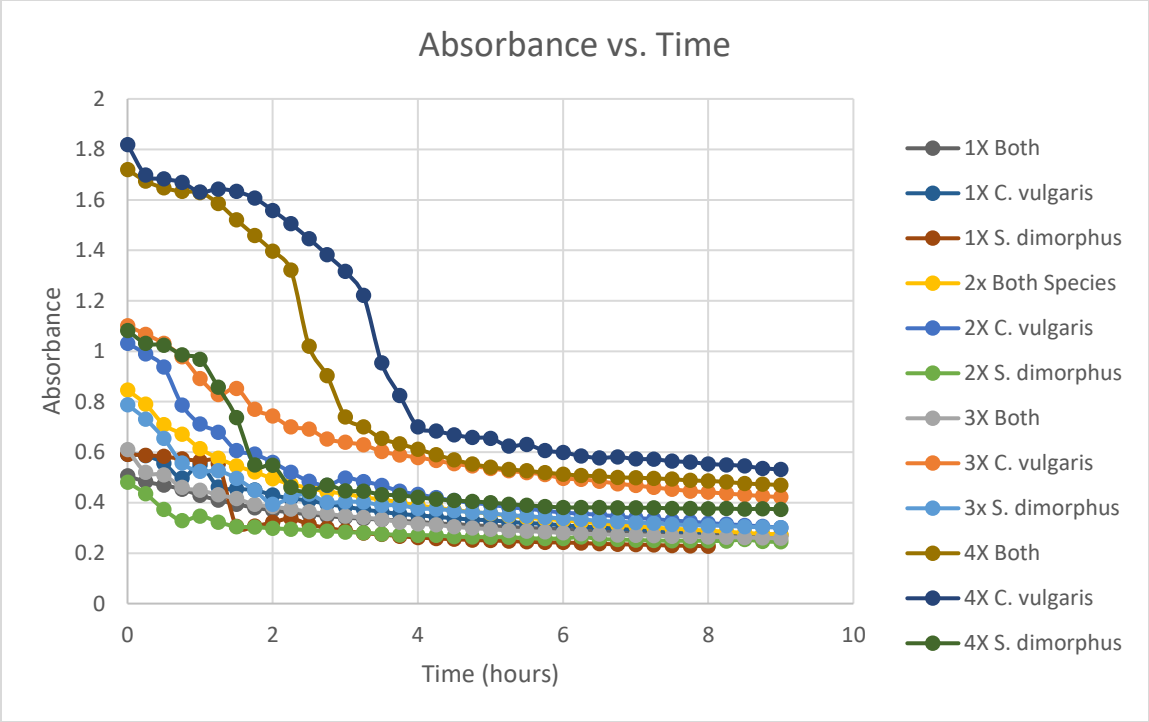

Figure 1: The absorbance value recorded over 9 hours of sedimentation for all trials.

Table 2: Recovery percentage from sedimentation for all trials.

Syn. WW. Conc.	Algal Species	30 min % Recovery	1 Hour % Recovery	4 Hour % Recovery	9 hour % Recovery
1x	Both	7.3	15.2	36.0	45.5
	C. vulgaris	5.9	7.1	40.8	52.5
	S. dimorphus	1.4	4.4	55.7	61.6
2x	Both	16.2	27.4	54.0	67.6
	C. vulgaris	9.1	30.9	58.0	70.8
	S. dimorphus	22.5	27.8	43.7	49.1
3x	Both	16.4	26.4	48.2	56.7
	C. vulgaris	6.4	19.0	47.4	59.9
	S. dimorphus	16.9	33.5	52.2	60.8
4x	Both	4.2	5.3	64.4	71.7
	C. vulgaris	7.5	10.3	61.5	69.6
	S. dimorphus	5.4	10.5	61.1	65.2

Table 3: The results of a two tailed T-tests for recovery efficiency in all trials.

Both species vs. *C. vulgaris*	P Value	Both Species vs. *S. dimorphus*	P Value	*C. vulgaris* vs. *S. dimorphus*	P value
1x	0.970	1x	0.807	1x	0.842
2x	0.962	2x	0.698	2x	0.692
3x	0.818	3x	0.783	3x	0.646
4x	0.975	4x	0.973	4x	0.944

Lipid and Chlorophyll Production

Table 4 shows the fluorescence intensity for lipid and chlorophyll in all trials. When grown together, lipids produced by the combined species fell in between the level produced by single species. The lipid intensity for 4x trial is the only trial that combined species produced less lipid or chlorophyll than those of the two single species. These values are nearly identical to those for the monoculture *C. vulgaris* and is mostly like due to minimal growth of *S. dimorphus* in that trial. S. *dimorphus* always produced the smallest amount of lipids with the highest amount of chlorophyll, while *C. vulgaris* had the highest values of lipids but lowest of chlorophyll. With the exception of *S. dimorphus* in the 2x trial, as concentration of wastewater increased, so did chlorophyll production in the algae. Lipid production did not increase with wastewater concentration as consistently as chlorophyll production. For both species and *C. vulgaris*, the lipid production does not show a trend in relation to wastewater concentration. However, *S. dimorphus* shows a consistent increase in lipid production as wastewater concentration increases. Table 5 shows that for the 3x concentration, there is a significant difference in lipid concentration between both species and *C. vulgaris* trials ($p = 0.011$). However, in all other trials for both lipid and chlorophyll values, there is no significant difference between both species and *C. vulgaris*.

Table 4: The fluorescence intensity of the chlorophyll and lipid in all trials.

Synthetic WW Conc.	Algal Species	Chlorophyll		Lipids	
		Average	Std. Dev.	Average	Std. Dev.
1X	Both Species	2001	152	1284	500
	C. vulgaris	1930	39	2130	223
	S. dimorphus	2839	229	374	32
2X	Both Species	2269	151	2197	148
	C. vulgaris	2467	94	2751	461
	S. dimorphus	1569	18	400	14
3X	Both Species	3442	172	1868	92
	C. vulgaris	2807	59	3106	55
	S. dimorphus	4446	36	516	33
4X	Both Species	3666	19	2162	285
	C. vulgaris	3771	135	2219	127
	S. dimorphus	5342	132	693	20

Table 5: P Values for Two-Tailed T Test Comparing Both Species vs. *C. vulgaris* for Chlorophyll and Lipid Production.

Synthetic WW Conc.	Chlorophyll	Lipid
1x	0.50	0.082
2x	0.14	0.204
3x	0.10	0.011
4x	0.31	0.775

Biomass Production

The concentration of total solids increased as wastewater concentration increased, with the exception of the 3x concentration (Table 6). A single factor ANOVA test was performed and a p value of 0.99 was obtained. Within each trial, there is no significant difference between the biomass concentrations of the different species within the trial.

Table 6: Total Solids Concentration for All Trials.

Synthetic WW Conc.	Algal Species	Total Solids (mg/L)
1X	Both	434
	C. vulgaris	368
	S. dimorphus	351
2x	Both	890
	C. vulgaris	922
	S. dimorphus	946
3x	Both	823
	C. vulgaris	855
	S. dimorphus	823
4x	Both	1074
	C. vulgaris	1045
	S. dimorphus	1013

Algae Flocculation

Particle size and distribution of all trials were evaluated using a Coulter Counter (Table 7). The mean and medium particle size in flasks with both species shifted to sizes between the two single algae species. The only trial that does not exhibit this trend is the 1x concentration. The multiple peaks for a given algae species in Figures 2 and 3 indicate possible

bacteria contamination, whose average cell size is smaller than that of algae. Figure 5 shows a slight shift in the peak of *C. vulgaris* to a larger particle sized peak for both species. This shows possible floc formation between both of the species forming intermediate sized particles. In Figure 4, the low particle count for both species agrees with the low OD values shown in Figure 1.

Table 7: The particle size statistics for each trial.

Syn. WW Conc.	Algal Species	Particle Count	Mean (µm)	Median (µm)	S.D. (µm)
1x	Both Species	34685	3.496	3.026	1.021
	C. vulgaris	43287	3.841	3.849	2.199
	S. dimorphus	29450	4.579	4.434	1.381
2x	Both Species	55208	4.49	4.574	1.145
	C. vulgaris	90658	4.054	3.899	1.06
	S. dimorphus	8198	6.234	6.24	2.182
3x	Both Species	24215	4.423	3.876	1.895
	C. vulgaris	106626	3.647	3.582	0.941
	S. dimorphus	33965	5.093	5.075	1.203
4x	Both Species	73738	3.918	3.666	1.25
	C. vulgaris	79160	3.701	3.385	1.291
	S. dimorphus	28737	5.187	5.234	1.408

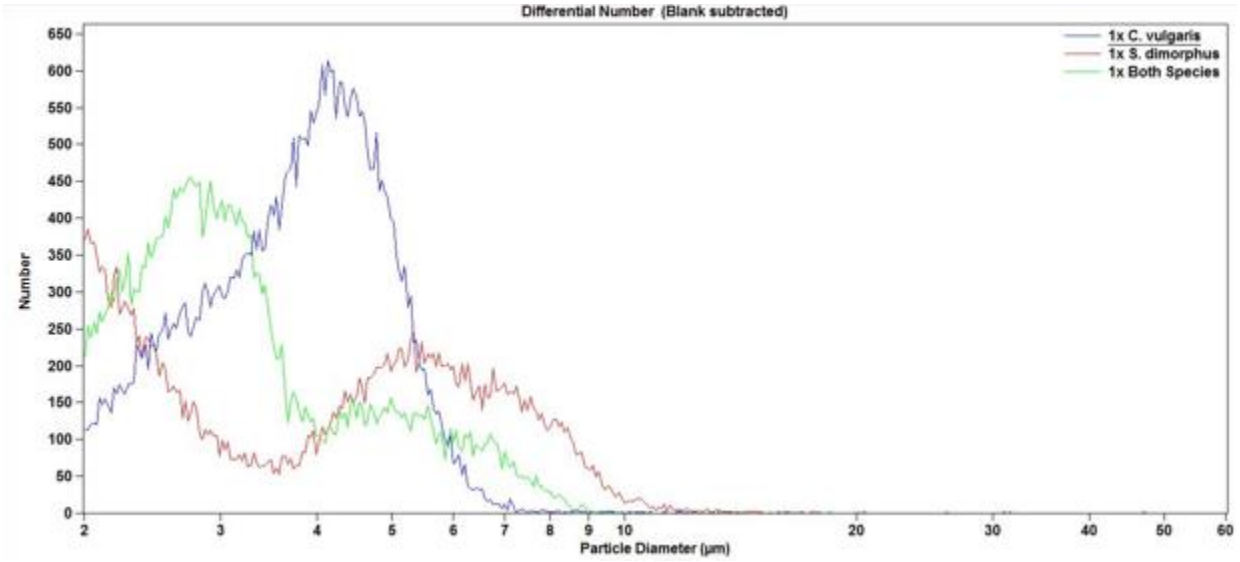

Figure 2: Particle Size and Distribution in 1x Synthetic Wastewater

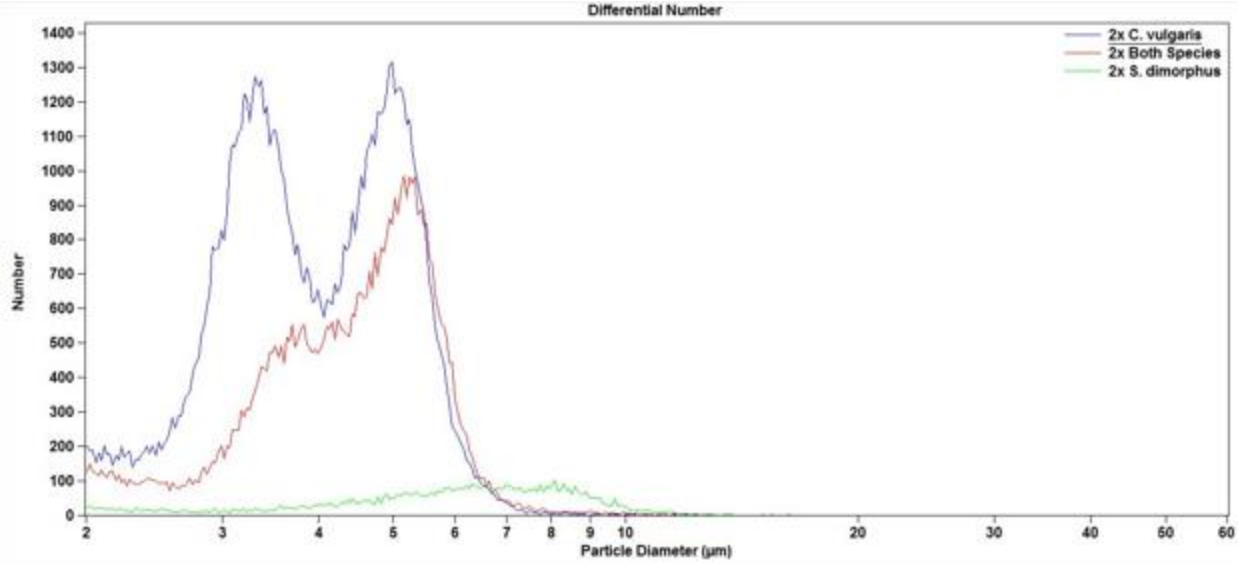

Figure 3: Particle Size and Distribution in 2x Synthetic Wastewater

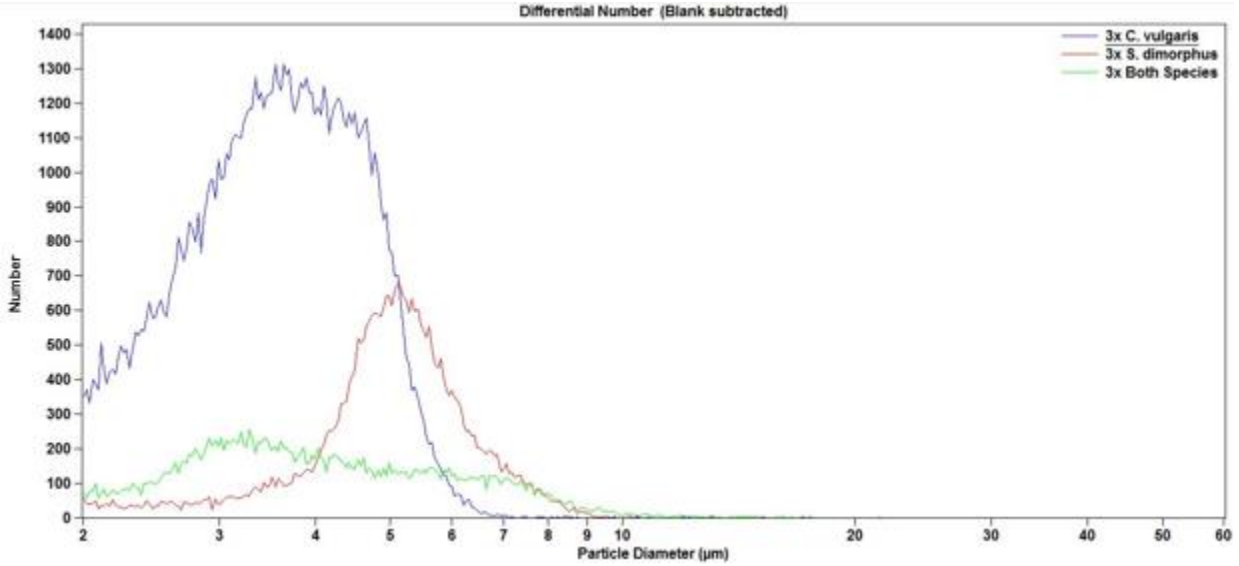

Figure 4: Particle Size and Distribution for 3x Synthetic Wastewater

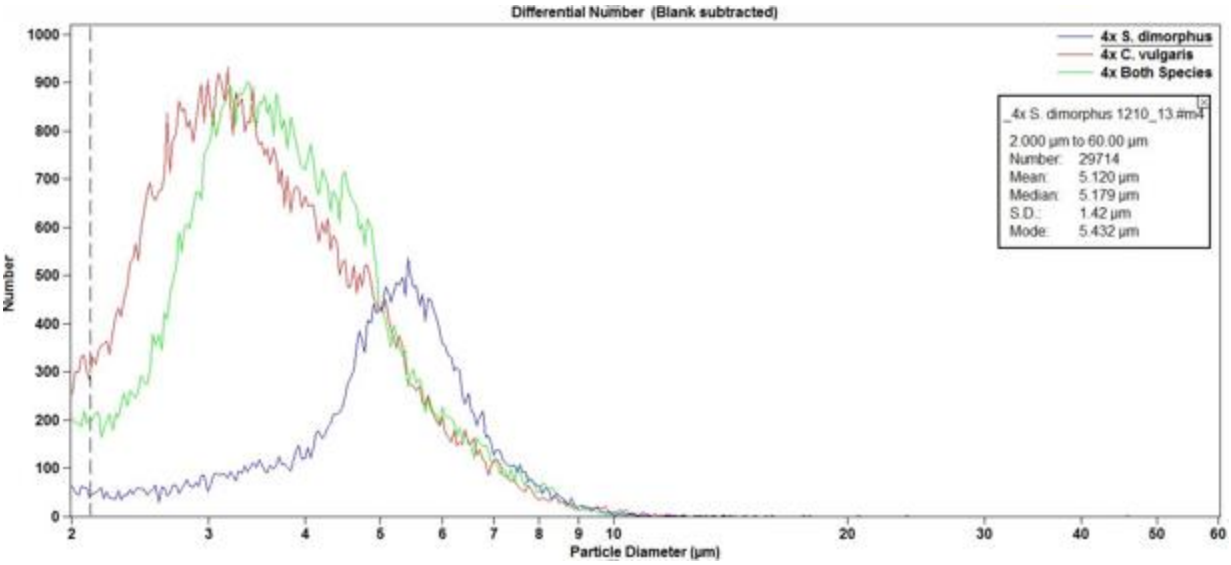

Figure 5: Particle Size and Distribution for 4x Synthetic Wastewater

Discussion and Future Opportunities

The results of this study differed from the expected. Growing the two species of algae together did not result in an increase in harvesting efficiency as was hypothesized. One possible explanation for this result is that the combination of the two species does not result in a significant shift to a larger average particle size. The coulter counter results in Table 5 show that even though the average particle size does shift to be slightly larger, this shift is not enough to improve the algae sedimentation. The particle size could also be affected by the bacteria contamination, decreasing the overall floc size. The effect of sedimentation to the clarity of the water is also visibly apparent, as figure 6 shows an example of algae-filled cuvette before and after sedimentation for 9 hours. Also, the recovery percentage of monoculture *S. dimorphus* did not differ from that of *C. vulgaris* so it is unlikely that it would increase when combining both species, unless due to an unforeseen synergistic effect.

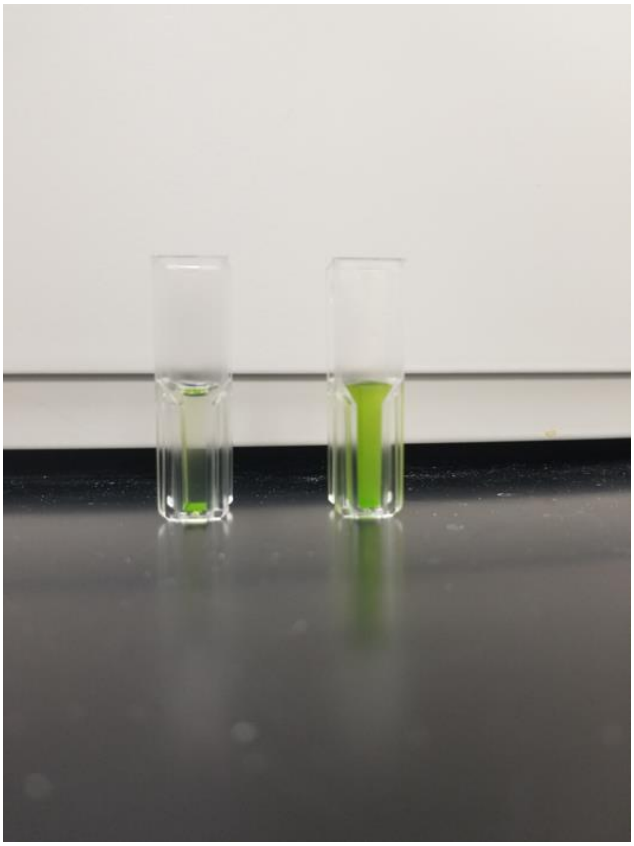

Figure 6: Sedimentation of the combined species in 4x wastewater before and after 9 hours in cuvettes.

However, this experiment does show that neither species strongly outcompetes the other in growth when both are present in synthetic wastewater. In all of the trials except the 4x synthetic wastewater, the flask with both species has chlorophyll and lipid readings approximately in the middle of values for the individual species in the same trial. For algae production in wastewater, both lipid production and sedimentation efficiency are important parameters. While the dominance of C. vulgaris can promote a higher lipid production, the growth of auto-flocculation species will help ensure the sedimentation of both algal cells. As a result, it is essential to identify an auto-flocculation species that also has high lipid yields.

The results of this experiment show as concentration of wastewater increases, there is lower return on biomass yield. If the final goal is to produce as much algal biomass per nutrients in the wastewater, it might be beneficial to dilute the concentration of wastewater. A possible explanation for this is as the biomass increases in the flasks, the growth becomes so dense that light cannot transmit effectively, limiting the ability of the algae to utilize those resources. However, at each wastewater concentration, the three species (single and combination) reach approximately the same level of biomass concentration.

Comparing to similar experiments, the initial sedimentation rate is similar or slightly greater than the previously found values for *C. vulgaris* combined with *A. falcatus* (10.4% recovery/hr) and *S. obliquus* (18.7% recovery/hr) (Salim, et al., 2011). However, the initial sedimentation of *C. vulgaris* alone found in this experiment varies greatly from the 13.6 % recovery/hr and 20.4 % recovery/hr found in the same article. Using the 30 minute recovery, this experiment found initial sedimentation of 11.8%/hr, 18.24%/hr, 12.72%/hr, and 14.95%/hr for the 1x, 2x, 3x, and 4x concentrations respectively.

According to Manheim and Nelson, the growth stage of *C. vulgaris and Scenedesmus* has a great effect on the settling of these microalgae, with *C. vulgaris* settling better in early growth stages but *Scenedesmus* species settling better in early and late growth stages. The conditions used in this experiment were comparable to the late growth stages used by Manheim and Nelson. It is possible that higher recovery efficiencies could be achieved by allowing sedimentation to happen in the early growth phase for both species. This would reduce the amount of nutrients removed, reducing its impact in wastewater treatment.

It has also been shown that certain species of bacteria can help to increase the flocculation of *C. vulgaris. Flavobacterium, Terrimonas, and Sphingobacterium* have been

shown to play a crucial role in forming flocs to increase the recovery of *C. vulgaris* (Lee, et al., 2013). It is possible that the introduction of these bacterial species could increase the recovery efficiencies found in this experiment by forming a basis to easily coagulate the two species together.

Though the results were unexpected, there are still a variety of future projects that can benefit from this experiment. They include investigating the effectiveness of the algae to treat the wastewater as well as including more replicates to ensure the results of this experiment. Though the auto-flocculation with *S. dimorphus* did not yield expected results, other species of microorganisms could be tested for the auto-flocculation efficacy.

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