A Cultured Revolution: Growing Algae in Wastewater for Ethanol Production

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Abstract

Bioethanol produced from algae may provide a sustainable alternative to petroleum and conventional biofuels. Algae present a superior option because they require minimal inputs and can be grown in non-potable water. Our study evaluated the potential of municipal wastewater as a growth medium for three local algal species and their ability to produce bioethanol. We grew *Penium margaritaceum, Scenedesmus qudricauda,* and *Spirogyra communis* in six different media treatments with varying concentrations of wastewater. After the growth period, we quantified the total production of biomass per treatment and produced ethanol from each species. We found that *S. quadricauda* and *P. margarticaeum* were most productive in the 25 and 50% wastewater treatments, respectively. *S. quadricauda* produced twice the amount of ethanol per gram of dry weight as *P. margaritaceum. S. communis* was not analyzed because of foreign algal contamination. The high nutrient content of wastewater promoted algal growth in lower concentrations, but had inhibitory effects in higher concentrations due to a number of factors. Culturing methodologies need to be optimized before large-scale integration with wastewater treatment is feasible.

Introduction

It is necessary to find renewable alternatives to fossil fuels that can fulfill the same roles while significantly reducing greenhouse gas emissions. Current renewable energy technology focuses predominantly on the generation of electricity, which accounts for less than one third of global energy consumption (Stephens *et al.* 2010). The remaining portion of energy production is derived from fossil fuels, creating an unsustainable reliance that contributes to global climate change. The development of liquid biofuels that are renewable and more efficient may provide a successful alternative to conventional petroleum-derived fuels (Demirbas 2011). Recently, the use and distribution of first generation biofuels—fuels produced from crop plants— has grown more prominent within the transport sector (Brennan and Owende 2010). However, biofuels produced from crop plants, such as corn and sugarcane, prove both inefficient and unfeasible for a number of reasons (Chisti 2008). A new generation of biofuels derived from algae can avoid these issues while providing a sustainable source of fuel.

The major constraint of the first generation biofuels industry is competition with the agricultural sector (Brennan and Owende 2010). As global population continues to increase, the

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demand for food will become more pressing, creating conflict over the use of arable land. If food crops are prioritized, biofuels will not have a significant impact on energy production. However, if biofuel crops are produced on a large scale, food crops will be displaced and global food security will be negatively impacted (Stephens *et al.* 2010). Biofuels derived from crop plants are also unsustainable because of the intensive agricultural inputs needed to support them (Brennan and Owende 2010). Further, familiar environmental factors such as drought and seasonal fluctuation make production inconsistent and unreliable (Demirbas 2011). Production of biofuels from algae presents a sustainable alternative to both first generation biofuels and fossil fuels (John *et al.* 2010).

The use of algae for biofuels is advantageous because they are easily cultivated, more productive and have a lower environmental impact than conventional fuels. Algal cultivation requires few inputs: a liquid medium, nutrients, CO_2 and a source of sunlight (Stephens *et al.* 2010). Because of these requirements, algae need not compete with agriculture for arable land (Demirbas 2011). Algae have a continuous growth cycle and can be cultivated in non-potable water, providing consistent production without using freshwater resources (Brennan and Owende 2010). Algae are also capable of producing higher yields in shorter time periods than conventional crops, with certain species capable of doubling their biomass over a period of 24 hours (Chisti 2008). Bioethanol is readily biodegradable and produces less air pollution after combustion than petroleum fuel (John *et al.* 2011). Furthermore, algae fix CO_2 through photosynthesis and can serve as a carbon sink, reducing atmospheric CO_2 levels (Brennan and Owende 2010).

Another benefit of algal production is that it can be integrated into the wastewater treatment process, enhancing sustainability and creating value added byproducts. The ability of

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algae to assimilate nutrients, specifically nitrogen (N), phosphorus (P) and some metals, creates the potential for use in the nutrient removal stage of wastewater treatment. Laboratory studies of sewage and urban wastewater treatment using the species *Scenedesmus quadricauda*, *Botryococcus braunii*, and *Chlorella vulgaris* show significant rates of N and P removal (Martinez *et al.* 2000; Sawayama *et al.* 1995; Lau *et al.* 1995) This could replace conventional removal methods like diffused aeration, reducing energy input while yielding algal biomass (Pittman *et al.* 2011).

Bioethanol is produced through the saccharification and fermentation of carbohydrates and sugars (John *et al.* 2011). Carbohydrates and sugars found in the cell material of algae are broken down enzymatically in solution. This sugar-rich supernatant is then fermented by aerobic organisms that produce ethanol (Brennan and Owende 2010). Research has indicated that several algal species in the genera *Chlorella*, *Scenedesmus* and *Spirogyra* have high carbohydrate contents amenable to bioethanol production (John *et al.* 2011; Eshaq *et al.* 2011).

The use of local resources for fuel production can decrease external inputs, making the process simpler and more efficient (Demirbas 2011). Identification of viable native algal species can localize bioethanol production and improve its sustainability (Odlare *et al.* 2011) Coupling the use of indigenous algal species with local wastewater as a growth medium can further enhance sustainable production (Pittman *et al.* 2011). Local cultivation could also harness energy from otherwise problematic annual algal blooms and turn it into a source of renewable fuels (Odlare *et al.* 2011).

Our study examined the use of municipal wastewater as a medium for culturing three algae local to the Saratoga Watershed. We predicted that as the concentration of wastewater increased, total algal growth would also increase. Our second goal was to evaluate the potential

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of each species to produce ethanol. We predicted that ethanol production would vary based on cell physiology. Our findings provide valuable data on the use of wastewater as a growth medium for native species of algae. If wastewater can support significant algal growth, an abundant waste material can be turned into a source of local energy.

Methods

We chose three charophycean green algae species native to the Adirondack Region: *Penium margaritaceum, Scenedesmus quadricauda,* and *Spirogyra communis* (North American Diatom Ecology Database 1996, Figure 1, Appendix A). Charophycean green algae are single-celled organisms with a high fraction of carbohydrates in their cell structure, making them suitable for ethanol production. We obtained algal cultures from Carolina Biological Supply Company and the Skidmore College Biology Department. We obtained anaerobic digestate effluent ("wastewater") from the belt press of the Gloversville-Johnstown Joint Wastewater Treatment Facility in Fulton County, New York, on 2/15/12 and stored it at 4°C until used.

Four Methods for Wastewater Filtration and Sterilization

1. We used commercial pure diatomaceous earth powder and cotton balls placed above a series of coffee filters inside of a 2" PVC pipe 5' in length. This technique decreased wastewater turbidity from 301 nephlometric turbidity units (NTU) to 141 NTU (Hach 2100P Turbidimeter). Upon further examination we found that diatomaceous earth had passed into the filtered wastewater. We considered the turbidity level after filtration to be too high and pursued the alternative methods described below.

2. We used the same PVC pipe set-up with coffee filters and cotton balls without

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diatomaceous earth. This resulted in filtered wastewater with a turbidity reading of 48 NTU. We still considered this turbidity level to be too high and abandoned the method.

3. We attempted to sterilize the wastewater through a heat pasteurization process. We found that heating the wastewater transformed it to a highly turbid state that could not be corrected through cooling. We suggest that applying heat caused an inert polymer (Slack Chemical, Stayfloc 6532), added to the wastewater in the municipal treatment process as a thickening agent (Bevington 2012), to become activated and turn the solution cloudy. We abandoned this method.

4. We used a 0.3 micron water filter (Katadyn, Hiker model) made for recreational camping. This filter sterilized the water and reduced turbidity to a level of 4 NTU, which we considered acceptable for culturing. We used this method to support our experimental work.

Algal Set-Up

We cultured each species separately in six different treatments: five varying concentrations of wastewater and deionized water- 25% WW, 50% WW, 75% WW, and 100% WW. The sixth treatment used Woods Hole Standard, a commercial growth media, as a reference. Wastewater was composed of almost three times the amount of Total Nitrogen (TN) and twice the amount of Total Phosphorous (TP) as Woods Hole Standard growth media (Table 1). Deionized water demonstrated low nutritional value across the board (Table 1).

Table 1. Nutrient analysis of total phosphorous, total nitrogen and pH of deionized water, wastewater and Woods Hole media

Growth Medium	TP (mg/l)	TN (mg/l)	pH
DI H2O	0.564	0.056	6.23
Wastewater	1.572	112.616	7.29
Woods Hole	0.867	38.612	7.13

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Analysis of heavy metals showed no detectable levels of zinc, lead or copper in deionized water. Wastewater had about twice the amount of zinc as Woods Hole Standard (WW Zn=0.061 mg/l, WHS Zn=0.03 mg/l, Table 2, Appendix C) and both had similar amounts of lead and copper (Table 2, Appendix C).

We cultured the algae in 1L sterile intravenous bags (Kangaroo Pouch, Sherwood Medical) hung in rows from a PVC structure. Each bag contained 450mL of growth solution and 50mL of algal base culture for a total volume of 500mL. We maintained the bags under continuous light (Sunlight Supply Inc., Sun Blaze TH50-48, 54W, 6500°K) for 14 days at a temperature of 24°C. We ran two trials of each treatment. During the first trial we let the bags sit for one week without agitation; during the second week we manually agitated each bag daily through the end of the trial period. During the second trial we manually agitated each bag once daily for the entire 14-day growth period.

Growth Analysis

At the end of the growth period we centrifuged the contents of each bag and froze the pure algal biomass at -80°F. We then freeze-dried the biomass (Labconco FreeZone 4.5) to remove all moisture and measured the final dry weight. All wastewater treatments of *S. communis* were contaminated with a foreign algal species across trials, hence we could not accurately analyze or describe the contributions of the species. Specifically, we could not evaluate the isolated biomass of *S. communis* and therefore discontinued its use in the experiment.

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After the growth period, we centrifuged each centrifuged each culture and froze the pure algal biomass in a -80°F freezer. We then thawed the biomass in a freeze-dryer and recorded the total weight of each species.

Carbohydrate Assay

We created 5 standard glucose dilutions (20ug/ml, 50ug/ml, 80ug/ml, 110ug/ml, and 140ug/ml of glucose) according to the methods of Hellebust and Craigie (1978) and analyzed them with a spectrophotometer at 485nm (Spectronic Instruments, Spectronic 401). We measured out 50ug/ml of *S. quadricauda* and *P. margaritaceum*, under the same protocol as the standards (Hellebust and Craigie 1978) and analyzed them with the spectrophotometer. We plotted the corresponding absorbencies on the standard curve to estimate the glucose concentration of each sample (Figure 2, Appendix B). We used this information to estimate the available fermentable sugars in each species.

Ethanol Production and Analysis

We re-suspended the dry mass of each species in five different water dilutions: 0.05%, 0.1%, 0.2%, 0.5%, and 1% algal biomass. We added 0.01g/100ml of the enzymes pectinase, macerase, and cellulysin to each dilution and incubated them overnight at 36°C in order to degrade the cell wall. We added 1mg of a yeast fermenting agent (Fleishmann's ActiveDry Yeast) to each dilution, fastened a lid for air tight conditions and let them ferment for 48 hours. We measured final ethanol production using an Agilent Technology GC 7986 gas chromatograph with a HP-5 column and FID detector (Inlet: 250 °C, Detector: 200 °C, Flow rate: 3.0 mL/min, Split ratio: 25:1). We held the column temperature at 60 °C for 2 minutes and increased to 110 °C at a rate of 20 °/min. We used these data to make a standard curve to compare the ethanol production per gram for both *P. margaritaceum* and *S. quadricauda* from individual treatments (Figures 3 and 4, Appendix A).

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Results

Spirogyra communis Contamination

Spirogyra communis cultures were contaminated by a foreign algal species in both trials and could not be used in our analysis. After the 11th day of growth we observed abnormal coloration and cell suspension characteristics in several of the *S. communis* growth bags. Upon further microscopic inspection we confirmed the presence of a small spheroid algal species, possibly of the genus *Chlorella* (Figure 2, Appendix A). *S. communis* treatments that contained wastewater showed signs of contamination, while the control the deionized water and standard media treatments did not. None of the *S. quadricauda* or *P. margaritaceum* cultures showed indications of contamination. The presence of contamination in all *S. communis* treatments with wastewater suggests algal contamination via wastewater.

Growth Analysis

Scenedesmus quadricauda produced the largest amount of biomass when grown in the 50% wastewater treatment (0.175g/500ml, Figure 1). The second most productive treatment was cultured in 25% wastewater (0.1343g/500ml, Figure 1). We used the total mass of *S. quadricauda* grown in 100% Woods Hole Standard (0.0758g/500ml) as a reference point of growth. In comparison to the wastewater treatments, the culture grown in Woods Hole Standard was only more productive than the 100% deionized water treatment (Figure 1). The culture grown in 100% deionized water demonstrated the lowest growth with a final dry weight of 0.0137 g/500ml (Figure 1).

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Fig 1. Dry weight (g) of *S. quadricauda* by treatment. The horizontal line depicts the total dry mass of *S. quadricauda* when grown in standard Woods Hole Medium and serves as a reference point.

Penium margaritaceum produced the largest amount of biomass when grown in the 25% wastewater treatment (0.0986g/500ml, Figure 2). The second most productive treatment was 50% wastewater, which produced only slightly less biomass (0.0924g/500ml, Figure 2). The smallest amount of biomass was produced in the 100% deionized water treatment (0.0159g/500ml, Figure 2). Again, we cultured *P. margaritaceum* in 100% Woods Hole Standard and used this treatment as a reference point for the five other treatments. Cultures grown in the Woods Hole Standard treatment (0.0916g/500ml, Figure 2) were less productive than the 25% and 50% wastewater treatments.

We found that the *P. margaritaceum* cultures grown in 75% and 100% wastewater treatments produced large amounts of dry weight (100% wastewater = 0.0546 g/500ml, 75% wastewater = 0.0452g/500ml, Figure 7). However, when examined qualitatively these treatments showed little sign of algal growth. They were pink in color and when centrifuged formed pellets

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of red and white matter. This matter may account for some of the recorded biomass of the 75% and 100% *P. margaritceum* treatments. The 25% and 50% wastewater treatments did not have evidence of this material, and this material was not found in any cultures of *S. quadricauda*.

Fig 2. Dry weight (g) of *P. margaritaceum* by treatment. The horizontal line depicts the total dry mass of *P. margaritaceum* when grown in standard Woods Hole Medium and serves as a reference point.

In inter-specific comparisons, *S. quadricauda* demonstrated greater biomass production than *P. margaritaceum* across all treatments except for 100% Woods Hole Standard and 100% deionized water (Figure 2). Although both *S. quadricuada* and *P. margaritceum* produced the most biomass when cultured in 50% and 25% wastewater treatements, final total biomass for *S. quadricuada* was more than twice that of *P. margaritaceum* in 50% wastewater and about 1.5X the biomass of the 25% treatment. (Figure 2).

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Fig 3. Comparison by species of total dry weight (g) per treatment.

Nutrient Analysis

The post-growth analyses of media composition demonstrated that there was limited decrease in nutrients over the growth period. Starting total phosphorous (TP) in the 25% wastewater treatment of *P. margaritaceum* was 0.816 mg/l (Table 2, Appendix C). After the growth period, TP in the supernatant of the same treatment increased by half its original volume (TP after=1.262, Table 2, Appendix B). TP of the 50% wastewater treatment of *S. quadricauda* before algal growth was 1.07 mg/l (Table 2, Appendix C). After the growth period TP in the supernatant of the same treatment had increased by 75% its original volume (TP after= 1.77 mg/l, Table 2). Total nitrogen (TN) in the 25% wastewater treatment for *P. margaritaceum* was 28.20 mg/l before growth. After growth TN levels had decreased by 39% its original volume (TN after= 17.305 mg/l, Table 2, Appendix C). TN levels in the 50% wastewater treatment of *S. quadricauda* only decreased by 0.16mg/l over the 14-day growth period (Table 2, Appendix C).

The supernatant of *P. margaritaceum* grown in 25% WW demonstrated increases of at

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least 50% in Zn, Pb, and Cu over the 14 day growth period (Table 3, Appendix C). Final concentrations of heavy metals in the supernatant of *P. margaritaceum* in the 25% WW treatment never exceeded 0.241 mg/l and the metal of the highest concentration was lead (Table 3, Appendix C). The supernatant of *S. quadricauda* grown in 50% WW demonstrated increases of at least 50% in Pb, and Cu over the 14-day growth period (Table 3, Appendix C). However, the concentration of Zn in solution showed little change over the same period (Zn in solution before= 0.31 mg/l, Zn in solution after= 0.039 mg/l, Table 3, Appendix C). Concentrations of heavy metals in solution never exceeded 0.231 mg/l.

Carbohydrate Analysis

We found that *P. margaritaceum* produced almost twice as much glucose per unit biomass (100ug/ml) as *S. quadricauda* (48.2 and 24.5 ug/ml, respectively). Therefore, *P. margaritaceum* has a higher cellular composition of carbohydrates than *S. quadricauda* (Figure 4, Appendix B).

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Ethanol Production

Fig 4. Comparison of ethanol produced per gram of dry weight in *P. margaritaceum* and *S. quadricuada*.

Scenedesmus quadricauda produced twice as much ethanol (g/ml) per gram of biomass as *P. margaritaceum* (Figure 4) Based on these numbers, we expect that the 25% wastewater treatment of *S. quadricuada* would produce the highest amount of ethanol of all treatments, projected at .21 ug/ml of ethanol. Furthermore, based on the total biomass measured per treatment, we expect that *S. quadricuada* would produce more ethanol than *P. margaritaceum* in every treatment (Figure 5). Even though *P. margaritaceum* produced a greater total biomass than *S. quadricuada* in 100% WHS and 100% deionized water treatments, we expect *S. quadricuada* to produce up to 4X the amount of ethanol per gram based on our standard curve (Figure 11). In treatments where total biomass is similar between species, such as 25% wastewater and 100% wastewater, we expect *S. quadricuada* would produce at least 3X the amount of ethanol per gram (Figure 5).

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Fig 5. Comparison of ethanol produced per gram of algal dry weight between *S. quadricuada* and *P. margaritaceum*.

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Discussion

Algal Growth

Penium margaritaceum and *Scenedesmus quadricauda* demonstrated similar growth patterns based on percentage of wastewater in solution. For both species the 25% and 50% wastewater treatments demonstrated the highest growth rates while 100% deionized water and 75% wastewater were the least productive. Originally we predicted that as the concentration of wastewater increased, the productivity of each treatment would also increase, however, we did not see this in our 75 and 100% wastewater treatments. This suggests that the high nutrient content of wastewater promoted algal growth in lower concentrations, but had inhibitory effects in higher concentrations due to a number of factors.

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Effects of Wastewater

The Gloversville-Johnstown Joint Wastewater Treatment Facility receives waste from a variety of users, which creates wastewater with variable physical, chemical, and biological characteristics. Due to the large input of organic waste, municipal wastewater often contains high levels of particulate organic matter (CITE). These particles are rich in nutrients needed for algal growth such as nitrogen and phosphorus and promote productivity (Wang 2009). Our media composition analysis shows that wastewater had almost three times the amount of TN and almost twice the TP as the Woods Hole Standard media. This suggests that the wastewater provided supplemental nutrients to algae grown in wastewater treatments. Algae grown treatments with lower concentrations of wastewater, such as our 25% and 50% wastewater treatments, were able to maximize the benefits of the added nutrients without being subjected to the negative impacts that may come with higher concentrations of wastewater. Although higher wastewater concentrations may have provided more nutrients, they also contributed to increased turbidity and possible bacterial contamination. Further, high wastewater concentrations may have provided nutrients in excess of the amounts required by the growing algae. Therefore, our findings that algae grown in 25% and 50% wastewater treatments produce the most biomass do not support our initial hypothesis that algal growth would increase with wastewater concentration.

Because of its high nitrogen and phosphorous composition, wastewater must undergo an extensive nutrient removal process before it is discharged into natural water bodies. Currently, the majority of nutrient removal in the wastewater treatment process is done by aerobic microorganisms (Pittman *et al.* 2011). However, algae can serve as both a nutrient sink and

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bioethanol feedstock, making them an attractive alternative to aerobes. As algae grow they uptake nutrients such as nitrogen and phosphorous from solution and assimilate them into their cellular structure. Therefore, if grown and harvested in a contained environment, algae can be used as an active source of nutrient removal as well as a feedstock for ethanol production.

Because of algae's nutrient removal potential, we expected to find decreased levels of nitrogen and phosphorus in solution at the end of the 14-day growth period. However, in our most productive treatments we only found a substantial decrease in TN for *P. margaritaceum* grown in 25% wastewater, and a marginal decrease for *S. quadricauda* in 50% wastewater. Both species demonstrated increased levels of total phosphorous after the growth period. We suggest that *S. quadricauda* was absorbing and retaining nitrogen, but it was senescing at a rate equal to its growth. Therefore, the nitrogen assimilated into the cell structures was returned to solution at the same rate as it was being absorbed and thus did not change the total nitrogen levels. Further, we suggest that *P. margaritaceum* may have been senescing less rapidly due to slower growth rates and was able to store nutrients in live cells for longer periods. Because phosphorous is not a limiting nutrient it may have been released into solution via senescence faster than it was assimilated by each species.

Certain concentrations of heavy metals such as copper, lead and zinc can be toxic to algae. These metals can inhibit growth, extend the lag phase of cultures, and disrupt necessary cell processes including photosynthesis, respiration, and cell division (Franklin 2000). However, our metals analysis shows that the most productive treatments of *S. quadricauda* and *P. margaritaceum* had higher amounts of Cu, Pb and Zn in their supernatant than 100 % wastewater. Because these treatments were still highly productive in the presence of these metals, we suggest that the metal concentration did not reach a threshold sufficient to induce

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metal toxicity. We were unable to measure the quantity of metals in other treatments because of financial limitations, so we cannot draw conclusions as to the kinetics of metals accumulation based on wastewater concentration. It is possible that treatments with more wastewater accumulated higher concentrations of metals and were thus affected by toxicity, however we do not have the data to support this. Thus, we conclude that treatments with more wastewater were not limited by increased levels of heavy metals, but by some other factor such as bacterial contamination or turbidity.

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Unfiltered wastewater is often turbid because of the high levels of suspended particulate matter in solution. High levels of turbidity significantly decrease the distance at which light can penetrate through a solution. This light-inhibition is detrimental to the growth of organisms that need sunlight to photosynthesize and produce sugars. A study on turbidity in the Illinois River demonstrated that native autotrophic algal species became less productive as turbidity levels increased (Wang 2009). Although turbidity can be decreased via filtration, particles smaller than 0.3 microns are often unavoidable in very turbid solutions. The wastewater used in our experiment also contained a polymer that gels with increased temperatures (Bevington 2012). We suggest that treatments with high concentrations of wastewater may have contained higher concentrations of this polymer and thus had higher levels of turbidity. This turbidity may have caused the productivity of our 100% and 75% wastewater treatments to decline due to increased light inhibition.

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Spirogyra + Chlorella Contamination

The *S. communis* wastewater treatments showed a pattern of contamination with *Chlorella sp.* consistent between both trials. We conclude that the 100% standard media and

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100% deionized water treatments did not become contaminated because they did not contain any wastewater. Because of this contamination, we were unable to obtain accurate growth data for *S. communis* could not evaluate wastewater growth trends.

Previous bio-prospecting research has shown that several *Chlorella* species are commonly found in wastewater and show productive growth rates (Zhou *et al*. 2011). Our results confirm these findings and suggest that *S. communis* may have been out competed by a *Chlorella* species present in the wastewater treatments. This out competition may be due to the fact that *S. communis* is a relatively large filamentous alga with slower growth rates than the small spheroid *Chlorella* species. Alternatively, the *Chlorella* species may have been better suited to the nutrient-rich environment of the wastewater and less affected by turbidity and heavy metals. Regardless, the growth of *Chlorella sp.* in our trials indicates that it is a potentially viable species for algal production in this wastewater and should be evaluated further.

Ethanol Production

Our data demonstrate that the morphology of both *S. quadricauda* and *P. margaritaceum* may influence their ability to be fermented and produce ethanol. Our results show that *S. quadricauda* produce more ethanol per gram of dry weight then *P. margaritaceum*. Surprisingly however, we found that *P. margaritaceum* actually had a sugar composition twice as large as *S. quadricauda*. This suggests that although *S. quadricauda* has a lower overall sugar composition, the sugars it does have may be more accessible for fermentation in some way. Therefore, after the cell wall is degraded, *S. quadricauda* still provides greater availability of sugars to yeast cells than *P. margaritaceum*.

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We used a series of cellulase enzymes to break down the cell walls of each species. The success of these enzymes to degrade the cell wall is dependent upon the molecular structure of the cell. Previous studies have shown that *Scenedesmus obliquus* has a difficult cell wall to break down, but this can be remedied through a short pre-treatment (Miranda *et al*. 2012). There is little research on *P. margaritaceum* as a source of ethanol. However, the species has a high pectin composition and intricate lattice structure within its cell wall that make it difficult to disrupt (Domozych *et al.* 2006; 2007). We infer that the structure of *S. quadricauda* cell walls may be easier to degrade than those of *P. margaritaceum* and therefore provide more access to fermentable sugars.

The physiology of each algal species may also affect its ability to be fermented. *S. quadricauda* is a small spheroid organism only about ⅛ the volume of *P. margaritaceum*. *P.margaritaceum* on the other hand, is much larger and cylindrical (Domozych 2007). Because of its size and shape *S. quadricauda* provides more surface area per gram of weight than *P. margaritaceum*. We suggest that these physical characteristics allow *S. quadricauda* cells to be degraded more rapidly than *P. margaritaceum* when pretreated using an identical technique. The sugars within the cell structure of *P. margaritaceum* might be equally accessible if treated with higher concentrations of degrading enzymes or left to incubate for longer (Miranda *et al.* 2012).

Sources of Error

There are six methodological aspects of our study that may have affected our results. First, the experimental conditions in which the algae were grown may have been flawed or inconsistent between trials. In the first trial we did not agitate the bags daily, allowing algal cells to settle to the bottom of the culture, reducing their light access and productivity. Second,

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our 24-hour photoperiod may have negatively impacted overall production, as it has been shown that most algae benefit from a dark period (Lee and Lee 2001). Third, the algae were grown in airtight bags without supplemental inputs of CO_2 and may have been limited by $C0_2$ availability (Brennan & Owende 2010). Fourth, the inability to properly sterilize cultures made it difficult to prevent contamination. We had to abandon all data from *S. communis* trials because of contamination by a foreign algal species and 75% and 100% *P. margaritaceum* treatments demonstrated possible bacterial infection. Fifth, we created standard curves of ethanol with biomass cultured in standard growth media, rather than fermenting biomass from each experimental culture. This means that our ethanol production data assumes there is no difference in ethanol yield between standard media and wastewater-grown algae, which may not be the case. Sixth, the sample size was not large enough to make statistically robust comparisons.

Local Production & Integration

Our results show that some algal species native to the Saratoga Watershed can be successfully cultured in wastewater and processed into bioethanol. However, there are several issues that need to be addressed before this can be integrated into the wastewater treatment process on a large scale. Filtration is difficult and costly because the high amounts of suspended particles clog filter membranes easily. However, wastewater needs some form of particle removal or it will be too turbid to promote successful algal growth. Sterilization of wastewater in large volumes through heat pasteurization or UV radiation would be energy intensive and possibly precluded by polymer additives. This means that a realistic large-scale application would have to exclude filtration and sterilization, making it susceptible to turbidity and various forms of biological infection. Lastly, full-scale integration would require the treatment of large

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volumes of wastewater, which would most likely occur in outdoor algal ponds (Christensen and Sims 2011). This means that algal growth would be impacted by daily sunlight exposure and ambient temperature, factors that would be limiting in the winter months in upstate New York. This could potentially be remedied by greenhouse cover structures, but this would be another added cost to production.

Given these considerations, the most realistic approach to integration would be to co-locate a small bioethanol facility with an existing wastewater treatment plant. The constant, on-site availability of an effective wastewater growth medium would simplify the production process and eliminate the need for commercial growth media. Reductions in turbidity could be achieved simply and without energy input in settling tanks. Small volumes of this wastewater could then be diverted for algal growth in indoor photobioreactors or greenhouse-covered ponds. The resulting biomass could then be converted to bioethanol for use in facility vehicles, eliminating fuel costs. The algal waste residue from the ethanol production process has also been shown to be a suitable feedstock for anaerobic digestion (Park et al. 2012), which generates methane-rich biogas. If located with a treatment plant that uses anaerobic digestion, waste from the ethanol production process could be used to generate heat and electricity. This could be integrated to power various phases of production such as biomass drying, ethanol distillation, and supplemental lighting. This capacity to recover energy from waste residue would help to create a sustainable and efficient closed-loop algal bioethanol production system.

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Appendix A: Images

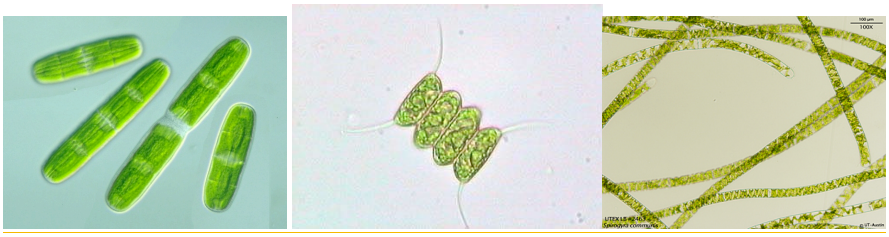

Fig 1. Left to right: *Penium margaritaceum*, *Scenedesmus quadricauda,* and *Spirogyra communis*

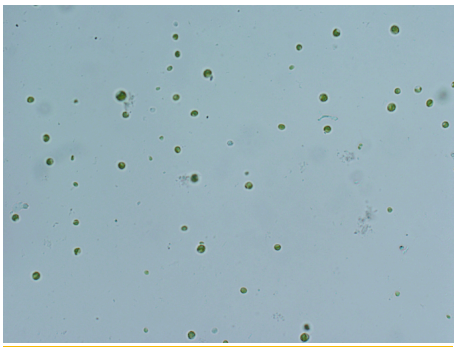

Fig 2. Foreign algal species, possibly *Chlorella sp.*

Appendix B: Standard Curves and Carbohydrate Analysis

Fig 1. Standard curve of glucose concentration read at 485nm.

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Fig 2. Percent ethanol produced per % grams of *S. quadricauda*/ml as analyzed with the GC-Mass Spec.

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Fig 3. Percent ethanol produced per % grams of *P.margaritaceum*/ml as analyzed with the GC-Mass Spec.

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Fig 4. Carbohydrate composition of each species when started with 100ug/ml of dried biomass.

Appendix C: Media Composition Analysis

Table 1. Nutrient analysis of total phosphorous, total nitrogen and pH of deionized water, wastewater and Woods Hole media.

Treatment	TP (mg/l)	TN (mg/l)	pH
DI H2O	0.564	0.056	6.23
Wastewater	1.572	112.616	7.29
Woods Hole	0.867	38.612	7.13

Table 2. Nutrient analysis of total phosphorous and total nitrogen of the supernatant of the most productive algal treatment per species before and after the growth period.

Treatment	TP (mg/l)	TN (mg/l)
P. margaritaceum 25% WW Before	0.816	28.196
P. margaritaceum 25% WW After	1.262	17.305
S. quadriquada 50% WW Before	1.06755	56.336
S. quadriquada 50% WW After	1.766	56.178

Table 3. Heavy metals analysis of Zinc, Lead, and Copper of the supernatant of the most productive algal treatment per species before and after the growth period.

Treatment	Zn (mg/l)	Pb (mg/l)	Cu (mg/l)
P. margaritaceum 25% WW Before	0.01525	0.04225	0.04
P. margaritaceum 25% WW After	0.034	0.241	0.233

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S. quadriquada 50% WW Before	0.0305	0.1205	0.08	BD= Below Detection
S. quadriquada 50% WW After	0.039	0.231	0.192	
Wastewater	0.061	0.169	0.16	
DiH2O	BD	BD	BD	
Woods Hole (WHS)	0.03	0.165	0.154	

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