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Wastewater polishing by a channelized macrophyte-dominated wetland and anaerobic digestion of the harvested phytomass

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Constructed wetlands (CW) offer a mechanism to meet increasingly stringent regulatory standards for wastewater treatment while minimizing energy inputs. Additionally, harvested wetland phytomass subjected to anaerobic digestion can serve as a source of biogas methane. To investigate CW wastewater polishing activities and potential energy yield we constructed a pair of secondary wastewater-fed channelized CW modules designed to retain easily harvestable floating aquatic vegetation and maximize exposure of water to roots and sediment. Modules that were regularly harvested averaged a nitrate removal rate of 1.1 g N m^{-2} d^{-1}; harvesting, sedimentation and gasification were responsible for 30.5%, 8.0% and 61.5% of the N losses, respectively. Selective harvesting of a module to maintain dominance of filamentous algae had no effect on nitrate removal rate but lowered productivity by one-half. The average monthly productivity for unselectively harvested modules was 9.3 ± 1.7 g dry wt. m^{-2} d^{-1} (±SE). Cessation of harvesting in one module resulted in a significant increase in nitrate removal rate and decrease in phosphate removal rate. Compared to the influent, the effluent of the harvested module had significantly lower levels of estrogenic activity, as determined by a quantitative PCR-based juvenile trout bioassay, and significantly lower densities of *E. coli.* In mixed vertical-flow reactors anaerobic co-digestion of equal dry weight proportions of harvested aquatic vegetation, wine yeast lees and dairy manure was greatly improved when the manure was replaced with the crude glycerol by-product of biodiesel production. Remaining solids were vermicomposted for use as a soil amendment. Our results indicate that incorporation of constructed wetlands into an integrated treatment system can simultaneously enhance the economic and energetic feasibility of wastewater and organic waste treatment processes.

Keywords: Anaerobic co-digestion, aquatic weeds, biofuel, biogas, constructed wetland, denitrification, endocrine disruptors, methanogenesis, nutrient removal.

Introduction

Constructed wetlands have been increasingly employed in recent decades to carry out wastewater polishing activities, including removal of residual nitrate, phosphate, estrogenic compounds and pathogenic microorganisms.[1,2] Declines in nitrate across wetlands occur primarily as a consequence of its conversion into N_2 gas by heterotrophic denitrifying bacteria dependent on organic compounds from the vegetation.[3] In contrast, removal of phosphate in wetlands occurs primarily through assimilation and sorption to soil and sediment.[2] Organic pollutants, such as endocrine disrupting compounds, are mineralized or otherwise inactivated by a combination of biological and physiochemical activities present in constructed wetlands.[1] Pathogens adapted for an enteric existence encounter a range of stresses in wetlands that negatively impact their survival.[4]

Management of wetlands may include regular harvesting of vegetation to maintain their hydraulic characteristics. Subjection of the harvested biomass to anaerobic digestion is a relatively simple means to extract usable energy from the material. Higher yields of methane are generally gained by co-digesting a mixture of substrates.[5-8] The solids remaining after anaerobic digestion can be applied directly as a soil amendment or may be further stabilized by aerobic composting prior to application to the soil.[9]

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To examine the potential for integration between wastewater polishing and anaerobic digestion and thereby improve the overall efficiency of wastewater treatment we have constructed an experimental demonstration system that consists of three primary components: (1) a pair of secondary wastewater-fed constructed wetland modules designed to maintain floating phytomass; (2) a pair of vertical downflow anaerobic digestion (AD) reactors; and, (3) a series of raised beds for application of composted digestate from the AD reactors. For this report we investigated removal of nitrate, phosphate, *Escherichia coli*, and estrogenic activity from water that flows through the wetland modules. In addition we monitored production of phytomass from the wetland and assessed its suitability as an AD feedstock when combined with wine lees and dairy manure or crude glycerol from biodiesel production.

Materials and methods

Wetland design and biological composition

In the summer of 2007 two parallel experimental wetland modules were constructed (R.S. Duckworth Construction, Sebastopol, CA) on the grounds of the City of Santa Rosa Laguna Treatment Plant (Fig. 1A). Water flows by gravity through each module, composed of three 6-m long channels lined with ethylene propylene diene monomer (EPDM) sheeting. Each top channel has a width of 2.4 m, and the two sets of bottom channels have widths of 1.8 m. From top to bottom, the channels have depths of 20 cm, 12 cm and 46 cm, respectively. Outflow from each channel exits through a standpipe surrounded by an 8 to 20-cm deep weir to select for maintenance of floating vegetation. All channels were stocked with mosquitofish (*Gambusia affinis*). The top and middle channels were stocked with vegetation native to local waterways, including filamentous algae (*Oedogonium* sp.), duckweeds (*Lemna* spp. and *Spirodela* sp.), mosquito fern (*Azolla filiculoides* Lam., hereafter "azolla") and the bottom channel with water pennywort (*Hydrocotyle ranunculoides* L.f., hereafter "hydrocotyle"). Other algae and cyanobacteria that later became apparent in the channels included species of the genera *Spirogyra*, *Hydrodictyon*, *Pediastrum, Anabaena*, *Pseudoanabaena*, and various pennate diatoms.

Wetland operations

The system was initiated with tertiary wastewater, but to avoid potential confounding influences of chlorination the source was changed to secondary wastewater beginning in March 2008. Source water was pumped from 7 a.m. to 8 p.m. daily through adjustable valves into both modules to give an approximate one-day retention time, assuming idealized plug flow fluid movement through the channels. Average flow rates for specific experimental periods described in this study are provided in Table 1.

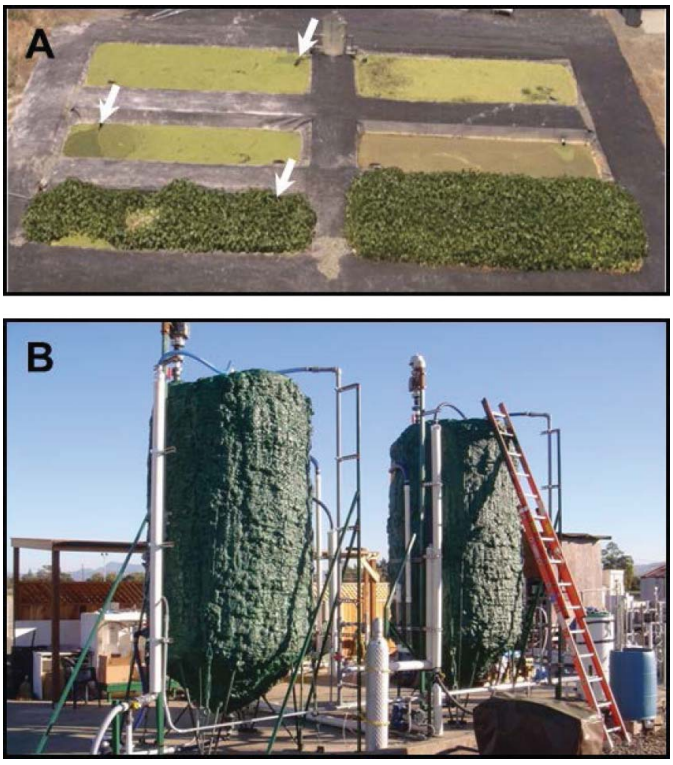

Fig. 1. Study sites on the grounds of the City of Santa Rosa Treatment Plant. (A) Aerial view of wastewater-fed constructed wetland with arrows indicating direction of flow of secondary wastewater into the channels for the west module, photo taken 28 July 2009 by L. Small. (B) Experimental AD reactors (color figure available online).

Small aquatic vegetation was harvested with a pool skimmer net, and the larger hydrocotyle was harvested by hand. The modules were harvested 1 to 3 times weekly depending on the season to maintain a growth state with the exception of a 130-d period beginning in April 2008 (Table 1) in which harvests were conducted 4 to 5 times per week to maintain >95% algal coverage in the west module, and the aquatic vegetation in the east module was indiscriminately harvested. Equivalent conditions were re-established in both the modules following a treatment period by reciprocal transfers of vegetation and sediment as described in Hare.[10] For one year, beginning in March 2009 (Table 1), there was no harvesting from the east module, yet indiscriminate harvesting from the west module continued.

Harvesting protocol depended on vegetation type. During the period in which algal dominance was maintained in the west channels mats were harvested to leave 60–70% coverage by removal of randomly distributed patches. In harvesting channels containing mixed vegetation near complete coverage remained even after harvesting since the duckweeds and azolla that remained immediately spread to occupy the newly opened water surfaces. Therefore,

Table 1. Constructed wetland module harvesting protocols and flow rates during the experimental periods described in this report.

		Harvesting protocol; flow rate (mean ± SE)[b]	
Period	*Experimental monitoring*[a]	*West module*	*East module*
12 April 2008–20 August 2008	Algae vs. mixed vegetation effect on nitrate removal	Selectively harvested to maintain algal dominance; 247.9 ± 22.0 L m^{-2} d^{-1} ($n = 17$)	Unselectively harvested; 241.8 ± 22.0 L m^{-2} d^{-1} ($n = 17$)
20 August 2008–23 September 2008	Equilibration period (data not used for analysis)	Unselectively harvested, vegetation exchanged between modules; 339.4 ± 12.8 L m^{-2} d^{-1} ($n = 5$)	Unselectively harvested, vegetation exchanged between modules; 343.2 ± 13.1 L m^{-2} d^{-1} ($n = 5$)
23 September 2008–18 March 2009	Nitrate removal	Unselectively harvested; 142.7 ± 23.3 L m^{-2} d^{-1} ($n = 25$)	Unselectively harvested; 136.6 ± 24.6 L m^{-2} d^{-1} ($n = 25$)
18 March 2009–16 March 2010	Harvest vs. no harvest effect on nitrate removal	Unselectively harvested; 260.7 ± 12.0 L m^{-2} d^{-1} ($n = 49$)	Not harvested; 265.0 ± 11.9 L m^{-2} d^{-1} ($n = 49$)
3 November 2009–19 October 2010	Phosphate removal	Unselectively harvested; 248.5 ± 10.0 L m^{-2} d^{-1} ($n = 24$)	Not harvested; 262.5 ± 10.0 L m^{-2} d^{-1} ($n = 24$)
10 June 2011–17 November 2011	Total coliforms and *E. coli*	Unselectively harvested; 252.0 ± 3.0 L m^{-2} d^{-1} ($n = 15$)	Unselectively harvested; 231.7 ± 3.0 L m^{-2} d^{-1} ($n = 15$)

[a]The nitrate and phosphate concentrations in the influent during periods in which their removal rates were monitored were 14.9 ± 3.4 mg N L^{-1} (mean ± SD, $n = 96$) and 3.12 ± 0.87 mg P L^{-1} (mean ± SD, $n = 24$), respectively.
[b]Average daily flow rate ± SE includes the 13 h of flow and 11 h of no flow.

harvesting was carried out to achieve a post-harvest coverage of 59 ± 9.1 g dry wt. m^{-2} (mean ± SE; n = 3)[10], similar to the 61 g dry wt. m^{-2} value reported by Koles et al.[11] to allow for maximum continuous production of a duckweed culture.

Physicochemical analyses

Sampling and environmental sensors

Water samples were collected at least 24 h after harvesting. Samples of module influent and effluent were initially obtained as 200 mL grab samples. On 31 March 2009 an autosampler (Sigma 900 Max Portable Sampler) was placed at the outflow of each module. On the day before conducting chemical analyses each autosampler was programmed to collect 150 mL samples every 15 min into an ice-cooled receptacle over the course of the 13 h that water flowed into the module.

Sediment was sampled by pressing a 10 cm-diameter silicone-edged PVC pipe to the channel bottom and suctioning off the pipe contents into sampling containers. The samples were stored at 4°C for up to 1 week before conducting density and dry weight analysis.

For calculation of evapotransporation rates, solar radiation, air temperature, relative humidity and wind speed data were downloaded from the California Irrigation Management Information System (CIMIS) automated weather station located 2 km north of the modules.[12]

Water nutrient analysis

Samples were analyzed for N-nitrate by the Laguna Environmental Laboratory using EPA Method 300.0 (SOP Rev. 4) on a Dionex Ion Chromatograph (Model 2200). Detailed procedures for determining average influent nitrate concentration and normalizing effluent grab and composite nitrate concentration data are described in Hare.[10]

For ammonia analysis, composite 24-h influent and 13-h effluent samples were preserved in sulfuric acid and ammonia was measured by the Laguna Environmental Laboratory using the selective electrode method with known addition (Standard Methods 20th Ed. 4500 - NH_3 E). Sampling was conducted on 6 and 20 October 2009, 17 November 2009, and 23 February 2010. Phosphate concentration was determined from 25- or 50-fold diluted samples using a Dionex Ion Chromatograph (series 4000 I, AS14A 3mm IONDAN analytical column).

Biomass elemental composition

Analysis of solid samples for nitrogen and carbon content was carried out using a CE Elantech 1112 elemental analyzer. Percent nitrogen and carbon was determined according to standard combustion and thermal conductivity methods (AOAC 990.03 and AACC 46-30) on a Flash EA 1112 Elemental Analyzer calibrated with aspartic acid (10.52% N, 36.09% C). Samples were analyzed for total phosphorus using the molybdenum-ascorbic acid method following combustion at 550°C for 4 h and hydrochloric acid digestion.

Biological characteristics

Productivity

To evaluate net productivity (i.e., biomass produced per day per square meter of surface area), biomass was weighed each time the modules were harvested. To estimate the dry weight equivalents of the harvested biomass, samples of the predominant species were taken weekly throughout spring 2008 and oven dried at 105°C until constant weight was reached.

Wetland vegetation composition

The abundance of each plant species was assessed weekly by visual examination of each channel. Percent cover (visual estimates) was recorded for each of the following categories: algae, duckweeds, azolla, hydrocotyle, as well as, in the unharvested channels, terrestrial plants. This assessment was made on the same day that nitrate and phosphate concentration samples were collected. Photographs were also taken this same day. Species composition data was used to determine productivity and N-assimilation, as each had a different wet to dry weight ratio and percent nitrogen composition.

Bacterial enumeration

Most probable number (mpn) enumeration of denitrifying bacteria in module sediment and surface-associated with whole plants of azolla and duckweed was carried out by 10-fold serial dilution of settled sediment samples and plant rinsates into nitrate broth (Difco, New Jersey) containing an inverted Durham tube to detect gas production. Total coliform and *E. coli* bacteria were enumerated by mpn analysis using Quanti-Tray 2000 (IDEXX Inc., Westbrook, ME) of module influent and effluent samples that were obtained at least one day following harvest.

Mosquito monitoring

The modules were monitored monthly using 0.35 L dipper cups for the presence of mosquito larvae by technicians from the Sonoma-Marin Mosquito Control District. Mosquitofish maintained self-sustaining populations in harvested channels. Monthly dipper samples did not reveal any mosquito larvae in harvested channels for the duration of the experiment (S. Miller, personal communication). Build-up of sediment and detritus in the unharvested channels prevented mosquitofish access to mosquito

larvae, thereby necessitating application of Bt insecticide into these channels.

Fish bioassay for estrogenic activity

Water sampling

Samples for the estrogenic activity bioassay were collected as composites on 9 July 2009, with a renewal sample collected on 11 July 2009, using an autosampler as described in previously from the module that had been under a regular harvest regime since project inception. Samples were stored at 4°C overnight.

Bioassay conditions

Procedures to detect estrogenic activity via a vitellogenin (VTG) mRNA bioassay were based on those of de Vlaming et al.[13] and were approved by the Committee for Human and Animal Subjects at San Francisco State University. Upon arrival at the laboratory juvenile Rainbow trout (Thomas Fish Company, Anderson, CA) were acclimated by floating the shipment bags for several hours in 15°C holding tanks before releasing into the holding tanks.

After 2 d fish were randomly distributed amongst each treatment in triplicate into 2.0-L glass beakers containing 1.5 L of the test medium. The beakers had been acid washed, rinsed with deionized water and then rinsed with each test solution. Experimental controls were prepared with synthetic hard water (pH 8.16, hardness 125 ppm, alkalinity 3.50 mEq/L, and conductivity 352 μS) and included positive controls of 2, 5 and 10 ng L^{-1} synthetic estrogen ethynylestradiol (EE2) (MP Biomedicals, Solon, OH, USA) as well as a water-only negative control. Methanol was used to dissolve the powdered EE2 to give a final concentration of 0.001% methanol in the spiked controls; preliminary experiments demonstrated that methanol at this concentration gave no significant difference in VTG mRNA expression compared to the water-only exposure (data not shown), consistent with previously published results[13].

Debris was aspirated from the beakers daily. After 60 h the treatment media were renewed following an established procedure:[14] 0.5 L was decanted from each beaker and replaced with 0.5 L of the same treatment of freshly prepared synthetic hard water medium or newly sampled module water followed by pouring off 1.0 L and adding back 1.0 L of new medium. The fish were fed once during the five day exposure period with Aquamax Trout Food (Purina, St. Louis, MO, USA), prior to the medium renewal event.

Liver removal and RNA extraction

The trout were sacrificed and livers harvested following the procedures of de Vlaming et al.[13] The harvested livers (~10 mg each) were immediately fixed in RNAlater® Tissue Collection: RNA Stabilization Solution (Applied Biosystems, Carlsbad, CA, USA). Livers of all three fish from a given beaker treatment were combined and treated subsequently as a single sample to account for variability in VTG mRNA production among individuals. Livers were then stored at -80°C until ready for RNA extraction.

The fixed frozen liver tissue was transferred to 0.5 mL TRI Reagent® Solution (Ambion, Austin, TX, USA) and homogenized for 4 min at 30 cycles s^{-1} in a Retsch MM300 bead beater with 3.2 mm grinding beads. Homogenized samples were incubated for 5 min at room temperature. Then 200 μL of chloroform (without isoamyl alcohol) was added, the samples mixed vigorously, and incubated for 15 min at room temperature. Samples were centrifuged for 15 min at 12,000 *g* at 4°C and 200 μL of the aqueous phase transferred to a tube containing 500 μL isopropanol. Samples were vortexed and incubated for 10 min at room temperature and then centrifuged at 12,000 *g* for 8 min at 4°C. The supernatant was removed and the RNA pellet washed with 1 mL 75% ethanol and incubated at room temperature for 10 min. Tubes were then centrifuged for 10 min at 12,000 *g*. The supernatant was aspirated and excess ethanol allowed to evaporate. The RNA was then resuspended in 100 μL RNAse-free water (Qiagen, Hilden, Germany) and stored at −20°C.

Reverse transcriptase quantitative PCR

Relative quantification of VTG mRNA was carried out with an Applied Biosystems Prism 7000 Sequence Detection System using the VTG primers and 18S rRNA (as an internal control for RNA applied) primers and Q-RT TaqMan® PCR design described by de Vlaming et al.[13] The instrument was calibrated using Applied Biosystems 7000 Sequence Detection Systems Spectral Calibration Kit.

Anaerobic digestion

Two 5.7 m^3 circulation-mixed anaerobic digestion reactors (Fig. 1B), constructed based on the vertical flow design of Biljetina et al.[15] suitable for the digestion of buoyant aquatic vegetation, were operated in the downflow mode continuously at 35°C beginning 28 March 2010 with a 4.4 m^3 culture volume. Reactor initiation, optimization, and monitoring procedures have been described previously.[16] Both reactors were fed every other day to achieve a 60-day hydraulic retention time with a 142 L mixed feedstock containing 3.5% (wt/vol), as dry material, approximately equal proportions of red wine lees (Hanna Winery, Santa Rosa CA), aquatic vegetation, and dairy manure or crude glycerol waste by-product from biodiesel manufacture (Yokayo Biofuels, Ukiah CA).

The aquatic vegetation component consisted of material harvested from the constructed wetland combined

with an invasive aquatic weed, Uruguayan primrose-willow (*Ludwigia hexapetala*, C/N ratio = 23.7 ± 2.4; mean ± SE, n = 5) harvested from invaded riverine wetlands along a reach of Laguna de Santa Rosa that flows through the treatment plant property. The effluent slurry removed from the AD reactors just prior to feeding was emptied into a settling bin and vermicomposted for use as a soil amendment. Biogas was passed through a biological sulfide scrubber and used to power an on-site electrical generator.

Biogas production from co-digestion of aquatic vegetation was determined by excluding it from the feedstock mix and calculating the depression in output from both reactors. For the purpose of calculations vegetation-free biogas production was considered to begin upon the point at which macroscopic fibrous matter was no longer apparent in the digestate, which corresponded to 65 d following the cessation of aquatic vegetation feeding in one reactor and 75 d in the other.

Vermicomposting

Digestate from the reactors was drained just prior to feeding into a 14.2 m^2 settling bin to allow for evaporation and infiltration of water into the ground. Periodically the settled digestate was mixed at a 20:1 vol/vol ratio with grape seed pomace and layered into approximately 1 m × 1.8 m piles, adding ∼5 cm per addition. The piles, containing earthworms, were built up over the course of a month to a height of approximately 22 cm upon which time they were allowed to mature. Elemental composition of a 9-sample grid composite from a mature compost pile that had not been supplied with material for 5 months was performed by the Laguna Environmental Laboratory.

Statistical analyses

We used general linear mixed models using SAS Proc Mixed (version 9.1, SAS Institute, Cary, NC) to assess the influence of harvesting regimes on net primary productivity and nitrate removal efficiency. Confidence intervals and p-values are of measurements taken over time from the same module, conditioned on an appropriate covariance model to account for non-independence among observations. To allay concern that any observed effects were due to module and not treatment, we analyzed nitrate removal rate and net productivity data for any effect of module from 30 September 2008 to 18 March 2009, when the modules were being treated identically. We found that module had no effect on nitrate removal rate ($F_{1,22.4} = 0.02$, $p = 0.878$) or productivity ($F_{1,23} = 0.01$, $p = 0.934$).

To account for the repeated measures in these analyses, we compared the fit of six candidate covariance models (autoregressive, autoregressive moving average, compound symmetry, heterogeneous autoregressive, unstructured, and Toeplitz) and retained the simplest autoregressive structure (ar(1)) in the final models because it had the smallest corrected Aikake's Information Criterion (AICC) value.[17] In addition to having the smallest AICC value, the autoregressive covariance model was a logical choice based on the nature of the data; two immediately adjacent measurements should indeed be the most highly correlated, as the treatment community in the module one week influences the treatment community of the next week. We assessed model fit through visual inspection of residual plots and used log and square root transformations as needed to normalize the distribution of residuals.

To assess the effect of harvesting regime on phosphate removal we used a two-way ANOVA (JMP version 8.0.2, SAS institute, Cary, NC). Harvesting regime and date were used as grouping factors and change in phosphate concentration across a module as the response variable. Due to lack of replication, we designated dates as blocks and specified the block as a random effect. Data were antilog transformed and tested for normality using the Shapiro-Wilk test. For presentation and comparison purposes, the ANOVA results were back transformed. We assessed heterogeneity of variance using Levene's test.

Results

Harvestable productivity and nitrate removal efficiency of unselectively harvested modules

Figure 2 shows the seasonal variation in the monthly mean nitrate removal rate and productivity of unselectively harvested modules in relation to the mean monthly evapotranspiration rates and minimum air temperatures. From May 2008 to Dec 2009 the average of the monthly mean nitrate removal rates and productivities were 1050 ± 115 mg N m^{-2} d^{-1} and 9.28 ± 1.67 g dry wt. m^{-2} d^{-1}, respectively (means ± SE; n = 12). The transition from February to March gave the largest increase in mean harvestable productivity (from 2.37 to 10.91 g m^{-2} d^{-1}, or 360%), with September to October exhibiting the largest decrease (from 10.74 to 5.81 g m^{-2} d^{-1}, or 46%). Variations in the nitrate concentration of the influent (Table 1) did not significantly affect the nitrate removal rate of the modules.[10]

Effect of vegetation type on wetland nitrate removal rate

Harvestable productivity of the unselectively harvested module containing mixed vegetation was significantly higher than that of the module selectively harvested to maintain dominance of filamentous algae ($F_{(1,44.6)} = 34.23$, $p < 0.0001$; Fig. 3).

Vegetation type had no effect on nitrate removal rate ($F_{(1,6.7)} = 0.18$, $p = 0.69$). The average nitrate removal rate of the algae dominated module was 1440 ± 276 mg N-NO_3^- m^{-2} d^{-1} and that of the mixed vegetation module was 1350 ± 235 mg N-NO_3^- m^{-2} d^{-1} (means ± 95% CI; Fig. 3).

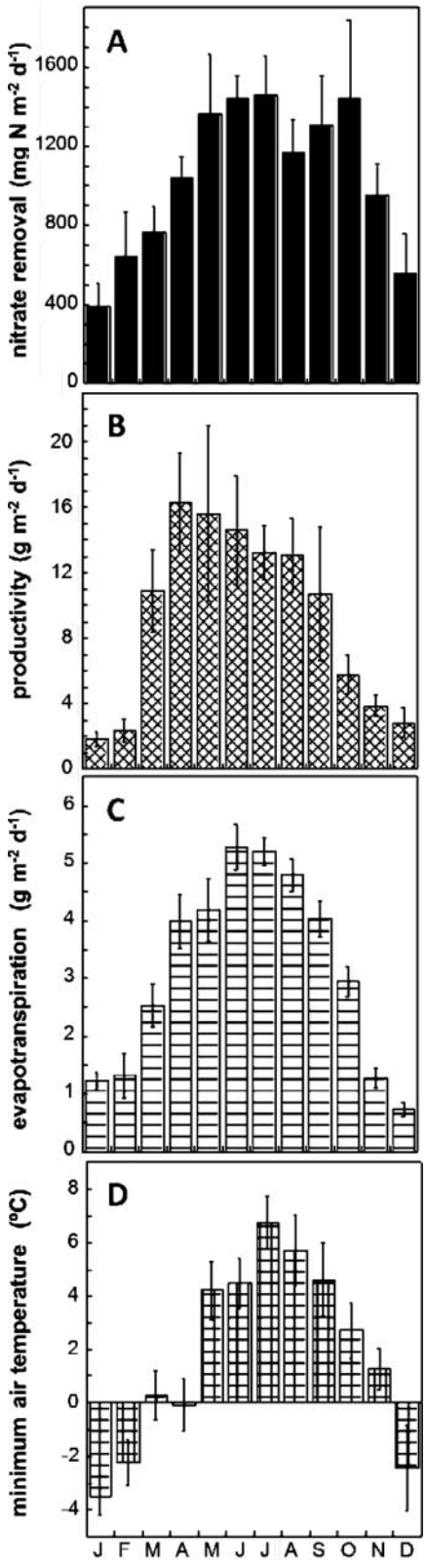

Fig. 2. Monthly means of (A) nitrate removal rate, (B) productivity, (C) evapotranspiration, and (D) minimum air temperature. Error bars indicate 95% confidence intervals; all data are from unselectively harvested modules from 13 May 2008 to 1 December 2009 (A: n = 95; B, C, D: n = 279).

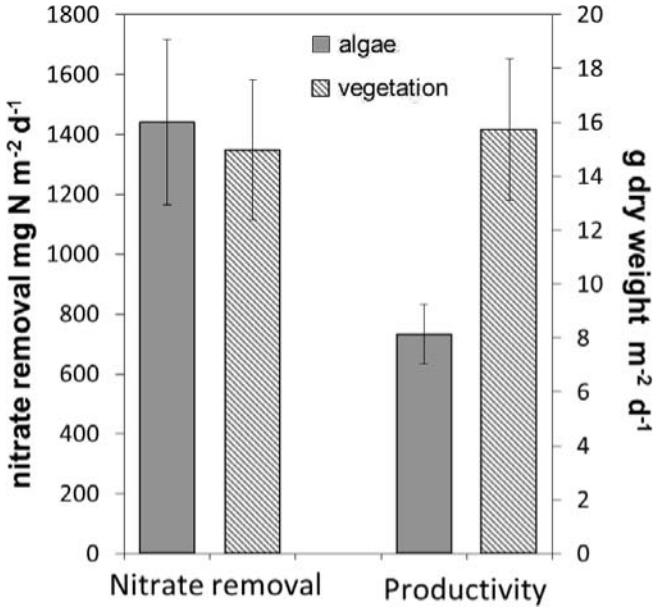

Fig. 3. Differences in mean nitrate removal rate and productivity in the selectively harvested algal-dominated module compared to the unselectively harvested module containing a mixed vegetation of aquatic macrophytes and algae; 18 May 2008 to 19 August 2008. Error bars indicate 95% confidence intervals (n = 30).

Effect of harvesting on wetland nutrient removal rate

Nitrate removal

Cessation of harvesting in the east module resulted in gradually increasing nitrate removal rate relative to the regularly harvested west module ($F_{1, 39} = 11.20$, $p = 0.0018$). Over the course of this experiment, from March 2009 to March 2010, the nitrate removal rate of the harvested module was 1070 ± 93.1 mg N m^{-2} d^{-1} compared to 1230 ± 79.5 mg N m^{-2} d^{-1} of the non-harvested module (means ± 95% CI). The divergence in the nitrate removal rates of the two modules became more apparent beginning in October 2009.[10]

Assimilation into harvested biomass accounted for 326 ± 15.3 mg N m^{-2} d^{-1} (mean ± SE, n = 144), or 30.5% of the total nitrogen removed from the water in the harvested module. Approximately 8.0% of the nitrogen removed over the course of this experiment was stored in the sediment, leaving approximately 61.5% of the nitrogen to have been removed by other means, most likely bacterial denitrification. Denitrifying bacteria were cultured from sediment at 3.2 ± x 10^7 mpn g^{-1} dry wt. (n = 14) and from rinses of whole plants of azolla and duckweeds at 1.4 ± 0.37 × 10^6 and 6.0 ± 3.6 × 10^6 mpn g^{-1} dry wt., respectively (n = 4; means ± SE). Ammonia in both the influent and effluent was typically not detectable and did not exceed 0.3 mg L^{-1} throughout the course of the study (data not shown).

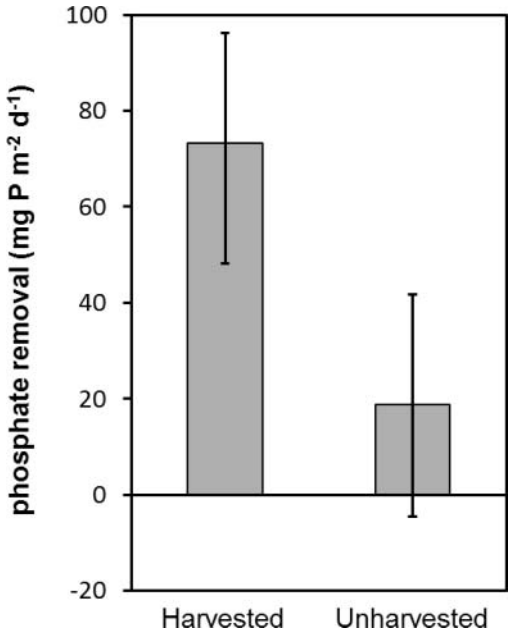

Fig. 4. Phosphate removal rates of harvested and unharvested modules; 3 November 2009 to 19 October 2010. Error bars indicate 95% confidence intervals (n = 48).

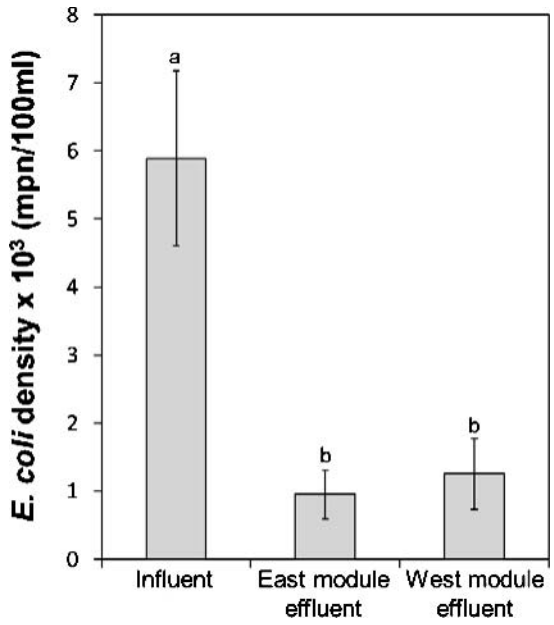

Fig. 5. Changes in *E. coli* densities across unselectively harvested modules; 10 June to 17 Nov 2011. Means (± SE) having the same letter are not significantly different from each other (n = 11).

Phosphate removal

The mean phosphate removal rate of the harvested west module was significantly higher than that of the unharvested east module ($p < 0.0001$; Fig. 4). Estimated phosphate removed by harvesting phytomass (60.7 mg P m^{-2} d^{-1}) and by sedimentation (15.6 mg P m^{-2} d^{-1}) when combined account for 104.2% of the measured 73.3 mg P m^{-2} d^{-1} phosphate removal rate of the module.

E. coli and total coliforms

Passage of the wastewater through either module during a period of regular harvesting was found to significantly lower the levels of *E. coli* in the water (Fig. 5). Levels of total coliforms were not significantly different between the influent and effluent of either module (not shown).

Removal of estrogenic activity

In July 2009 a VTG mRNA bioassay was conducted to assess the change in estrogenic activity caused by passage of water through the unselectively harvested west module operating under a one-day retention time. The estrogenic activity of the module influent was found to be significantly higher ($p < 0.05$) than that of the module effluent, which was not significantly different from the water-only control (Fig. 6).

Anaerobic digestion

Consistent with other studies,[7] initial laboratory-scale testing of our available substrates revealed that co-digestion of multiple substrates gave the highest yields on a per weight

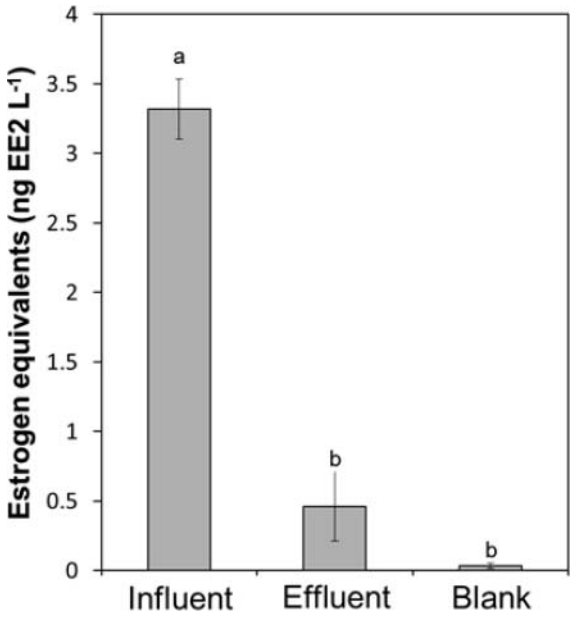

Fig. 6. EE2-equivalence of water from influent and effluent of the unselectively harvested module and the water-only blank treatment. Significance was determined by a Tukey-Kramer MSD test. Means (± SE) having the same letter are not significantly different from each other ($n = 4$).

Table 2. Performance of anaerobic digestion reactors fed with a 1:1:1 mixture of aquatic vegetation, wine lees and dairy manure or crude glycerol.

	Feedstock mixture containing:	
Characteristic	*Dairy manure**	*Crude glycerol**
Methane yield (m^3 kg^{-1} VM)	0.28 ± 0.03 (*2*)	0.53 ± 0.03 (*2*)
Methane concentration (%)	61.3 ± 3.5 (*40*)	61.3 ± 4.2 (*46*)
Volatile solids reduction (%)	47.7 ± 3.6 (*12*)	67.5 ± 5.0 (*12*)
Volatile acids reduction (%)	90.8 ± 0.5 (*13*)	87.4 ± 2.0 (*8*)
Feed pH	4.66 ± 0.01 (*4*)	4.60 ± 0.11 (*8*)
Digestate pH	7.36 ± 0.09 (*63*)	7.16 ± 0.06 (*46*)
Feed alkalinity (mg L^{-1} $CaCO_3$)	2151 ± 213 (*17*)	488 ± 68 (*8*)
Effluent alkalinity (mg L^{-1} $CaCO_3$)	4241 ± 264 (*17*)	2475 ± 110 (*8*)
Effluent NH_3 (mg L^{-1})	409 ± 82 (*53*)	214 ± 87 (*46*)

*Mean values from both reactors operated under the same conditions, ± range for methane yield, ± SD for methane concentration, effluent pH, and effluent NH_3 and ± SE for others; *n* in parentheses.

basis.[16] The replacement of dairy manure with crude glycerol in the feed mix provided to the AD reactors resulted in a substantial increase in methane yield (Table 2). To determine the contribution of the aquatic vegetation breakdown to the output of the AD reactors we subsequently excluded this component from the feed, resulting in a 0.23 m^3 drop in methane production per kg volatile solids phytomass omitted for both reactors. After 110 days of phytomass-free feed, a precipitous decline in pH began in the first reactor, leading to a near cessation of biogas output within 10 days. To prevent this fate in the other reactor, addition of aquatic vegetation to the feedstock mix was resumed.

Vermicompost

Mature vermicompost generated from a 20:1 (vol/vol) digestate:grape seed pomace mixture had 57.1± 1.0% volatile solids (mean ± SE; n = 4) and contained, on a dry weight basis, 0.6% total Kjeldahl nitrogen, 1.6% potassium and 0.6% phosphorus. Concentrations of US EPA-regulated metals were well below ceiling concentration limits for land application:[18] 64 mg kg^{-1} copper, 0.04 mg kg^{-1} mercury, 16 mg kg^{-1} nickel, 170 mg kg^{-1} zinc; arsenic, cadmium, lead, and selenium were not detected.

Discussion

Wastewater polishing

The average annual nitrate removal rate of 1070 mg N $m^{-2}d^{-1}$ found in this study compares favorably to values reported of other aquatic treatment systems in California, which range from 522 to 800 mg N m^{-2} d^{-1}.[19,20] Our observed nitrate removal rate corresponds to a 4.2 mg N L^{-1} decline through the system operating under a 1-day retention time, which would result in an approximately one-third reduction in nitrate concentration for typical municipal wastewater treatment plant effluents.[2] The estimated 61.5% contribution of gasification to nitrate removal in harvested modules was consistent with other studies.[21,22]

It is likely that increased deposition of detritus in the non-harvested module stimulated gasification via denitrification to a degree that compensated for or even surpassed the contribution of N removed by harvesting;[23] provision of sufficient organic carbon is often the rate limiting step for denitrification activity.[24] The lower C:N ratio in the harvested module sediment (Table 3) provides evidence that the harvested module was more carbon limited than the unharvested module. Moreover, the more complete surface coverage of biomass in the unharvested module provided

Table 3. Nitrogen, carbon and phosphorus composition of oven-dried samples obtained from the constructed wetland modules[a].

Sample	*% N*	*% C*	*C:N ratio*	*(n)*[b]	*%P*
Algae	4.60 ± 0.21	31.2 ± 2.66	6.75 ± 0.30	(5)	0.847 ± 0.029 (*n* = 3)
Duckweed	5.09 ± 0.06	36.1 ± 0.22	7.11 ± 0.07	(41)	0.830 ± 0.015 (*n* = 3)
Azolla	4.35 ± 0.19	38.6 ± 0.50	9.07 ± 0.48	(11)	0.678 ± 0.149 (*n* = 25)
Hydrocotyle	4.17 ± 0.27	35.9 ± 0.57	8.77 ± 0.50	(7)	1.007 ± 0.040 (*n* = 3)
Sediment (harvested module)	2.61 ± 0.06	16.2 ± 0.34	6.21 ± 0.09	(11)	0.476 ± 0.040 (*n* = 8)
Sediment (unharvested module)	2.42 ± 0.12	19.4 ± 0.74	8.04 ± 0.16	(8)	0.464 ± 0.011 (*n* = 9)

[a]Samples for N and C analysis were collected monthly from November 2008 to December 2009; samples for P analysis were gathered in February and March 2010; mean ± SE.
[b]Number of samples taken for N and C analysis only.

insulation that kept the channels warmer on average (a 1.6°C difference during two intensively monitored weeks in October 2009) than the harvested channels[10] and could have contributed to the increased rate of denitrification in the unharvested module.

Phosphate removal by the wetland modules was minimal, typical of other constructed wetlands.[25] Our observed phosphate removal rate from a harvested module corresponds to a 0.29 mg P L^{-1} decline when operating under a 1-day retention time, which would lower phosphate levels in typical municipal wastewater treatment plant effluents by less than 10%. There are several potential reasons for this observed limited phosphate removal. In contrast to its pronounced role in the nitrogen cycle, volatilization is only a minor contributor to the phosphorus cycle[2] and, furthermore, plants assimilate substantially less phosphorus than nitrogen (Table 3).

Additionally, wetland phosphate removal rates decline substantially within a year after initiation;[25] for our study phosphate analysis was conducted two years into wetland operations. Furthermore, the EPDM lining of our modules prevented phosphate removal that would otherwise occur through sorption to soil. For further removal of phosphate, we are currently investigating passage of effluent through steel slag, a high-surface area material with a strong capacity for binding phosphate that has be shown to substantially lower concentrations of phosphate in municipal wastewater treatment plant effluent.[26]

Our finding that passage of wastewater effluent through a harvested constructed wetland module lowers estrogenic activity to levels not significantly different from an estrogen-free synthetic aqueous medium is consistent with that of a previous study of an unharvested channelized wetland that utilized a plasma VTG protein bioassay.[27] The modified quantitative VTG mRNA bioassay we employed requires less labor, resources and shorter exposure times than does the plasma VTG bioassay.[13] Estrogenic activity in municipal wastewater effluent is due primarily to the presence of natural estrogens and EE2.[28,29] Though we did not investigate the mechanism for dissipation of these compounds, others have reported that a combination of biodegradation and sorption are responsible for their removal from wastewater fed streams and constructed wetlands.[30,31]

Although *E. coli* densities declined substantially across harvested modules, levels in the effluent still exceeded that recommended for surface water by the US EPA. Therefore, further disinfection of the water would be required in most applications when treating secondary wastewater. The lack of an effect on total coliforms was not unexpected since members of this group are known to reproduce in wetland environments.[4]

Co-digestion of wetland-derived phytomass

Methane yields and volatile solids reductions from the feedstock mixture when operating with crude glycerol as a co-substrate were within the upper range of typical values reported for anaerobic digestion.[32,33] Inclusion of crude glycerol in AD reactors is known to result in synergistic increases in production in co-digests.[34] One probable benefit of glycerol to the AD culture was the higher C:N ratio of 20 it imparted to the feed compared to the C:N ratio of 12 of the manure-containing feedstock. The optimal C:N ratio for anaerobic digestion has been reported to be 20 to 25.[8] Excluding aquatic vegetation from the feed increased the C:N ratio to 28. Various factors could have accounted for the eventual collapse of the anaerobic digestion culture in one of reactors during the phytomass-free feeding regime, including the declines in available N, buffering capacity, and colonizable surface area formerly provided by the vegetative material.

Approximately 84% and 72% of the total biogas production occurred within the first day of the 2-day feeding cycle in the manure-fed and glycerol-fed reactors, respectively (data not shown), indicating that the culture could have likely tolerated a shorter retention time. In support of this possibility, a vertical downflow reactor fed with a 1:1 mixture of water hyacinth and human biosolids gave methane yields and total solids reductions similar to ours but operating at only an 11-day hydraulic retention time.[15]

Based on the observed contribution of 0.23 m^3 CH_4 kg^{-1} volatile solids from the aquatic vegetation component of the feedstock, which is similar to methane yields from other vegetative feedstocks,[32] 563 m^2 of wetland surface area would need to be harvested regularly to generate one cubic meter of methane per day via anaerobic digestion. Production of biomass for energy generation cannot alone justify the allocation of space for constructed wetlands; the world per capita consumption of methane is 1.26 m^3 CH_4 d^{-1}[35] and photovoltaic solar panels generate approximately 56 times more energy than can be released by combustion of methane produced from the daily yield of an equivalent surface area of aquatic vegetation subjected to anaerobic digestion (assuming 5.2 kWh m^{-2} d^{-1} and a solar panel conversion efficiency of 20%).

Rather, methane production and other benefits of anaerobic digestion are best viewed as helping to offset the costs of wastewater polishing operations. Similarly, an economic analysis of aquatic plant harvesting from natural wetlands found that, though not sufficient to cover costs of weed control, the combined economic value of methane and soil amendment from anaerobic digestion would make harvesting a more cost-effective and sustainable alternative to herbicide treatment of aquatic weeds.[36]

Conclusions

Passage of secondary wastewater through the channelized constructed wetlands operating on a 1-day retention time brought about significant reductions in nitrate, estrogenic activity and *E. coli* levels. Although regular harvest-

ing of the modules did not increase, and in fact slightly decreased, nitrate removal rate, the benefits of modest phosphate removal and production of methane and soil amendment from harvested phytomass can help to offset wetland management costs. Moreover, we demonstrated that anaerobic co-digestion can simultaneously improve the sustainability of wastewater treatment and processes that generate organic wastes, including wine, dairy and biodiesel production.

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