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Title: Production of *Daphnia* zooplankton on wastewater-grown algae for sustainable conversion of waste nutrients to fish feed

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**Abstract**

This study investigates the upcycling of nutrients in anaerobic digestate via algal biomass to zooplankton which is a natural fish feed. We tested the viability of digestate-grown *Chlorella sorokiniana* as a feed for the large-bodied generalist zooplankter, *Daphnia,* and found that in comparison to *Daphnia* growth on *Ankistrodesmus* sp., an established feed, digestate-grown *C. sorokiniana* led to 1.5 to 14 fold greater *Daphnia* population growth. A sterol analysis of *C. sorokiniana* found 4-6 mg/g of the sterol, ergosterol, and nearly double the α-linolenic acid content of *Ankistrodesmus*. Sterols and α-linolenic are often-limiting nutrients in *Daphnia* diets. We also tested other factors hypothesized to influence nutrient transfer from algae to *Daphnia*, including algal feed concentration, sterol supplementation, and the presence of digestate bacteria in the algal feed. The presence of bacteria and exogenous cholesterol had no significant impacts on *Daphnia* growth. The higher feed concentration (5 mg C/L) led to 3 times higher *Daphnia* growth than the low feed concentration (1.5 mg C/L) even though the latter concentration has frequently been used by other researchers. Finally, we determined that the feed conversion ratio of algae to *Daphnia* fell in the range of 0.19-0.31 and that trophic transfer of carbon was 25-28% while that of nitrogen was 29-34% in this un-optimized system. These values compare favorably to livestock feed conversion efficiency but additional losses will occur when *Daphnia* are fed to fish. These results show that cultivation of *Daphnia* on digestate-grown algae is feasible.

**Key Words:** anaerobic digestate, nutrition, phytoplankton, trophic transfer

## 1. Introduction

When disposed of improperly, high nutrient waste can act as a pollutant, spurring eutrophication and harmful algal blooms (Glibert, 2017; Mallin and Cahoon, 2003). The environmental costs of inadequately-treated waste are often economically expensive as well, threatening the fishing industry, tourism, and human health (Ger et al., 2016). Research suggests algal cultivation on high nutrient waste may be a sustainable method of wastewater treatment because algae can assimilate inorganic nutrients efficiently and their biomass can be harnessed for commercial purposes (Chaump et al., 2018; Cho et al., 2013). For example, researchers have cultivated algal biomass on wastewater for the production of biofuels (Salama et al., 2017), however, harnessing algal biomass for cultivation of zooplankton fish feed is potentially more lucrative. Where algal biofuels suffer from low selling prices (Davis et al., 2011; Quinn and Davis, 2015), high-protein fish meal has, over the past five years, fluctuated between $1,300-$1,600 per metric ton (WorldBank, 2020). Upgrading wastewater nutrients to zooplankton also directly reintegrates nutrients, such as nitrogen and phosphorus, back into the food production system.

Use of algae to treat anaerobic digestates has gained particular attention given the potential for rapid algal growth and nutrient removal from diluted anaerobic digestates (Bohutskyi et al., 2016; Passero et al., 2015). However, use of high-strength anaerobic digestates without any dilution can be toxic to algae. Many researchers have diluted the anaerobic digestate 10-30 fold with water to mitigate its toxicity to algae (Cho et al., 2013; Franchino et al., 2016). We recently developed a pretreatment method using aerobic bacteria instead of dilution to reduce algal growth inhibition on digestate (Wang et al., 2019b). In fact, we found this approach to yield more rapid algal growth than the dilution approach.

High transfer efficiencies of wastewater nutrients to algae, and efficient trophic transfer from algae to zooplankton is of great importance to commercialization of this feed production process. Our recent study showed that *Chlorella sorokiniana* was well-adapted to growth on pretreated municipal sludge anaerobic digestate (Wang et al., 2020). However, there is little existing research on the viability of feeding zooplankton with algae that was grown on high-strength digestate. The zooplankton *Daphnia* is a commercial fish feed and these organisms exhibit a very high feed conversion efficiency (Pauw et al., 1981). *Daphnia* are also model organisms frequently used to assess aquatic toxicity (Nikunen and Miettinen, 1985) making them an appropriate model organism to test the feed-value of algae grown on wastewater. That said, *Daphnia* also exhibit impressive natural variation and resilience to a range of algal feeds, including toxic cyanobacteria (Chislock et al., 2013a; Chislock et al., 2013b; Tillmanns et al., 2008).

The trophic transfer of nutrients from algae to *Daphnia* has been tied to both feed abundance and nutritional quality (Taipale et al., 2014). Studies have shown suppression of reproduction and somatic growth in *Daphnia* that are fed algae lacking in certain fatty acids and sterols, which *Daphnia* are unable to synthesize de-novo (Elert et al., 2003; Koch et al., 2012). Prior research suggests that eicosapentaenoic acid (EPA) is the primary limiting polyunsaturated fatty acid (PUFA) for somatic growth and reproduction of *Daphnia* (Becker and Boersma, 2005; Martin-Creuzburg and von Elert, 2009; Müller-Navarra et al., 2004). α-linolenic acid (ALA) has also been identified as a non-substitutable fatty acid because *Daphnia* cannot synthesize ALA but can convert ALA to EPA (Becker and Boersma, 2005; Martin-Creuzburg and von Elert, 2009; Müller-Navarra et al., 2004; von Elert, 2002). Cholesterol, which is required for membrane stabilization, is also necessary for growth and reproduction (Hassett, 2004). *Chlorella*

*sorokiniana*, an algae strain we identified in previous research to be well-adapted to growth on pretreated digestate, was also rich in protein and some omega3 fatty acids, and therefore showed promise as a feedstock for zooplankton-based fish feed (Wang et al., 2020). However, its sterol content was uncertain.

The objective of this study was to test the hypothesis that *Chlorella sorokiniana* grown on pretreated anaerobic digestate is a viable feed for the model zooplankton *Daphnia*. We compared *Daphnia* population growth between organisms fed a standard algae-based feed, *Ankistrodesmus*, and those fed *Chlorella sorokiniana* cultivated on digestate. We also tested whether the presence of wastewater bacteria (from digestate pretreatment) mixed into the algae influenced *Daphnia* growth. Our second objective was to determine whether we could increase *Daphnia* growth by manipulating factors including feed abundance and sterol supplementation. We hypothesized that *Daphnia* would benefit from the high omega3 fatty acid content produced by *C. sorokiniana* under high-nutrient conditions as documented in Wang et al. (2020), and that *Daphnia* growth could be further enhanced through exogenous sterol addition. The findings of this study lay the groundwork for a novel, scalable and cost-effective method for upcycling anaerobic digestate nutrients into fish feed.

## 2. Material and methods

### *2.1 Experimental conditions*

*Daphnia* population growth experiments were conducted to measure the growth and trophic transfer efficiency of *Daphnia* that were fed algae cultivated on pretreated municipal anaerobic digestate. In the experimental treatments, *Daphnia* were fed the chlorophyte, *Chlorella sorokiniana* (UTEX 2805). *C. sorokiniana* feed was grown on two different batches of pretreated

municipal sludge anaerobic digestate. In the first batch, *C. sorokiniana* were cultured on a low-strength municipal anaerobic digestate (~400 mg/L ammonium). In the second batch, *C. sorokiniana* were cultured on a higher strength municipal anaerobic digestate (~1,500 mg/L ammonium). These differences in strength are due to the natural temporal variation in digestate collected from the wastewater treatment plant in Columbus, GA. These digestates and the algae cultivation methods have been described in detail in a previous publication (Wang et al., 2020).

Two batch growth experiments (17-20 days) were conducted with *Daphnia* fed *C. sorokiniana* that were cultivated on low and high strength pretreated digestate, respectively. Each experiment tested six experimental conditions to investigate factors hypothesized to influence nutrient transfer from algae to *Daphnia*: algae type, algae feed concentration, sterol supplementation, and presence of bacteria from the pretreated digestate (Table 1). This experiment was conducted first with *C. sorokiniana* grown on low-strength anaerobic digestate and then again with *C. sorokiniana* grown on high-strength anaerobic digestate. *Ankistrodesmus* sp. was used as the feed for the control group. While our previous study showed that *C. sorokiniana* grown on pretreated anaerobic digestate was rich in fatty acids, it had no precedent as a *Daphnia* feed. Conversely, the green algae *Ankistrodesmus* is an established *Daphnia* feed, with a growth-saturating concentration of 1.5 mg C/L for juvenile *Daphnia* (DeMott et al., 2010; Lampert, 1978; Sarnelle and Wilson, 2005). Thus, *Daphnia* were fed *Ankistrodesmus* in one of the treatments as a positive control. We also investigated the effects of algae feed that contains residual wastewater bacteria since this is likely in a full scale system. We therefore fed *Daphnia* with *C. sorokiniana* grown on sterile-filtered (0.2 μm) anaerobic digestate (no bacteria) whereas the remaining treatments received *Chlorella sorokiniana* grown on nonsterile anaerobic digestate (containing bacteria). The established feed concentration of 1.5 mg C/L (FR1.5) was held

constant for the control and for three of the five experimental conditions. To test whether growth and trophic transfer efficiency could be increased by higher feed rates, two *Daphnia* cultivation conditions received a high feed concentration of 5 mg C/L (FR5). Informed by a prior study suggesting cholesterol supplementation increased egg production in copepods (Hassett, 2004), we hypothesized that sterol supplementation might also facilitate *Daphnia* population growth. A treatment of *Daphnia* at the standard and high feed rates were supplemented weekly with 10 μg of cholesterol (+ Chol) which was the same concentration used in the study by Hasset (2004).

*2.2 Feed preparation*

The chlorophyte, *C. sorokiniana* (UTEX 2805), was originally isolated from a wastewater treatment plant (de-Bashan et al., 2008). As detailed in the methods from Wang et al. (2020), *C. sorokiniana* was cultured on sterile and nonsterile municipal sludge anaerobic digestate that had been pretreated with aerobic bacteria (activated sludge) for 96 hours. The digestates were collected from a mesophilic anaerobic sludge digestor at the South Columbus Water Resources Facility (Columbus, GA, USA). Activated sludge bacteria were collected from the aeration basins in this same facility and used for the digestate pretreatment process. Sterile digestate was prepared by filtration down to 0.2 μm whereas non-sterile digestates were filtered to 0.7 μm. *Ankistrodesmus* was maintained in 1 L bottles filled with BG11 medium. *C. sorokiniana* was cultured in 200 ml bubble column photobioreactors as previously described (Wang et al., 2019a). All algal cultures were maintained at room temperature and provided with 0.5 vvm of air supplemented with 2% $CO_2$. Fluorescent growth lamps (170 mmol photons/$m^2$/s) were operated on a 16:8 light/dark cycle. After five days of cultivation, algal cultures were harvested by centrifugation at 4696 x g for 5 minutes and the supernatant was decanted. The algal pellet was

153 washed twice with $dH_2O$ and resuspended in filtered (~1 μm) water obtained from an
154 oligotrophic lake in Alabama (Lake Martin) to achieve a concentration of 1 mg of dry weight
155 algae per ml. This stock algae feed was stored at 4 °C and used over the course of the *Daphnia*
156 feeding study. All cultures were handled in a biosafety cabinet using sterile technique to prevent
157 contamination with laboratory organisms.
158 Because cholesterol is insoluble in water, cholesterol aliquots were made using the
159 protocols described by Hassett (2004) by homogenizing increasing dilutions of cholesterol in a
160 beadruptor (OMNI) twice for 30 s at a speed of 8 m/s. Homogenized aliquots were stored in a -
161 $20^{o}$ C freezer, and rehomogenized with the beadruptor before supplementation at 10 μg/L.
162
163 *2.3 Daphnia culture conditions and sampling*
164 All experiments were conducted with *Daphnia* clones isolated from a eutrophic lake. At the start
165 of the batch experiment, 10 *Daphnia* were introduced into each bottle filled with 250 mL
166 autoclaved and filtered (~1 μm) lake water. Algae and cholesterol were added to bottles per the
167 experimental design (Table 1). Bottles were kept at room temperature (~25 °C) under ambient
168 room lighting. *Daphnia* populations in each bottle were counted daily before sampling through
169 careful observation. When *Daphnia* populations were above 30, rough counts were taken
170 multiple times and averaged. Rough counts were verified by multiple counters. In the second
171 experiment (using *C. sorokiniana* grown on high-strength digestate), the number of ovigerous
172 *Daphnia* were also recorded daily. Every other day, the bottles were swirled to resuspend the
173 algal feed and 10 ml samples were taken from each replicate by serological pipette, with care not
174 to remove any *Daphnia* from the populations. The samples were used to measure pH, ion
175 content, and optical density of each replicate. Optical density of each sample was measured at

680 nm with a spectrophotometer (SpectraMax M2) using cuvettes. The samples were measured in technical triplicates and averaged. Using a regression of optical density and feed concentration specific to the algal species, the optical density of the samples was used to calculate the feed amount required for each replicate to maintain the feed concentration of each treatment at either 1.5 or 5 mg C/L. The carbon content of *Ankistrodesmus* sp. and *C. sorokiniana* were 44% and 46% on a dry weight basis, respectively, according to CHNS analysis using a vario MICRO cube Elemental Analyzer (Elementar). After feeding, each bottle was replenished to 250 ml as needed with autoclaved lake water. After 17-19 days, *Daphnia* were harvested from each bottle using a sieve and freeze dried. The *Daphnia* wet and dry weights were recorded to calculate biomass. The freeze-dried samples underwent elemental analysis (CHNS).

*2.4 Fatty acid analysis in algae*

Gas chromatography and mass spectrometry was used to determine the concentration of omega3 fatty acids in the algae feeds. The fatty acid profile of the *C. sorokiniana* biomass was reported previously (Wang et al., 2020), but the same analysis was carried out in this study for the *Ankistrodesmus* feed material. Lipids were extracted from freeze dried algae using a modified Folch method as previously described (Wang et al., 2019a). Lipid extracts were dried and transesterified with 1 M methanolic HCl and analyzed by gas chromatography and mass spectrometry (GC-MS) using methods previously described (Higgins et al., 2014) with modifications. Nonadecanoic acid was added to lipid extracts as an internal standard. Samples were injected into an HP 6890 GC coupled to an HP 5970 mass spectrometer. A Restek RXI-5Sil MS column (Length: 30m, ID: 0.25mm, $d_f$: 0.25µm) was used for separation with the following oven temperature program: Initial Temperature: 100 °C, 2 minutes; ramp to 240 °C in 10 minutes

(rate: 14 ℃/ min); hold at 240 ℃ for 47 minutes. Helium was used as the carrier gas with a flow of 6.4 ml/min and a split ratio of 8:1 (0.8 ml/min on column). Peaks were integrated using the ChemStation software and concentrations of individual fatty acids were determined from the internal standard plus recovery factors obtained from an analytical canola oil external standard (Sigma). Peak area response for a range of FAMEs were also determined using a mixture of FAME standards (Sigma). The following equation was used to calculate unknown fatty acid concentrations in samples:

$$C_x = A_x \left(\frac{C_{IS}}{A_{IS}}\right) \left(C_{RS} \left(\frac{A_{RS}}{CvF_{IS}}\right) \left(\frac{RF_B}{RF_x}\right)\right) \qquad \text{Eq. 1}$$

Where C is the concentration, A is the integrated peak area, CvF is conversion factor of concentration/area, RF is the response factor or slope of concentration to area. The subscript X represents the unknown fatty acid, the IS subscript represents the internal standard, and the RS subscript is the recovery standard and B is a benchmark fatty acid (18-2 n6). This method accounts for varying responses and recoverability of the different fatty acids in the mixture.

*2.5 Sterol analysis by liquid chromatography with mass spectrometry*

Cholesterol was measured in the algal lipid extracts by LCMS using a method adapted from Nagy et al. (2006). Cholesterol analysis was conducted on a Shimadzu Prominence UPLC coupled to a Shimadzu LCMS2020 single quadrupole mass spectrometer. An Accucore RPMS (C18), 2x100 mm column was used. 85:15 v/v MeOH/water was used for phase A and isopropyl alcohol was used for phase B under a flow rate of 0.3 ml/min. The HPLC gradient was as follows: 0-10 min ramp 0% to 90% B, 10-12 min hold 90% B, 12-13 min ramp 90%-0% B, 13-17 min hold 0% B. MS detection parameters were as follows: Positive mode, DUIS with 4 uA current, and Scan mode from 100-1000 m/z per second. Standards of cholesterol (RT, 7.2 min

and quantification m/z, 369) and ergosterol (RT, 6.7 min and quantification m/z, 379) were used for quantification. For both sterols, the dominant positive ion was a molecular fragment representing the loss of the negatively charged OH group. The parent ion with an H+ adduct was also observed for both of these sterols but this ion was weaker and was not used for quantitation.

*2.6 Elemental mass balance and trophic transfer*

Trophic transfer efficiency was determined by calculating the feed conversion ratio (FCR) alongside C and N transfer rates from algae to *Daphnia*. FCR was calculated as the ratio of the total amount of algae fed (dry weight basis) divided by the wet weight of *Daphnia* harvested from each treatment. The carbon and nitrogen content of the algae and *Daphnia* were measured using a CHNS elemental analyzer. Due to instrument malfunction part way through the run, total nitrogen assays (HACH) were used to re-analyze nitrogen content of the algal biomass in a subset of the samples. This method has been described previously (Wang et al., 2020).

To complete the mass balance on nitrogen, the nitrogen ion concentrations in the water samples including ammonium, nitrate, and nitrite, were measured. Ion concentrations were measured on a Prominence Liquid Chromatography (LC) system coupled with a conductivity detector (Shimadzu, Japan). Measured ions also included sodium, potassium, calcium, magnesium, chloride, phosphate, and sulfate, based on a published method (Chaump et al., 2018). Briefly, A Dionex IonPac CS12 column (4 × 250mm, Thermo science) and a Dionex IonPac AS22 column (4 × 250mm) with suppression (Dionex CERS 500 4 mm and Dionex AERS 500 4 mm, respectively) were used for ion separation. Acidic eluent (20 mM methanesulfonic acid) was used on the CS12 column, and basic eluent (4.5 mM sodium carbonate and 1.4 mM sodium bicarbonate solution) was used on the AS22 column.

245

246 *2.7 Data analysis and statistics*

247 Experiments were all conducted in biological triplicate. For non-time course data, statistical one-
248 way ANOVA of treatments and two-way ANOVA for experimental variables (feed rate,
249 cholesterol, bacteria, algae type), were carried out in R with the 'lattice' package and 'multcomp'
250 package and followed by Turkey's HSD test with $p < 0.05$ considered significant. For time-
251 course data associated with the *Daphnia* growth curve, a repeated measures ANOVA of
252 treatments coupled with Tukey's HSD test was used. Means and standard deviations were
253 calculated in Microsoft Excel.

254

255 **3. Results**

256 *3.1 Daphnia growth*

257 *Daphnia* populations grew as well or better on the wastewater-grown *C. sorokiniana* than they
258 did on the standard feed algae, *Ankistrodesmus* sp. (Figure 1). Final *Daphnia* counts at the end of
259 the batch experiments were 1.5 to 14 times higher in the experimental diets compared to the
260 control diet (*Ankistrodesmus* sp.), regardless of whether the *C. sorokiniana* feed was cultivated
261 on low strength or high strength digestate. There was also no significant difference between
262 treatments fed with *C. sorokiniana* grown on sterile filtered digestate versus algae that contained
263 wastewater bacteria. Treatments maintained with a high feed concentration of 5 mg C/L of *C.*
264 *sorokiniana* had faster rates of *Daphnia* population growth than treatments maintained at 1.5 mg
265 C/L of *C. sorokiniana* ($p < 0.001$, Fig.1). In the first experiment, where *C. sorokiniana* were
266 cultivated on low strength digestate, the peak population of the *Daphnia* fed 5 mg C/L of *C.*
267 *sorokiniana* was three times larger than the treatment fed 1.5 mg C/L and fourteen times larger

than those fed with *Ankistrodesmus sp.* (Fig. 1A). The second experiment, where *C. sorokiniana* were cultivated on high strength digestate, showed similar trends: the high feed treatment had a peak population five times larger than the standard feed treatment of *C. sorokiniana*, and twelve times larger than the *Ankistrodesmus* control (Fig. 1 B). Nitrite and nitrate levels in the *Daphnia* cultures (potential metabolic waste products) remained steady throughout the experiment despite the increases in population density (Figure S1 and S2). Ammonium concentrations increased and scaled with population size (Figure S3). Increases in ammonium may have influenced some of the population decline observed after day fifteen of the second experiment, although concentrations remained below acute toxicity levels for *Daphnia* (EPA, 2013).

Addition of cholesterol to the *Daphnia* cultures appeared to have a small suppressive effect on growth but the effect was not statistically significant (Figure 1, $p = 0.055$). A count of ovigerous *Daphnia* taken during the second experiment suggested possible differences in reproductive timing for treatments supplemented with cholesterol, however. Within treatments of the same feed rate, those supplemented with cholesterol appeared to have less *Daphnia* carrying eggs during the first half of the experiment (Figure S4).

On the 9$^{th}$ day of *Daphnia* growth in the first experiment, there was an air conditioning failure in the building. This heat shock led to increased room temperature of 27-32 ℃ under high humidity conditions. This reduced *Daphnia* populations in all treatments fed 1.5 mg C/L, especially those growing on *Ankistrodesmus*, while slowing the growth rate of *Daphnia* cultures fed 5 mg C/L (Figure 1 inset). However, all cultures were able to recover from this shock event and continued growing.

The dry weights of the *Daphnia* populations taken at the end of the experiment showed significant differences in weight between the high and low feed conditions, regardless of feed

type ($p < 0.001$, Figure 2). The high feed rate resulted in 2.2-4.7 times higher final biomass than the corresponding low feed rate. These results also confirmed findings from the rough *Daphnia* counts that digestate-grown *C. sorokiniana* was not harmful to *Daphnia* biomass production when compared to the established *Daphnia* feed, *Ankistrodesmus* sp. Unlike the daily counts, which had more room for error but also finer time-course resolution of population size, the final dry weight showed no significant difference between treatments maintained at the same feed concentrations, regardless of whether the *Daphnia* were fed *C. sorokiniana, Ankistrodesmus sp.* or supplemented with cholesterol. From the similarity in *Daphnia* dry weight obtained from the two experiments it appeared that the strength of the anaerobic digestate used for algae cultivation did not impact *Daphnia* population growth. This analysis also showed that the harvested *Daphnia* dry matter content was 6-7%.

*3.2 Fatty acid and sterol content of feed algae*

Analysis of the fatty acid and sterol content in the algae feed suggested that there were notable differences in nutrient content between *Ankistrodesmus* sp. and *C. sorokiniana* (Figure 3). *C. sorokiniana* contained nearly twice the fatty acid content per mg of dry algae compared to *Ankistrodesmus* sp., which translated to higher total PUFA concentrations and twice as much ALA supplied to *Daphnia*. Cholesterol was not found in either algae. Instead, ergosterol was detected in *C. sorokiniana* but not in *Ankistrodesmus* sp. (Figure 3B). *Scenedesmus*, a relative of *Ankistrodesmus*, are known to produce fungisterol (Martin-Creuzburg and Merkel, 2016) and a small peak matching the expected m/z of fungisterol was detected (m/z 383.4 and retention time of 7.4 min). However, no external standard of fungisterol was purchased to confirm the identity of this peak.

The different *C. sorokiniana* cultivation methods – on high and low strength digestate, axenic and mixed with bacteria, resulted in very similar fatty acid contents. There was no discernable difference in total fatty acid, PUFA and ALA content among treatments of *C. sorokiniana* (Figure 3, $p > 0.05$). However, *C. sorokiniana* cultivated on high strength digestate contained a little more than half the ergosterol of *C. sorokiniana* cultivated on low strength digestate (Figure 3B, $p < 0.001$).

### *3.3 Trophic Transfer*

The feed conversion ratio was calculated as the dry weight of algae fed throughout the experiment to the wet weight of *Daphnia* harvested at the end of the experiment. Lower values indicate more efficient feed conversion. Overall, the feed conversion ratio of treatments ranged between 0.19 and 0.31 in the second experiment (Figure 4). Feed conversion ratios were not significantly different among treatment groups, including between the high and low feed rates (Figure 4, $p > 0.05$).

The relative consistency in trophic transfer between low and high feed was demonstrated again in a mass balance model developed to trace the nitrogen and carbon through the experimental system. This trophic transfer model was based on data from the second experiment which used the high strength digestate for algae cultivation. Algal additions into the system contributed 100% of the input carbon, assuming minimal algal growth within the *Daphnia* culture. In both the high and low feed models, between a quarter and a third of the carbon ended up in the *Daphnia*, about five percent remained in algae, and the vast majority, 67-70%, either entered the aqueous phase or left the system, presumably as $CO_2$ (Figure 5). Most of the nitrogen input (>60%) came from the lake water and remained in the water as ions. It is presumed that

this nitrogen (mostly nitrate) was not used by *Daphnia*; only nitrogen from algae was available for assimilation. Of the nitrogen entering in the algal biomass, the high feed rate led to 34% nitrogen assimilation into *Daphnia*, whereas the low feed rate led to 29% assimilation.

## 4. Discussion

### *4.1 Daphnia growth and nutrition*

*C. sorokiniana* grown on pretreated anaerobic digestate was not found to be harmful to *Daphnia* when provided as a feed. In fact, *Daphnia* growth using this algae exceeded the growth in populations fed *Ankistrodesmus* sp., despite the latter's use as a standard *Daphnia* feed (DeMott et al., 2010; Lampert, 1978; Sarnelle and Wilson, 2005). These trends were consistent regardless of the strength of the digestate used to grow the algae and regardless of whether wastewater bacteria were present in the algal feed.

More significant for *Daphnia* growth than feed type was feed concentration. *Daphnia* are especially sensitive to water quality, including high-nutrient conditions (Cowgill et al., 1986). Consequently we initially had concerns about overfeeding *Daphnia*, which has been shown to result in population decline and mortality (Jakobsen and Johnsen, 1987; Jensen and Hessen, 2007; Martínez-Jerónimo et al., 1994; Porter et al., 1982). However, *Daphnia* populations fed the high feed concentration of 5 mg C/L grew 2.2-4.7 times more than those fed 1.5 mg C/L. These findings contrasted with previous feed studies, which identified 0.6 mg C/L as the limiting incipient level of feed concentration for somatic growth (Lampert, 1978). However, Pajk et al. (2012) cautions that changes in temperature and food quality strongly effect dietary requirements of *Daphnia*, so *Daphnia* raised on different conditions and feeds may have notable variation in dietary constraints. Additionally, many of the previous studies that identified an incipient

limiting level of feed concentration for *Daphnia* measured somatic growth instead of populational growth and were conducted in the *Daphnia's* habitat, where variables such as temperature, predation and nutrient composition of feed were not held constant (Jakobsen and Johnsen, 1987; Martínez-Jerónimo et al., 1994; Porter et al., 1982). Because *Daphnia* have been used primarily as model organisms in ecological and water-quality studies, there was little motivation to alter natural feeding habits or feed availability. The results from our study suggest that *Daphnia* populations raised in a controlled settings may thrive on much higher feed concentrations than the standard feeding rate for *Daphnia* (1.5-2 mg C/L). It is even possible that they can tolerate feed concentrations even higher than 5 mg C/L, especially with feeds high in PUFAs and sterols.

That both experiments shared the same trends in *Daphnia* populational growth was encouraging considering the combination of methodological and incidental variation that distinguished the first and second experiment. In addition to different digestates used to cultivate the feed algae, *Daphnia* cultures in the first experiment experienced heat-shock following a building-wide air-conditioning malfunction. This unplanned event reduced the reliability of our results from the first experiment. Despite high variability within treatments, the maintenance of trends of populational growth between the treatments strengthens our confidence that *Daphnia* can survive on wastewater-grown algae and that the higher feed concentrations increased populational growth and perhaps its resilience to shock events as well.

The nutritional composition and abundance of limiting nutrients such as omega3 fatty acids and sterols may explain some of the differences in populational growth between treatments that varied only in feed type or in feed concentration. Compositional analysis of the algal feeds showed that *C. sorokinaina* contained twice the total fatty acids as *Ankistrodesmus*, and twice the

383 concentration of EPA. The greater populations observed in *Daphnia* fed digestate-grown *C.*
384 *sorokinaina* compared to *Ankistrodesmus* sp. were consistent with other studies that have
385 observed enhanced *Daphnia* growth on algae and cyanobacteria supplemented with EPA
386 (Windisch and Fink, 2018).This difference in fatty acid and sterol content between algal species
387 can be explained in part by natural variation among algal species, but the high levels of fatty
388 acids and sterol in *C. sorokiniana* may also be a result of growing the algae in nutrient-rich
389 digestate. *C. sorokiniana* (and many other green algae) are known to produce predominantly
390 polar lipids that are rich in PUFAs including ALA, when cultured under high-nutrient conditions
391 (Higgins et al., 2015; Negi et al., 2016). Our results also showed that the strength of the digestate
392 altered the production of ergosterol in *C. sorokiniana*, with higher strength digestate suppressing
393 production.

394 It was initially surprising that cholesterol supplementation had no benefit and even
395 seemed to inhibit *Daphnia* growth slightly. Increased dietary sterols in *Daphnia magna* and *D.*
396 *galleta* have been linked to an increase in somatic and populational growth, although the degree
397 of change is minimal when the basal feed is already high in sterols (Martin-Creuzburg et al.,
398 2005). We therefore hypothesize that sterols were not limiting for growth because the
399 concentration of ergosterol in the *Chlorella* was sufficient. Additionally, the cholesterol was not
400 added to the feed but to the water following sonication, so it is possible that the method of
401 cholesterol supplementation did not translate to higher ingestion of cholesterol by *Daphnia*. It is
402 also possible that the inhibition of growth in cholesterol-supplemented treatments was due to the
403 presence of impurities in the cholesterol supplement. In LCMS of the cholesterol supplement, an
404 unidentified peak was present in the sample suggesting possible contamination. Nevertheless,

405 sterol supplementation is unlikely to provide benefits so long as the wastewater-grown algae
406 have sufficient sterol content, as was the case in this study.
407
408 *4.2 Practical implications*
409 Our results reinforce the point that *Daphnia* are highly-efficient converters of algae-based feed
410 into body mass. The observed FCR of <0.31 was substantially lower than established livestock
411 production modes. Finfish are among the most efficient animals with FCR typically ranging from
412 1-2.5 depending on feed quality and environmental conditions (Fry et al., 2018). Part of the
413 reason FCR appears so low in this study is the high water content of *Daphnia* (~93%). Assuming
414 dry algae is converted to wet *Daphnia* at an FCR of 0.25 and dry *Daphnia* is converted to fish
415 biomass at an FCR of 1, then the overall FCR of the entire process (dry algae to wet fish
416 biomass) is just under 4. This is similar to the conversion efficiency of pigs (2.7-5) but lower
417 than that of chickens (1.7-2) (Fry et al., 2018). More meaningful than FCR alone is the carbon
418 and nitrogen transfer from algae to *Daphnia*. Here, we found that, without any system
419 optimization or *Daphnia* improvements through breeding, 25-28% of carbon and 29-34% of
420 nitrogen from the algae was assimilated by *Daphnia*. Because nitrogen is often used to estimate
421 crude protein content of feed materials, we can infer that the protein conversion efficiency was
422 also 29-34%. This compares favorably to animal protein production with values typically falling
423 in the range of 15-20% (Fry et al., 2018), but again, additional losses will occur when
424 zooplankton are consumed by fish. When compared to aquaculture or livestock production, it is
425 still important to consider that all of the nutritional inputs to this system were derived from waste
426 whereas traditional animal production utilizes synthetic fertilizers to produce plant-based feed.
427 Future studies should investigate whether nutrient transfer from wastewater to *Daphnia* is viable

on a larger scale, and whether feed and environmental conditions can be optimized to promote trophic transfer efficiency from algae to *Daphnia*.

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## Tables

Table 1: Experimental treatments for *Daphnia* cultures.

| **Treatment abbreviation** | **Feed type** | **Algal growth medium** | **Feed concentration** | **Cholesterol Supplement** |
|---|---|---|---|---|
| Ank FR1 | *Ankistrodesmus* | BG11 | 1.5 mg C/L | None |
| Ax C. Sor (FR1) | *Chlorella sorokiniana* | Sterile-filtered digestate | 1.5 mg C/L | None |
| C. Sor (FR1) | *C. sorokiniana* | Digestate | 1.5 mg C/L | None |
| C. Sor (FR1) + Chol | *C. sorokiniana* | Digestate | 1.5 mg C/L | 10 μg/L |
| C. Sor (FR2) | *C. sorokiniana* | Digestate | 5.0 mg C/L | None |
| C. Sor (FR2) + Chol | *C. sorokiniana* | Digestate | 5.0 mg C/L | 10 μg/L |

**Figure captions**

Figure 1. Populational growth of *Daphnia* varies by feeding rate. (A) Experiment 1 *Daphnia* were fed for 19 days using *Chlorella sorokiniana* (C. Sor) grown on low-strength anaerobic digestate. The use of axenic *C. sorokiniana* (Ax C. Sor), a low feeding rate of 1.5 mg C/L (FR1.5), a high feeding rate of 5 mg C/L (FR5), and cholesterol (Chol) supplementation were tested. Inset of Days 6-11 show impact of heat shock (*) on Day 9. (B) Experiment 2 *Daphnia* were grown under the same treatments as experiment 1 except that *C. sorokiniana* feed algae were grown on high-strength anaerobic digestate. Error bars are ±SD; n=3. Statistical significance was tested using repeated measures ANOVA with random factor for bottles followed by a Tukey HSD post hoc test ($p < 0.05$).

Figure 2. Dry weight of *Daphnia* varies by feeding rate. (A) Experiment 1 *Daphnia* were fed for 19 days using *Chlorella sorokiniana* (C. Sor) grown on low-strength anaerobic digestate. The use of axenic *C. sorokiniana* (Ax C. Sor), a low feeding rate of 1.5 mg C/L (FR1), a high feeding rate of 5 mg C/L (FR2), and cholesterol (Chol) supplementation were tested. (B) Experiment 2 *Daphnia* were grown under the same treatments as experiment 1 except that *C. sorokiniana* feed algae were grown on high-strength anaerobic digestate. Error bars are ±SD; n=3. Statistical significance was tested using one-way ANOVA followed by a Tukey HSD post hoc test ($p < 0.05$).

Figure 3. Fatty acid and sterol content in algal feeds (μg/mg). Total α-linolenic acid (A), ergosterol content (B), total polyunsaturated fatty acid content (C), total fatty acid content (D). Ank = *Ankistrodesmus*, C. Sor = *Chlorella sorokiniana*, LS = low strength digestate, HS = high strength digestate, Ax = axenic algae-based feed were grown on sterile-filtered anaerobic

592 digestate. Statistical significance was tested using one-way ANOVA followed by a Tukey HSD
593 post hoc test, ($p < 0.05$); error bars are ± SD, n = 3.
594
595 Figure 4. Feed conversion rate in experiment 2 where *Daphnia* were fed for 17 days using
596 *Chlorella sorokiniana* (C. Sor) grown on high-strength anaerobic digestate. The use of axenic *C.*
597 *sorokiniana* (Ax C. Sor), a low feeding rate of 1.5 mg C/L (FR1.5), a high feeding rate of 5 mg
598 C/L (FR5), and cholesterol (Chol) supplementation were tested. Error bars are ±SD; n = 3.
599 Statistical significance was tested using one-way ANOVA followed by a Tukey HSD post hoc
600 test ($p < 0.05$).
601
602 Figure 5. Mass balance of C and N using data from experiment 2 (*C. sorokiniana* grown on high-
603 strength anaerobic digestate), for both low feed (left panel) and high feed (right panel)
604 concentrations for *Daphnia*. Negative values are the result of imperfect mass closure.
605
606 Figure S1. Nitrite concentration (mg/L) in water samples taken throughout experiment 1 (top)
607 and 2 (bottom). Bars are standard deviation based on 3 biological replicates.
608
609 Figure S2. Nitrate concentration (mg/L) in water samples taken throughout experiment 1 (top)
610 and 2 (bottom). Bars are standard deviation based on 3 biological replicates.
611
612 Figure S3. Ammonium concentration (mg/L) in water samples taken throughout experiment 1
613 (top) and 2 (bottom). Bars are standard deviation based on 3 biological replicates.
614
615

Figure S4. Ovigerous *Daphnia* count by treatment over 17 days in Experiment 2. *Daphnia C. Sor* treatments fed algae grown on vacuum filtered wastewater. Treatment labels are described in Table 1; *bars*, ±SD; n=3. Using Repeated Measures ANOVA followed by a Tukey HSD post hoc test ($p < 0.05$), $p < 0.0001$ for FR1.5 vs FR5, $p = 0.056$ for cholesterol supplementation, $p = 0.07$ for algae type and 0.97 for axenic vs non-axenic algae feed.