

## ARTICLE OPEN

## Algal–bacterial synergy in treatment of winery wastewater

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There is significant potential for employing algae in tertiary wastewater treatment, however, little is known about the contribution of algae–bacteria synergy toward treatment performance. This study demonstrates potential synergy in the treatment of three winery wastewater samples. Two strains of green algae, *Auxenochlorella protothecoides* and *Chlorella sorokiniana* were tested and each removed > 90% of nitrogen, > 50% of phosphate, and 100% of acetic acid in the wastewater. Both algae strains grew significantly faster on wastewaters compared to growth on minimal media. Organic carbon in the wastewater apparently played a limited role in algal growth enhancement. When cultured on sterile-filtered wastewater, *A. protothecoides* increased soluble COD loadings in two of the three wastewaters and *C. sorokiniana* secreted an insoluble film. Culturing algae with the native wastewater microbial community negated the secretion of algal photosynthate, allowing for simultaneous reductions in COD and nutrient concentrations. Both algae species stimulated bacterial growth in a strain-specific way, suggesting unique responses to algal photosynthate. Cofactor auxotrophy for thiamine, cobalamin, and biotin is widespread among algae and these cofactors are typically obtained from bacteria. Sequencing the wastewater microbial community revealed bacteria capable of synthesizing all three cofactors while liquid chromatography with mass spectrometry (LCMS) and bio-assays revealed the presence of thiamine metabolites in the wastewaters. These cofactors likely increased algal growth rates, particularly for *A. protothecoides*, which cannot synthesize thiamine de-novo but can salvage it from degradation products. Collectively, these results demonstrate that bacteria and algae provided synergistic growth benefits, potentially contributing to higher levels of wastewater treatment than either organism type alone.

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## INTRODUCTION

Many agricultural and food processing operations are increasingly employing anaerobic digestion to reduce organic carbon loads in wastewaters while producing methane-rich biogas.<sup>1</sup> However, digesters do a poor job of removing inorganic nutrients such as ammonium and phosphate, which are largely retained in the effluent, and can pollute waterways, contributing to harmful algal blooms.<sup>2</sup> Compared to digesters, algae are not particularly effective at degrading complex organic molecules but are very effective at sequestering nitrogen and phosphorus from wastewaters, including those from anaerobic digesters.<sup>3,4</sup> Algal biomass cultivated on wastewater can also be used for biofuel and bioproduct synthesis.<sup>5,6</sup> Given the complimentary nature of these two technologies, cultivation of algae on anaerobic digester effluent could lead to high-quality wastewater effluent that has low concentrations of both organic and nutrient pollutants. High-quality effluent holds the potential for re-use in irrigation.

Numerous studies exist on the use of algae to treat various types of wastewaters including those from dairies,<sup>7</sup> piggeries,<sup>8</sup> wineries,<sup>9</sup> slaughterhouses,<sup>10</sup> and municipalities.<sup>11,12</sup> The fact that algae can grow successfully on such chemically diverse wastewaters indicates the potential scalability of algal wastewater treatment. However, significant hurdles remain including potential negative interactions among native wastewater organisms and algae. The vast majority of studies on algal wastewater treatment focus on simple reporting of reductions in nutrient loads with little investigation into why a particular algae strain or mixed culture was effective. Moreover, numerous studies fail to explain if the

wastewater was sterilized prior to algae cultivation; thus it is difficult to determine the relative contributions of algae and bacteria toward wastewater treatment performance.

In addition to measuring changes in water quality parameters in the present study, we also investigated how green algae and native wastewater organisms interact in the treatment process. Our past research has focused on interactions between green algae and bacteria in synthetic co-cultures. Thus, we were interested in understanding the extent to which our co-culture findings apply to a more complex wastewater environment. In previous studies, co-culturing the model organisms *Escherichia coli* and *Auxenochlorella protothecoides* led to synergistic interaction that increased algal growth rates and lipid production under mixotrophic conditions.<sup>13,14</sup> Those same studies also showed that co-cultures could more rapidly remove organic and nitrogen species from the medium, potentially benefiting wastewater treatment. Further study revealed that much of the synergy was due to exchange of particular thiamine metabolites between the bacteria and algae.<sup>15</sup> We hypothesized that similar synergy exists among many potential pairs of organisms due to widespread cofactor auxotrophy in algae<sup>16</sup> and the important role of bacteria in providing these cofactors to algae in nature.<sup>17</sup> In the present study, the potential for cofactor symbiosis between green algae and organisms present in winery wastewater was investigated. The relative contributions of algae and the native wastewater community toward the observed changes in water quality parameters were also studied.

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## RESULTS

### Algal wastewater treatment

Two strains of green algae, *A. protothecoides* and *C. sorokiniana*, were cultured in photobioreactors filled with sterile-filtered winery wastewater collected at three different times during the grape crush season. These wastewaters were categorized as “pre-crush,” “crush,” and “post-crush” in correspondence to the time in the wine production season when they were collected relative to the production of wine grape juice (grape crush). The winery employed a digester-based treatment system that yielded a wastewater low in organic content but high in nutrients, particularly nitrogen, phosphate, and sulfate (Table 1). The majority of nitrogen was in the form of ammonium. When cultured on sterile-filtered wastewater, both algae strains were effective at removing > 90% of total nitrogen, > 50% of phosphate, > 12% of sulfate, and all detectable acetic acid from the three wastewater types. *C. sorokiniana* in particular could remove all detectable nitrogen and phosphate in the crush and post-crush wastewaters within a span of 4 days. *A. protothecoides* could not metabolize the small quantities of nitrite and nitrate found in the pre-crush wastewater but was generally very effective at ammonium removal (> 90%).

When *A. protothecoides* was grown on sterile-filtered wastewater, soluble organic material was secreted into the wastewater as observed by the increase in chemical oxygen demand (COD) loads in the pre- and post-crush wastewaters after algae cultivation. These compounds were also likely secreted into the crush wastewater but the net effect on COD was over-shadowed by algal consumption of acetate in the wastewater. *C. sorokiniana* also secreted material but it appeared to be polymeric in nature, forming a white film that pelleted with the algal cells and thus was not captured by the soluble COD assay.

The effect of algal treatment on removal of ions that contribute to salinity including chloride, sodium, potassium, magnesium, and calcium was modest or non-existent. Potassium removal was statistically significant for *A. protothecoides* cultures where removal levels were consistently between 30–50 mg/L regardless of the wastewater source. In the pre-crush wastewater, *A. protothecoides*

appeared to increase the calcium concentration, however, this effect was more likely due to limited instrument sensitivity at such low concentrations.

### Growth rates of algae on wastewater

All three wastewater types supported robust algae growth (Fig. 1a). In all cases, algae grew more rapidly on sterile-filtered wastewaters than they did on minimal media (controls). In the case of *A. protothecoides*, growth rates on wastewater were more than 13-fold greater than that on N8-NH<sub>4</sub> minimal medium. N8-NH<sub>4</sub> medium contains 257 mg/L ammonium, which is 1.3–2.2-fold higher than that found in the three wastewaters. *C. sorokiniana* growth on wastewater was roughly double that on N8 minimal medium, which contained 614 mg/L as nitrate. More detail on minimal media composition can be found in the Methods section.

### Algal–bacterial complementarity in wastewater treatment

Thus far, results pertain to algae cultivated on sterile-filtered wastewater in order to understand algal interaction with chemical constituents in the wastewater. However, real treatment systems will include a complex mixture of bacteria along with algae. Additional culture studies were carried out to better understand the impact of the native wastewater microbial community on algal growth and wastewater treatment performance. In this experiment, algae were cultured on both sterile-filtered wastewater and on raw un-sterilized wastewater. Additional reactors were filled with raw wastewater without algae to understand the role of the native bacterial community in wastewater treatment performance. Acting alone, the bacterial community further reduced the COD loads in the wastewater by 36–60% over a 5-day period (Table 2). However, this community had significantly lower removal rates for nitrogen, phosphate, and sulfate compared to algae. By culturing the bacteria together with algae, significant reductions in both COD and nutrients were achieved. Significantly, the bacterial community appeared capable of consuming the organic photo-synthate generated by *A. protothecoides*: culturing the algae on sterile-filtered wastewater significantly increased COD loads by

**Table 1.** Changes in wastewater nutrient concentrations in response to algal treatment

	Pre-crush			Crush			Post-crush		
	Initial <sup>a</sup>	<i>A. proto</i> <sup>b</sup>	<i>C. soro</i>	Initial	<i>A. proto</i>	<i>C. soro</i>	Initial	<i>A. proto</i>	<i>C. soro</i>
COD <sup>c,d,e</sup>	157 (22) b	219 (34) a	124 (15) b	298 (26) a	178 (12) b	136 (8) c	154 (18) b	229 (17) a	138 (30) b
Acetate	–	–	–	344 (36)	–	–	–	–	–
Total N	142 (14) a	7 (6) b	–	147 (9) a	12 (5) b	–	79 (22) a	1 (1) b	–
Ammonium	173 (5) a	7 (12) b	–	204 (20) a	17 (15) b	–	114 (6)	–	–
Nitrite	19 (3) a	18 (3) a	–	–	–	–	–	–	–
Nitrate	28 (7) a	22 (< 1) a	–	–	–	–	–	–	–
Phosphate	88 (4) a	43 (2) b	38 (1) b	46 (8)	–	–	44 (12)	–	–
Sulfate	87 (2) a	73 (1) b	63 (1) c	135 (4) a	117 (3) b	105 (3) c	54 (3) a	37 (1) b	33 (1) b
Chloride	40 (5) a	43 (2) a	36 (1) a	41 (5) a	43 (< 1) a	36 (< 1) a	36 (9) b	50 (1) a	29 (1) b
Potassium	80 (20) a	45 (5) b	57 (8) ab	383 (31) a	334 (10) b	363 (18) ab	486 (22) a	445 (9) b	449 (31) b
Sodium	396 (41) a	387 (25) a	436 (13) a	96 (23) a	106 (5) a	105 (5) a	97 (6) a	98 (3) a	104 (9) a
Magnesium	8 (< 1) ab	8 (1) a	7 (< 1) b	293 (28) a	306 (3) a	275 (29) a	173 (15) a	162 (12) a	157 (12) a
Calcium	7 (1) b	15 (2) a	6 (1) b	22 (2) a	22 (< 1) a	23 (1) a	27 (4) a	30 (2) a	22 (2) a
pH	8.5	7.5	7.5	8.8	7.5	7.5	8.5	7.5	7.5

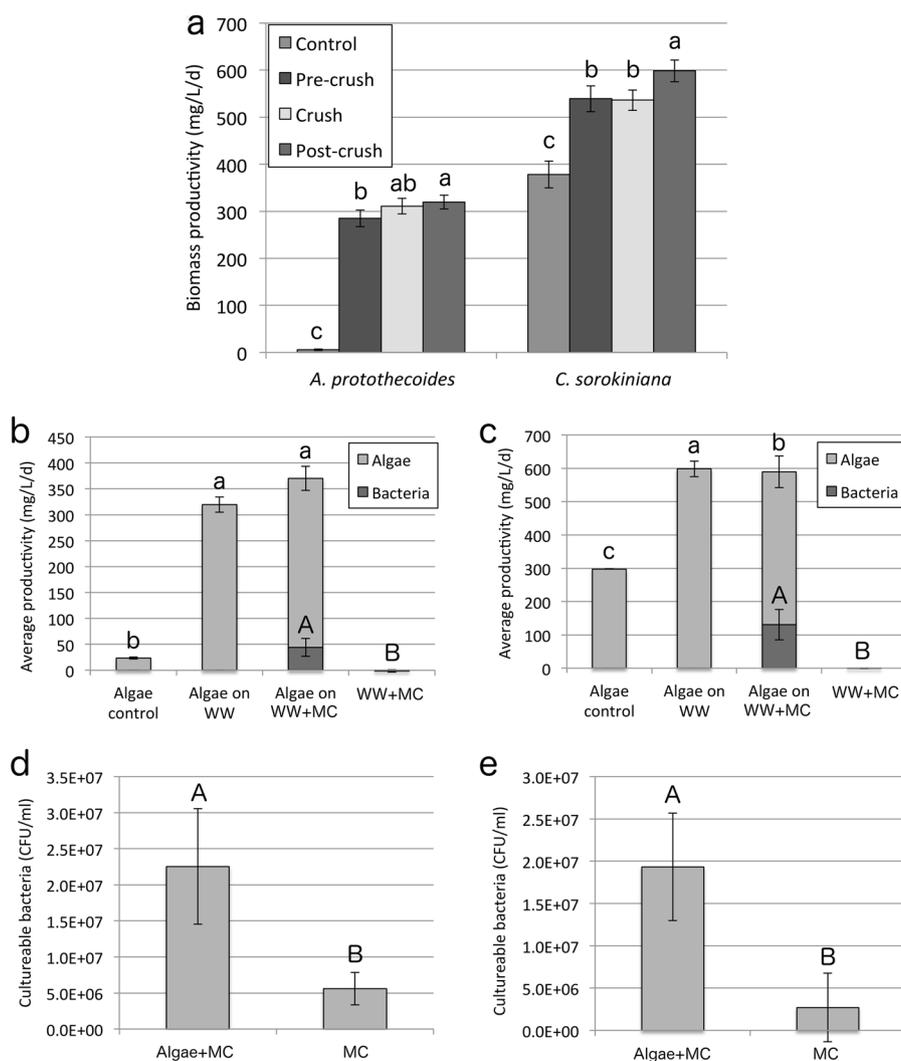
<sup>a</sup>Initial concentrations refer to filtered effluent from the oxidation tank of the winery treatment process

<sup>b</sup>Columns labeled *A. proto* and *C. soro* refer to concentrations of nutrients after 4 or 5 days of treatment with *A. protothecoides* and *C. sorokiniana*, respectively

<sup>c</sup>All concentrations are in mg/L except for pH

<sup>d</sup>Values in parenthesis are standard deviations based on  $n = 3$  (post-crush) or 4 (pre-crush and crush)

<sup>e</sup>Within each wastewater type, values followed by the same letter within a row are not significantly different at the 0.05 level



**Fig. 1** Growth of algae and bacteria on wastewater. **a** Average dry weight growth rate of *A. protothecoides* over 5 days and *C. sorokiniana* over 4 days using sterile-filtered pre-crush, crush, and post-crush winery wastewaters. Control cultures were grown on minimal media under autotrophic conditions. Parts **b–e** show microbial (MC) and algal growth on post-crush wastewater. **b** Cultivation of *A. protothecoides* on minimal medium (control), on sterile-filtered wastewater, and on raw wastewater with the native microbial community. The far right bar shows net growth of the native microbial community without algae. **c** Cultivation of *C. sorokiniana* under the same treatments as described for *A. protothecoides*. **d** Total cultureable non-algae organisms based on plate counts in the presence and absence of *A. protothecoides*. **e** Cultureable organisms in the presence and absence of *C. sorokiniana*. For all panels, error bars denote standard deviation ( $n = 3$  for post-crush and  $n = 4$  for pre-crush and crush wastewater). Lowercase letters denote significance at the 0.05 level for algae, whereas uppercase letters are used for comparison of bacterial populations

44% ( $p < 0.001$ ), whereas the mixed culture reduced COD loads by 38% ( $p < 0.001$ ). Neither the community nor algae were particularly effective at removing chloride, potassium, sodium, magnesium, or calcium ions.

#### Impact of algae on bacterial populations

Two approaches were utilized to quantify the total bacterial population in the post-crush wastewater: quantitative PCR and plate counts. The former quantifies total (living and dead) bacteria through quantitation of a segment of the 16S ribosomal RNA (rRNA) gene, and the latter captures information on viable counts of culturable organisms. Both methods showed that the presence of algae significantly increased the bacteria population size (Fig. 1b–e). Total bacterial growth rates based on PCR quantitation were  $\sim 44$  mg/L/day in the presence of *A. protothecoides* and  $\sim 130$  mg/L/day in the presence of *C. sorokiniana* compared to almost no detectable growth in the absence of algae (Fig. 1b,c).

Likewise, Colony-forming units (CFU) counts of non-algae organisms were 4–7 times higher in treatments with algae than those without (Fig. 1d, e).

Cultivation of algae on post-crush wastewater resulted in faster growth than cultivation on chemical medium, regardless of whether filtered or raw wastewater was used. The presence of bacterial cells in the raw wastewater had no significant impact on *A. protothecoides* growth but led to a 23% decline in *C. sorokiniana* growth (Fig. 1b, c) compared to sterile-filtered wastewater. However, *C. sorokiniana*'s growth rate on raw wastewater was significantly higher than that on minimal medium.

To better understand the impact of aerobic treatment and algae on the microbial community in the raw wastewater, flow-cell sequencing of the V3–V4 region of the 16S rRNA gene was employed on the Illumina MiSeq platform. Mapping reads to operational taxonomic units (OTUs) revealed that the native wastewater community was composed primarily of organisms

**Table 2.** Percent changes in nutrient concentrations in response to treatment by algae and wastewater microbial community

	<i>A. protothecoides</i>			<i>C. sorokiniana</i>		
	Algae	Algae + MC	MC <sup>a</sup>	Algae	Algae + MC	MC
COD <sup>b,c</sup>	44 (11) a	−38 (6) b	−36 (14) b	−8 (20) a	−70 (9) b	−60 (5) b
Acetate	−	−	−	−	−	−
Total N	−99 (1) b	−100 (0) b	−16 (17) a	−100 (0) b	−100 (0) b	16 (22) a
Ammonium	−100 (0) b	−100 (0) b	−14 (1) a	−100 (0) b	−100 (0) b	−16 (4) a
Nitrite	−	−	−	−	−	−
Nitrate	−	−	−	−	−	−
Phosphate	−100 (0) b	−100 (0) b	1 (1) a	−100 (0) b	−100 (0) b	20 (2) a
Sulfate	−32 (2) b	−33 (1) b	0 (0) a	−39 (5) b	−33 (5) b	2 (5) a
Chloride	5 (< 1) a	1 (2) b	4 (1) ab	−1 (7) a	1 (6) a	3 (2) a
Potassium	−6 (4) a	−4 (4) a	2 (3) a	−13 (5) a	−12 (6) a	0 (4) a
Sodium	−2 (6) a	6 (6) a	12 (7) a	0 (7) a	1 (13) a	3 (4) a
Magnesium	1 (9) a	−1 (5) a	2 (3) a	−1 (12) a	−5 (7) a	0 (4) a
Calcium	18 (13) a	6 (4) a	4 (3) a	8 (13) a	−6 (7) a	3 (2) a

Negative values show percent reduction and positive values show percent increase from initial wastewater conditions

<sup>a</sup>MC stands for microbial community

<sup>b</sup>Values in parenthesis are standard deviations based on at least  $n = 3$  biological replicates

<sup>c</sup>Within an algae species, values with the same letter in the same row are not statistically different at the 0.05 level

**Table 3.** Phylum level microbial community composition

	<i>A. protothecoides</i> <sup>a</sup>			<i>C. sorokiniana</i>		
	Original MC <sup>b</sup>	Adapted MC <sup>c</sup>	Adapted MC + algae	Original MC <sup>b</sup>	Adapted MC <sup>c</sup>	Adapted MC + algae
Actinobacteria	0.39 b <sup>d</sup>	0.63 b	4.52 a	0.45 a	0.79 a	0.80 a
BRC1	0.10 a	0.27 a	0.15 a	0.11 a	0.24 a	0.09 a
Bacteroidetes	33.43 a	18.41 b	30.25 a	29.84 a	22.38 b	28.33 ab
Chloroflexi	0.16 a	0.05 b	0.07 ab	0.15 a	0.03 a	0.09 a
Elusimicrobia	0.64	−	−	0.58 a	0.01 b	−
Firmicutes	31.66 a	1.89 b	0.50 b	33.61 a	2.24 b	0.98 b
Lentisphaerae	0.19 a	−	−	0.06 a	< 0.01 b	< 0.01 b
OD1	0.03 a	1.11 a	0.85 a	0.04 a	0.53 a	0.94 a
Proteobacteria	22.98 b	71.75 a	61.18 a	22.56 b	68.17 a	55.81 a
Spirochetes	0.40	−	−	0.44 a	< 0.01 b	< 0.01 b
Synergistetes	5.97 a	0.47 b	0.03 b	7.41 a	0.59 b	0.24 b
TM7	1.11 a	0.06 b	0.01 b	2.14 a	0.10 b	0.01 b
Tenericutes	0.09 a	< 0.01 b	−	0.09	−	−
Verrucomicrobia	1.09 a	2.76 a	2.10 a	0.63 a	2.35 a	11.38 a
WPS-2	0.33 a	< 0.01 b	0.02 b	0.53 a	0.02 b	< 0.01 b
WS6	0.15	−	−	0.21 a	< 0.01 b	−
Other	0.35	0.12	0.13	0.33	0.10	0.04
Unassigned	0.85	1.94	0.42	0.84	2.44	1.29
Shannon diversity	4.74 a	3.78 b	4.60 a	4.86 a	4.00 b	4.19 b
Richness	968 a	682 ab	509 b	858 a	689 ab	506 b
Evenness	0.69 a	0.58 b	0.74 a	0.72 a	0.61 b	0.67 ab

<sup>a</sup>The algae strain was only added to one set of bioreactors but all bioreactors under this heading were part of the same batch

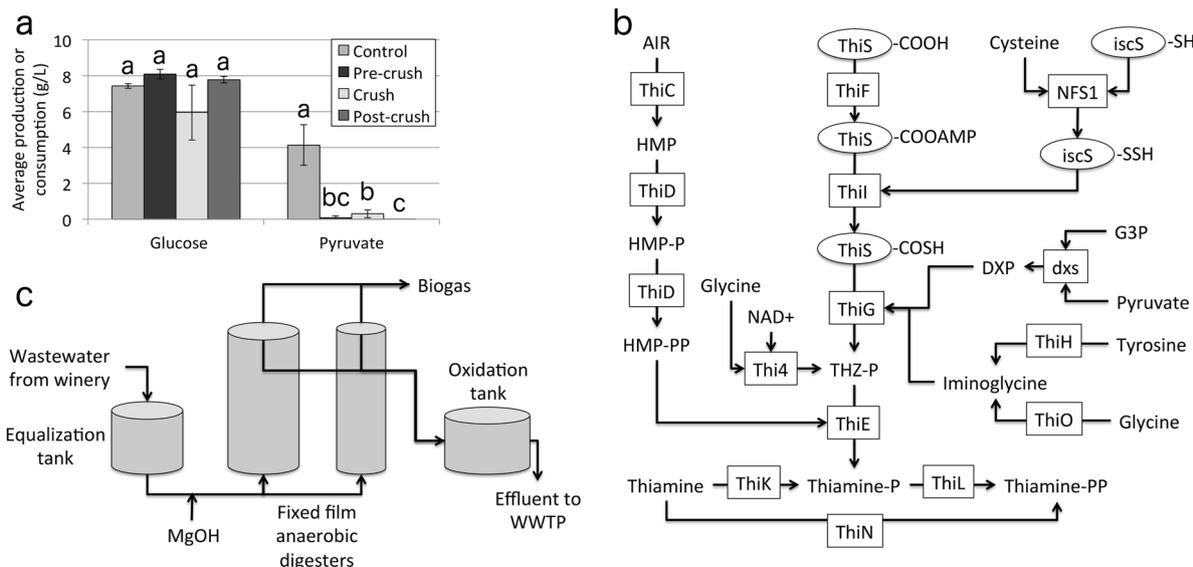
<sup>b</sup>Original community refers to microorganisms present in the wastewater prior to cultivation in aerated bioreactors

<sup>c</sup>Adapted community refers to microorganisms present after 4–5 days of culture in aerated bioreactors without algae

<sup>d</sup>Within an algae species, values with the same letter in the same row are not statistically different at the 0.05 level based on  $n = 3$  replicates

from the phyla Bacteroidetes, Firmicutes, Proteobacteria, and Synergistetes (Table 3). Cultivation of this community in the raw wastewater for 5 days in aerated bioreactors (without algae) led to a large shift in the microbial structure. This bioreactor-adapted

community exhibited dramatic reduction in the relative abundances of Firmicutes and Synergistetes and an increase in Proteobacteria. When *A. protothecoides* was cultured in wastewater with the bacterial community, the relative abundances of



**Fig. 2** Thiamine metabolites in winery wastewater. **a** Bio-assay for useable thiamine metabolites. *A. protothecoides* was cultured on minimal medium and wastewaters supplemented with 8 g/L glucose. Glucose consumption and pyruvate secretion were measured after 5 days of culture. Error bars denote standard deviation ( $n = 3$ ) and bars with the same letter are not significantly different at the 0.05 level. **b** Thiamine biosynthesis pathway using KEGG gene abbreviations. **c** Schematic of the wastewater treatment process employed at the winery. Wastewater from the plant's crushing and washing operations is pumped to the equalization tank where the acidogenesis process begins. The anaerobic digesters utilize an expanded granular sludge bed to help retain biomass inside the digesters. Digester effluent then flows to an aerated oxidation tank prior to discharge to the local municipal wastewater treatment plant. This effluent was used for algae culture studies

Bacteroidetes and Actinobacteria were higher than the bioreactor-adapted community without algae. In co-culture with *C. sorokiniana*, the relative abundances of Bacteroidetes and Verrucomicrobia nominally increased but were not statistically significant. Given these changes, SIMPER analysis was performed using the Vegan package in R to determine OTUs that contributed most to dissimilarity among treatments. This revealed specific OTUs that increased in relative abundance in response to algae (Supplementary Table S1). In the presence of *A. protothecoides*, members of the genera *Sediminibacterium* (OTU 7), *Devosia* (OTU 8), *Luteolibacter* (OUT 19), and of the order BD7-3 (OTU 16), all increased in relative abundance. In the presence of *C. sorokiniana*, a different set of organisms increased in relative abundance including members of Cryomorphaeae (OTU 3), *Phenylobacterium* (OTU 6), and BD7-3 (OTU 18).

We also searched the sequence data for signs of indicator bacterial pathogens, which is important for water re-use applications. The post-crush wastewater showed zero abundance for members of *Escherichia*, *Salmonella*, *Shigella*, and *Vibrio*. Very low relative abundance levels (<5) of *Arcobacter* were detected in the post-crush wastewater but were not detected after 5 days of culture in aerated bioreactors, regardless of the presence of algae.

#### Wastewater thiamine metabolites

We were interested in better understanding why algae grew more rapidly on wastewaters than they did on minimal medium. Past research has demonstrated that bacterial secretions can dramatically increase *A. protothecoides* growth through provision of thiamine metabolites. Given the diverse bacterial populations in the wastewaters and rapid *A. protothecoides* growth on wastewater, the presence of thiamine metabolites merited investigation. Two methods were used to determine if thiamine metabolites were present in the wastewaters: targeted liquid chromatography with mass spectrometry (LCMS) and a bio-assay. Direct measurement of five thiamine metabolites in the three wastewaters by targeted LCMS proved challenging due to instability of these metabolites in the wastewater. For instance, near complete

destruction of thiamine monophosphate (TMP) and thiamine pyrophosphate (TPP) was observed within a few hours of exogenous addition of these metabolites to the wastewater (data not shown). Thiamine can degrade into a variety of products,<sup>18</sup> of which, two potential candidates are 4-methyl-5-thiazoleethanol (THZ) and (4-amino-2-methyl-5-pyrimidine) methanol (HMP). Without accounting for the low recovery factors, quantifiable thiamine and HMP were observed in all three wastewaters. When correcting for recovery factors, concentrations of these two metabolites were in the range of 12–104 pM thiamine and 12–23 pM HMP (Table S2). These concentrations are far lower than the roughly 20 nM level required to fully alleviate thiamine limitation in *A. protothecoides* based on our previous experiments.<sup>15</sup> With an observed *A. protothecoides* growth rate on wastewater of ~300 mg/L/day, some thiamine limitation is possible but previously published dose–response studies suggest thiamine metabolite concentrations on the order of 4 nM.<sup>15</sup>

In addition to LCMS analysis, a more general biological assay for total salvageable thiamine metabolites was carried out using *A. protothecoides*. Under thiamine-limited conditions, culture of *A. protothecoides* on glucose leads to secretion of pyruvate due to suppressed pyruvate dehydrogenase activity.<sup>15</sup> *A. protothecoides* was, therefore, cultured in thiamine-free chemical medium and on all three sterilized wastewaters supplemented with 8 g/L glucose. Pyruvate secretion was measured by high-performance liquid chromatography (HPLC). The control culture secreted roughly 4 g/L pyruvate over the 5-day culture period. Wastewater cultures secreted minimal pyruvate (Fig. 2a). The crush wastewater resulted in a small (0.3 g/L) but statistically significant secretion of pyruvate ( $p = 0.045$ ). These results suggest the presence of salvageable thiamine metabolites in all three wastewaters.

#### Bacterial community cofactor synthesis capability

Based on a survey of 306 species, roughly half of all algae are likely auxotrophs for one or more B-vitamin cofactor, particularly cobalamin (B12), thiamine (B1), and biotin (B7).<sup>16</sup> In nature, algae typically obtain these cofactors from exudates of bacteria, and

**Table 4.** PICRUSt-predicted thiamine synthesis gene abundance from wastewater microbial community

Gene ID <sup>b</sup>	KEGG orthology	Gene name	<i>A. protothecoides</i> <sup>a</sup>			<i>C. sorokiniana</i>		
			Original MC <sup>c</sup>	Adapted MC <sup>d</sup>	Adapted MC + algae	Original MC <sup>c</sup>	Adapted MC <sup>d</sup>	Adapted MC + algae
ThiC	K03147	Phosphomethylpyrimidine synthase	2248 a <sup>e</sup>	1892 ab	1000 b	1537 a	1640 a	1131 a
ThiD	K00941	Hydroxymethylpyrimidine kinase	4787 a	4809 a	2882 a	3317 a	4134 a	2831 a
ThiE	K00788	Thiamine-phosphate pyrophosphorylase	3560 a	3487 a	2677 a	2483 a	3201 a	2443 a
ThiL	K00946	Thiamine-monophosphate kinase	2020 a	2490 a	2204 a	1301 a	2298 a	2168 a
ThiN	K00949	Thiamine pyrophosphokinase	1376 a	234 b	321 b	1006 a	216 b	170 b
ThiK	K07251	Thiamine kinase	14 a	< 1 b	1 b	10 a	1 b	–
Thi4	K03146	Thiamine thiazole synthase	284 a	15 b	1 b	208 a	17 b	4 b
ThiG	K03149	Thiazole synthase	1722 a	1749 a	947 a	1123 a	1480 a	1062 a
ThiH	K03150	2-Iminoacetate synthase	2290 a	349 b	294 b	1546 a	245 b	292 b
ThiO	K03153	Glycine oxidase	507 b	1218 a	471 b	341 b	1104 a	715 ab
ThiI	K03151	Thiamine biosynthesis sulfur relay system	272 a	157 b	29 c	237 a	106 b	10 c
dxs	K01662	1-Dioxy-D-xylulose-5-phosphate synthase	2923 a	2638 a	2343 a	2048 a	2420 a	2231 a
ThiF	K03148	Sulfur carrier protein ThiS adenyltransferase	436 a	7 b	2 b	263 a	8 b	3 b
iscS, NFS1	K04487	Cysteine desulfurase	2839 a	3141 a	3268 a	2045 a	2905 a	2768 a
ThiS	K03154	Sulfur carrier protein	2091 a	1749 a	901 a	1408 a	1550 a	1143 a
Total			27,370 a	23,935 a	17,340 a	18,873 a	21,323 a	16,972 a

<sup>a</sup>The algae strain was only added to one set of bioreactors but all bioreactors under this heading were part of the same batch

<sup>b</sup>Gene IDs are based on the KEGG orthology and match those shown in Fig. 2b

<sup>c</sup>Original community refers to microorganisms present in the wastewater prior to cultivation in aerated bioreactors

<sup>d</sup>Adapted community refers to microorganisms present after 4–5 days of culture in aerated bioreactors without algae

<sup>e</sup>Within an algae species, values with the same letter in the same row are not statistically different at the 0.05 level based on  $n = 3$  replicates

B-vitamin abundance is governed by the size and composition of the bacterial community. To understand the capability of the wastewater microbial community to synthesize these three cofactors given their relevance to algae growth, the metagenomic prediction tool, PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States), was applied to predict the likely abundance of key genes associated with synthesis pathways for thiamine (Fig. 2b), cobalamin (Supplementary Fig. S1), and biotin (Supplementary Fig. S2) within the wastewater microbial community.

PICRUSt analysis revealed the likely presence of complete biosynthesis pathways for all three B-vitamin cofactors within the microbial communities (Table 4, Supplementary Tables S3 and S4). Interestingly, the native microbial community typically exhibited the highest total abundance of cofactor synthesis genes, whereas the community adapted to the presence of algae typically had the lowest abundance. However, this trend was not consistent across all genes within each pathway.

An interesting result emerged in the analysis of cobalamin synthesis genes that revealed a shift from a partially anaerobic community to a more fully aerobic community after cultivation in the bioreactors. The native wastewater community had moderate abundance of genes corresponding to CbiE (K03399) and CbiT (K02191), which are unique to the anaerobic cobalamin synthesis pathway. Communities from the aerated bioreactors had almost no abundance of these genes but instead had high abundance of genes corresponding to CobN, CobS, and CobT, which are unique to the aerobic synthesis pathway. These results suggest a shift toward aerobic metabolism during culture in the bioreactors, regardless of the presence of algae.

## DISCUSSION

Culturing green algae with the native wastewater community resulted in the greatest removal of both COD and nutrient pollutants from the wastewater. In general, algae were effective at removing nutrients, whereas the microbial community consumed residual organic material including photosynthate secreted by algae. Ordinarily, algal photosynthate contributes to organic loads in wastewaters but the bacterial community could reverse this otherwise negative outcome, resulting in a net decrease in COD loads. This result is encouraging because it showed that not only were the algal and bacterial populations compatible but they also exhibited complementarity. However, none of the biological treatments could fully eliminate detectable soluble COD. The presence of algae also led to a significant increase in the total bacterial population size. This is further evidence that algal photosynthate likely provided organic carbon that supported bacterial growth. The shift in bacterial population composition in response to specific algae species also suggests an adaptation of the community to the specific photosynthate of each species. However, further research is required to characterize the photosynthate and elucidate bacterial metabolism of specific secretions.

One concern that may arise from this result is the potential for increased growth of wastewater pathogens due to the presence of algae. This was not specifically tested in this study. However, our previous result from co-culturing *A. protothecoides* with *E. coli* showed that *E. coli* populations were relatively unaffected by the presence of algae.<sup>13</sup> Research on algal treatment of municipal wastewater has shown that algae cultivation can reduce coliform population size.<sup>19,20</sup> It is also well-established that anaerobic digester treatment can suppress human pathogen population

sizes,<sup>21,22</sup> however, recent literature suggests that many pathogens enter a viable but non-culturable state during digestion.<sup>23</sup> Thus, it would be important to implement a pathogen monitoring program for wastewater treated by anaerobic digestion followed by algae cultivation. This is particularly relevant if the water will be re-used in agricultural settings.

Acting alone or in conjunction with bacteria, algae were able to remove significant portions of nitrogen, phosphorus, and sulfur nutrients from the wastewaters. This finding is consistent with the results of other research efforts in which algal treatment led to complete or nearly complete nutrient removal from wastewater.<sup>8–10</sup> Within this broad observation, an interesting trend emerged. The uptake ratios of total nitrogen and phosphate in algae cultures were nearly 3:1 in the pre-crush and crush wastewaters but were 1.8:1 in the post-crush wastewater. This was true for both algae species. The same N:P uptake ratio was observed in post-crush wastewater regardless of whether algae were cultured alone or in the presence of the microbial community. The difference in N:P uptake ratio is most likely due to the lower initial N:P ratio of the post-crush wastewater. The Redfield ratio suggests algal N:P ratios of 16:1 on a molar basis,<sup>24</sup> which translates to a total nitrogen:phosphate ratio of 2.4. In reality, algae exhibit flexible nutrient uptake and elemental composition based on environmental conditions.<sup>25</sup> For example, it is well-established that many algae species, including *A. protothecoides* and *C. sorokiniana*,<sup>14</sup> respond to nitrogen limitation by reducing protein and increasing lipid production,<sup>26</sup> thereby reducing the nitrogen content of their biomass.

In contrast to nutrient and organic matter removal, none of the biological treatments were effective at removing ions that frequently contribute to salinity such as sodium and chloride. Salinity is an important factor in cases where stakeholders, such as the collaborating winery in this study, wish to utilize wastewater for irrigation.<sup>27,28</sup> The wastewaters in the present study were highly variable across different parts of the crush season with sodium concentrations varying from 97 to over 400 mg/L. Without substantial dilution, these concentrations generally exceed acceptable limits for fruit crop irrigation.<sup>29</sup> The water may be acceptable for saline-tolerant crops such as sunflowers and sugar beets.<sup>29</sup> Chloride concentrations were consistently <70 mg/L and are generally considered safe for all plants.

Given improved algae growth on wastewater compared to minimal medium, we were interested in understanding the source of this growth promotion. Faster-growing algae not only increase nutrient removal rates but also facilitate more cost-effective production of biofuels from wastewater-grown algae.<sup>30</sup> Because growth enhancement could be achieved in sterile-filtered wastewater, (bio)chemical constituents in the wastewater were the likely source of growth promotion. Increased algae growth on wastewater is frequently attributed to mixotrophic growth due to organic material found in wastewaters.<sup>31</sup> However, relatively low (<300 mg/L) COD levels were measured in the winery wastewaters. The crush wastewater had the highest COD level and also contained 344 mg/L residual acetic acid. Both algae strains consumed all acetate within 24 h but given the low concentrations, mixotrophy is unlikely to fully explain the robust algae growth on the three wastewaters, particularly in the case of *A. protothecoides*. In a previous study, the addition of 500 mg/L of acetate to an axenic *A. protothecoides* culture only increased growth rates by 2.4-fold<sup>13</sup> compared to the roughly 13-fold increase observed on winery wastewater. Nutrient abundance also cannot explain the growth promotion, as the N8-NH<sub>4</sub> minimal medium had higher ammonium and phosphate concentrations than all three wastewaters.

Our previous research on algal–bacterial interaction revealed important synergies related to thiamine metabolism that resulted in faster algae growth and lipid production.<sup>15</sup> While the presence of live bacteria in wastewater did not improve algae growth in this

study (and slightly repressed it in the case of *C. sorokiniana*), it is likely that bacterial exudates in the wastewater had a positive effect on algae growth, particularly in the case of *A. protothecoides*. Such exudates persist in water even after removal or death of the organisms that created them, thus co-culture with live bacterial cells is not necessarily a prerequisite for improved algae growth. Past research on a model co-culture system showed that exudates from *E. coli* contained thiamine metabolites, which could increase *A. protothecoides* growth rates 10–30-fold.<sup>15</sup> That work also showed that *A. protothecoides* could obtain similar benefits from degraded thiamine products. Therefore, detecting thiamine metabolites in exudates from wastewater bacteria were of particular interest in the present study. The bio-assay and LCMS results suggest that the winery wastewaters contained utilizable thiamine metabolites, despite significant degradation. This further underscores our previous finding that *A. protothecoides* can salvage thiamine from degradation products. *C. sorokiniana* is not a thiamine auxotroph but did show modest growth enhancement when provided with exogenous thiamine in previous research.<sup>15</sup> Thus provision of cofactors by the wastewater community could have partially contributed to the increased growth of this species on wastewater. Nevertheless, other mechanisms could also play a role in algal growth promotion on wastewater: bacterial provision of algal-growth-promoting hormones<sup>32,33</sup> or the provision of other non-thiamine cofactors.

We did not specifically assay for other vitamin cofactors but predicted metagenomes from PICRUSt analysis suggest that the wastewater microbial communities contained complete synthesis pathways for thiamine, biotin, and cobalamin. These cofactors have been identified as particularly relevant to algae: in a survey of 306 algal species, 51% required cobalamin, 22% required thiamine, and 6% required biotin.<sup>16</sup> In nature, algae typically obtain these cofactors from bacteria, as indicated by a number of studies.<sup>17,34</sup> Paerl et al.<sup>35</sup> demonstrated the potential of *Pseudomonas* sp. to transform TPP into a form useable by the green algae *Ostreococcus lucimarinus*, a known thiamine auxotroph. In another study, marine bacteria were shown to produce a thiazole precursor used by *Ostreococcus* species to synthesize thiamine.<sup>36</sup> These findings are analogous to our past findings in co-cultures of *E. coli* with *A. protothecoides* and indicate the widespread importance of cofactor symbiosis in promoting algae growth.

Complete cofactor synthesis pathways in the microbial community may not be required to support auxotrophic algae growth due to the prevalence of partial synthesis and salvage pathways in algae such as those for thiamine mentioned previously. With regard to cobalamin, bacteria in consortia are known to remodel corrinoids obtained from other species,<sup>37</sup> however, studies on algae are limited. One recent study has shown that some eukaryotic algae can convert pseudocobalamin into cobalamin when provided with 5,6-dimethylbenzimidazole.<sup>38</sup> In light of salvage mechanisms, we predict that the winery wastewater could support a wide range of algae species, including a variety of B-vitamin auxotrophs, which are estimated to comprise half of algae species.<sup>16</sup> This opens the possibility of engineering a process that employs specific algae species with desired co-product characteristics. It also allows for cultivation of algal polycultures, which hold the promise of greater process stability through species complementarity.<sup>39</sup> Synergies between algae and bacteria should be a top consideration when designing suspended-cell processes for wastewater treatment.

## METHODS

### Wastewater collection and preparation

Wastewater was obtained from a treatment plant located at a large winery in California (Fig. 2c). This on-site plant primarily treats wastewater from the winery's crushing and tank washing operations. The effluent from the

on-site treatment plant is then sent to a local municipal wastewater treatment plant for additional treatment at a significant cost to the winery.

Wastewaters were collected in clean plastic containers from a sampling port exiting the oxidation tank at the winery's facility. Wastewater was kept on ice for transport back to the lab at UC Davis. Thereafter, wastewaters were immediately frozen at  $-20^{\circ}\text{C}$  until use. Thawed wastewater was used directly for the non-axenic cultures. Sterile wastewaters were prepared by centrifuge to remove large particulates followed by filtration first through a  $0.45\ \mu\text{m}$  filter and then through a  $0.2\ \mu\text{m}$  sterile filter to remove bacteria.

### Strain selection

*A. protothecoides* (UTEX 2341)<sup>40</sup> and *C. sorokiniana* (UTEX 2805) are model green algae and were selected for this study. Both strains grow rapidly and remove ammonium and phosphate from culture medium, important criteria for nutrient removal from wastewater. *C. sorokiniana* was isolated from a wastewater treatment pond in Mexico and is also capable of utilizing nitrate.<sup>41</sup> *C. sorokiniana* accumulates intracellular starch, which has value for biofuel production from wastewater-grown algae.<sup>42</sup> *A. protothecoides* accumulates neutral lipids and past work revealed that *E. coli* stimulated increased acetate and nutrient uptake by this strain.<sup>13,14</sup> There was interest in whether wastewater bacteria could stimulate a similar response.

### Culture experiments on wastewater

A series of culture experiments were carried out in which algae were cultured on sterile-filtered wastewater and also raw wastewater using the specific procedures described in the following section. In the first set of experiments, *A. protothecoides* and subsequently *C. sorokiniana* were cultured on sterile-filtered pre-crush, crush, and post-crush wastewater alongside autoclaved minimal medium as a control. These experiments revealed the "algae-only" contribution to wastewater treatment. Subsequently, the post-crush wastewater was selected for the study of algae growth on raw wastewater to better understand interactions with live bacteria. The reason post-crush wastewater was selected was to ensure that freshly collected wastewater was used, given the time-sensitive nature of its microbial community. In this round of culture experiments, first *A. protothecoides* and then *C. sorokiniana* were cultured on sterile filtered and raw post-crush wastewater. An additional set of reactors containing only raw wastewater (and its associated community) was also included in each of these experiments. Finally, algae cultured on autoclaved minimal medium served as a control for algae growth. Four biological replicate cultures were used per experimental treatment for pre-crush wastewaters. Three biological replicates per experimental treatment were used for post-crush wastewaters. These values were found to give sufficient power based on previous culture studies on winery wastewater. For all culture experiments, cultures were handled using sterile technique and samples were taken daily for optical density, pH, and HPLC analysis of the filtered ( $0.2\ \mu\text{m}$ ) wastewater. Additional samples were taken at culture harvest for total nitrogen and COD analysis. For cultures in raw wastewater, additional samples were taken on the first and last day of the culture period for viable bacteria enumeration by plating.

### Algae and bacterial culture procedures

Algae pre-cultures were established in sterile 1 L bioreactors from colonies scraped from ATCC #5 agar plates. *A. protothecoides* was cultured on  $\text{N8-NH}_4$  medium<sup>13</sup> and *C. sorokiniana* was cultured on N8 medium<sup>42</sup> until cell density reached roughly OD550 of 0.2, which corresponds to 0.11 and 0.13 g/L for *A. protothecoides* and *C. sorokiniana*, respectively. Pre-cultures were supplied with 400 mL/min sterile-filtered 2%  $\text{CO}_2$  mixed with air and illuminated by T5 growth lamps (10,000 lux operating on a 16:8 light/dark cycle). Algae were allowed to settle in the bioreactor for 24 h and residual medium was removed first by aspiration and then by centrifugation and decanting of supernatant. Algae pre-cultures were then used to inoculate a set of 12 sterile 300 mL hybridization tubes containing 200 mL of various wastewaters or control media. Inoculation density was roughly OD550 of 0.16. Full factorial designs were used with a minimum of three biological replicates for each treatment.

Hybridization tubes were suspended in a temperature-controlled water bath ( $28^{\circ}\text{C}$ ) and mixed by magnetic stir bar ( $\sim 150\ \text{rpm}$ ). The same illumination scheme as the stock cultures was used. A mixture of 2%  $\text{CO}_2$  and air was sterile filtered and supplied to each reactor at a rate of 125 mL/min. Cultures were grown to log phase (4–5 days) and reactors were rotated to new incubator positions each day. At the end of the culture

period, cells were harvested by centrifugation at 6000 rcf for 5 min. Algae harvested by centrifuge were washed three times with  $\text{dH}_2\text{O}$  to remove medium components and then freeze-dried to obtain a final "harvestable" dry weight.

### Viable bacterial counts in wastewater

For non-axenic algae cultured on raw post-crush wastewater, additional samples were taken on the first and last day of the culture period for plating on ATCC #5 agar. This agar contains a rich variety of metabolites from yeast extract, beef extract, and tryptose and can support a range of bacteria and algae. Samples were plated at a range of dilutions and CFUs were counted to determine viable and culturable organisms. Only non-axenic species were tabulated (algae typically take  $> 5$  days to appear on plates).

### High-pressure liquid chromatography analysis of wastewater

HPLC and ion chromatography for wastewater analyses were carried out on a Shimadzu Prominence instrument (20 series) equipped with a 10 series pump. Organic acid analysis, including acetic and pyruvic acids, was carried out using an Aminex 87H column (Bio-Rad). Mobile phase was 5 mM sulfuric acid in MilliQ water with flow rate set to 0.6 mL/min. Shimadzu RID (10A) and PDA (20A) detectors were used. Suppressed cation chromatography was performed using a  $4 \times 250\ \text{mm}$  CS14 column (Dionex), CERS500 suppressor, and Shimadzu conductivity detector (CDD-10AVP) based on the method described by Yu et al.<sup>43</sup> Mobile phase was 10 mM methanesulfonic acid in MilliQ water with a flow rate of 1 mL/min. Anion chromatography was performed using a  $4 \times 250\ \text{mm}$  AS22 column (Dionex), AERS500 suppressor, and conductivity detector. Mobile phase was a solution of 4.5 mM sodium carbonate and 1.5 mM sodium bicarbonate in MilliQ water. Chromatograms were analyzed in LC Solutions software (v. 1.23).

### COD and nitrogen assays

Samples were collected at the beginning and end of the culture period and filtered ( $0.2\ \mu\text{m}$ ) to remove cells. Each assay kits for COD (high range) and total nitrogen (low range) were used and sample vials were read in a DR/890 colorimeter. Procedures followed the manufacturer's instructions; distilled water was used for blanks and dilutions.

### Extraction of thiamine metabolites and targeted LCMS

Sterile-filtered wastewater (35 mL) was freeze-dried and extracted with methanol to recover thiamine metabolites per a previously established procedure.<sup>15</sup> Recovery standards were prepared with known concentrations of five thiamine metabolites: thiamine-HCl, TMP, TPP, HMP, and THZ. Recovery rates for TMP, TPP, and THZ were low (Supplementary Table S1) so C18 solid-phase extraction approach was employed based on the method developed by Okbamichael and Sañudo-Wilhelmy for B-vitamin concentration in seawater.<sup>44</sup> This method worked reasonably well for standards prepared in phosphate buffer but substantial losses of all metabolites were observed for recovery standards prepared in wastewater. Thus, the original methanol extraction method was used for the final analysis of thiamine and HMP, which had higher recovery efficiencies than the other metabolites.

Targeted LCMS analysis of thiamine metabolites in wastewater extracts was performed on a 1200 series LC (Agilent) coupled to an AB Sciex 4000 QTrap LC/MS/MS system. A HILIC method was used for separation and MRM setpoints were established to quantify thiamine, TMP, TPP, HMP, and THZ in the samples as described previously.<sup>15</sup>

### Bio-assay for determination of useable thiamine metabolites

A bio-assay for useable thiamine was carried out using *A. protothecoides*. This organism secretes large quantities of pyruvate into the culture medium when cultured on glucose in the absence of useable thiamine metabolites. This is due to limited functionality of its pyruvate dehydrogenase complex in the absence of the cofactor TPP. For this study, *A. protothecoides* was grown on all three wastewaters, supplemented with 8 g/L glucose. As a positive control, it was also cultured on  $\text{N8-NH}_4$  medium (which contains no thiamine) supplemented with 8 g/L glucose. Culture samples were obtained daily, centrifuged, and the supernatant was filtered through  $0.2\ \mu\text{m}$  filters. Filtered samples were analyzed by HPLC to quantify glucose uptake and pyruvate secretion.

## DNA extraction and qPCR for bacterial analysis

DNA was extracted from harvested cultures grown on post-crush wastewater. These cultures included raw wastewater prior to bioreactor culture, raw wastewater after bioreactor culture (without algae), and algae cultured in raw wastewater. DNA extraction was performed on harvested freeze-dried biomass using the Fast DNA Spin Kit (MP Biomedicals). Procedures followed recommendations in the kit. Specifically, 1.5 mg of freeze-dried biomass was suspended in 200  $\mu$ L of distilled water and vortexed. Since the goal of this extraction was to recover bacterial DNA for quantification and sequencing, 1 mL of the bacterial cell lysis buffer in the Fast DNA Spin Kit was then added to the biomass suspension. Lysing matrix A tubes were used to homogenize the samples twice in a FastPrep instrument (Savant) at 6 m/s for 30 s. Homogenized samples were incubated at room temperature for 30 min per the manufacturer's recommendation for dry biomass. Remaining steps followed kit instructions.

A small portion (1  $\mu$ L) of the extracted DNA was used to perform quantitative PCR on a segment of the 16S rRNA gene. The goal of this analysis was to quantify the total bacterial fraction of biomass in samples where algae were cultured in raw wastewater. Similar analysis on co-cultures of *A. protothecoides* and *E. coli* has been employed in past work with validated results.<sup>13</sup> In that case, an *E. coli*-specific primer was used. In the present analysis, the goal was amplification of total bacterial 16S rRNA genes and thus only conserved gene regions could be used for primer development. The challenge with this approach is that amplification of conserved regions can also result in amplification of algal chloroplast genes. This contamination could be minimized through use of the 799F-mod3 (5'-CMGGATTAGATACCCCKGG-3') and 926R (5'-CCGTCAATTCMTT-TRAGTTT-3') primers as recommended by Hanshaw et al.<sup>45</sup> The 799F-mod3 primer has been shown to effectively resist amplification of chloroplast 16S rRNA genes with < 1% chloroplast amplification in a 50–50 mock mixture of chloroplast and bacterial DNA.<sup>45</sup> Quantitative PCR was carried out on a StepOnePlus instrument (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). Template DNA was diluted tenfold and 2  $\mu$ L was added to the reaction tube. Reaction volume was 25  $\mu$ L and primer concentrations were 100 nM. The thermocycler ran for 35 cycles: 95 °C for 15 s, 47 °C for 15 s, and 72 °C for 45 s. PCR product size (~130 bp) was confirmed by gel electrophoresis using a 2% agarose gel with a 100 bp ladder. Threshold cycle methodology was employed to quantify 16S rRNA genes using serial dilutions of previously amplified 16S rRNA gene product. In all, 16S rRNA gene quantity was correlated to the dry weight of bacterial cell mass in the raw wastewater (without algae). Using this correlation, the total bacterial population in the mixed alga-bacteria samples was quantified. One assumption of this approach is that the bacterial community was relatively unaffected by the presence of algae. Microbial community analysis revealed some statistically significant population shifts in response to algae but the magnitudes of the shifts were not dramatic (see Table 3).

## 16S rRNA gene sequencing and microbial community analysis

The remaining extracted DNA was submitted to CD Genomics (Shirley, NY) for flow-cell sequencing on an Illumina MiSeq instrument. In all, 300 bp paired-end reads were obtained for the V3–V4 region (~250 bp) of the 16S rRNA gene. Reads were quality-filtered and demultiplexed by CD Genomics using a proprietary software pipeline. The resulting FASTA files were then loaded into QIIME (Quantitative Insights Into Microbial Ecology, v. 1.9.1) for removal of chimeric sequences with usearch v. 6.1. The cleaned FASTA file was then used for OTU picking against the Greengenes database (v. 13.5) at 97% identity. Two different OTU picking procedures were used: closed-reference OTU picking was used for subsequent PICRUSt analysis, whereas open-reference OTU picking was used for microbial community analysis. Supplementary Table S5 shows mapping rates for both methods. Singletons and chloroplast hits were removed from the dataset prior to further analysis. The Vegan package (v. 2.4–1)<sup>46</sup> in R was used to calculate Shannon diversity, richness, and evenness of microbial communities. Vegan was also used to calculate the contributions of specific OTUs to Bray-Curtis dissimilarity using SIMPER analysis.

## PICRUSt prediction of metagenomes

The metagenome prediction tool PICRUSt (v. 1.0.0) was used to infer gene functions available to the wastewater microbial community. This tool determines genes likely to be present in a reference OTU tree (from Greengenes) through ancestral state reconstruction and the use of a

reference genome database.<sup>47</sup> Closed-reference OTUs picked in QIIME were subsequently imported into PICRUSt. Because only 70% of the 209,580 total sequences mapped to reference OTUs at 97% identity, the PICRUSt analysis is likely to be conservative. One of the most important metrics for accurate metagenome predictions is the similarity between environmental organisms and the suite of reference genomes used in the PICRUSt algorithm. This dissimilarity can be quantified by the nearest sequence taxon index (NSTI) value with values below 0.18 resulting in reasonably accurate predictions.<sup>47</sup> Of the 12 samples analyzed in this study, all but one had an NSTI below 0.18 with an average score of 0.15 (Supplementary Table S6).

## Statistical analysis

Basic statistics including mean, standard deviation, and independent group 2-sided *t*-tests were performed in Microsoft Excel. ANOVA and multiple comparison tests were performed in R (v. 3.1.1)<sup>48</sup> using the “car” and “agricolae” packages. Homogeneity of variance was tested using both Levene's test and the Brown and Forsythe test. Where these tests showed significance, data were transformed using established methods<sup>49</sup> to reduce heteroscedasticity prior to application of ANOVA. No data points from culture replicates were discarded from the datasets. Analytical assays were repeated additional times if an anomalous value was observed.

## Data availability

This Targeted Locus Study project has been deposited at DDBJ/ENA/GenBank under the accessions KBPZ00000000, KBQA00000000, KBQB00000000, KBQC00000000, KBQD00000000, KBQE00000000, KBQF00000000, KBQG00000000, KBQH00000000, KBQI00000000, KBQJ00000000, KBQK00000000, KBQL00000000, KBQM00000000, KBQN00000000, KBQO00000000. The versions described in this paper are the first versions, KBPZ01000000, KBQA01000000, KBQB01000000, KBQC01000000, KBQD01000000, KBQE01000000, KBQF01000000, KBQG01000000, KBQH01000000, KBQI01000000, KBQJ01000000, KBQK01000000, KBQL01000000, KBQM01000000, KBQN01000000, KBQO01000000. In addition, the chimera-cleaned multi-line FASTA file obtained from 16S rRNA sequencing is publicly accessible at (<https://www.dropbox.com/sh/a4ffenzaxx4ein/AAB7XIMJO5HL9CAHJz8btSha?dl=0>). Other data types are available upon request.

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## AUTHOR CONTRIBUTIONS

B.T.H. and J.S.V. designed the experiments. B.T.H. and S.J.C. conducted experiments. B.T.H. analyzed sequencing and LC datasets. I.G., P.S.F., and O.F. carried out LCMS analysis. B.T.H. wrote the manuscript. J.S.V. and S.J.C. also contributed to the manuscript.

## ADDITIONAL INFORMATION

**Supplementary information** accompanies the paper on the *npj Clean Water* website (<https://doi.org/10.1038/s41545-018-0005-y>).

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