NITRATE REMOVAL EFFICIENCY AND NET PRODUCTIVITY OF CHANNELIZED WASTEWATER-FED PLEUSTON WETLANDS

by

Caden Hare

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Date

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Key words: wastewater, treatment wetlands, polishing wetlands, natural treatment systems, aquatic treatment systems, nitrate removal efficiency, productivity, pleuston, algae, azolla, duckweed, hydrocotyle

ABSTRACT

Constructed wetlands have been emerging over the last 30 years as a good way to treat bodies of water with undesirable levels of pollutants in general, and of polishing municipal wastewater effluent (MWE) in particular. In this study, the nitrate removal efficiencies of two 37.2 m^2 Channelized Aquatic Scrubbers (CAS) in Santa Rosa, CA were compared with respect to vegetation type and harvesting regime. Nitrate removal by a filamentous algae dominated CAS did not differ significantly from a CAS containing a mixture of aquatic vegetation, but biomass production by the algae was roughly half that of the aquatic vegetation mix. Between 13 May 2008 and 1 December 2009 harvested channels of the CAS removed an average of 1050 ± 115 mg N m^{-2} d^{-1} (mean ± SE). The average influent and effluent nitrate concentrations of all channels during this time were 14.9 ± 0.4 mg L^{-1} and 9.9 ± 0.3 mg L^{-1}, respectively (mean ± SE). During this same time period, the average productivity of the vegetation in the CAS was 9.28 ± 1.67 g DW m^{-2} d^{-1} (mean ± SE). Denitrification was the predominant mechanism of nitrate removal, and removal was dependent on both temperature and evapotranspiration rates. Species of aquatic vegetation varied seasonally, with harvesting playing an important role in allowing species succession. Based on the results from this study and others like it, CAS are a promising way to meet increasingly stringent regulatory discharge limits on nitrate concentration while generating biomass that can be converted to electricity.

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Linden, the Col., Shaun Horne, and Kandis Gilmore all help harvest the CAS, and their help is much appreciated. Zane Knight, Meghan Parish, and Marianita Viera all

submitted undergraduate theses on the CAS, and their work has increased my understanding of the system. I must also thank Nels Worden, John Collins, Dr. Thomas Buckley, and Dr. Richard Whitkus of SSU for their assistance with this project. Nels Worden got involved at the inception of the project, and without his help determining nitrate, phosphate, and metal concentrations, it is doubtful that we would have been able to persuade the City of Santa Rosa to fund Phase I of the project. Bob Duckworth, of R.S. Duckworth Construction, was essential in designing and building Phase I. John Kozlowski, another fellow graduate student, is essential to Phase II, and I am grateful to him for all his hard work.

This brings us to the City of Santa Rosa. With the exception of Dell Tredinnick, my boss, who got us our funding for Phase I, and Nicole Dorotinsky, who helped get us our funding for Phase II, I will not have sufficient space to explain why I am listing the people that I am. It will simply have to be taken on faith that these names deserve to be here: Miles Ferris, Dan Carlson, Lynn Small, Joe Schwall, Randy Piazza, Shirlee Johnson, Martin St.George, Cindy Larkin, Matt McGarey, Denise Cadman, Bill Gardner, Ron Laufer, Sabrina Temple, Jeff Villerot, Bob Cauthen, Aaron De La Montanya, Ike Estiqueta, Robert Frederick, Cole Keller, Sean Murphy, Al Phelps, Chris Puder, Paul Tynan, Mike Wellington, JJ Daigle, John Pilger, Dan Silveria, Bob Bertolucci, Jeff Bittner, Paul Critchett, Eric Huse, Karl Righetti, Jason Bishop, Tony Guillory, Mary Ellen Lazzarini, Jama Giammona, Shelley Carter, Melissa Jones, Leslie Hunziker, Janet Reisner, Cory Jones, Albert Carranza, Rachel McCormick, Juan Mora, Walter Norosky, Raul Ramirez, Lisa Seale, Kathy Stokes, Anna Veit, Melanie White-Lam, Heather Gourley, Renae Gundy, Larry Moreda, Chris Murray, Bruce Taylor, Roy Throop.

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I. Introduction

Treated Municipal Wastewater Effluent (MWE) contains levels of nutrients, including nitrate, that can support dense growth of aquatic vegetation. Entry of nutrients from MWE and other anthropogenic sources into natural waterways can result in eutrophication during which algae and aquatic plants grow excessively. Subsequent decomposition of this vegetation can lead to hazardously low dissolved oxygen levels in the water and resultant fish kills. During this process, invasive plants may outcompete native species and sometimes, as in the case of *Ludwigia* spp. invasion of the Laguna de Santa Rosa, create habitat for mosquitoes. I seek to turn this liability of MWE into an asset by designing a system for cultivating aquatic plants and algae in the wastewater effluent prior to release, thereby removing the nitrate in a controlled fashion and avoiding the possible impacts of eutrophication. Such a system would simultaneously generate biomass that could be harvested and converted into a usable energy source. The feasibility of such an approach depends on key biological aspects of this aquatic system including its seasonal nitrate removal efficiency and the nature of the accumulated biomass.

Oswald and Golueke (1960) presented a model plan for wide-scale production of energy based on algal photosynthesis. Wastewater-cultivated algae would capture solar energy and the harvested biomass would be converted in part into methane in anaerobic digesters. Combustion of this methane would generate electricity and CO_2 flue gas that could be infused back into the algal medium to enhance growth. A nearly two decade-long study by the U.S. National Renewable Energy Laboratory's Aquatic Species Program concluded that cultivation in municipal or agricultural wastewater effluent

would be the only economically feasible approach for large-scale production of algae for production of fuels, including biodiesel (Sheehan et al., 1998). Recent research by various algal biodiesel startup companies is aimed at expanding the list of feasible approaches to include production in brackish water, but it remains to be seen whether this will be economically viable. Regardless, growing algae outdoors using wastewater as a growth medium makes a great deal of sense as it minimizes costly nutrient inputs, provides a cheap source of fresh water, and eliminates the need to use electricity to power indoor lighting (Clarens et al., 2010; Sheehan et al., 1998).

One major impediment to implementation of an algae-based energy crop has been the difficulty of efficiently harvesting microalgae from the culture ponds. As microalgae are generally too small (<10 μm) to be harvested either untreated or with simple filtration devices either a centrifuge or chemical flocculants must be used. Unfortunately, the large amount of energy required for the first option and the purchasing cost associated with the second option make neither attractive in a large scale operation. There is some promise from suspended air flotation (Wiley et al., 2009), but as with many aspects of the technology emerging around microalgae growth and harvesting, it has yet to be proved economically viable. The other major impediment to implementation has been the extreme difficulty of maintaining a monoculture in a large system (Reed et al., 1995; Sheehan et al., 1998; Vasudevan and Briggs 2008).

Floating mats of aquatic vegetation, termed pleuston, are a potential alternative to microalgae for integrated wastewater scrubbing and bioenergy production. Depending upon the species, growth mechanism, and climate, productivities of microalgae (2.5 – 72 g DW m^{-2} d^{-1}) and pleuston species (0.25 – 24.7 g DW m^{-2} d^{-1}) can be similar (Chisti

2007; Lakshman 1987; Mulbry et al., 2008). Pleuston, however, are much easier to harvest than microalgae; the simple process of scooping by hand, rake, or net can easily harvest floating mats, and there are a variety of ways in this process could be mechanized (Bagnall et al., 1987). Additionally, by their nature, pleuston are a consortium of species and are therefore more resistant to stresses than microalgal monocultures (Charudattan 1987; Kadlec and Wallace 2009; Stevenson 1996).

Pleuston help to purify their aqueous growth medium by removing nitrogen and phosphorous (Davis et al., 1990), binding metals (Vymazal 1995), and metabolizing organic contaminants such as pharmaceuticals and pesticides (Rose et al., 2006; Semple et al., 1999). Bacterial denitrification and anammox, which collectively metabolize NO_3^-, NO_2^-, and NH_4^+ to gaseous N_2, are responsible for the majority of nitrogen loss from wetlands with N-assimilation by aquatic vegetation and seepage making up the remainder (den Camp et al., 2006; Kuenen 2008; Xue et al., 1999). Bacterial activity is significantly enhanced in the presence of a consortium of aquatic vegetation (Bachand and Horne 1999b; Sirivedhin and Gray 2006). In this manuscript "denitrification" will be taken to have the more general meaning of processes, predominantly microbial, internal to the system that reduce the entering nitrate to a gaseous form.

To better understand natural treatment systems and further the possibility of more extensive adoption of such systems in water treatment, in this study I have addressed the following questions:

> *1) What are the percentages of lipid, carbon, and nitrogen in the predominant pleuston species?*

2) What influence do environmental factors such as temperature, solar radiation, relative humidity, and wind speed have on net productivity and the efficiency of nitrate removal?

3) What are the relative contributions of N-assimilation and denitrification to the nitrate removal capacity of the system?

4) What are the effects of harvesting regime on net productivity and nitrate removal efficiency of a module?

II. Study System

In the summer of 2007 two near identical experimental Channelized Aquatic Scrubbers (CAS) were constructed on the grounds of the Laguna Treatment Plant (LTP). Water flows by gravity through each module, which is divided into three 6 m long channels lined with EPDM plastic 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. The channels were inoculated with filamentous mat-forming algae (*Oedogonium* sp.), duckweed (*Lemna* spp. and *Spirodela* sp.), azolla (*Azolla filiculoides*), and hydrocotyle (*Hydrocotyle ranunculoides*) native to local waterways and stocked with mosquito fish. Other algae that later became apparent in the channels included species of genera *Spirogyra*, *Hydrodictyon*, *Anabaena*, *Pseudoanabaena*, *Pediastrum*, and various pinnate diatoms.

From July 2007 to March 2008, chlorinated tertiary-treated wastewater flowed into both modules from an existing pressurized water system on-site (see Appendix I). From March 2008 to the present, secondary-treated wastewater has been pumped for 13 hours each day through adjustable valves into both modules from the LTP channel that

connects the clarifiers to anthracite coal filters (see Appendix II for a brief description of the stages of wastewater treatment). From 13 May 2008 to 16 March 2010, the average flow rate into each module was 232.6 ± 13.5 L d^{-1} m^{-2} (mean ± 95% CI; n = 190, average daily flow rate includes the 11 hours of zero flow). For the periods from 13 May 2008 to 21 October 2008 and 28 May 2009 to 16 March 2010, the average calculated retention time in the CAS was 0.94 ± 0.02 days (mean ± SE, n = 134). For the periods from 28 October 2008 to 2 December 2008 and 24 March 2009 to 19 May 2009, the average calculated retention time in the CAS was 1.58 ± 0.06 days (mean ± SE, n = 30). From 9 December 2008 to 17 March 2009, the average calculated retention time in the CAS was 4.22 ± 0.39 days (mean ± SE, n = 28). Actual retention times were less than calculated retention times as calculations assume complete plug-flow conditions and do not account for decreasing channel volume over the length of the experiment due to soil shifting.

III. Materials and Methods

Harvesting

With the exception of the summer of 2008, when we harvested more frequently, we harvested the modules three times a week, and never within the 24 hours prior to sampling. Small aquatic vegetation was harvested with a pool skimmer, and hydrocotyle was harvested by hand, first cutting the perimeter of the harvested sections with grass clippers.

During the spring of 2008 we established differing conditions in the west and east modules. Initially, both modules contained a naturally evolving pleuston of aquatic

vegetation. In April, in the west module, we established a pleuston composed almost entirely of mat-forming algae. From 12 April 2008 to 20 August 2008, the west module was selectively harvested to maintain the highest percent cover of algae possible, while the aquatic vegetation in the east module was indiscriminately harvested. This experiment with selective harvesting will be referred to as "algae vs. aquatic vegetation." Data analyzed for this experimental period were gathered between 13 May 2008 and 19 August 2008. Experimental periods and harvesting protocols are listed in Table 1.

In the 97 days of testing algae vs. aquatic vegetation we harvested 63 times. Harvesting during this time was executed based upon the degree of surface area cover, and with only a few exceptions, harvesting of both modules was conducted at the same time. Once the algae in the west channels were confluent, 30 to 40 percent of the cover was removed in patches. The harvesting protocol for channels covered predominantly in duckweed and azolla differed from that for channels covered predominantly by filamentous green algae due to the different growth habits exhibited. Duckweed and azolla both have a tendency to spread out across the surface of a channel, such that as long as individual fronds sufficient to cover the surface are present, they will do so. Much of this diffusion happens immediately, with the remainder generally occurring over the rest of the day. To harvest aquatic vegetation, then, it is necessary to use vegetation density rather than surface cover area to determine the proper ending point of harvesting. Harvesting was therefore carried out until a consistent density was reached, as estimated visually. To quantify this post harvesting density, after harvesting on each of three dates (9^t December 2009, 8 February 2010, and 10 March 2010) a 20.1 cm internal diameter pipe was placed in five different locations in the channels. At each sampling location, a

perforated thin aluminum tray (modified loaf pan) was used to collect all of the vegetation within the pipe. The vegetation was then dried at 105°C to constant weight and reweighed. The post harvesting density of aquatic vegetation was determined to be 59 ± 9.1 g DW m^{-2} (mean ± SE; n = 3), which closely matches the density of 1.25 kg fresh weight m^{-2} (61 g DW m^{-2}) reported by Koles et al. (1987) to allow the maximum production of a duckweed culture.

From 20 August 2008 to 23 September 2008, we reestablished equivalent conditions in both modules by ceasing selective harvesting and exchanging aquatic biomass between modules (Fig. 1 arrow). We then treated both modules identically until 18 March 2009, when we stopped harvesting the east module altogether and continued harvesting the west module as before. As this experiment tested the effect of harvesting on nitrate removal, I will refer to it as "harvest vs. no-harvest." Data analyzed for this experimental period were gathered between 24 March 2009 and 16 March 2010.

Flow Rate

Flow rate was measured at the time of each harvest. From March 2008 to February 2009, flow rate was measured continuously by two impeller flowmeters (SeaMetrics SPX 050) with two flow computers (SeaMetrics FT420W) displaying the instantaneous flow rate and the total flow volume. At the time of each harvest these values were recorded. Before March 2008 and again after February 2009 due to impeller clogging, flow rate was measured using the bucket method, during which the volume of

water flowing into a container and the time in which the water flowed were measured simultaneously using a bucket and a stopwatch, respectively.

Species Composition

Species representation was assessed weekly by visual examination of each channel. Percentages of surface coverage were recorded of the following categories: algae, duckweed, azolla, hydrocotyle, and terrestrial plants. This assessment was made on the same day that nitrate concentration samples were collected. Photographs were taken this same day. Species composition data was used to determine productivity and N-assimilation, as each group of species has a different wet to dry weight ratio and percent nitrogen composition. The effect of the dominant vegetation type (algae, azolla, duckweed or hydrocotyle) on productivity was analyzed on a per-channel basis by isolating the productivity data only for those periods in which one vegetation type covered at least 80% of a channel's surface area and comparing the means. The effect of the dominant vegetation type in the first and second channels (hydrocotyle was always present in third channels) on nitrate removal efficiency was analyzed on a per-module basis by isolating the nitrate removal efficiency data of modules that had at least 80% of their upper two channels covered by the same vegetation type and comparing the means.

Nitrate Concentration

All samples were analyzed for N-nitrate using EPA Method 300.0 (SOP Rev. 4) on a Dionex Ion Chromatograph (Model 2200) in the Laguna Environmental Laboratory. Treatment plant operators collect a one day composite sample of final wastewater effluent three times per week, including the day of our CAS effluent sampling and two days prior to our day of sampling. We average these two values as our CAS influent value. The autosamplers (Sigma 1600 Automatic Liquid Sampler, Hach #6507) collecting the composite samples are programmed flow proportionally to collect 125 mls every 7 minutes at the maximum flow of 75 mgd from midnight to the following midnight.

Until 31 March 2009, all CAS effluent samples taken were grab samples. Sets of grab samples taken at periodic intervals over the course of a day were taken on 27 May, 21 October, and 9 December 2008 to ascertain the possible effect of grab sample time on nitrate concentration (Fig. 2).

Two autosamplers (Sigma 900 Max Portable Sampler, Hach catalog number 8992) were set up at the end of March 2009, one at the outflow of each module. Each autosampler was programmed to collect 150 ml samples every 15 minutes over the course of the 13 hours that water flows into the CAS (7 a.m. to 8 p.m.). From 31 March 2009 to 27 October 2009, the autosamplers collected composite samples, and two sets of grab samples were taken manually at different time points over the course of the day. From these data, a standard curve was plotted by hand (Fig. 3) to establish the relationship between the time the grab sample was taken and the difference between its

value and the value of the composite sample. Ideally, we would have been able to fit a mathematical function to the data, but perhaps due to the inherent complexity of the system, no functions fit satisfactorily and we therefore elected to fit the curve by hand. Grab sample nitrate concentration values from 13 May 2008 to 31 March 2009 were then corrected by applying the correction factor for a particular time as determined from the standard curve. After 31 March 2009, the composite values were used for all CAS effluent data.

Ammonia Concentration

Composite CAS 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). Ammonia in CAS influent was determined from composite samples of secondary treatment plant effluent taken by plant operators on the same day. Sampling was conducted on the 6th and 20th of October 2009, the 17th of November 2009, and the 23rd of February 2010.

Productivity

To evaluate net productivity, 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. Net productivity can therefore be expressed

either as g wet weight m^{-2} d^{-1} or g dry weight (DW) m^{-2} d^{-1}; in this paper I use DW exclusively.

Environmental Variables

To collect water temperature, pH, dissolved oxygen, conductivity, and turbidity data, Datasondes (YSI model 6000s) were deployed in the modules periodically over the course of the experiment. The Datasondes measure temperature using a stainless steel strain gage Thermistor sensor (± 0.4 °C), dissolved oxygen percent saturation using a rapid-pulse polaro-graphic sensor (± 2% air saturation), conductivity using a 4 electrode cell (± 1% of reading, + 0.05 mS cm^{-1}), and pH using a glass combination electrode (± 0.2 units).

Solar radiation, air and soil temperature, precipitation, and wind speed data was downloaded from the California Irrigation Management Information System (CIMIS) automated weather station located at Brown Farm, two kilometers north of LTP. The weather station measures total solar radiation using a Li-Cor high stability silicon photovoltaic detector (model LI200S, ±1% error over 360 degrees at 45 degrees elevation), soil temperature using a Fenwal Electronic UUT51J1 thermistor (model 107b, ±0.4 °C over -33 to 48 °C), air temperature and relative humidity using a Fenwall Thermistor/HUMICAP H-sensor (model HMP35C, ±2% RH (0-90% RH), ±5% RH (90-100%), ±0.1 °C over -24 to 48 °C range), wind speed using a Met-One magnet activated reed switch three-cup anemometer (model 014-A, 1.5% or 0.11 m sec^{-1} (0.25 mph), and

precipitation using a Texas Instruments magnetic reed switch tipping-bucket rain gauge (model TE525MM, ±1% at 5 cm hr^{-1} or less).

Evapotranspiration is a summation variable that describes the combined water loss to the air from evaporation and transpiration. It is calculated using values for solar radiation, air temperature, wind speed, and relative humidity data (see appendix III).

Biomass Composition

From December 2008 to December 2009, three grab samples were collected monthly from each separable type of vegetation present in each channel. Grab samples of the same vegetation and channel were combined, transported to Sonoma State University, dried at 105 °C until constant weight, and ground with a mortar and pestle. Analysis of samples for percent nitrogen and percent carbon was carried out in the laboratory of Dr. Tim Nelson at Seattle Pacific University using a CE Elantech 1112 elemental analyzer. Percent nitrogen 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 (%N 10.52). Only 17.7% of the N content of azolla was assumed to be derived from nitrate uptake with the balance of N assimilated coming from the N_2-fixing cyanobacterium in plant leaf cavities (Ito and Watanabe 1983, Sah et al. 1989).

Lipid characterization was performed at the United States Department of Agriculture Agricultural Research Service Eastern Regional Research Center (USDA-ARS ERRC). Soxhlet hexane extractions of biomass were used to gravimetrically

measure percent lipid. The lipid fraction obtained through these extractions was analyzed on silica Thin Layer Chromatography (TLC) plates using 80:20:1 hexane: diethyl ether: acetic acid with standards of monoacyl, diacyl, and triacylglycerols (MAG, DAG, and TAG, respectively), free fatty acids (FFA), and fatty acid methyl esters (FAME) to determine the relative presence of these compounds. *In situ* transesterifications (I.S.T.) were performed with 0.1 N NaOMe in MeOH as described by Haas et al. (2004). All procedures done at the USDA analyzed biomass sampled from the CAS from April to July 2008.

Mosquitoes

The modules are regularly monitored using 12-oz dipper cups for the presence of mosquito larvae by technicians from the Sonoma Marin Mosquito Control District.

Statistical Analyses

We used general linear mixed models (SAS proc mixed ver. 9.1) to assess the influence of 1) environmental variables on net primary productivity and nitrate removal efficiency of the modules; 2) selective harvesting (to influence vegetation type in the "algae vs. aquatic vegetation" experiment) on net primary productivity and nitrate removal efficiency; and 3) harvesting per se ("harvest vs. no-harvest" experiment) on nitrate removal efficiency. A major caveat of the latter two analyses is that these experiments were not replicated as there were only two modules (one assigned to each

treatment level), thus p-values and confidence intervals must be interpreted with caution as they are derived from measurements over time from the same module, albeit conditioned on an appropriate covariance model to account for non-independence among observations. Furthermore, statistical inferences are restricted to these two modules only as they do not constitute a true statistical sample. To help assuage concern that any effects we saw were due to module and not treatment, we analyzed nitrate removal efficiency 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 efficiency ($F_{1,22.4} = 0.02$, $p = 0.878$) 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 (Littell et al., 2006). 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 CAS one week influences the treatment community of the next week. Because many of the environmental variables we considered for the first analysis may be interrelated and thus collinear (e.g., insolation and air temperature), we tested for multicollinearity by examining variance inflation factors in an ordinary least squares regression analysis and excluding those with a score above 5 (Stine 1995). We assessed model fit through visual

inspection of residual plots and used transformations as needed to normalize the distribution of residuals. We used log and square root transformations, respectively of the productivity data and the nitrate removal efficiency data, except in the case of the harvest vs. no-harvest segment, where we used untransformed data.

We examined different environmental variables (evapotranspiration, solar radiation, minimum air temperature, precipitation, and wind speed) for use as predictor variables in our first analysis. We began our analysis with fully saturated models and then proceeded to remove one by one all predictor variables and interactions between predictor variables with p-values above 0.05, starting with the least significant parameter. To prepare our environmental data for use in the models, we had to decide what range of time we should include. Of our response variables, nitrate removal efficiency was measured weekly, and productivity at the slightly higher resolution of three times weekly. To make the predictor variables best match this time scale, we used weighted averages of the four days leading up to and including the day of sampling. Taking evapotranspiration as an example, if E_0 is the evapotranspiration on the day of sampling, and E_{-1} is the evapotranspiration on the day before the day of sampling, etc, then:

$$E_a = (0.4 * E_0) + (0.3 * E_{-1}) + (0.2 * E_{-2}) + (0.1 * E_{-3})$$

where E_a is the weighted average evapotranspiration. For minimum air temperature, we used the absolute minimum of this same four day period in our models.

IV. Results and Discussion

Preliminary study

During this period we established that the CAS could reliably keep effluent nitrate concentrations below the regulatory discharge limit of 10 ppm (see Appendix 1).

Biomass Composition

In regularly harvested modules, samples of pleuston biomass collected between December 2008 and December 2009 showed percent dry weights of algae (n = 5), azolla (n = 17), duckweed (n = 37), and hydrocotyle (n = 24) at 6.05 ± 0.50 %, 5.34 ± 0.19 %, 4.89 ± 0.14 %, and 6.51 ± 0.19 %, respectively (mean ± SE). These values were used in the calculations of productivity, percent carbon, and percent nitrogen.

A sample of algae collected on the 3rd of July 2008, freeze dried, and ground with a mortar and pestle was 5.32 ± 0.007% lipid (mean ± SD, n = 2). Thin Layer Chromatography (TLC) of this extracted lipid showed a predominance of free fatty acids (FFA), with triacylglycerols (TAG) present but significantly less (Fig. 4, lanes 12 and 13). TLC of *in situ* transesterification (I.S.T.) of this sample of algae showed complete transesterification of the TAG, but the remaining presence of FFA (Fig. 4, lanes 1 - 4). This suggests that using an acid-catalyzed process might result in a higher amount of fatty acid methyl esters (FAME, also known as biodiesel) than the base-catalyzed process used here. Incorporating a first step to convert the TAG to FFA would probably maximize the yield of such an acid-catalyzed I.S.T. However, the 5.32% lipid present in

our algae was significantly less than the 14.5% lipid average of green filamentous algae and was below the reported range of 11.8 – 16.1 % lipid (Stevenson 1996). Our value aligns more closely with the Huggins et al. (2004) finding of a 1.5% lipid content in a biofilm containing *Oedogonium* and other chlorophytes, diatoms, and cyanobacteria. While acid-catalyzed I.S.T. of algae containing high levels of FFA is worth investigating, application of the process using algae available from our system for biofuel production would not be energetically feasible due to its low lipid content.

As Table 2 shows, in the CAS from December 2008 to December 2009, our mean percent N ranged from 4.17 to 5.09, depending on the type of vegetation. These values are statistically equivalent to the values obtained from the USDA-ARS, where sampled oven and sun dried algae was 4.41 ± 0.05 % N (mean ± 95% CI, n = 6), and sampled oven dried hydrocotyle was 4.42 ± 0.05 % N (mean ± 95% CI, n = 3) (data not shown). Our calculated C:N ratios ranged from 6.8 to 9.1, which is significantly below the reported ideal C:N ratio of 25-30 for anaerobic digestion (Ward et al., 2008). The problem of low C:N can be remedied by codigestion with higher C:N substrates (Bouallagui et al., 2009). Since February 2010, we have been operating two 1500 gallon anaerobic digesters with feedstock consisting of 40% aquatic vegetation, 40% dairy manure, and 20% winery lees. The C:N ratio of dairy manure ranges from 9.3 – 33.4, with a median value of 16.1 (Pettygrove et al., 2010). Local winery lees have a C:N ratio of 11.69 ± 0.32 (mean ± 95% CI; n = 4). Our combined C:N ratio is low enough that we may encounter reduced methanogenesis due to ammonia inhibition (Chen et al., 2008; Cuetos et al., 2008), but we have yet to witness inhibitory ammonia levels either in the lab or in our 1500 gallon digesters (John Kozlowski, personal communication).

Species Composition: Succession

As in other studies (e.g. Kadlec 1987), aquatic vegetation in the modules has undergone succession (Fig. 1, Fig. 5). Following its introduction to channel E3 in February 2008 and to channel W3 in August 2008, hydrocotyle steadily displaced the pleuston then growing in the bottom channels (Fig. 6d, f). Hydrocotyle appeared to suffer with the frost (Fig. 7c), and its productivity diminished significantly in the winter months, but it was never displaced. In March 2008, filamentous green algae (Fig. 5b), the culture we most heavily initially established, dominated both modules. In April 2008, following the initiation of unselective harvesting in the east module, duckweed and azolla began to outcompete the algae (Fig. 6a, b, e). In August 2008, azolla dominated the surface area in channels E1 and E2 until the appearance of fungus and azolla weevils (*Stenopelmus rufinasus)* (Fig. 7b), at which point algae displaced it (Fig. 1). In February 2009, in all four top channels (W1, W2, E1, and E2), azolla became dominant and was subsequently displaced by duckweed (May 2009) without, however, the occurrence of fungal infection or weevil infestation. Again in February 2010, azolla became dominant in W1 and W2 (the east module was not being harvested at this time, and lands plants dominated (Figs. 8e, f and 9e, f)).

The causal sequences of these displacements will never be entirely clear due to the preponderance of contributing factors. It does seem clear, however, that one important factor is height relative to the surface of the water. It is apparent upon visual inspection that duckweed and azolla can grow on top of and shade out the algae (Roijackers et al., 2004). Interestingly, Whitehead (1987) reported that algae sometimes grew over and submerged duckweed fronds, thereby gaining dominance in the culture. In

the CAS, however, and in general, duckweed consistently gains the upper hand; duckweed is in fact often employed in wastewater treatment specifically to preclude algal growth (Kadlec and Wallace 2009; Reed et al., 1995). Moreover, rain events heavily favor duckweed and azolla over algae, as they remain buoyant while algae sinks below the surface, thereby allowing the duckweed and azolla to spread into the space left by the algae and making it harder for algae to reemerge. Azolla, in turn, appears to be able to shade out duckweed (Fig. 6c, e), though with the arrival of higher irradiances and temperatures in June, duckweed appears to displace azolla in spite of its height disadvantage. It is possible that such temperature and/or irradiance stress in azolla helped bring about the fungal infection and weevil infestation of August 2008. Though correlation is never sufficient, the ubiquitous presence of the fungus and sporadic distribution of the weevils do at least point towards the potential importance of the fungus in this instance. This would also be consistent with the report by Charudattan (1987) that of the most important pathogens of aquatic plants (fungi, bacteria, viruses, and nematodes), fungi are the most common and can have "immense destructive capacity".

As Kadlec (1987) found, areas of open water with an abundance of nutrients and without heavy wind disturbance favor the development of a thick duckweed mat. The west end of channel W2 seemed to experience heavier winds than the other channels, and while azolla was able to establish a confluent mat even in extremely windy conditions, duckweed was sometimes blown to the east side of the channel. Lemna Technologies, Inc. dealt with this issue by establishing a floating grid structure that prevented the duckweed from being blown too far (U.S. Patent # 5,096,577). In the CAS, even the gentle flow of water into W2 (never greater than 15 L min^{-1}) often appeared too turbulent

for full duckweed establishment (Fig. 5a, darker circle of open water visible in bottom middle), at least at the densities maintained by the harvesting regimen.

Despite heavy aphid infestation (Fig. 7d) and cold temperatures (Fig. 7c), hydrocotyle has never been displaced, presumably due to its height advantage (Fig. 6d, f) and cold tolerance. This is consistent with the NASA study in Mississippi (Wolverton and McCaleb 1987) that reported that while water hyacinth could not function in (or survive) extreme winter temperatures, artificial marsh systems with hydrocotyle and duckweed could. It is unclear to what extent the hydrocotyle might be dormant here in the winter, and what effect such a dormancy might have on its associated microbial community. The lowest recorded water temperature in any channel, 4.5 °C, occurred at 7:32 a.m. on 5 February 2008 in W1. The lowest recorded water temperature in either 3rd channel was 5.6 °C, also on 5 February 2008 (at 8:47 a.m.), but that was at the bottom of the deepest water, beneath the zone hydrocotyle occupies. The lowest recorded water temperature recorded in the surface zone of the 3rd channels was 6.9 °C, recorded at 10:16am on 14 December 2008. As is common, Watson et al. (1987) found significant changes in system performance in winter in Iselin, PA (40° 56' N, 79° 39' W, average summer maximum air temperature 24.3 °C, summer min. 9.5 °C, winter max. 9.5 ° C, winter min. -4.8 °C) that they attributed in part to plant dormancy. Cal Lemke of the University of Oklahoma reports that hydrocotyle goes dormant at approximately 5 °C (Lemke 2005); as Stowell et al. conclude (1981), low temperatures in winter may cause aquatic plants to become dormant, which might decrease support to associated bacterial communities, but further research in this area is needed.

Duckweed is well acclimated to winter, and has been found to vegetate even at 1 °C (Wolverton 1987a). When temperatures get too low for duckweed it forms turions ("winter buds") which sink due to a high specific gravity. In this way duckweed can survive even harsh winters and then float to the surface in the spring; this capability obviates the need to reseed a treatment pond in order to maintain annually-recurring cultures of duckweed (Reed et al., 1995). Azolla is also well adapted to winter as it can vegetate at 5 °C (Janes 1998a) and produce sporocarps capable of overwintering in the sediment and releasing germinating gametophytes in the spring (Janes 1998b; Toia et al., 1987).

Harvesting plays an important role in species succession, as it frees up space in which to compete and keeps the level of competition closer to the surface of the water (Tchobanoglous 1987; Whitehead 1987). The effect of not harvesting is discussed in more detail on p. 27.

Species Composition: Influence on Nitrate Removal Efficiency and Productivity:

The relationship of dominant vegetation type to nitrate removal efficiency and productivity are presented in Figs. 10 and 11, respectively. Seasonal variability in system performance confounds statistical analysis since certain vegetation types tend to dominate during certain times of the year (see above section). In the case of nitrate removal efficiency, the data is further obfuscated by the constant presence of hydrocotyle in the third channels. Since nitrate removal efficiency is only measured on a per-module basis, the effect of hydrocotyle may be masking a differential effect between the vegetation

types. Taking the above into account, there is no apparent effect of vegetation type on nitrate removal (Fig. 10). In the case of productivity, while we were able to examine the data on a per-channel basis, the confounding effect of seasonal variability may still have either masked or enhanced differences in productivity according to vegetation type (Fig. 11). The only significant difference we could detect was the much higher productivity of azolla compared to duckweed. However, as periods of azolla dominance occurred from February to August, and those for duckweed from June to January, this difference could well have been at least partially a seasonal effect; in laboratory studies, duckweed generally has a higher productivity than azolla (DeBusk and Ryther 1987; Lakshman 1987). Hydrocotyle had the largest standard deviation of any of the vegetation types at least in part because, due to its year-round dominance in the third channels, data used for hydrocotyle dominance included its lower winter productivities along with its higher summer productivities. To get a true comparison of productivity between vegetation types we would have to measure vegetation growth in the lab under standardized environmental conditions. While our productivity values fall within the reported ranges for the vegetation we are cultivating (Lakshman 1987), and the ability of hydrocotyle to be more productive than duckweed in the summer is consistent with the literature (DeBusk and Ryther 1987; Reddy and DeBusk 1987), a measure of comparative productivity independent from environmental effect is outside the scope of this study.

Mosquitoes

Periodic dipper samples have not revealed mature mosquito larvae. This observation of self-sustaining mosquito control contrasts with typical constructed wetlands, which require active management of mosquito populations (Knight et al., 2003). Mats of azolla are known to decrease both oviposition and adult emergence of mosquitoes (Rajendran and Reuben 1991; Wagner 1997), which accounts for its common name, "mosquito fern". Additionally, the harvesting of aquatic vegetation avoids the potential build up of 'pockets' or sections that mosquito fish cannot reach (Tchobanoglous 1987). The presence or absence of mosquitoes is no small matter, as it may determine whether or not an aquatic treatment system will be permitted.

Algae vs. Aquatic Vegetation: Nitrate Removal Efficiency

After completing a preliminary study that demonstrated that the CAS could indeed keep nitrate below regulatory discharge levels (Appendix I), the first question I addressed was whether selectively harvesting one of the modules to maintain a predominant culture of filamentous algae would affect the nitrate removal capacity of the system relative to harvesting a module unselectively. In other words, I set out to determine whether CAS should be operated with algae specifically or with aquatic vegetation more generally. This was of course highly relevant to the rest of the work we were to do, as it would establish the operating procedure moving forward.

Using data from 13 May 2008 to 19 August 2008 we found that vegetation type has no effect on nitrate removal efficiency ($F_{(1, 6.7)} = 0.18$, $p = 0.69$). The nitrate removal

efficiency of the algae module was 1440 ± 276 mg $N\text{-}NO_3^-$ m^{-2} d^{-1} (mean ± 95% CI). The nitrate removal efficiency of the aquatic vegetation module was 1350 ± 235 mg $N\text{-}NO_3^-$ m^{-2} d^{-1} (mean ± 95% CI) (Fig. 12). The lack of a significant difference between the two modules is perhaps even more clearly shown in Fig. 13; the nitrate removal efficiency does vary over time, but the two modules perform nearly identically at each particular time.

Algae vs. Aquatic Vegetation: Productivity

In addition to the effect of vegetation type on nitrate removal efficiency, we investigated the effect of vegetation type on productivity. Using data from the 13th of May 2008 to the 19th of August 2008, we found that the productivity of aquatic vegetation was almost double that of algae ($F_{(1,44.6)} = 34.23$, $p < 0.0001$). The productivity of algae was 8.14 ± 1.11 g m^{-2} d^{-1} (mean ± 95% CI). The productivity of aquatic vegetation was 15.7 ± 2.64 g m^{-2} d^{-1} (mean ± 95% CI) (Fig. 12).

The amount of biomass produced was highly relevant because a major intention of the present study was to maximize the eventual creation of bioenergy from biomass produced during phase I. After determining that algae and aquatic vegetation removed equivalent amounts of nitrate, and that aquatic vegetation actually out-produced algae, we proceeded to the next period of our investigation. During this period, we first re-equilibrated the two modules, and then gathered data to determine the effect of environmental variables on the performance of the modules.

Environmental Effects: Nitrate Removal Efficiency

To determine the influence of environmental factors on the performance of the modules, it was important to include as wide a temporal range of data as possible. Towards this end, data from a module was applied to this analysis during all periods in which it contained aquatic vegetation that was unselectively harvested. We found using a mixed model that minimum air temperature had a significant effect on nitrate removal efficiency ($F_{(1, 89.9)} = 16.86$, $p < 0.0001$). Evapotranspiration also had a significant effect on nitrate removal efficiency ($F_{(1, 86.5)} = 10.55$, $p = 0.0017$). The interaction effect between evapotranspiration and minimum air temperature did not have a significant effect on nitrate removal and was therefore removed from the model ($F_{(1,83.2)} = 3.54$, $p = 0.0632$). The equation for the square root of nitrate removal efficiency is:

$$N = 25.15 + (1.536 * E_t) + (0.7744 * T_{min})$$

N = the square root of nitrate removal efficiency (mg N m^{-2} $d^{-1})^{1/2}$

E_t = Evapotranspiration (mm d^{-1})

T_{min} = Minimum air temperature (°C)

As is evident in the slope of the response curve in Fig. 14, minimum air temperatures below 0 °C and evapotranspiration rates below 2.5 mm d^{-1} have the largest effect on nitrate removal efficiency. Once the minimum air temperature is above approximately 0 °C, nitrate removal efficiency appears to respond more strongly to increasing evapotranspiration rates (up to 4 mm d^{-1}) than it does to further increases in minimum air temperature. The nitrate removal efficiency decreases slightly with increasing evapotranspiration rates above approximately 5 mm d^{-1}, perhaps due to

photoinhibitory processes in the aquatic vegetation, or to water temperatures higher than is optimal for the CAS bacterial community adapted to denitrification processes.

Overall Mean Nitrate Removal Efficiency

To describe the overall nitrate removal capabilities of the CAS we examined the monthly means of the same segment of data as was evaluated for environmental effects on nitrate removal efficiency. Fig. 15 exhibits each monthly mean in relation to the mean monthly evapotranspiration rate, and the mean monthly minimum air temperature. The average of the monthly mean nitrate removal efficiencies during this period was 1050 ± 115 mg N m^{-2} d^{-1} (mean ± SE; n = 12). This mean compares favorably with comparable aquatic treatment systems (Table 3). Kelly Wetlands, located about five km from our study site, exhibited a mean nitrate removal efficiency of 625 mg N m^{-2} d^{-1} (Smith 1990, as cited in Bachand and Horne 1999a). The Arcata Wetlands, located in Humboldt County, averaged 800 mg N m^{-2} d^{-1} (pers. communication from Gearheart as published in Bachand and Horne 1999a). The Prado Wetlands, located in Orange County, CA, averaged 522 mg N m^{-2} d^{-1} (Bachand and Horne 1999a; Reilly et al., 1999).

It is too soon to be sure, but it seems likely that the nitrate removal efficiency of each module is increasing over time. If future studies do indeed determine that this is true, it would be consistent with the observation that constructed wetlands often require 2-3 years to operate at their full potential (Reed et al., 1995). Bavor et al. (1987) also found an increased capacity of their shallow lagoon-macrophyte systems to remove nutrients over time.

Environmental Effects: Productivity

Using data from the same periods of time as for examining the effect of environmental variables on nitrate removal efficiency, we tested the effect of these same environmental variables on productivity. We found that evapotranspiration had a significant effect on productivity ($F_{(1, 275)} = 133.7$, $p < 0.0001$). Minimum air temperature also had a significant effect on productivity ($F_{(1, 267)} = 27.08$, $p < 0.0001$). Furthermore, the interaction between evapotranspiration and minimum air temperature had a significant effect on productivity ($F_{(1, 269)} = 13.05$, $p = 0.0004$). The equation for productivity is:

$$P = 0.335 + (0.146 * E_t) + (0.0471 * T_{min}) - (0.00926 * E_t * T_{min})$$

P = Productivity ($g\ m^{-2}\ d^{-1}$)

E_t = Evapotranspiration ($mm\ d^{-1}$)

T_{min} = Minimum air temperature (°C)

It is interesting that the interaction between evapotranspiration and minimum air temperature is highly significant for productivity ($p = 0.0004$), but not for nitrate removal efficiency ($p = 0.0632$). It is possible that because bacteria are predominantly responsible for the nitrate removal, and because bacteria operate on time scales shorter than that of aquatic plants, that the period of four days as examined for our environmental variables (see Materials and Methods) was too long for an interaction effect to be seen for nitrate removal. Unfortunately, our budget did not permit us to do the near continuous monitoring of nitrate concentration over an extended period of time that would have been required to test this possibility.

As is evident from the slope of the response surface in Fig. 16, productivity showed the greatest positive response to increasing evapotranspiration in the range between 1 and 3 mm d^{-1}. Increasing evapotranspiration in the range between 3 and 5 mm d^{-1} also increases productivity, but not as sharply. As with nitrate removal efficiency, increasing evapotranspiration above approximately 5 mm d^{-1} actually slightly decreases productivity. When minimum air temperatures were below -2 °C, untransformed productivity values never rose above 5 g m^{-2} d^{-1}. Between -2 and 0 °C, untransformed productivity values increased steeply to as much as 25 g m^{-2} d^{-1}. Above 0 °C, evapotranspiration appears to influence productivity more strongly than does minimum air temperature.

Overall Mean Productivity

To describe the overall productivity of the CAS we examined the monthly means of the same segment of data as was evaluated for environmental effects on productivity (Fig. 15b). The mean of the monthly mean productivity during this period was 9.28 ± 1.67 g DW m^{-2} d^{-1} (mean ± SE; n = 12). February to March saw the biggest increase in mean productivity (from 2.37 to 10.91 g m^{-2} d^{-1}, or 360%), with September to October exhibiting the largest decrease in mean productivity (from 10.74 to 5.81 g m^{-2} d^{-1}, or 46%). Modeling was done (see above) to better define these relationships.

Harvest vs. No-Harvest

The non-harvested module had a higher nitrate removal efficiency than the harvested module ($F_{1, 39} = 11.20$, $p = 0.0018$) (Fig. 17). The nitrate removal efficiency of the harvested module was 1070 ± 93.1 mg N m^{-2} d^{-1} (mean ± 95% CI). The nitrate removal efficiency of the non-harvested module was 1230 ± 79.5 mg N m^{-2} d^{-1} (mean ± 95% CI). The difference between the nitrate removal efficiencies of the two modules began to show more obvious divergence beginning in October 2009 (Fig. 18).

Using the species composition data, percent nitrogen data, harvesting data, and dry weight data, it was possible to calculate the amount of nitrogen removed with each harvest. Normalizing this to the area of the CAS and the time since the last harvest permitted us to determine that 326 ± 15.3 mg N m^{-2} d^{-1} (mean ± SE, n = 144), or 30.5 percent of the total nitrogen removed by the harvested module, was removed through assimilation by the aquatic vegetation. Knight (2010) found that an average of 85.5 mg N m^{-2} d^{-1}, or 8.0 percent of the total nitrogen removed during this time segment, was stored in the sediment in the harvested module, a little more than half of the 150 mg m^{-2} d^{-1} reported by Gumbricht (1993b). As the channels are lined to prevent seepage, this leaves denitrification to account for 658.5 mg N m^{-2} d^{-1}, or 61.5 percent of the nitrogen removed, which is consistent with other studies (Gumbricht 1993a; Watson et al., 1987). The sediment carbon to nitrogen ratios (C:N) in the harvested module and unharvested module were 6.21 ± 0.09 (n = 11) and 8.00 ± 0.15 (n = 8), respectively (mean ± SE) (Knight 2010).

Cessation of harvesting did presumably lower assimilatory N removal despite continued vertically-oriented vegetative growth, but it is conceivable that increased deposition of detritus in the non-harvested module stimulated denitrification to a degree that compensated for or even surpassed the contribution of N removed by harvesting (Bastviken et al., 2005). An insufficient amount of carbon is known to substantially hamper the amount of denitrification, and the addition of carbon can therefore play a crucial role in determining the outgoing nitrate concentration (Hume et al., 2002). Carbon availability plays an essential role in Biological Nutrient Removal (BNR), a relatively new wastewater treatment process that optimizes denitrification activity to achieve levels of effluent nitrate concentrations below those afforded by traditional activated sludge systems, and external carbon addition is often required (Li et al., 2002). The lower C:N ratio in the harvested module sediment (Knight 2010) supports the hypothesis that the harvested module was more carbon limited than the unharvested module.

To further investigate the effect of organic carbon supply from aquatic vegetation to denitrifying populations, it would be best to establish a control module in which no vegetation was allowed to grow. To achieve this, it would be necessary to prevent light from entering the module, as simply removing all harvestable biomass would allow unharvestable microalgal blooms to dominate. We intend to pursue this line of study, resources permitting, at the conclusion of the current phosphate study. Bavor et al. (1987) established two control trenches, one with gravel, and one with open water. They found that while the trench with open water performed significantly less well than their other trenches, the trench with gravel performed almost as well as the trenches with both

gravel and macrophytes, though they expected the trenches with macrophytes to increasingly perform better as the systems matured. As Wolverton (1987a; 1987b) describes, there is a symbiotic relationship between microorganisms and plants in growing in wastewater effluent which allows for synergistically enhanced degradation of organic contaminants. Metabolites and waste products from microorganisms can be used as a nutrient source for the plants, and vice versa, which prevents the eventual slowing down of the chemical reactions breaking down the contaminants due to product accumulation.

In conjunction with the effect of added carbon, it is likely that the increased trend in nitrate removal by the unharvested module resulted from a steady increase in temperature relative to the harvested module. Due primarily to metabolic processes in activated sludge systems, water enters the modules at a temperature above that which would be expected due to ambient environmental conditions. As much as possible, it is important to take the effect of temperature on denitrifying activity into account when comparing results with other studies as well as within our own study (Table 3). Unfortunately, I did not collect water temperature data extensively enough to allow me to add it to the models as an independent variable. What I have done instead is use the data that we possess to explore the potential range of effects of temperature differences.

The direct effect of not harvesting a CAS module is for the channels to accrue a thick mat of vegetation (Figs. 8 and 9). An indirect effect of this thick mat is to decrease the heat exchange between the water in the channels and the air. Moreover, the elevated temperature of the water entering the modules means that this decreased heat exchange translated into higher temperatures in the non-harvested module than in the harvested

module. To model the potential effect of temperature on the rate of denitrification, a type of Arrhenius equation is used:

$$r_{D,\,T1} = r_{D,\,T2} \bullet \theta^{(T1 - T2)}$$

r_D = the rate of denitrification

θ = the temperature coefficient

T_1 = the first temperature

T_2 = the second temperature

The Arrhenius equation in general is used to establish the temperature dependence of the rate of a chemical reaction. In our case, what is important is not the actual rate of denitrification, but rather the ratio between the denitrification rate at one temperature and the denitrification rate at another temperature. In other words, we are interested in the percentage increase possible in denitrification rate with a specific increase in temperature. Over the course of an intensive sampling event from 12 to 26 October 2009, a significant temperature difference was observed between the harvested and the unharvested modules (Fig. 19). It appears that the west source (harvested) loses more heat than the east source (unharvested), and that the west output loses more heat, and loses it more quickly than the east output. It is important therefore to determine what effect this temperature could have had on denitrification rate, and thereby determine its potential contribution to any difference in efficiency of nitrate removal.

The difference between the average temperatures in the outflow of the two modules during the intensive sampling event of October 2009 was 1.6°C. To determine the effect this could possibly have on the rate of denitrification, a temperature coefficient (θ) must be ascertained. As the procedure to derive this experimentally is outside the

scope of this research, I used values established in the literature. Three published studies have experimentally determined a θ value for the range of our operating temperatures during this period. Timmermans and Van Haute (1983), using methanol as a carbon source and a range of temperatures from 6 - 30°C, found a θ of 1.13. Christensson et al. (1994), using a range of temperatures from 15 - 25°C, found a θ of 1.11 when using methanol as a carbon source, and a θ of 1.12 when using ethanol. Carrera et al.(2003), using a methanol mixture as a carbon source and a range of temperatures from 6 - 25°C, found a θ of 1.10. Inserting the average θ value of 1.115 into the Arrhenius equation tells us that the rate of denitrification at 21.4°C is 1.19 times that at 19.8°C. In other words, we would expect based on that temperature difference that the non-harvested module should show a denitrification rate 19% higher than that in the harvested module. However, the temperature difference is less dramatic at different locations. As the incoming water flows through the channels, it loses its added heat. At the end of the first two channels, during this same two week period, the difference in average temperatures was only 1.17°C. At this smaller difference in temperature, the unharvested module should show a 14% increase in denitrification rate over its harvested counterpart. It makes sense given the greater insulation present in the non-harvested module that the temperature differential between modules would gradually increase as the water flows through the modules.

In addition to an increasing east - west water temperature differential over space, I think probable just such an increasing temperature differential over time. The increasing difference in nitrate removal efficiencies shown between modules over time (Fig. 18) could be due either to a steadily rising relative temperature dependent increase in

denitrification, a gradual increase in carbon availability from accumulated decomposing biomass in the non-harvested module, or some combination of these two possibilities. A study of phosphate levels now ongoing will include more continuous water temperature data and should further illuminate the possibility of the effect of seasonal temperature differentials. At the conclusion of the study, scheduled for October 2010, biomass remaining in the channels will be weighed and analyzed for N content, and that in turn should elucidate the role of N-assimilation in the performance of the unharvested module.

Kadlec (1987) describes two regions of treatment in wetland systems: saturated, and unsaturated. In a saturated zone, such as in the unharvested modules, nutrients released from decaying plant matter equal or exceed new nutrient assimilation into plant biomass. We therefore tested for ammonia levels in the CAS effluent. No value higher than 0.3 mg L^{-1} was found in any of the samples, and no significant differences were found between harvested module effluent, unharvested module effluent, and CAS influent (data not shown). This result demonstrates that the nitrate being removed from the unharvested module is not simply coming out the other end as ammonium. It is likely that anammox plays a significant role in the gasification of ammonium released through biomass decomposition in the unharvested module (den Camp et al., 2006; Kuenen 2008).

The primary reason for performing the harvest vs. no harvest experiment was to determine the effect of harvesting on nitrate removal efficiency. Aside from its effect on water quality, the primary benefit of harvesting frequently is the accrual of more biomass for bioenergy production. Conversely, the primary drawback to harvesting frequently is the energy cost associated with harvesting. Energy limitations and bioenergy production

goals can be site-specific and should be taken into account in addition to concerns pertaining exclusively to water treatment when establishing a harvesting regime in a larger system.

V. Conclusions

CAS are an efficient way to remove nitrate from wastewater effluent compared to other systems; denitrification is the primary mechanism of N removal. Evapotranspiration and minimum air temperatures are the best way to predict CAS performance; freezing appears to have a non-linear effect. Modules containing algae and modules containing aquatic vegetation remove nitrate equivalently, though aquatic vegetation is more productive. The unharvested module removed N slightly more efficiently than the harvested module, possibly due to higher temperatures and higher availability of carbon, and this difference became more apparent as the modules matured. Harvested modules showed a cyclical pattern of species succession throughout the experimental periods, whereas the unharvested module showed a linear succession toward land plants. The harvested biomass is not suitable for biodiesel production; anaerobic digestion of the biomass has been shown to be a feasible means to transform harvested biomass into energy.

Appendix I

From July 2007 to March 2008, chlorinated tertiary-treated wastewater flowed into both modules from an existing pressurized water system on-site (see Appendix II for a brief description of the stages of wastewater treatment). During this period we established that the CAS could reliably keep effluent nitrate concentrations below the regulatory discharge limit of 10 ppm (Fig. A1). Values for nitrate concentrations entering the CAS are the final treatment plant effluent composite samples described on p. 8. Values for nitrate concentrations leaving the CAS are grab samples and are not corrected for time of day sampled. Species composition during this time was not recorded, nor was harvested biomass weighed.

The Laguna Treatment Plant does not chlorinate its effluent for distribution, but it does chlorinate effluent for on-site use to ensure smoothly operating pumps and to backflush the anthrocyte coal filters. Using standard methods (Total Residual Chlorine by DPD – FAS, S.O.P. Rev. 4, 20th ed. Standard Methods 4500 – Cl), the average chlorine concentration measured in the CAS influent between 25 January 2008 and 18 February was 3.09 ± 0.42 mg L^{-1} (mean ± SE, n = 7). During this same period, the average concentration of chlorine in the CAS effluent was 0.083 ± 0.033 mg L^{-1} (mean ± SE, n = 3), and the average concentration of chlorine at the end of the first channel was 0.39 ± 0.12 mg L^{-1} (mean ± SE, n = 3). Despite the levels of chlorine more appropriately suited to swimming pools, algae grew robustly in the CAS until the 10th of August, when algal mats in the top channels abruptly died due to a spike in chlorine levels resulting from an especially long backflush of the filters. This led us to seek the unchlorinated secondary water source that was used for the experiments that make up this thesis.

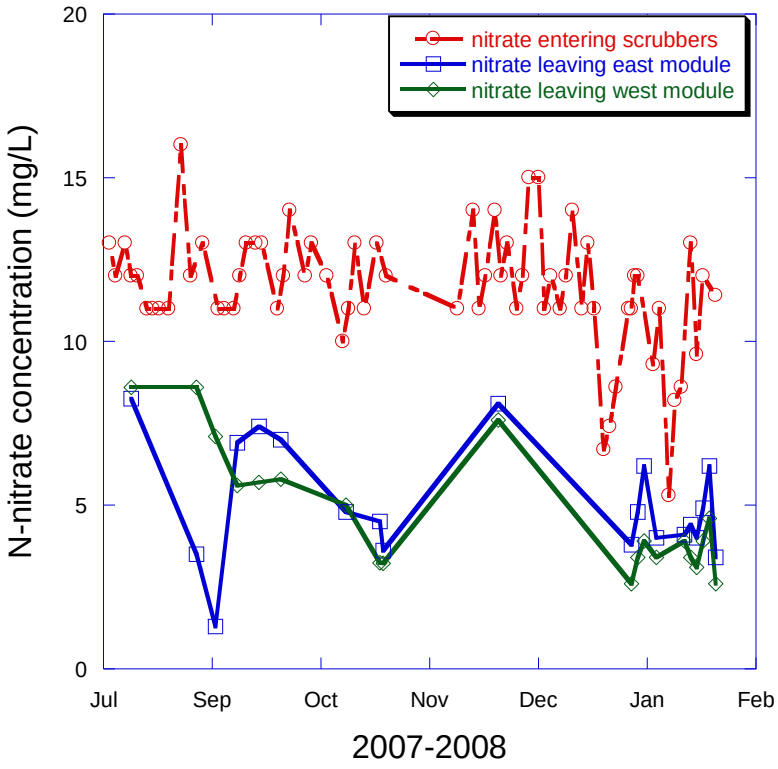


Fig. A1: Initial operation of the CAS on tertiary-treated MWE inflow demonstrated an ability to maintain N-nitrate levels below the regulatory limit of 10 mg ml^{-1}. The flow rate in both modules started off at 17 L min^{-1}. The significant drop in the east scrubber effluent nitrate concentration on the 28th of August and 3rd of September was due to the decrease in east module flow to 3.5 L min^{-1}. Between the 3rd of September and the 27th of September the flow rate averaged 9 L min^{-1} into each module, and after the 27th of September, the flow rate averaged 2.6 L min^{-1}.

Appendix II

Primary Treatment

Sewage from homes, business, and industry arrives at the treatment plant by passing through large bar screens that remove wood, paper, and plastics from the water. Sand and gravel then settle out in the grit tank and are removed. Clarification tanks allow lighter materials to float to the surface and be skimmed off. Heavier material, called biosolids, falls to the bottom and is pumped to anaerobic digesters. Bacteria in the digesters break solids down, creating methane gas.

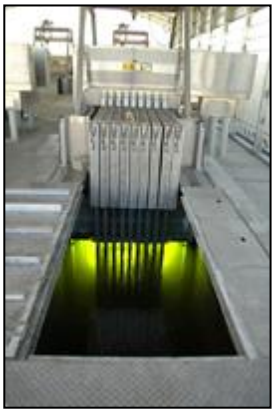
Methane powered generators serve as the source of energy for a sixth of the treatment process. Solids are digested for up to thirty days, reducing their volume by 50%. Following a dewatering process, biosolids are blended with greenwaste material to create compost, or they are applied directly to farmers' fields as fertilizer. A small quantity is sent to the landfill.

Secondary Treatment

After the majority of solids have been removed, water flows into aeration basins. The aeration basins are tanks injected with oxygen to stimulate the growth of microorganisms and their consumption of dissolved wastes. These microorganisms modify pollutants to reduce their impact on the environment.

As the water moves toward the next treatment phase, the microorganisms are removed in clarification tanks. As they settle to the bottom of the clarifiers, they are returned to the aeration basins to re-supply the self-sustaining population of microorganisms. Clean water continues on to further treatment.

Tertiary Treatment and Disinfection



The water flows through a four-foot bed of coal. This small, black, granular coal (like the type used in some fish aquariums) acts as a filter to trap fine suspended solids and some potential pathogens, or disease causing organisms. Finally, ultraviolet light (UV) removes bacteria and viruses by destroying their DNA, the genetic material needed to reproduce. The reclaimed water then leaves the plant, and is clean enough for many approved reuse purposes.

All information from http://ci.santa-rosa.ca.us/DEPARTMENTS/UTILITIES/TREATMENT/

Appendix III

Calculation of Evapotranspiration[1]

Variables Required

i. ea = Mean hourly vapor pressure (kPa)
ii. RH = Mean hourly relative humidity (%)
iii. Rn = Mean hourly net radiation (Wm^{-2})
iv. T = Mean hourly air temperature (Celsius)
v. U = Mean hourly wind speed at 2 meters (ms^{-1})
vi. Z = Elevation of the station above mean sea level (m)

Steps

1. **Convert temperature from Celsius to Kelvin**
   $T_k = T + 273.16$
2. **Saturation vapor pressure**
   $es = 0.6108 * \exp(T * 17.27/ (T + 237.3))$
3. **VPD - Vapor pressure deficit**
   VPD = es - ea (kPa)
4. **DEL - Slope of the saturation vapor pressure vs. air temperature curve at the average hourly air temperature**
   $DEL = (4099 * es)/(T + 237.3)^2$
5. **Barometric pressure**
   $P = 101.3 - 0.0115 * Z + 5.44 * 10^{-7} * Z^2$
6. **GAM - Psychrometer constant (kPa C^{-1})**
   GAM = 0.000646 (1 + 0.000946*T) P
7. **W - Weighting function**
   W = DEL/(DEL + GAM)
8. **FU2 - Wind function**
   For Rn<=0 (nighttime)
   FU2 = 0.125 + 0.0439U
   For Rn>0 (daytime)
   FU2 = 0.030 + 0.0576U
9. **NR - Convert Rn from Wm^{-2} to mm**
   NR = Rn/(694.5 (1-0.000946*T))
10. **Hourly ETo is approximately equal to RET**
    RET = W*NR + (1-W)VPD * FU2
11. **Daily ETo equals the sum of 24 hours RET (mm)**

[1] All information from http://www.cimis.water.ca.gov

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Tables

and

Figures

Dates	Experiments	Harvesting Protocol	
		West Module	East Module
4/12/2008 – 8/20/2008	Algae vs. Aquatic Vegetation, Environmental Effects	Selectively harvested to maintain algal dominance	Unselectively harvested
8/20/2008 – 9/23/2008	N/A	Unselectively harvested, vegetation exchanged between modules	Unselectively harvested, vegetation exchanged between modules
9/23/2008 – 3/18/2009	Environmental Effects	Unselectively harvested	Unselectively harvested
3/18/2009 – 3/16/2010	Harvest vs. No Harvest, Environmental Effects	Unselectively harvested	Not harvested

Table 1. Harvesting protocols observed during the experiments described in this thesis.

organism	% N	% C	C:N ratio	n
algae	4.60 ± 0.21	31.2 ± 2.66	6.75 ± 0.30	5
duckweed	5.09 ± 0.06	36.1 ± 0.22	7.11 ± 0.07	41
azolla	4.35 ± 0.19	38.6 ± 0.50	9.07 ± 0.48	11
hydrocotyle	4.17 ± 0.27	35.9 ± 0.57	8.77± 0.50	7

Table 2. Percent carbon and nitrogen of oven-dried samples (mean ± SE) collected monthly from November 2008 to December 2009. Samples analyzed by Dr. Tim Nelson, Seattle Pacific University.

study system	location (lat, lon)	N removal (mg N m^{-2} d^{-1})	summer (°C) high	summer (°C) low	winter (°C) high	winter (°C) low
Arcata Wetlands	40°86'N, 124°09'W	800	17.0	10.7	13.3	6.0
Prado Wetlands	33°94'N, 117°65' W	522	40.8	13.7	21.8	6.5
Kelly Farm Wetlands	38°42'N, 122°81'W	625	26.5	10.8	17.0	5.8
harvested CAS	38°37'N, 122°77'W	1070	26.5	10.8	17.0	5.8
unharvested CAS	38°37'N, 122°77'W	1230	26.5	10.8	17.0	5.8

Table 3. Comparative mean nitrate removal efficiencies from published data (Smith 1990, Bachand and Horne 1999a, Reilly et al. 1999). CAS data from harvest vs. no harvest study encompassing the time period between 24 March 2009 and 16 March 2010. All temperature data the mean of monthly average high and low air temperatures from the closest city to the study system (www.weather.com), with summer defined as May – Oct. and winter as Nov. – Apr.

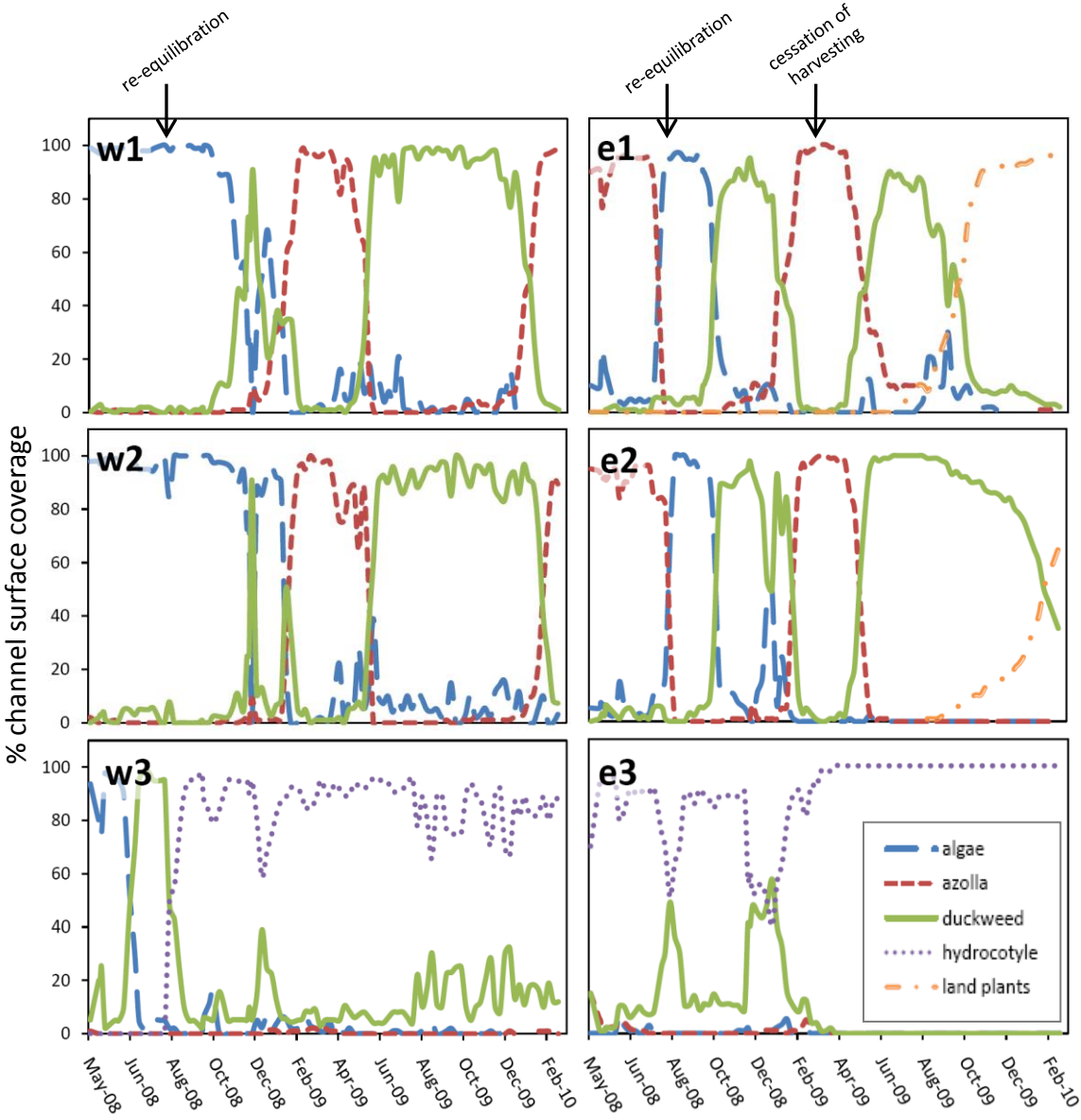

Fig. 1. Species composition as percentages of surface cover in the CAS from May 2008 to February 2010. "w" and "e" indicate west module and east module, respectively, while "1", "2", and "3" refer to the specific channel within a module. The first arrow indicates the beginning of the one month re-equilibration period between modules after the algae vs. aquatic vegetation experiment in August 2008, and the second arrow indicates the cessation of harvest in March 2009 in the east module that initiated the harvest vs. no harvest experiment.

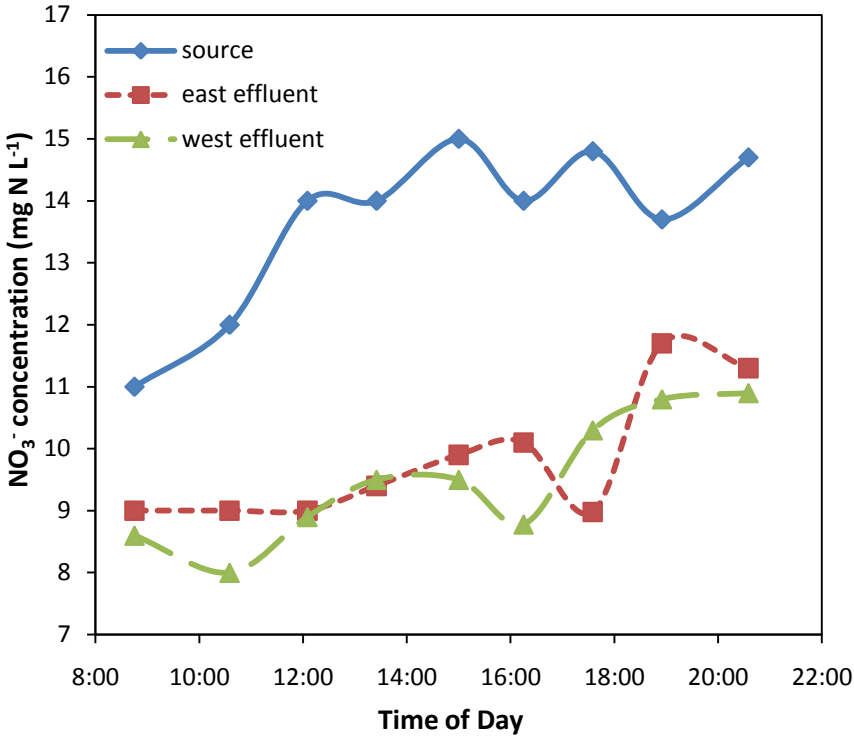

Fig. 2. Nitrate concentration of CAS influent and effluent over the course of a day. Data shown for the 27th of May 2008.

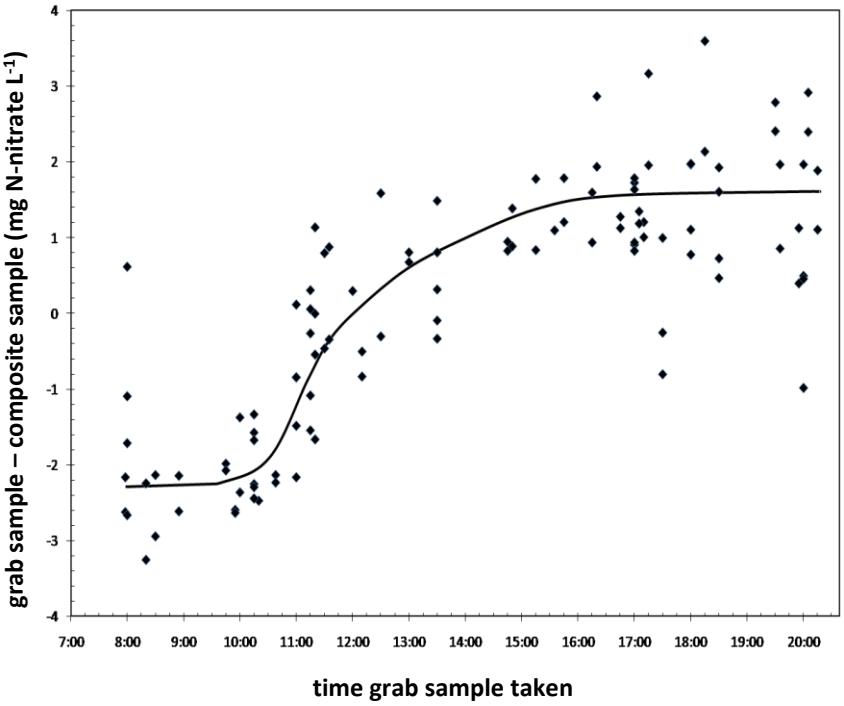

Fig. 3. Standard curve establishing the relationship between the time a grab sample was taken and the difference between its N-nitrate concentration value and the value of the composite sample from that same day. Grab sample values were then corrected by applying the correction factor for a particular time as determined by the standard curve. All effluent nitrate concentration data pictured was collected between the 31st of March 2009 and the 27th of October 2009.

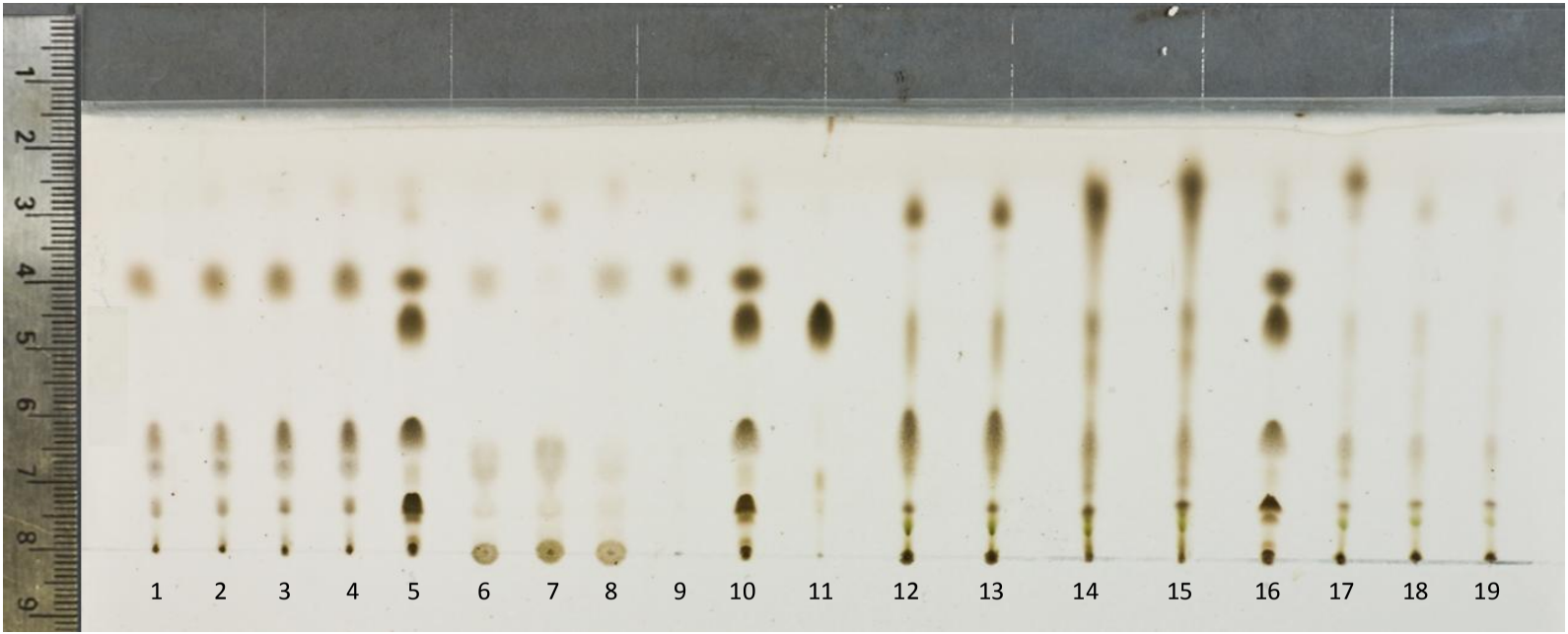

Fig. 4. Thin Layer Chromatography of algal lipid extraction and transesterification

1-4:	I.S.T. of SSU algae: lanes 1-2 = bead beat, lanes 3-4 = not bead beat (just coffee ground);FAME evident at the 4" mark
5, 10, 16:	standard: FAME at 4", TAG at 4.5", FFA at 6.25", DAG at 7.5", MAG at 8"
6-7:	I.S.T of different batches SSU algae; faint FAME evident at 4" mark, visible decomposition of sample in lane 7
8:	I.S.T. combination SSU algae and azolla; faint FAME evident at 4" mark
9:	FAME standard
11:	TAG standard
12-13:	soxhlet extraction SSU algae; pigment visible at 3", minimal TAG visible at 5", FFA visible at 6.25"
14-15:	soxhlet extraction Cal Poly. algae; pigment visible at 3", TAG visible at 5", FFA visible at 6.25"
17-19:	soxhlet extraction combination SSU algae and azolla with pretreatments: 17 bead beat (note extra fast moving spot probably accounts for extra weight on extract and is not TAG or FFA), 18 polytron homogenized, and 19 coffee ground

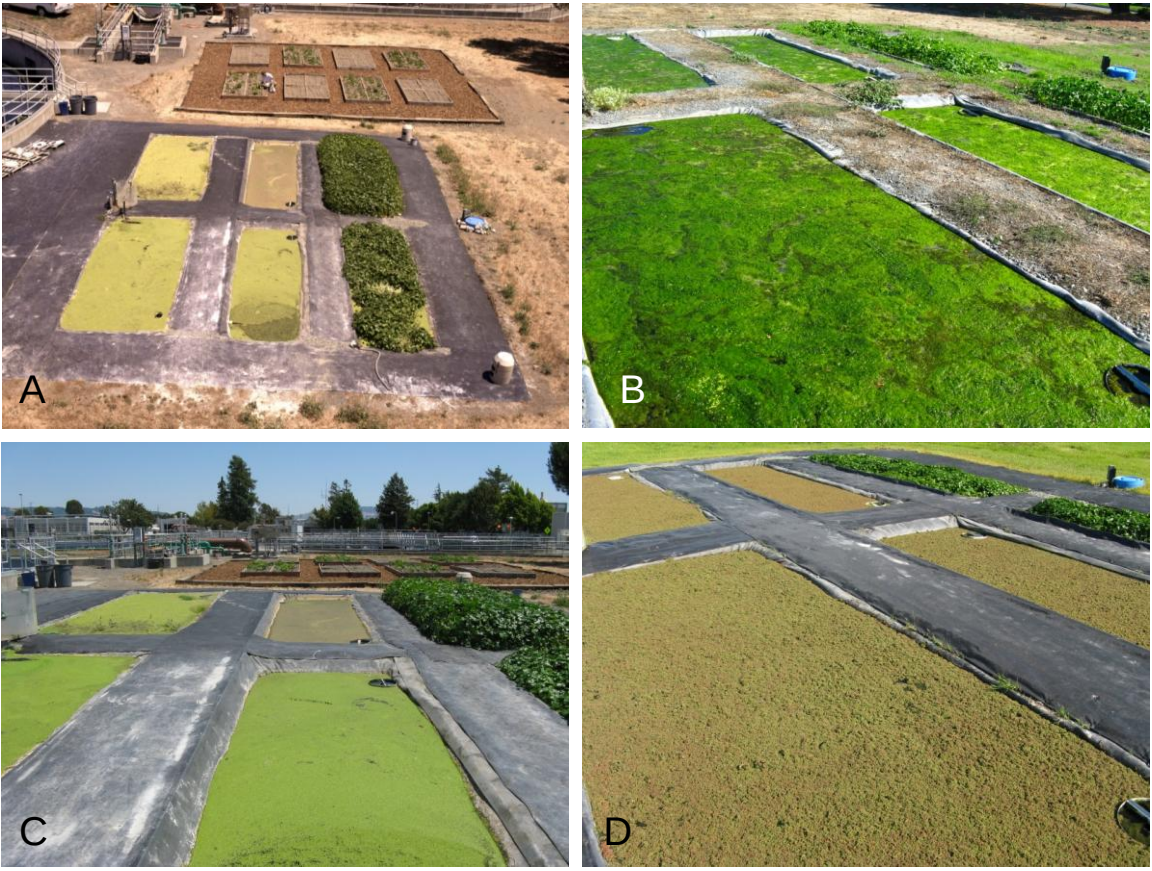

Fig. 5. Domination of the CAS by different pleuston in upper four channels (W1, W2, E1, E2); hydrocotyle dominant in all pictures in bottom two channels (W3, E3)
A) aerial view 27th July 2009, duckweed dominant; B) 8th October 2008, algae dominant; C) 28th July 2009, duckweed dominant; D) 24th March 2009, azolla dominant.

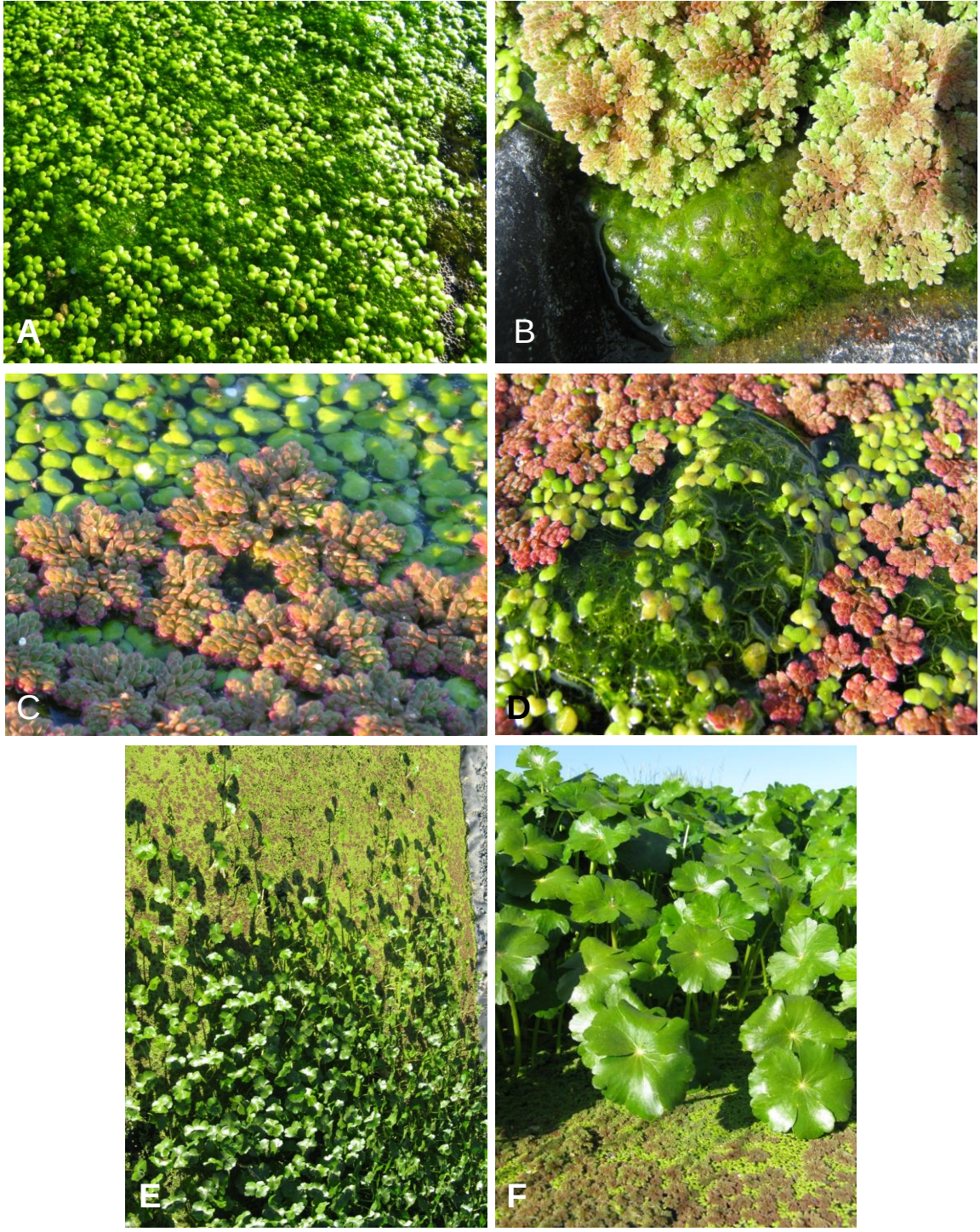

Fig. 6. Detailed views of pleuston shifts-in-progress. A) 7th Nov. 2008, W1, duckweed on algae; B) 31st Mar. 2009, W1, Al growing next to azolla after a harvest (algae did not expand); C) 9th Dec. 2008, E1, azolla on duckweed; D) 22nd Feb. 2008, W1, azolla and duckweed overlying algae; E) 1st May 2008, E3, hydrocotyle overlying azolla and duckweed; F) 1st May 2008, E3 detail, hydrocotyl overlying duckweed and azolla.

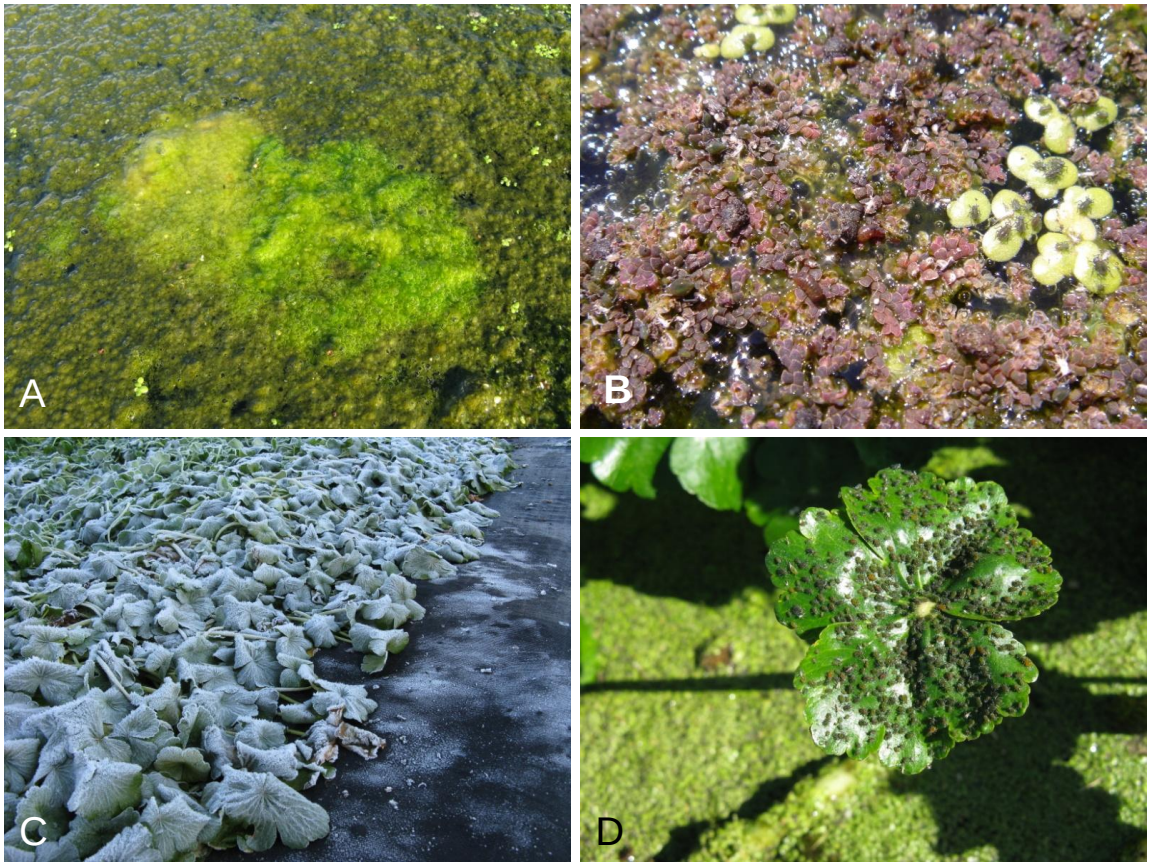

Fig. 7. Other factors affecting composition of the pleuston mat
A) 20th March 2008, W1: apparent decomposition of filamentous algae; B) 15th August 2008, E2: apparent fungal invasion of azolla, accompanied by the azolla weevil (*Stenopelmus rufinasus,* top right) and aphids; C) 9th Dec. 2009, E3: hydrocotyle with frost; D) 25th Aug. 2009, E3: hydrocotyle with aphids.

Fig. 8. Shifting vegetation cover in E1 post cessation of harvest.
A) 31st Mar. 2009, very thick mat of azolla; B) 19th May 2009, duckweed growing in crevasses of azolla mat; C) 23rd Jun. 2009, duckweed continues growing on top of azolla; D) 15th Sep. 2009, apparent decomposition of remaining visible azolla; E) 25th Aug. 2009, land plants sink into duckweed mat, allowing fresh growth of duckweed; F) 10th Nov. 2009, land plants dominating surface area.

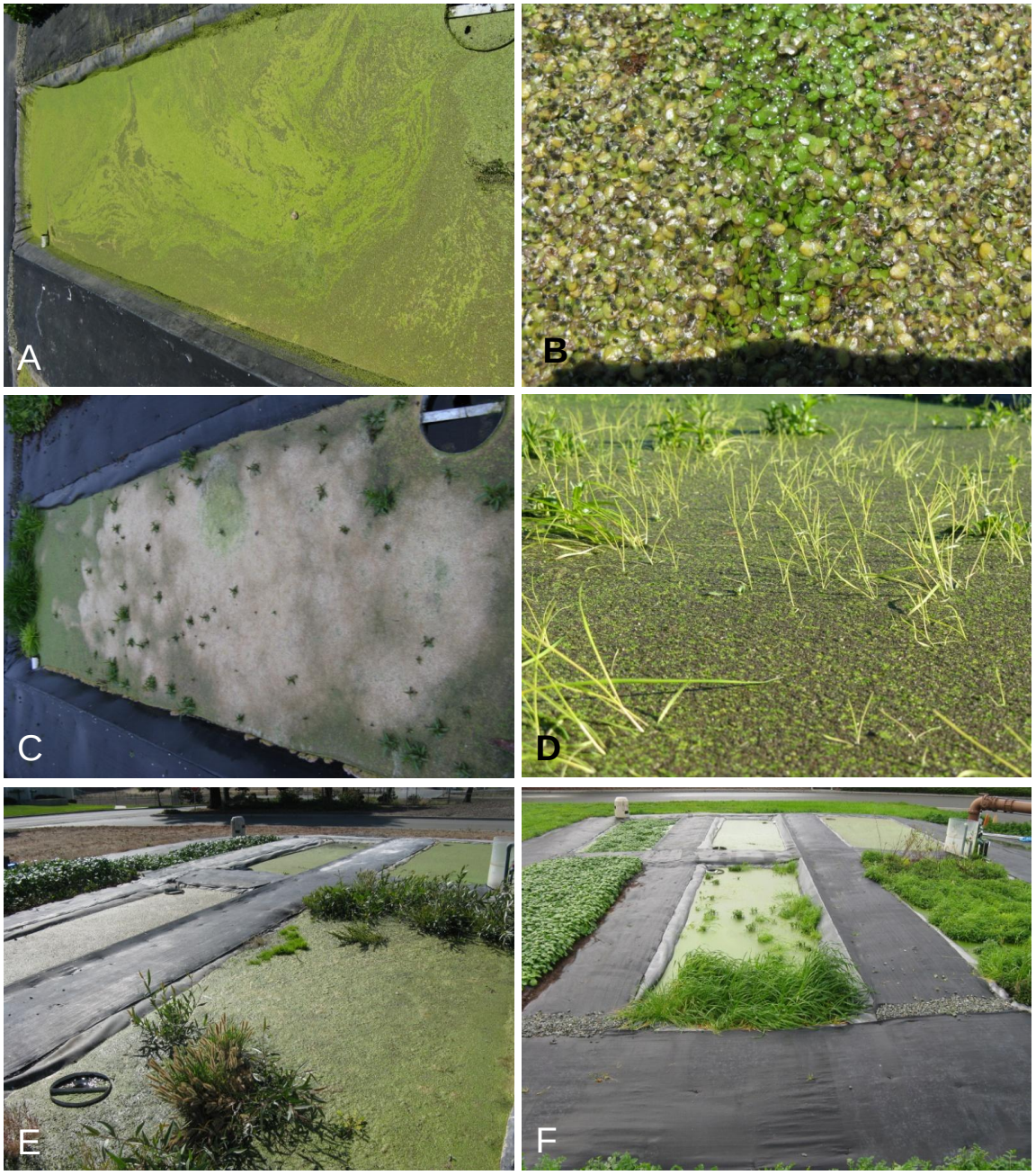

Fig. 9. Shifting vegetation cover in E2 post cessation of harvest.
A) 23rd Jun. 2009, browning thick mat duckweed; B) 28th Jul. 2009 detail, green duckweed present at inflow, aphids ubiquitous; C) 17th Nov. 2009, bleached duckweed mat; D) 22nd Dec. 2009, expanding growth of land plants; E) 1st Sep. 2009, land plant expansion in E1, land plants yet to colonize E2; F) 26th Jan. 2010, land plants starting to dominate surface area.

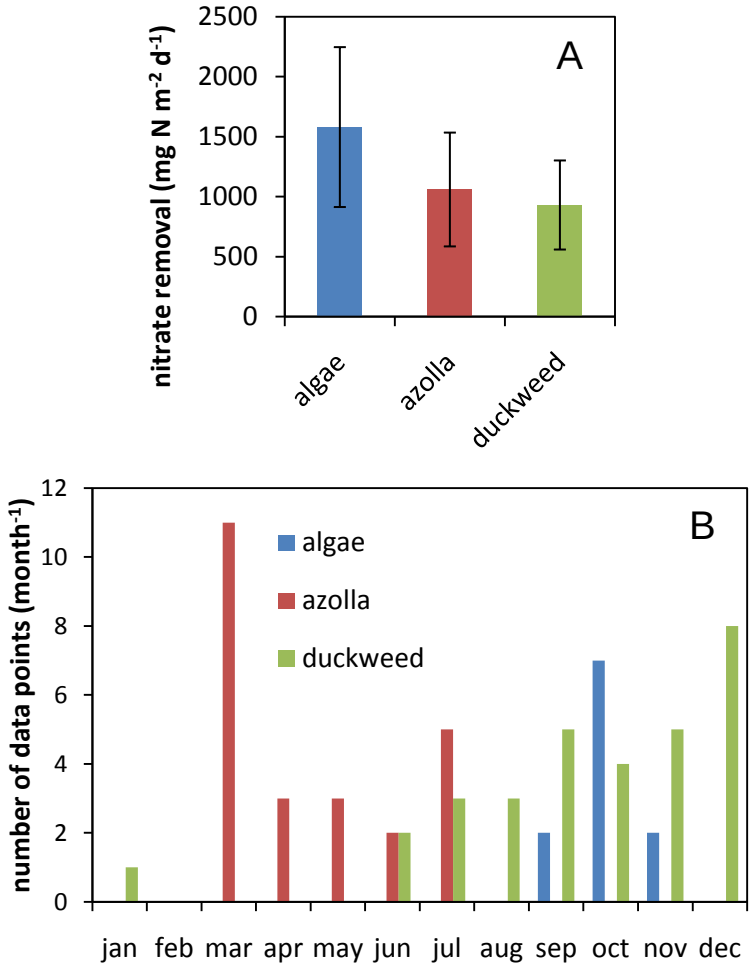

Fig. 10. Effect of dominant vegetation on nitrate removal efficiency on a per-module basis. Data was collected from 4 May 2008 to 9 March 2010 from unselectively harvested modules. Error bars show standard deviations (A). Bars indicate the number of data points chosen for each vegetation type (based on ≥80% coverage of the top two channels) and their temporal distribution (B).

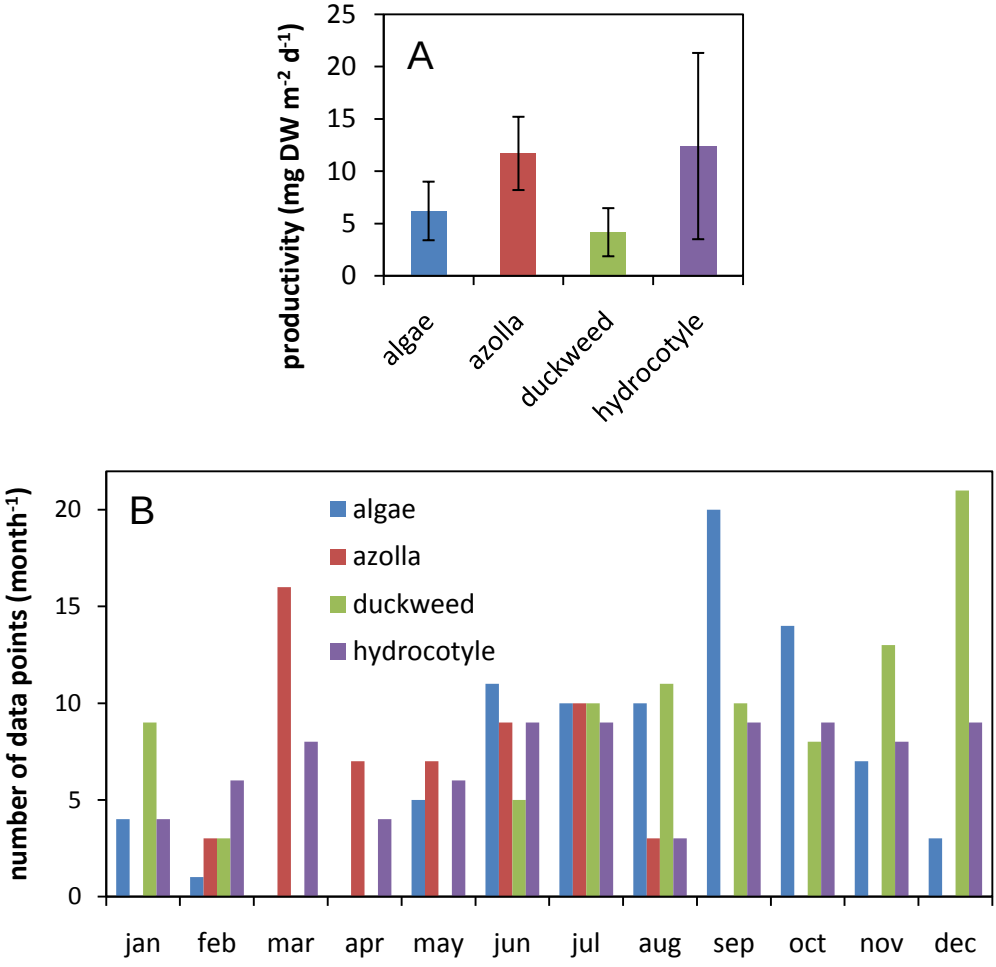

Fig. 11. Effect of dominant vegetation on productivity on a per-channel basis. Data was collected from 4 May 2008 to 9 March 2010 from unselectively harvested modules. Error bars show standard deviations (A). Bars indicate the number of data points chosen for each vegetation type (based on ≥80% coverage of a channel) and their temporal distribution (B).

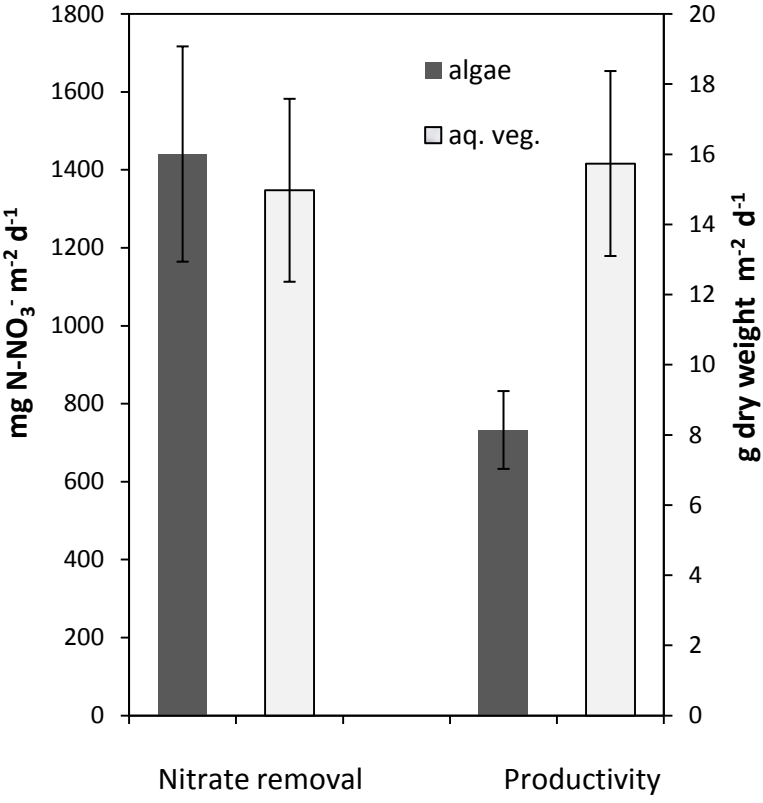

Fig. 12. Algae vs. aquatic vegetation: differences in mean nitrate removal efficiency and productivity. Data was used from 13 May 2008 to 19 August 2008 (n = 30). Error bars show 95% confidence intervals.

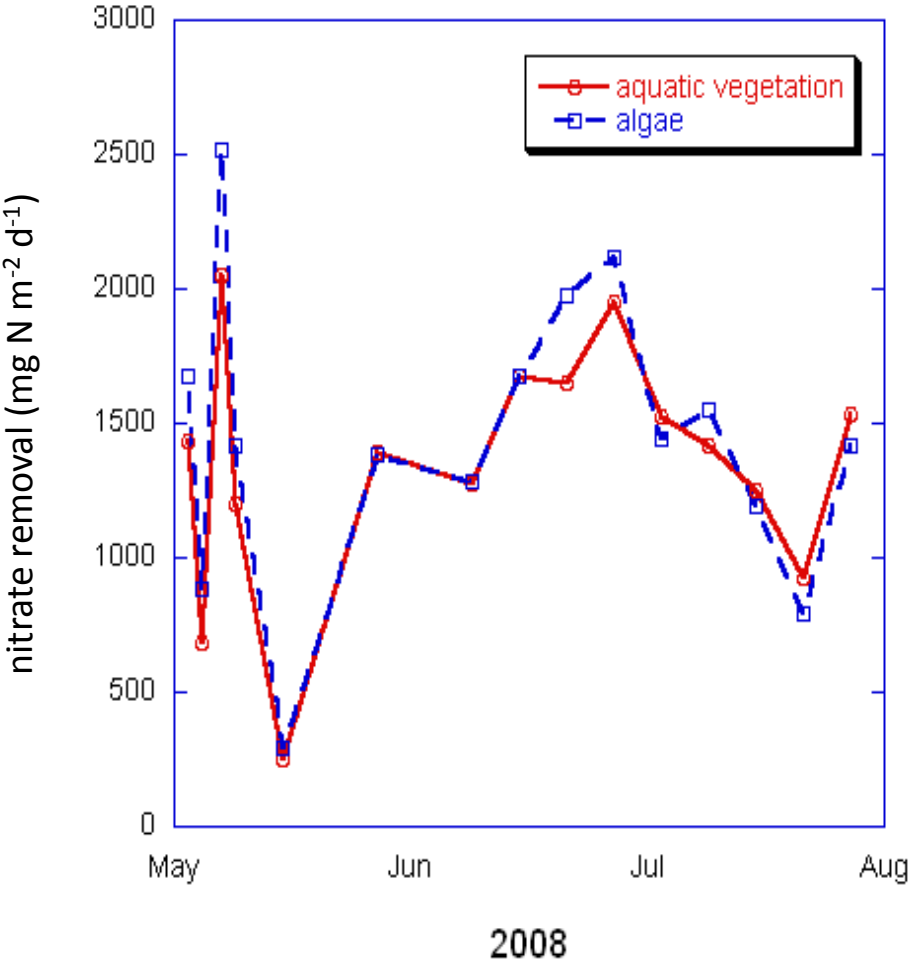

Fig. 13. Algae vs. aquatic vegetation: nitrate removal efficiency over time. Data was used from 13 May 2008 to 19 August 2008 (n = 30).

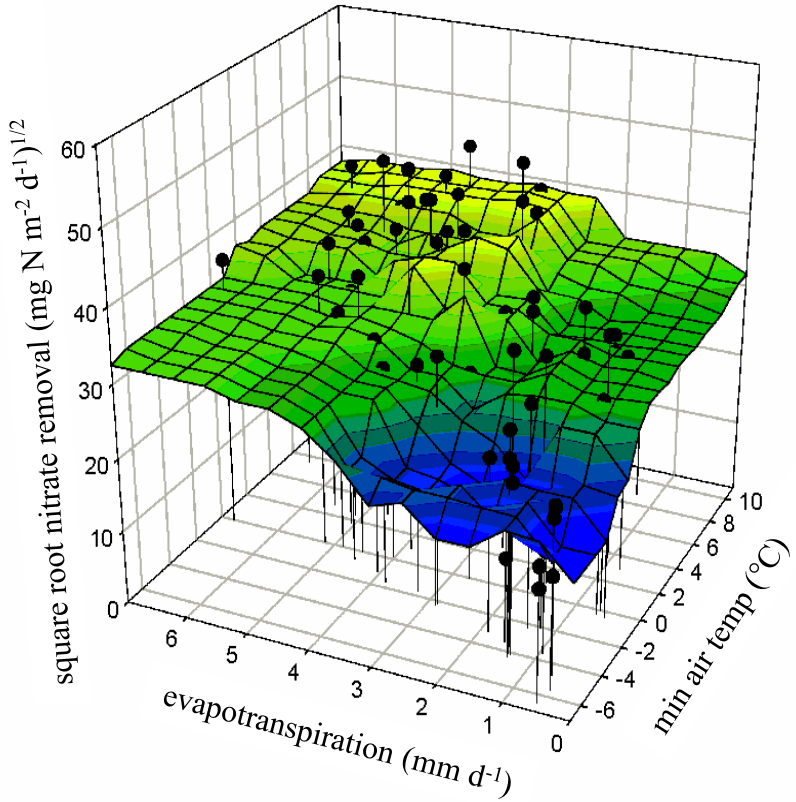

Fig. 14. Environmental effects on nitrate removal efficiency. Disregard the "wings" of the response surface where evapotranspiration is at its maximum and minimum air temperature is at its minimum, or vice versa, as there is no data to sustain them. Data was used from unselectively harvested modules from 13 May 2008 to 1 December 2009 (n = 95).

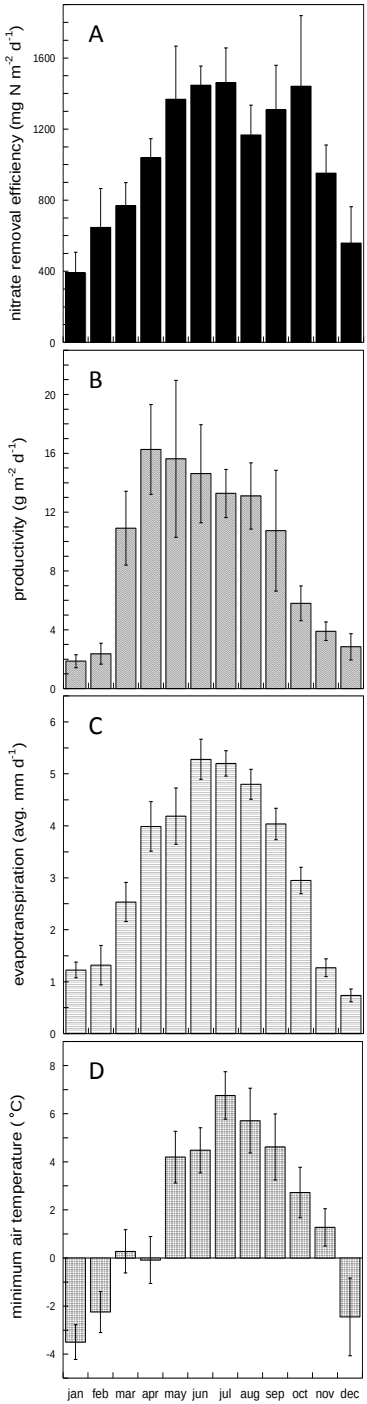

Fig. 15. Monthly means of nitrate removal efficiency, productivity, minimum air temperature, and evapotranspiration. Error bars indicate 95% confidence intervals; all data from unselectively harvested modules from 13 May 2008 to 1 December 2009 (A: n = 95; B, C, D: n = 279).

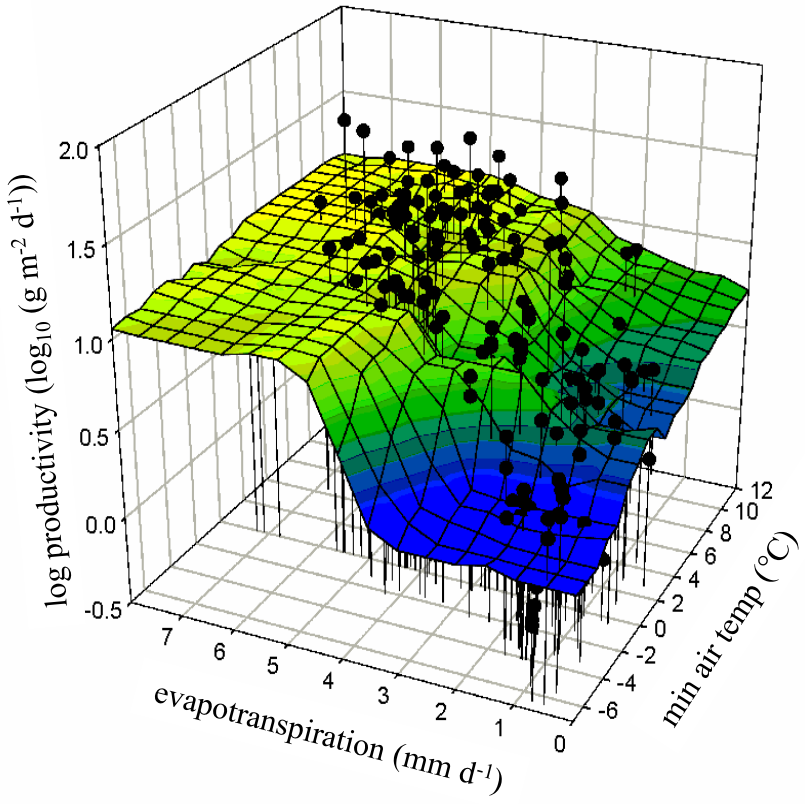

Fig. 16. Environmental effects on productivity. Data was used from unselectively harvested modules from 13 May 2008 to 1 December 2009 (n = 279).

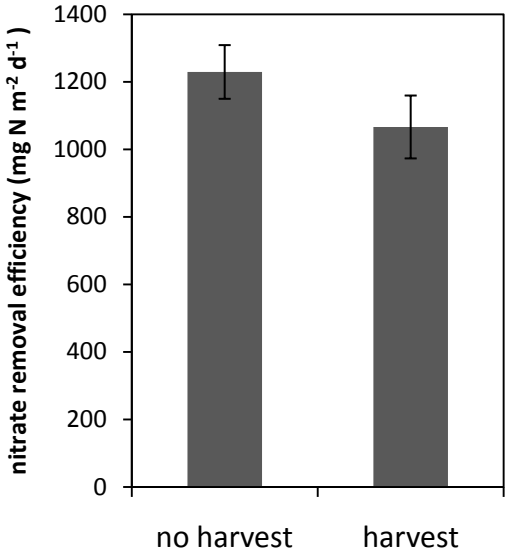

Fig. 17. Harvest vs. no harvest: overall mean nitrate removal efficiencies. Error bars indicate 95% confidence intervals. Data was used from 24 March 2009 to 16 March 2010 (n = 92).

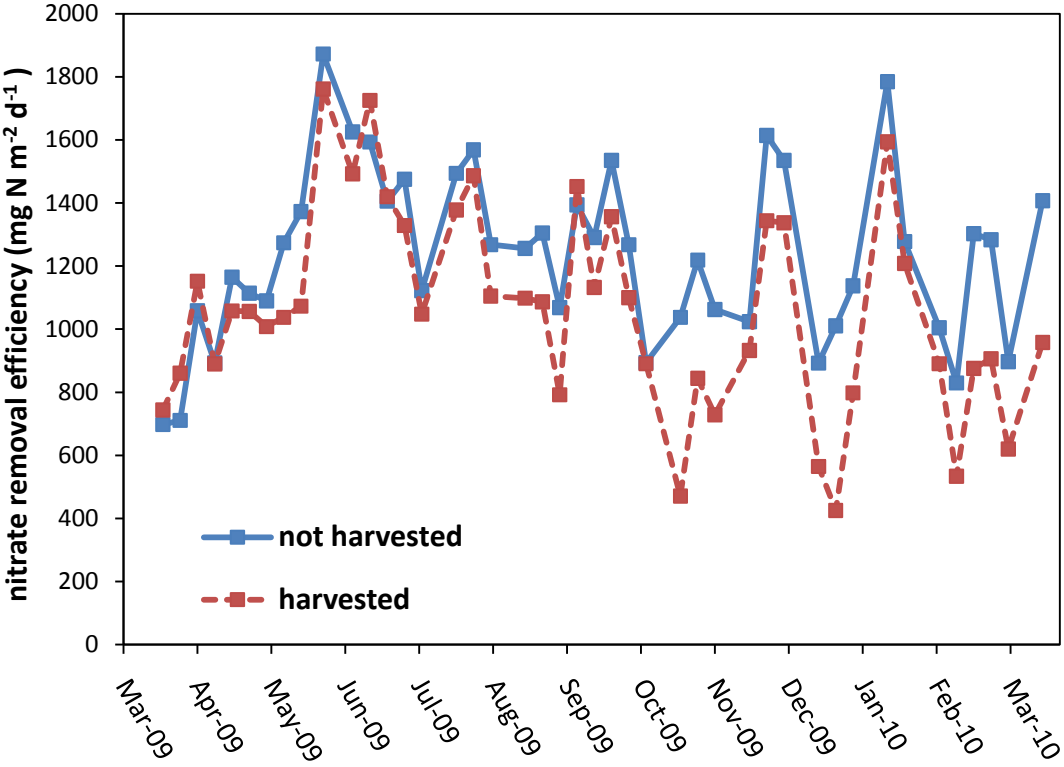

Fig. 18. Harvest vs. no harvest: nitrate removal efficiency over time. Data was used from 24 March 2009 to 16 March 2010 (n = 92).

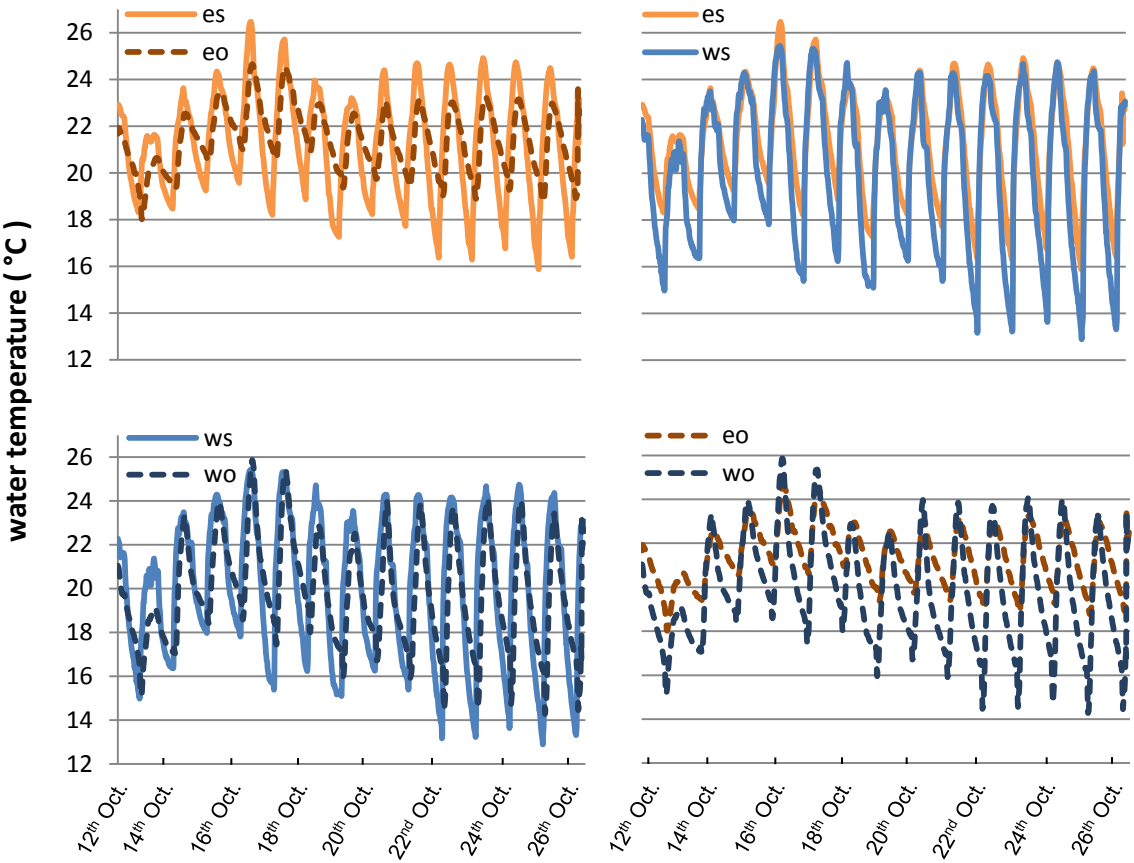

Fig. 19. Temperature difference between east (e) and west (w) modules measured every 15 minutes from the 12th to the 26th of October 2009. "s" designates water temperature at the end of the first channels, and "o" designates water temperatures at the end of the third channels.