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RR-118
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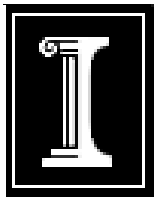

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Submitted to the
Illinois Sustainable Technology Center
Institute of Natural Resource Sustainability
University of Illinois at Urbana-Champaign
www.istc.illinois.edu

The report is available on-line at:
http://www.istc.illinois.edu/info/library_docs/RR/RR-118.pdf

Printed by the Authority of the State of Illinois
Patrick J. Quinn, Governor

Acknowledgements

This study was funded by the Illinois Sustainable Technology Center (formerly the Illinois Waste Management and Research Center, contract HWR05195) and Southern Illinois University Edwardsville (SIUE, 2004-2005 Funded University Research). The author acknowledges the contributions of Dr. Yanhong Zhang, assistant research director of the National Corn-to-Ethanol Research Center at SIUE, for assisting with the project development and experimental design; Dr. Kevin Johnson, professor of the Environmental Sciences Program at SIUE, and Benjamin Paulson, a former undergraduate student of the Department of Chemistry at SIUE, for GC/MS/MS analysis to identify chemical compounds from decomposed barley straw; Syed F. Jaffery, a former graduate student of the Department of Civil Engineering at SIUE, for laboratory research work; Cynthia Finau, a former graduate student in Department of Chemistry at SIUE, for assisting the laboratory sampling and analysis; Mr. Brent Vaughn, a laboratory specialist and lecturer in the Department of Civil Engineering at SIUE, for assisting with the lab set-up; Dr. Walter R. Hill, an aquatic ecologist at the Illinois Natural History Survey, for advising on algae ecology and conducting microscopic examination of algal species; and Ms. Nancy Holm of the Illinois Sustainable Technology Center for constructive comments and editorial assistance.

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Abstract

Wastewater lagoons and industrial ash ponds are engineered earthen basins for municipal or industrial wastewater treatment and ash solids removal. Many lagoons and ponds experience algal overgrowth in summer, resulting in the violation of discharge criteria, because algae form a major fraction of suspended solids. The use of chemicals such as copper sulfate to control algae increases the toxicity of the receiving water. Earlier studies have shown that decomposing barley straw appears to be a promising and inexpensive method of algal control in surface water bodies. However, there is little information on the effects of decomposing barley straw on algal growth. The purpose of this seed project was to identify key variables to guide further investigation of this subject. This project studied how water temperature, water characteristics (dechlorinated tap water vs. wastewater), barley straw loading rate, and the duration of barley straw application affect algal growth and water quality parameters. The study also identified chemical compounds from decomposed barley straw in water. Several batch reactors were used in the laboratory work. They were operated at 22°C or 27°C. Barley straw loading rates of 0.5, 1, 2, and 4 g/L were studied. Chlorophyll *a* concentration was measured to quantify the amount of algae. Microscopic examination characterized algal species in several selected samples.

This study found that decomposing barley straw at a loading rate of 4 g/L was effective in inhibiting the growth of *Anabaena* and *Oscillatoria* in dechlorinated tap water at 22°C. Loading rates of 2 g/L and 4 g/L were both effective (no significant differences at a 90% confidence level) in inhibiting the growth of *Anabaena* in water at 27°C. Each gram of barley straw produced 5.2 to 7 mg PO_4^{3-} and 1.4 mg NO_3^--N in nine weeks of decomposition in water at 22°C. For wastewater with existing algal populations at 22°C, a paired t-test revealed that, at a 90% confidence level, each of the tested loading rates (0.5, 1, 2 g/L) lowered algal growth. A loading rate of 2 g/L of barley straw resulted in a statistically more significant effect on algal growth than 0.5 g/L or 1 g/L of barley straw. However, the difference in the effect between 0.5 g/L and 1 g/L was not significant. For wastewater at 27°C, changes in algal species and variability in the values of chlorophyll *a* caused difficulty in determining the effects of barley straw on algal growth. The chemical compounds of 2,6-Dimethoxy-4-(2-propenyl)phenol and octanoic acid were below detection limit in the water samples in which 0.83 g/L of barley straw decomposed. Three other compounds were detected: 1,2,4-trimethyl benzene; 1,3,5-trymethyl benzene; and 1-ethyl-4-methyl benzene. These and several additional chemical compounds, including butylated hydroxytouluene and 2-methoxy-4-vinylphenol that were frequently found in the water samples in which 5.4 g/L of barley straw had decomposed, are good candidates for further investigation of their specific effects on algal growth.

Keywords: Wastewater treatment lagoon, algae, barley straw, chlorophyll *a*

1. Introduction

Wastewater lagoons are engineered earthen basins for municipal or industrial wastewater treatment. In the United States, more than 7,000 lagoon systems are in use for wastewater treatment (Crites and Tchobanoglous 1998). In Illinois, there are 400-500 active *National Pollutant Discharge Elimination System* (NPDES) regulated wastewater lagoon systems (IEPA 2003). Where large land areas are available, wastewater lagoons offer the benefits of low capital and operational costs, and requiring minimal operational skills. However, many wastewater lagoons experience algal overgrowth during summer months. Algae in wastewater cause an increase in total suspended solids (TSS) in lagoon effluents and this can result in the violation of effluent TSS discharge criteria (USEPA 1983). The negative impacts of excessive algae on receiving water quality also include: depleting dissolved oxygen in water due to biological decay of dead algae, destruction of the living environment of aquatic life such as fishes, blocking mechanical devices such as water gates and valves, and interfering with water treatment plant operation by plugging water filters. Traditionally, algicidal chemicals are used to control algal overgrowth in water bodies. For example, copper sulfate ($CuSO_4$) is widely used for algal control. However, the use of copper sulfate results in copper build-up in the sediments of lagoons and increases toxic effects on aquatic biota in the receiving water (AWWA 1999). In addition, copper sulfate must be applied regularly throughout summer because its control effect on algal growth is only temporary. The routine use of chemicals is costly to municipalities and industries. Therefore, it is imperative to develop a cost-effective alternative for algal control.

In recent years, decomposing barley straw in ponds, canals, or reservoirs has been studied and shown to be a promising and effective method for algal control. In England, it was found that decomposing barley straw inhibited algal growth in canals (Welch et al. 1990), aerated water bodies (Pillinger et al. 1994), and reservoirs (Everall and Lees 1996). In the U.S., there has been a growing interest in applying barley straw for algal control in fresh water or brackish river water (Terlizzi et al. 2002; Brownlee et al. 2003), wastewater treatment lagoons (Corley 2003a, b; Zhou 2006), and ash ponds of power plants (Smithson and Portz 2006). These studies revealed that TSS in lagoons' effluent appeared to be lower in the years of barley straw application, when the data were compared to those from non-application years. No deleterious environmental effects of barley straw on water systems were found from these studies (Ridge 1992; Corley 2003a, b; Zhou 2006).

Newman and Barrett (1993) described the effect of barley straw as being algistatic (preventing new growth of algae) rather than algicidal (killing already existed algae). The inhibitory effect of barley straw might be due to chemical compounds such as oxidized phenolics and hydrogen peroxide, which occur during the decomposition process (Everall and Lees 1997; IACR, 1999). It appears that good aeration, neutral to alkaline pH, and open sunlit water are essential for optimum algal control by barley straw (Everall and Lees 1997). The current understanding of the mechanism of decomposing barley straw affecting algal growth is still limited (Lembi 2003) and contradictory research findings have been reported. The study by Ferrier et al. (2005) showed that barley straw liquor inhibited the growth of three algal species, had no effect on other five algal species, and enhanced the growth of another four algal species instead. Boylan and Morris (2003) did

not find consistent inhibition effects of barley straw on algal growth in a 1-ha Midwestern pond during their 14-week study.

In order to use barley straw for cost-effective algal control, more in-depth understanding is needed about how barley straw effectiveness is influenced by water temperature, water characteristics, and loading rate. The duration of effectiveness per batch of straw also needs to be determined. Over-loading of barley straw will result in increased costs in materials and deployment, as well as cause problems of deoxygenating the water in the decomposition process of the barley straw. Under-loading or delayed removal of exhausted barley straw will compromise the effectiveness of algal control. Furthermore, chemical compounds released from the decomposing barley straw need to be identified and quantified. Such information will be useful for understanding the decomposition process of barley straw and assessing the impact on water quality.

The objectives of this research were:

(1) To investigate how water temperature, water characteristics (dechlorinated tap water vs. wastewater), barley straw loading rate, and its application duration affect algal growth
(2) To identify and quantify chemical compounds from the decomposition of barley straw in water

This project, supported by a seed grant from the Illinois Sustainable Technology Center (formerly the Illinois Waste Management and Research Center), aimed to generate preliminary results that could benefit further development of research projects on this subject.

2. Methodology

2.1. Experimental Design, Set-up, and Sample Collection

Algal Growth Studies

Experimental Design and Set-up: The experimental design and conditions for the algal growth studies are summarized in Table 1. Four runs of experiments were conducted. Two water temperatures were used in the studies: 22°C (room temperature) and 27°C. These are the typical lagoon water temperatures that occur in southern Illinois in spring/fall and summer, respectively. Two types of water were studied. The first was dechlorinated tap water (referred to as water in this report) in which algal species of *Anabaena* and *Oscillatoria* were added and tested in separate tanks. These species were obtained from Carolina Biological Supply Company as pure cultures and they are commonly found in Illinois water bodies (Walter R. Hill, personal communication, 2005). The second type was wastewater from a local wastewater treatment lagoon, in which existing algae in the wastewater were utilized. Four loading rates of barley straw (0.5, 1, 2 and 4 g/L) were chosen, based on other relevant studies (Martin and Ridge 1999; Newman and Barrett 1993; Pillinger et al. 1994; Pillinger et al. 1996).

The experiments were conducted in plastic tanks. The tanks were approximately 15-22 inches in length, 10-15 inches in width, and 6-9 inches in depth. The volume of water and wastewater used in the experiments varied from14 L to 23 L as indicated in Table 1.

Table 1. Experimental Design and Conditions

Run	Experimental Conditions	Water Temp.[1] (°C)	Loading Rate (g/L)	Initial Water Volume (L)
One	Control – *Anabaena* in water	21.1±0.5	0	23
	Treatment - *Anabaena* in water	21.2±0.4	4	23
	Control – *Oscillatoria* in water	21.2±0.5	0	23
	Treatment - *Oscillatoria* in water	21.2±0.5	4	23
Two	Control -Wastewater	22.3±1	0	23
	Treatment A - Wastewater	22.5±1	0.5	23
	Treatment B - Wastewater	22.4±1	1	23
	Treatment C - Wastewater	22.4±1	2	23
Three	Control - Wastewater	26.9±0.7	0	14
	Treatment A - Wastewater	27.9±0.5	2	16
	Treatment B - Wastewater	26.5±0.8	4	16
Four	Control - *Anabaena* in water	26.5±0.3	0	14
	Treatment A - *Anabaena* in water	26.8±0.3	2	16
	Treatment B - *Anabaena* in water	26.8±0.2	4	16

[1]Water temperatures were measured weekly. The reported temperature is the average and standard deviation of all measurements throughout the duration of each run.

Because ponds and lagoons typically have larger surface dimensions than water depths, tanks instead of flasks were used in this study to better simulate ponds and lagoons in which barley straw could be used for controlling algal population. A disk-type fine bubble air diffuser was placed in each tank for aeration and mixing. The applied air flow rate was 2,000 mL/min. (0.09-0.13 vol./vol.-min. based on initial water volume). This resulted in dissolved oxygen (DO) concentrations of at least 5 mg/L in the water or wastewater of all runs. A timer-controlled fluorescent natural light panel provided 16 h of daily light exposure at 600-700 foot-candles (i.e., 6,500-7,500 lux). A digital light meter (DLM2 by UEI Test and Measurement Instruments) was used to measure the light intensity. The experimental set-up is shown in Fig. 1.

Experimental Materials and Handling: Pure cultures of *Anabaena* and *Oscillatoria* were obtained from Carolina Biological Supply Company that also supplied the ALGA-GRO, an algal inoculation medium (Carolina 1978). In both Runs One and Four, the algal culture was incubated in ALGA-GRO medium in several 250 mL flasks at about 22^{o}C (room temperature) for 17 days prior to beginning the experiments in the tanks. Increased intensity of green color provided visual evidence of the increased amount of algae in the flasks. The algae grown in these flasks were combined and mixed thoroughly, then 550 mL of algal medium was introduced to each tank. One tank was used for each experimental condition shown in Table 1, which also shows the initial volume of tap water (dechlorinated by aeration and confirmed to have undetectable chlorine) in each tank. Bold's Basic Medium (Carolina 1978) was prepared in the SIUE Environmental Engineering Laboratory and was added to each tank for supplementing nutrients and trace elements in the tap water. The wastewater used in Run Two and Run Three already contained algae, organic matters, and nutrients, so the wastewater was not amended.

Fig. 1. Experimental Set-up

Barley straw used in the experiments was weighed after it was air-dried, then was loosely wrapped in fishing net before the straw was placed in water (or wastewater) in the tanks. The applied straw was allowed to decompose in the water tanks for 17 days at 22^{o}C in Run One and at 27^{o}C in Run Four, respectively, before the algae were added to start the experiments. Increased intensity of the brownish color was visual evidence that the barley straw was decomposing in water. In Runs Two and Three, the applied straw was placed in the wastewater tanks without the pre-decomposing process. Each run was conducted as a batch test: all of algae and barley straw were added at the beginning of the test.

Sample Collection and Handling: Water depth was measured and recorded before and after each sampling. Before each sampling, the water lost due to evaporation was replaced with distilled water. Liquid in each tank was mixed manually before a sample was taken. Samples taken from each tank were processed immediately following sample collections.

Nutrient Level Study

In addition to the four runs of algal growth studies, a Nutrient Level Study was conducted to determine nutrients produced from the decomposing process. A loading rate of 4 g/L barley straw was placed in 16 L of dechlorinated tap water, in which no algae were added. This experiment was performed to characterize the release potential of nutrients from the decomposed barley straw in water and was conducted only at 22^{o}C (room temperature), not at 27^{o}C, for nine weeks. Weekly samples were taken to measure for orthophosphate and nitrate concentrations.

Studies on Chemical Compounds from Decomposed Barley Straw

Experimental Design and Set-up: A separate plastic tank was set up in the Environmental Sciences Laboratory of SIUE for batch studies examining chemical compounds from decomposed barley straw. Barley straw was placed in 127 L of tap water to decompose at about 22^{o}C (room temperature). Although aeration was not applied to the tap water, the chlorine in the tap water should have been gone when the barley straw was introduced after the water had sat in the tank for at least one day. No algae were involved in this experiment. Water samples, collected weekly from this tank, were extracted and analyzed using gas chromatography (GC)/Mass Spectrometry (MS)/MS. Three batch studies were conducted. Batch One used 0.83 g/L of barley straw (weighed after it was air-dried and loosely wrapped in fishing net) and lasted for approximately three months from February to May of 2005. A total of 13 samples, taken weekly, were analyzed. There were no overhead lights or aeration throughout the Batch One experiment. During subsequent studies of Batch Two (May to August of 2005) and Batch Three (September to December of 2005), a light panel was mounted overhead of the water tank to provide 700-800 foot-candles (i.e., 7,500-8,600 lux) of 16 hours daily light exposure. For these two studies, a loading rate of 5.4 g/L of barley straw was used and porous aeration tubing provided 11,270 ml/min of air flow rate, or 0.089 volume air/volume water-min. (based on an initial water volume of 127 L) for aeration and mixing. No algae, nutrients, or other chemicals were added to any of the batches.

Sample Collection and Handling: Water depth was measured and recorded before and after each sampling. Before each sampling, the water lost due to evaporation was replaced with distilled water. Liquid in each tank was mixed manually before a sample was taken. Due to limited resources, duplicate tanks were not used and replicate samples were not run for most of the experiments. Samples collected from the tank were processed immediately after collection and then were preserved in a freezer until GC/MS/MS analysis was conducted.

2.2. Experimental Analysis and Quality Assurance

Algae and Water Quality Parameters: The chlorophyll *a* concentration was used to quantify algae and was determined using the spectrophotometric method (Standard Method 10200 H, APHA 1998). Briefly, approximately 50 mL of sample was centrifuged at 2,000-3,000×g for 20 minutes to separate the algal solids from the liquid. Each collected solid residue was ground in 90% acetone to extract chlorophyll *a*, which was measured for its optical density at 664 nm and 665 nm for subsequent calculation of the concentration (Standard Method 10200 H, APHA 1998). Next, selected samples were sent to the Center of Aquatic Ecology of the Illinois Natural History Survey to determine algal species by microscopic examination. Water temperature, DO (using an YSI 550-A portable DO meter), and pH were measured weekly when a sample was taken. NH_4^+-N was determined following the Selective Electrode Method (Standard Method 4500-NH_3 D, APHA 1998). The measurement of PO_4^{3-}, NO_3^--N, and chemical oxygen demand (COD) followed HACH Methods 8048, 8171, and 8000, respectively (HACH 1997). Hydrogen peroxide (H_2O_2) was determined using the iodometric titration method, where the samples were titrated using potassium iodide solution, thiosulfate solution and starch indicator. The runs in which these parameters were measured are described in the Results and Discussion section.

GC/MS/MS Analysis for Chemical Compounds: Analyzing chemical compounds from decomposing barley straw requires sample pretreatment and extraction prior to instrumental analysis. Samples taken from Batch One were analyzed for octanoic acid and 2,6-Dimethoxy-4-(2-propenyl)phenol. It has been postulated that these two compounds are important in inhibiting algal growth (Pillinger et al. 1994, Pillinger et al. 1996; Everall and Lees 1997). As part of the Batch One experiment, a calibration test was conducted to validate the recovery rate of octanoic acid and 2,6-Dimethoxy-4-(2-propenyl)phenol. Samples from Batch Two and Batch Three were taken in the same way as Batch One and were analyzed for all possible chemical compounds of significant concentrations.

For each batch study, one liter of collected water sample was filtered with a Whatman 1.5μm glass micro fiber filter. The filtration reduced the time of sample extraction from 12 hours to 3 hours. The filtrate was adjusted to be pH 3.5 before it was extracted by a Solid Phase Extractor (SPE). Five different cartridges from Agilent Technologies (Accubond) and one cartridge each from Alltech and Supelco were evaluated. The Accubond C-18 SPE cartridge was chosen because of its relatively high recovery. This method was validated by adding 10% methanol to a sample and adjusting it to pH 3.5. Sulpelco Visiprep™ tubing was used for connecting to the C-18 cartridge. The sample extraction was conducted with a vacuum, after which the compounds were eluted with 5ml 3:2 ethyl acetate:acetone solution, then analyzed in a Varian GC/MS with ion trap. Since the chemical compounds of interest were expected to be at trace levels, GC/MS/MS method was used because it can better detect

chemicals of low concentrations. The GC separation and identification were optimized using a J&W Scientific DB-5MS column for 4-allyl-2,6-dimethoxyphenol and a J&W Scientific Innowax column for octanoic acid. Proper excitation voltage, amplitude, and temperature were adjusted and set for GC/MS and GC/MS/MS.

Quality Assurance: At the beginning of the experimental program, several samples were sent to the Madison County Environmental Laboratory (a certified lab) for chlorophyll *a* measurement. Duplicate samples of the same source were also measured at the SIUE Environmental Engineering Lab for inter-laboratory comparison. The Madison County Environmental Lab was also visited to discuss and verify SIUE's chlorophyll *a* extraction and analysis method. The chlorophyll *a* and COD were measured in duplicate; the average of the measurements was reported. DO was measured in triplicate. The analysis methods of PO_4^{3-} and NO_3^--N are relatively simple. After confidence of analysis was established, a single measurement was used to determine PO_4^{3-} and NO_3^--N in most samples. The GC/MS/MS analysis, conducted in the SIUE Environmental Sciences Lab, included method validation and recovery rate test of known chemical compounds.

3. Results and Discussion

3.1. Effect of Barley Straw on Algal Growth in Water at 22°C (Run One)

The effects of decomposing barley straw on *Anabaena* and *Oscillatoria* growth (quantified by chlorophyll *a* concentration) in water at 22°C (Run One) are shown in Fig. 2 and 3, respectively. In these figures, the lines were derived from regression analysis to show the trend of changes, which were based on actually measured data shown as the points. The initial chlorophyll *a* concentration in each tank, after the incubated algae in flasks were introduced, was below the detection limit. Samples were not taken in weeks 0 and 2 because of the lack of visible algae in the water in the initial two weeks. The next samples were taken at 3½ weeks into the experiment and revealed that the chlorophyll *a* concentrations were 11 µg/L in the "treatment" tank (which used water with decomposing barley straw) and 462 µg/L in the "control" tank, respectively (Fig. 2). By 9½ weeks into the experiment, the chlorophyll *a* concentrations in the "treatment" tank of the *Anabaena* experiment rose to approximately 60 µg/L compared to 571 µg/L found in the "control" tank. Experiments with *Oscillatoria* yielded similar results. At 9½ weeks, the chlorophyll *a* concentrations in the "treatment" tank of the *Oscillatoria* experiment rose to 80 µg/L compared to 589 µg/L found in the "control" tank (Fig. 3). The "control" chlorophyll *a* concentrations in the first sample taken at 3½ weeks for both the *Anabaena* and the *Oscillatoria* experiments did not fit well with their respective general trends of algal growth. The high chlorophyll *a* concentrations of this first sample and the relatively low chlorophyll *a* concentrations of the next sample taken at 4½ weeks may be due to issues related to sampling or algal growth. Although water in the tanks was thoroughly mixed prior to each sampling, because the water volume in each tank was limited, one sample was taken for each sampling. Increasing the number of tank replicates in future studies could help to clarify uncertainties with the data.

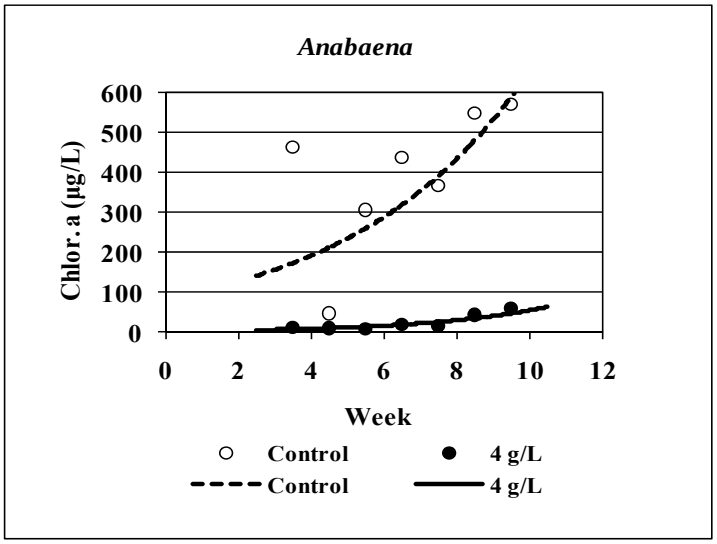

Fig. 2. Effect of Barley Straw on *Anabaena* Growth in Water at 22°C

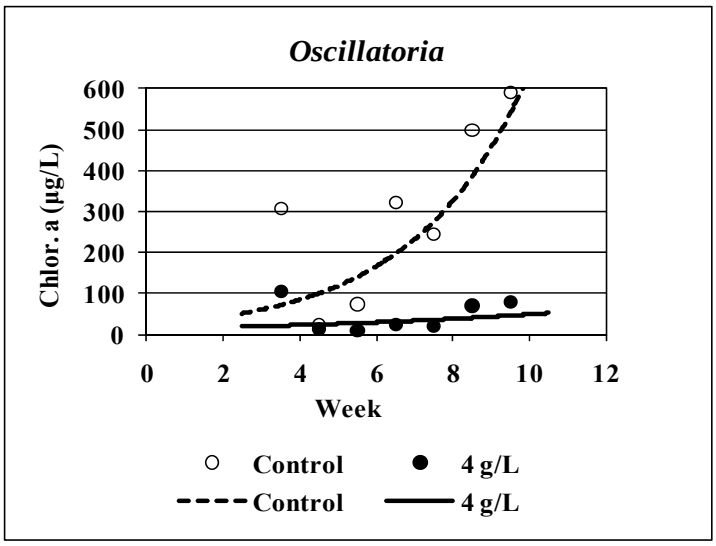

Fig. 3. Effect of Barley Straw on *Oscillatoria* Growth in Water at 22°C

The Bold's Basic Medium was added to each tank to provide pH buffer, nutrients (estimated 42 mg/L of NO_3^--N and 160 mg/L of PO_4^{3-}), and trace elements. Therefore, pH and nutrients were not likely limiting algal growth. The liquid color in the "treatment" tanks became dark brown due to the decomposition of the barley straw. Because the liquid depth in the tanks was less than 5 inches and was shallow enough to allow for light penetration, the blocking of the light by the brownish color of the liquid should be negligible (Gibson et al. 1990). Concern about any possible effect of light blockage on algal growth could be investigated further in future studies. The measured DO confirmed the aerobic condition of the liquid medium. The contact areas between the inside surface of each tank and liquid at the initial water depth was approximately 4.4 ft^2. Although some algae attached to the inside surface of tanks and to the barley straw, the amount of attached algae appeared to be limited and was comparable between the "control" and "treatment" tanks. After considering all of the factors, experimental results suggest that 4 g/L of barley straw inhibited the growth of blue-green algae (i.e., *Anabaena* or *Oscillatoria*) at 22°C.

3.2. Effect of Barley Straw on Algal Growth in Water at 27°C (Run Four)

The effect of barley straw on algal growth at 27°C was studied in Run Four (Table 1). Results are shown in Fig. 4. The chlorophyll *a* concentrations in the "control" and two "treatment" tanks were comparable during the initial four weeks of the experiment. Thereafter, algae in the "control" tank grew much more rapidly than those in the two "treatment" tanks. By the 10th week, the chlorophyll *a* concentration in the "treatment" tanks was approximately150 µg/L compared to 500 µg/L in the "control" tank. When the two tested loading rates (2 and 4 g/L of barley straw) were compared, no apparent difference was found in algal growth at 27°C.

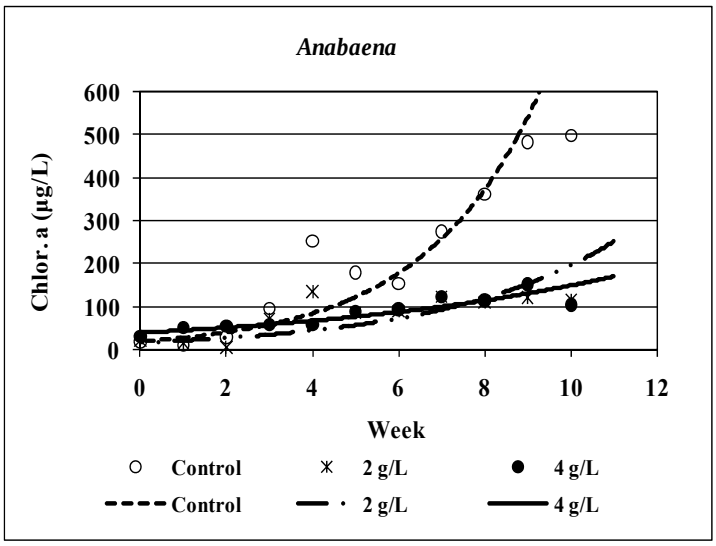

Fig. 4. Effect of Barley Straw on *Anabaena* Growth in Water at 27°C

The microscopic examination of samples taken at Week 4 revealed clumps of filamentous cyanophytes, probably *Phormidium*, in the sample taken from the "control" tank. These algae didn't appear to be *Anabaena* because they lacked a beaded appearance and heterocysts. Such algae may have grown because of impurity in the initial algal culture or have been introduced (e.g., from air) during the experiment. In the 4 g/L "treatment" tank, beaded and filamentous cyanophytes of unknown genus were found. They looked like *Anabaena* without heterocysts. The lack of heterocysts may be caused by the culture conditions. The *Anabaena*, inoculated at 22°C, was introduced to 27°C water in each tank without an acclimation period.

The lower chlorophyll *a* concentrations in the two "treatment" tanks compared to the "control" tank indicated the inhibitory effect of barley straw on algal growth at 27°C. The gradual increase of chlorophyll *a* concentrations in the "treatment" tank suggests the decline of the inhibitory ability due to the exhaustion of barley straw as the decomposition progressed. It appears the barley straw exhausted sooner at 27°C than at 22°C, likely due to more rapid decomposition at the higher temperature.

3.3. Characterizing the Decomposition Process of Barley Straw in Water

3.3.1. Decomposing Barley Straw in Water at 22°C without Algae

Results of this Nutrient Level Study are shown in Fig. 5. The increase in orthophosphate concentrations occurred after one week of decomposition, indicating that barley straw had already begun to break down. The orthophosphate concentrations increased from 2.2 mg PO_4^{3-}/L in the Week One sample to 20.6 mg PO_4^{3-}/L in the Week Two sample. Each gram of barley straw produced 5.2 mg to 7 mg of PO_4^{3-} during the nine weeks of decomposition.

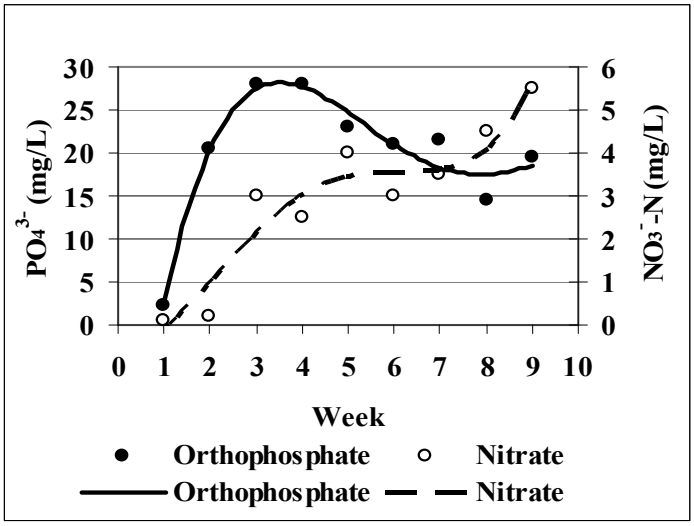

Fig. 5. Nutrients from 4 g/L of Decomposing Barley Straw in Water at 22°C

The nitrate concentration increased from 0.2 mg/L in the Week Two sample to 3 mg/L in the Week Three sample, and further increased to 5.5 mg/L at the end of the nine-week experiment. The nitrate likely originated from the organic nitrogen of the barley straw. Organic nitrogen changed to ammonia through mineralization, then to nitrate through nitrification under aerobic conditions. Each gram of barley straw produced approximately 1.4 mg NO_3^--N/g during the nine weeks of decomposition.

3.3.2. Decomposing Barley Straw in Water at 22°C and 27°C with Algae Present

The effect of decomposing barley straw on pH is summarized in Table 2. These samples were taken from Run One and Run Four of the algal growth studies. In general, pH variations were within 0.5 pH unit (standard deviation). The average pH in the "treatment" tanks of Run One was approximately one pH unit lower than the average pH in the "control" tank. No apparent difference in pH was found between the "control" and the "treatment" tanks of Run Four. Further studies are needed to better understand the factors causing these differences at 22°C and the different effects at the two temperatures of Run One and Run Four.

Orthophosphate concentrations in weekly samples are summarized in Table 3. The Bold's Basic Medium provided approximately 160 mg/L of PO_4^{3-} in each tank. It was expected that decomposing barley straw would add extra orthophosphate to the water (Fig. 5). The estimated orthophosphate concentrations were 174 mg/L in the 2 g/L "treatment" tank and 188 mg/L in the 4 g/L "treatment" tank, respectively. The data shown in Table 3 revealed, however, that PO_4^{3-} concentrations in each tank were lower than the estimated PO_4^{3-} concentrations. Factors for such lower PO_4^{3-} concentration may include: assimilation by algae for their growth; sorption of phosphate to weakly charged particles; and precipitation by binding with calcium and other metals in the tap water, which had hardness of

approximately 150 mg/L as $CaCO_3$. Calcium ions may react with orthophosphate to form hydroxyl apatite precipitate when pH is high, usually above 10 (Metcalf and Eddy 2003).

As shown in Table 2, pH measurements in the two "control" tanks of Run One were higher than 9, which likely created a favorable condition for phosphate precipitation, resulting in lower than expected orthophosphate concentrations. Conclusive assessments about the difference between the "control" and "treatment" in Run Four cannot be made because of the large variation in the measured concentrations of PO_4^{3-}.

Table 2. Effect of Decomposing Barley Straw on pH in Water

Run	Experimental Conditions	pH[1]	"Treatment" pH minus "Control" pH[2]
One	Control – *Anabaena* in water	9.4±0.5	
22°C	Treatment - *Anabaena* in water, 4 g/L	7.9±0.1	-1.4±0.5
	Control – *Oscillatoria* in water	9.2±0.4	
	Treatment - *Oscillatoria* in water, 4 g/L	7.9±0.2	-1.3±0.3
Four	Control - *Anabaena* in water	9.2±0.5	
27°C	Treatment A - *Anabaena* in water, 2 g/L	8.9±0.4	-0.3±0.3
	Treatment B - *Anabaena* in water, 4 g/L	8.8±0.3	-0.4±0.3

[1]pH was measured weekly. The reported pH is the average and standard deviation of all measurements throughout the duration of each run.
[2]The weekly pH of the "treatment" is subtracted from the corresponding weekly pH of the "control". The average and standard deviation of these differences were calculated throughout the duration of each run.

Table 3. Effect of Decomposing Barley Straw on Orthophosphate in Water

Run	Experimental Conditions	PO_4^{3-} (mg/L)	"Treatment" PO_4^{3-} minus "Control" PO_4^{3-} (mg/L)
One	Control – *Anabaena* in water	113±29	
22°C	Treatment - *Anabaena* in water, 4 g/L	147±22	40±23
	Control – *Oscillatoria* in water	95±17	
	Treatment - *Oscillatoria* in water, 4 g/L	137±24	42±8
Four	Control - *Anabaena* in water	146±39	
27°C	Treatment A - *Anabaena* in water, 2 g/L	144±42	-2±23
	Treatment B - *Anabaena* in water, 4 g/L	147±29	1±31

[1]PO_4^{3-} was measured weekly. The reported PO_4^{3-} is the average and standard deviation of all measurements throughout the duration of each run.
[2]The weekly PO_4^{3-} of the "treatment" is subtracted from the corresponding weekly PO_4^{3-} of the "control". The average and standard deviation of these differences were calculated throughout the duration of each run.

For the experiments where algae were present, nitrate and COD were measured on samples taken from Run Four (27°C), but not on samples taken from Run One (22°C). It is likely that the nitrate and COD at 27°C could be higher than that at 22°C. Results of nitrate and COD from Run Four (27°C) are shown in Fig. 6 and 7, respectively. The Bold's Basic Medium added approximately 41 mg/L NO_3^--N to each tank (mainly from sodium nitrate). The decomposition of the barley straw at 27°C for 17 days during the algal incubation period may have added another 1-2 mg/L nitrate to each tank (Fig. 5). The measured initial concentrations of nitrate were close to the estimation, but the concentration decreased during the experiment. The estimated nitrogen reduction due to nitrogen assimilation by algae for their growth was approximately 4-5 mg/L of N with a chlorophyll *a* concentration of 500-600 μg/L. Most of the reduction in nitrate was likely due to denitrification, which converted nitrate to nitrogen gas. Although aeration provided oxygen and mixing to each tank, conditions may have been anoxic inside the bundle of barley straw, which allowed denitrification to occur (Metcalf and Eddy 2003). Denitrification could have compensated for the decrease in alkalinity and pH resulting from the nitrification process.

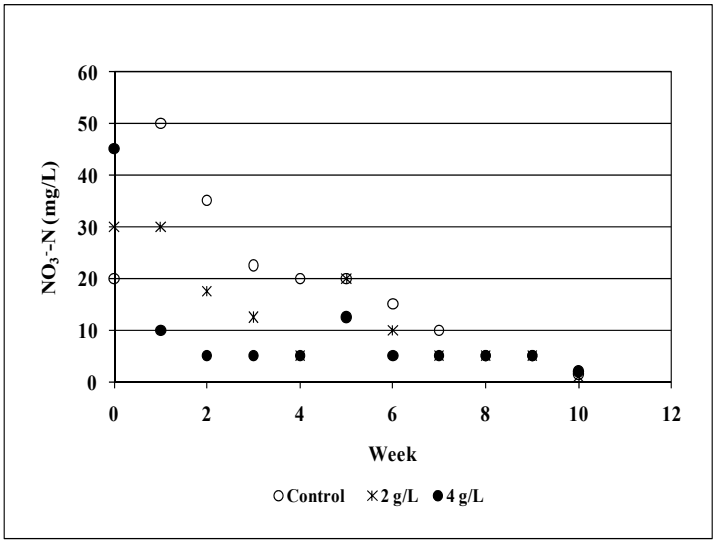

Fig. 6. Effect of Barley Straw on Nitrate Levels in Water at 27°C

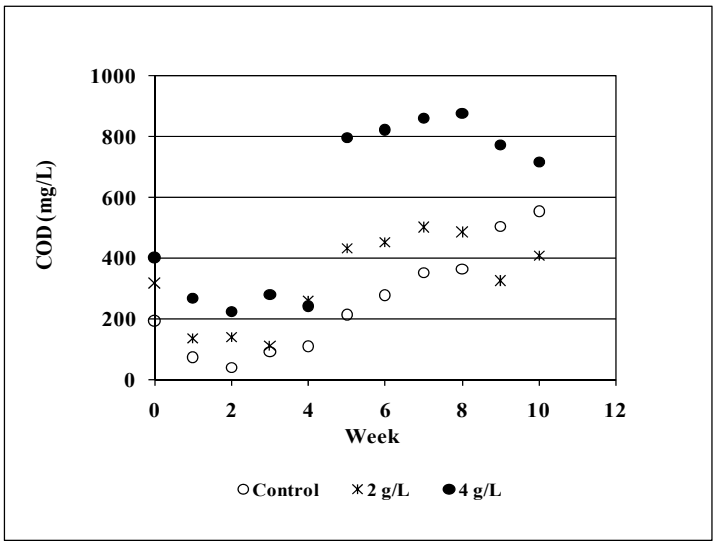

Fig. 7. Effect of Barley Straw on COD Levels in Water at 27^{o}C

COD measures the amount of organics in a sample. COD samples from each tank were taken in duplicates. Because total COD, not soluble COD, was measured, the sampling difficulties concerning uneven distribution of large solid particles in the tanks led to variations in some of the COD data. The COD standard deviations ranged from 2% to over 25% in some cases, with the majority of less than 15%. Given the difficulty in achieving thorough mixing prior to sampling due to solid particles, COD values of both duplicates were used to calculate the average values shown in Fig. 7. Despite these limitations, the COD data can still reveal valuable information about the decomposing process of barley straw. For Run 4, COD values in the "treatment" tanks were higher than those in the "control" tank, except in weeks 9 and 10 when the COD of the "control" was higher than that of the 2 g/L loading rate tank (Fig. 7). This may have been due to the substantial growth of the algae in the "control" tank during the last two weeks of the experiment (Fig. 4). Algal cells were probably the largest component of the organics measured in the "control", whereas products from decomposed barley straw could be the major fraction of the organics measured by COD in the "treatment" tanks, in which there were much less amounts of algal cells. COD in the tank of "4 g/L" was higher than COD in the tank of "2 g/L". In each tank, COD decreased during the first three weeks, then increased in the remaining seven weeks of the experiment. The initial decrease in COD appeared to coincide with the decrease in nitrate concentrations shown in Fig. 6. The process of denitrification needs readily available external carbon sources as electron acceptors (Metcalf and Eddy 2003). The organics in the water were likely used to meet the needs of denitrification, resulting in a reduction in COD. Fig. 5 shows that most of the barley straw decomposition occurred after approximately three weeks, which likely caused the increase in COD due to the release of organics from the breakdown of the straw. As mentioned above, the increase in COD for the "control" was probably due to the large increase of algal cells in the "control" tank.

3.4. Effect of Barley Straw on Algal Growth in Wastewater at 22°C (Run Two)

The effect of decomposing barley straw on algal growth in wastewater at 22°C (Run Two) is shown in Fig. 8. The barley straw was placed in wastewater without pre-decomposition. No other algal species were added into the wastewater other than those already present in the collected wastewater. Microscopic examinations were not conducted to characterize algal species for this run. The initial chlorophyll *a* concentration was 496 µg/L in all of the four tanks. The chlorophyll *a* concentrations in all of the tanks decreased to less than 53 µg/L after the initial four weeks of the experiment. The reduction in chlorophyll *a* concentrations could have been caused by several factors that may include algal adsorption to the inside surface of the plastic tanks, onto the barley straw (for the "treatment" tanks), co-precipitation with solids in the wastewater, or other unknown factors. The chlorophyll *a* concentrations gradually increased during the subsequent nine weeks of the batch test, indicating algal growth in each tank. By the 13th week, the chlorophyll *a* concentration in the "control" tank rose to 523 µg/L, which was comparable to those concentrations in other runs (Fig. 2 and 3). The chlorophyll *a* concentration in the "treatment" tanks also increased but to a lesser extent.

A paired t-test of chlorophyll *a* concentrations was conducted to compare "control" and "treatment" tanks. The t-test results, shown in Table 4, revealed that the use of barley straw significantly (at a 90% confidence level) lowered algal growth at each of the tested loading rates. When the three tested loading rates were compared, the effect of 2 g/L barley straw on algal growth was statistically more significant than that of 0.5 g/L or 1 g/L. There was no significant difference of effects between 0.5 g/L and 1 g/L. Experimental results indicate that the use of barley straw at the tested loading rates inhibited algal growth. The inhibition was diminished as the barley straw became exhausted.

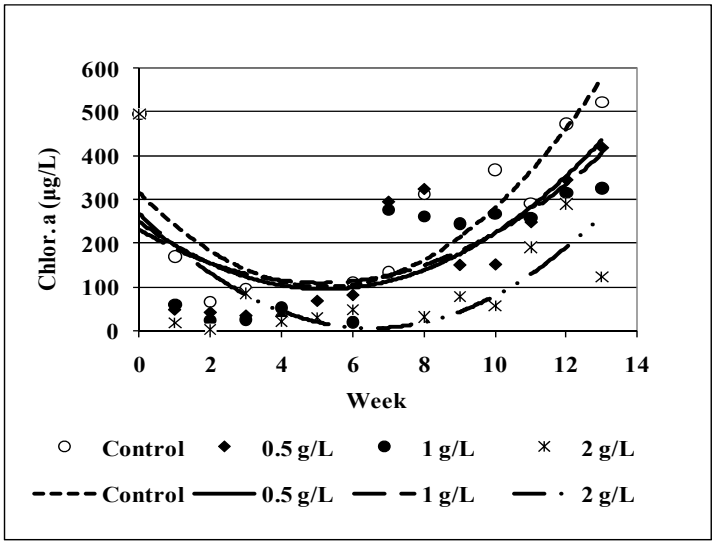

Fig. 8. Effect of Barley Straw on Algal Growth in Wastewater at 22°C

Table 4. Effect of Barley Straw on Algal Growth in Wastewater at 22°C: Paired t-test

Pair of Comparison	Sample size (n)	Calculated t	Critical t (α=0.10)	Former higher than latter at 90% confidence?
Control vs. 0.5 g/L	11	1.5	1.372	Yes
Control vs. 1 g/L	11	1.9	1.372	Yes
Control vs. 2 g/L	10	2.3	1.383	Yes
0.5 g/L vs. 1 g/L	12	0.2	1.363	No
0.5 g/L vs. 2 g/L	12	2.1	1.363	Yes
1 g/L vs. 2 g/L	11	2.1	1.372	Yes

3.5. Effect of Barley Straw on Algal Growth in Wastewater at 27°C (Run Three)

Results of the experiment on the effect of barley straw on algal growth in wastewater at 27°C (Run Three) are shown in Fig. 9. The rapid decline in chlorophyll *a* concentration during the first week was similar to what was found in Run Two (22°C) shown in Fig. 8. The 2 g/L of barley straw appeared to be effective in keeping chlorophyll *a* concentration lower than that in the "control" tank. However, because of large variations in measured chlorophyll *a* concentrations of this run, it was difficult to interpret the effect of barley straw at the given experimental condition. Instead, microscopic examination of samples taken during selected weeks of the experiment was used to evaluate the effect of the barley straw.

Cyanobacteria (blue-green algae) were predominant in the wastewater collected from a local treatment lagoon in late July 2005 and were present in these experiments. Two major types of algae were identified: (1) a filamentous form (ca. 5 μm diameter by 100-200 μm long) that resembled *Tychonema*; and (2) a small, round (ca. 5 μm diameter) colonial type that resembled *Microcystis*. Although the study also found some flagellated green unicells (division *Chlorophyta*) that resembled *Chlamydomonas*, these algae were not nearly as abundant as the two cyanobacteria. In addition, small amounts of other green taxa (e.g., *Ankistrodesmus*) were found.

At Week Five, a mostly pure culture of a heterocystous cyanobacterium (blue-green alga) was found in the sample taken from the "control" tank, the genus being either *Aphanizamenon* or *Anabaena*. In the sample taken from the 2 g/L "treatment" tank, clumps of diatoms with a few cyanobacterial filaments (*Oscillatoria/Lyngbya*) were found. The diatoms were primarily *Nitzschia* and naviculoids (boat-shaped diatoms with raphes, resembling *Navicula*). In the sample taken from the 4 g/L "treatment" tank, clumps of diatoms, almost entirely *Nitzschia*, were found. A few naviculoid diatoms and very few cyanobacterial filaments of the genus *Oscillatoria* were also found in the 4 g/L "treatment" tank. The barley straw could have brought about a shift in the algal population in the treatment tanks by causing changes in certain nutrient or other chemical concentrations or by inducing inhibitory effects on certain species. The blue-green algae may have been more susceptible to the effects of barley straw resulting in the diatoms becoming the dominant algae. Other factors may also have contributed to these observed changes in species.

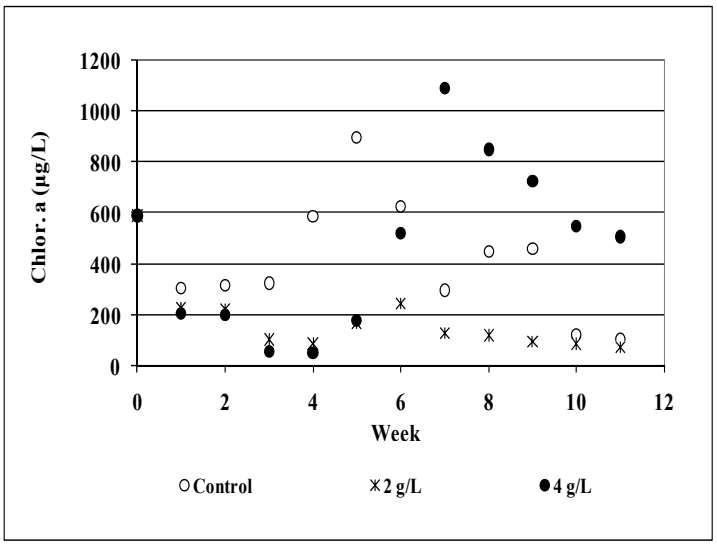

Fig. 9. Effect of Barley Straw on Algal Growth in Wastewater at 27°C

At Week Ten, live algae (mostly the diatom *Gomphonema* and a few *Navicula*, as well as *Oscillatoria*) were still present in the sample taken from the "control" tank, though at very low levels (Fig. 9). The shift to more diatoms in the "control" tank indicated that growth conditions for blue-green algae were not as favorable in this tank by Week Ten, similar to the shift seen earlier in "treatment" tanks. In contrast to the "control", no live algae were found now in the "treatment" tanks where 2 g/L or 4 g/L of barley straw was used (even though chlorophyll *a* measurements were 547 µg/L for 4 g/L loading rate). Microscopic examination revealed that the algae appeared to be dead; no additional tests were conducted to verify the algal status further. For future studies, a recovery test or phaephytin (degraded chlorophyll) test could be performed to confirm the viability of algae. The decline in algal population, as indicated in low chlorophyll concentrations of the "control" and 2 g/L tank, could have been due to depletion of certain nutrients by this time or other factors. In contrast, large amounts of nutrients from the decomposing barley straw in the 4 g/L tank may have helped to support high algal population growth in this tank during the experiment, resulting in high chlorophyll concentrations in the samples of weeks 7 and 8. However, a similar effect did not occur for the 2 g/L tank, which should have had some nutrients released. Therefore, nutrients may not be the reason for the observed increase of growth in the 4 g/L tank at Weeks 7 and 8. Alternatively, sampling or analytical problems may have contributed to the chlorophyll concentrations detected in samples from the 4 g/L tank. No increase in growth the 2 g/L tank was seen (Fig. 9), so these results were inconclusive. The study results indicated a negative effect on algae when barley straw was applied in wastewater, especially at 2 g/L. However, due to the changes of algal species and the variability of chlorophyll *a* values in these tests, it was difficult to draw conclusions on the inhibitory effects of barley straw on algae at 27°C. Replicate tanks and more nutrient and chemical analyses would help to better understand these growth patterns in the wastewater experiments.

3.6. Characterizing the Decomposition Process of Barley Straw in Wastewater

The effect of decomposing barley straw on pH of the tested wastewater (Run Two and Run Three) is summarized in Table 5. In general, pH variations were within 0.5 pH unit (standard deviation). The use of barley straw in wastewater appeared to have no major effect on pH, likely due to the buffering capacity in the wastewater. A field study that placed barley straw in a wastewater lagoon also found little effect on the pH of lagoon effluent (Zhou 2006).

The PO_4^{3-} concentrations of Run Two (22°C) are shown in Fig. 10. During the first four weeks of the test, higher PO_4^{3-} concentration correlated to larger amounts of barley straw used. However, when the sources of PO_4^{3-} were considered, namely those from wastewater and those from decomposing barley straw (Fig. 5), the measured concentrations seemed to be lower than estimated concentrations, especially the PO_4^{3-} concentrations in the 4 g/L "treatment" tank. Furthermore, PO_4^{3-} concentration in each "treatment" tank decreased and remained at approximately 1 mg/L from Week Five to Week 13. The decrease in PO_4^{3-} likely resulted from uptake by microorganisms and algae and co-precipitation with solids in the wastewater.

The PO_4^{3-} concentrations of Run Three (27°C) are shown in Fig. 11. PO_4^{3-} concentrations in the "control" tank were comparable to those found in Run Two (22°C). In contrast, from Week Three onward, PO_4^{3-} concentrations in both "treatment" tanks rose to 10-12 mg/L. Fig. 5 suggests that 4 g/L of barley straw would yield 20-25 mg/L of PO_4^{3-} in water at 22°C. It was expected that 2 g/L of barley straw would result in approximately half of the above mentioned amount, and PO_4^{3-} release at 27°C would be higher than PO_4^{3-} release at 22°C (Fig. 11). Chemical precipitation of PO_4^{3-} and its adsorption to particulates in water, as well as the uptake of PO_4^{3-} by algae and microorganisms, might cause the concentration of PO_4^{3-} in the 4 g/L "treatment" tank to be lower than the expected concentration.

Table 5. Effect of Decomposing Barley Straw on pH in Wastewater

Run	Wastewater Characteristics	pH[1]	"Treatment" pH minus "Control" pH[2]
Two	Control -Wastewater	8.5±0.3	
22°C	Treatment A – Wastewater, 0.5 g/L	8.5±0.3	0±0.2
	Treatment B – Wastewater, 1 g/L	8.5±0.3	0±0.2
	Treatment C – Wastewater, 2 g/L	8.4±0.3	-0.1±0.3
Three	Control - Wastewater	8.2±0.5	
27°C	Treatment A – Wastewater, 2 g/L	7.5±0.4	-0.7±0.4
	Treatment B – Wastewater, 4 g/L	7.5±0.5	-0.7±0.4

[1]pH was measured weekly. The reported pH is the average and standard deviation of all measurements throughout the duration of each run.
[2]The weekly pH of the "treatment" is subtracted from the corresponding weekly pH of the "control". The average and standard deviation of these differences were calculated throughout the duration of each run.

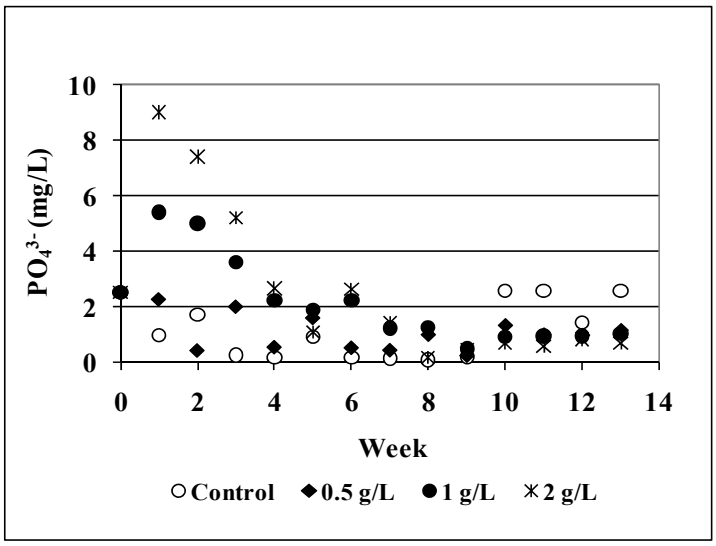

Fig. 10. Effect of Barley Straw on Orthophosphate Levels in Wastewater at 22°C

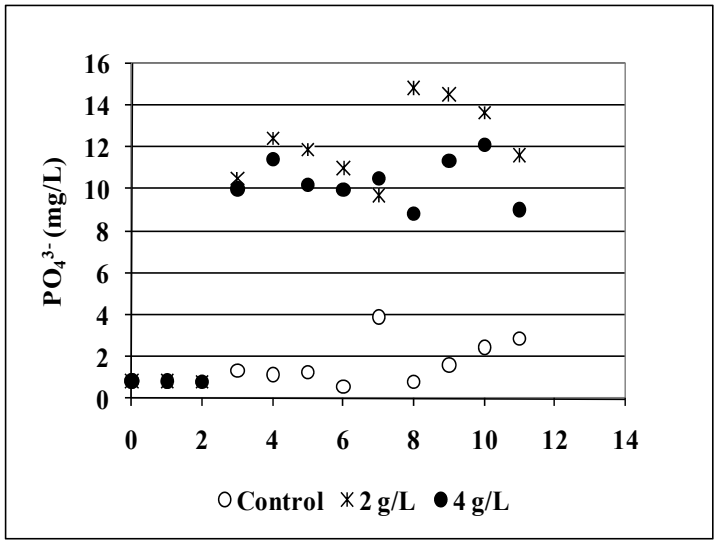

Fig.11. Effect of Barley Straw on Orthophosphate Levels in Wastewater at 27°C

The nitrate concentrations among the three tested barley straw loading rates at 22°C (0.5, 1, and 2 g/L) were comparable. All were higher than the nitrate concentrations in the "control" by approximately 1 mg/L (Fig. 12). Nitrate in Run Three (27°C, Fig. 13) showed comparable concentrations among the "control" and the two "treatment" during the first nine weeks of the test. The accuracy of the measurement was likely affected by the solids and algal debris in the wastewater, which could have interfered with the colorimetric measurement method of nitrate.

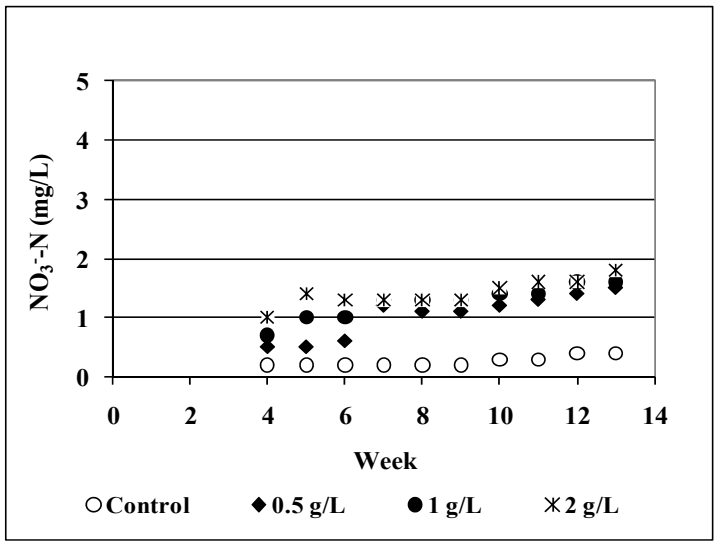

Fig.12. Effect of Barley Straw on Nitrate Levels in Wastewater at 22°C

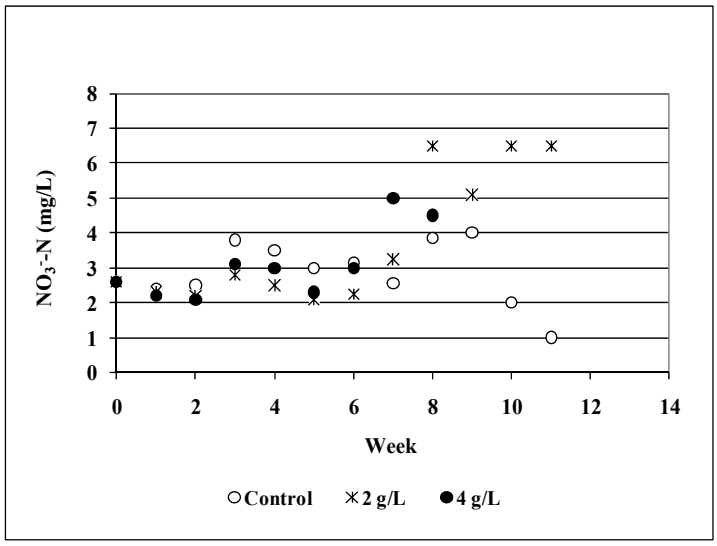

Fig. 13. Effect of Barley Straw on Nitrate Levels in Wastewater at 27°C

The measured COD for Run Two (22°C) and Three (27°C) are shown in Fig. 14 and 15, respectively. The measured COD included soluble COD, organic solids, and algal cells. COD decreased during the first several weeks and increased in the remaining weeks. The COD decrease was likely caused by organics degradation. The COD increase was likely due to the decomposition of barley straw in the "treatment" tanks. The algal cells may count for a relatively small fraction of the COD, because COD did not increase as much in the "control" even though there was an increase in algal population (Fig. 8). Ammonia was measured on samples taken from Run Two, Three and Four. All were less than 0.1 mg/L NH_4^+-N. Hydrogen peroxide was measured on samples taken from Run Two (Weeks 7, 9, 10, 12) and Run Three (Weeks 0, 2, 3, 5, 7, 9, 11). All were below the detection limit as well.

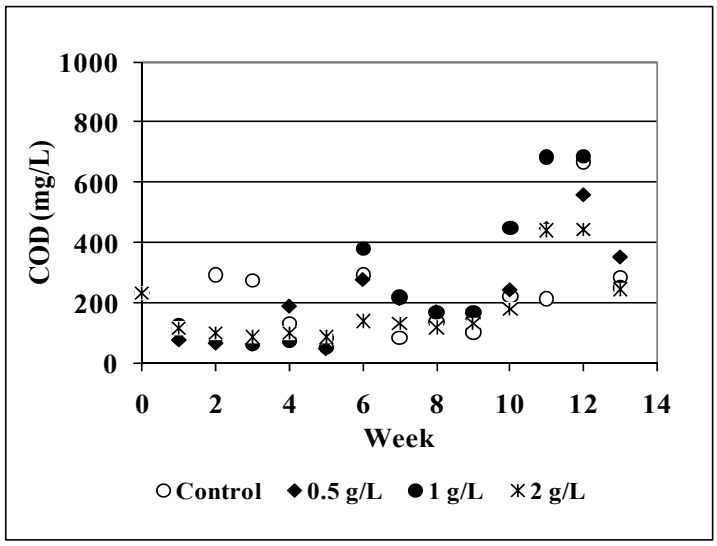

Fig. 14. Effect of Barley Straw on COD Levels in Wastewater at 22^{o}C

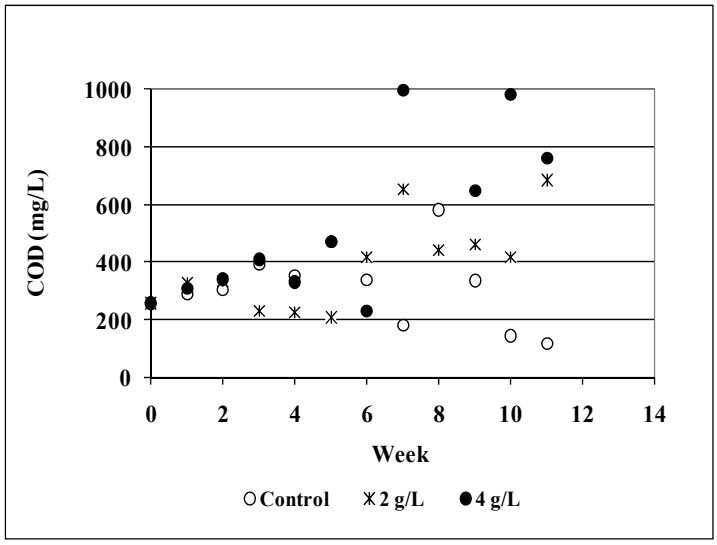

Fig. 15. Effect of Barley Straw on COD Levels in Wastewater at 27^{o}C

3.7. Chemical Compounds from Decomposed Barley Straw in Water at 22^{o}C

3.7.1. Chemical compounds from 0.83 g/L of barley straw decomposing in water

The Batch One experiment aimed to identify and quantify 2,6-Dimethoxy-4-(2-propenyl)phenol and octanoic acid from decomposing barley straw in water because of the postulated importance of these two compounds in inhibiting algal growth (Pillinger et al. 1994, Pillinger et al. 1996; Everall and Lees 1997). The calibration test of Batch One revealed that 4-allyl-2,6-Dimethoxyphenol (Fig. 16, the top graph) showed a major parent ion peak at 194 and a minor peak 91; octanoic acid (Fig. 16, the bottom graph) showed major

parent peaks at 60 and 73 and a minor peak at 145. For MS/MS, an ion of m/z 163 was used for the 4-allyl-2,6-Dimethoxyphenol and an ion of m/z 101 was used for the octanoic acid. The validation of the GC/MS/MS method indicated 73-74% recovery of these two chemical compounds with the C-18 cartridge (Table 6). The relatively low recovery is likely due to, in part, the differences in each compound's miscibility and only having one extraction.

However, 2,6-Dimethoxy-4-(2-propenyl)phenol and octanoic acid were not detected in any of the samples of the barley straw water extract from the Batch One study. The sample taken on March 9, 2005 (Week 4) is shown in Fig. 17 as an example. The graphs from the other samples were similar. There are several possible reasons why no significant amount of either chemical was found in the barley straw water: (1) the recovery of the two chemical compounds may be insufficient due to the complex matrix of the barley straw extract and the varied physical properties of the two compounds; (2) the lack of aeration and light in the initial three months may have hindered the decomposition and the release of chemicals; (3) the selected chemical compounds may have been present, but the amount was too low to be detected ; and (4) the time frame was too short to obtain adequate amounts of chemicals. Everall and Lees (1996, 1997) suggested that it could take months before barley straw decomposition released active and inhibitory chemical compounds.

Three other compounds were found from the GC/MS/MS analyses and are shown in Fig. 18. These compounds were not measured for actual concentrations. Instead, the relative ion counts were reported. These compounds should be investigated further in the future for properties that may affect algal growth.

3.7.2. Chemical compounds from 5.4 g/L of barley straw decomposed in water

In the Batch Two and Three experiments, barley straw of 5.4 g/L was used (6.5 times of what was used in Batch One) to determine if an increased loading rate would enable detectable concentrations of chemical compounds from the decomposed straw. The GC/MS/MS analysis did identify many additional chemical compounds, as shown in Fig. 19 (Batch Two) and Fig. 20 (Batch Three). These compounds were not measured for their actual concentrations. Instead, they are reported as relative ion count. Results revealed that more chemical compounds could be identified as the barley straw loading rate increased. Butylated hydroxytouluene and 2-methoxy-4-vinylphenol appeared in many of the samples taken during the 11 weeks of the Batch Three test. These persistently appearing compounds are good candidates for future investigation of their effects on algal growth.

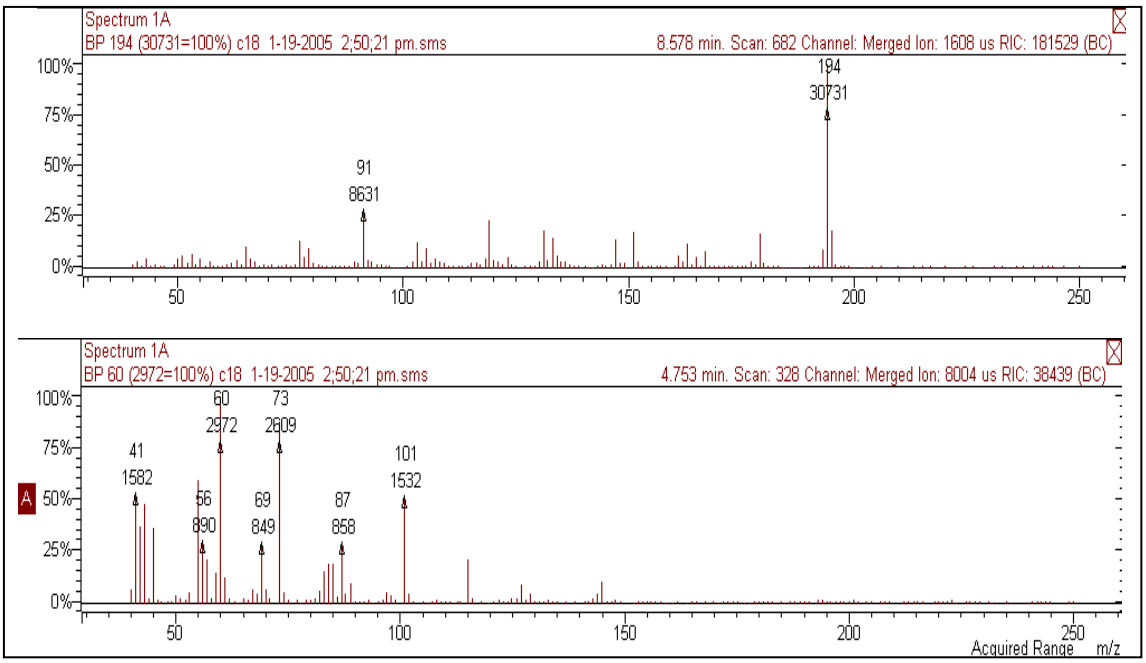

Fig. 16. Mass Spectrum of Chemical Compounds of Interest
Top Graph Shows 4-allyl-2, 6-Dimethoxyphenol; Bottom Graph Shows Octanoic Acid

Table 6. GC/MS/MS Recovery Rate of Selected Chemical Compounds

Compound	MS Peak Area Spike Check	MS Peak Area C-18 Cartridge	Recovery Rate
Octanoic Acid	6.85E+4	4.98E+4	73%
4-allyl-2,6-dimethoxy phenol	5.14E+4	3.81E+4	74%

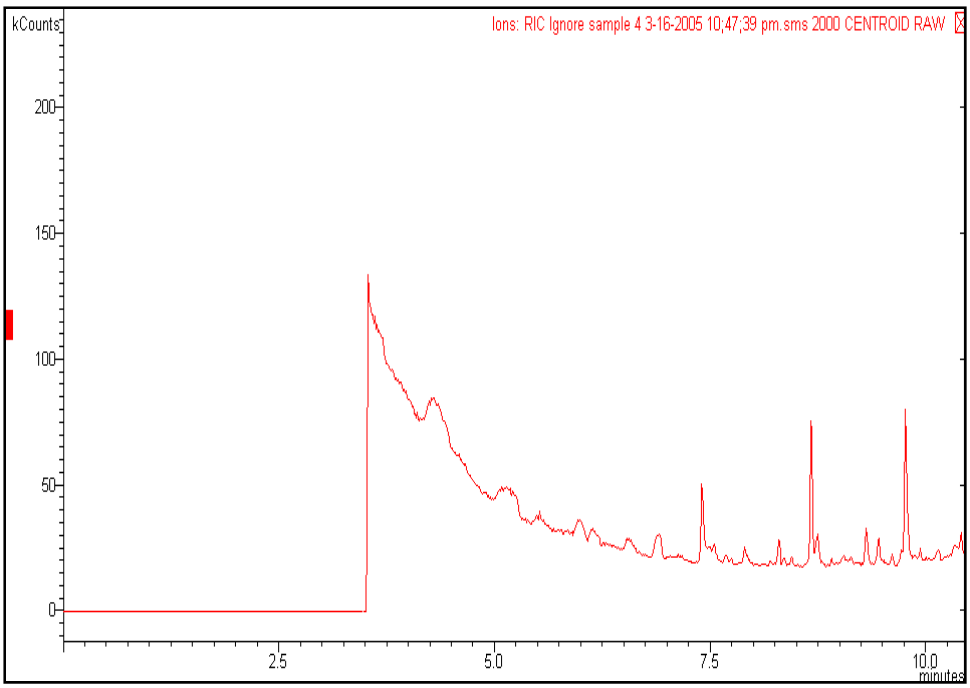

Fig. 17. Example Chromatogram of GC/MS/MS Analysis

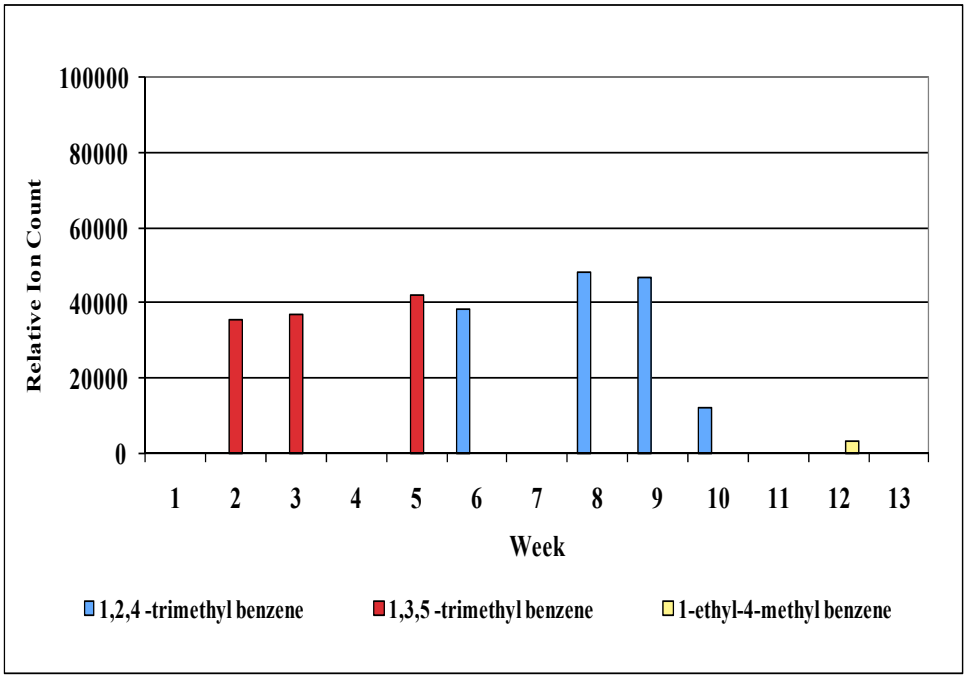

Fig. 18. Detected Compounds from Extract of 0.83 g/L Barley Straw (Batch One)

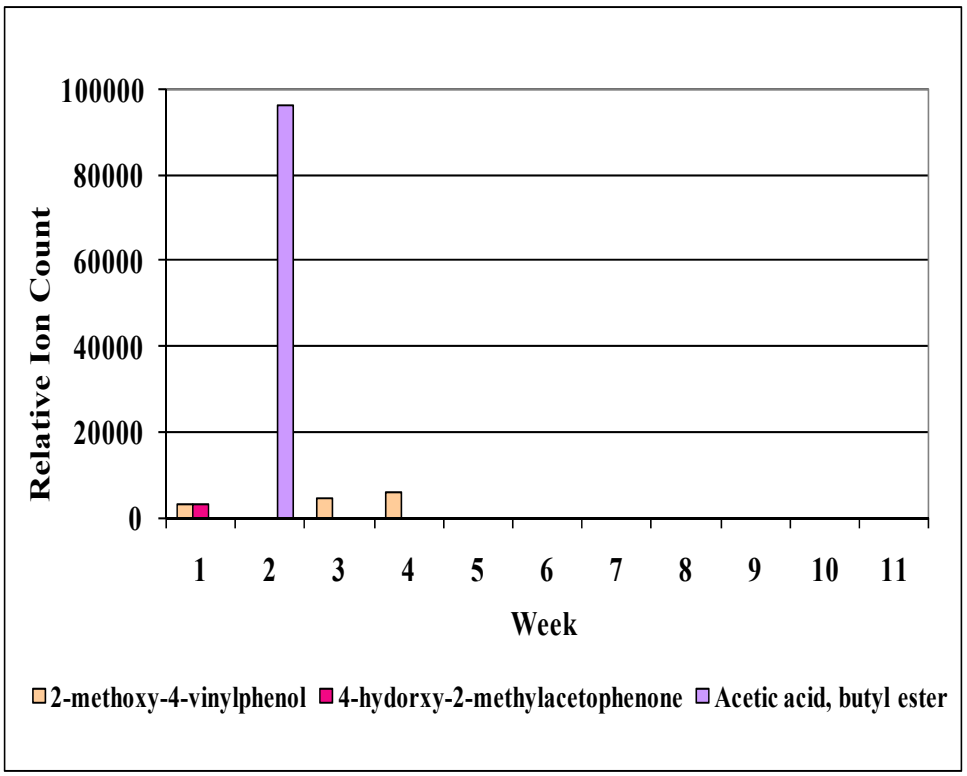

Fig. 19. Detected Compounds from Extract of 5.4 g/L Barley Straw (Batch Two)

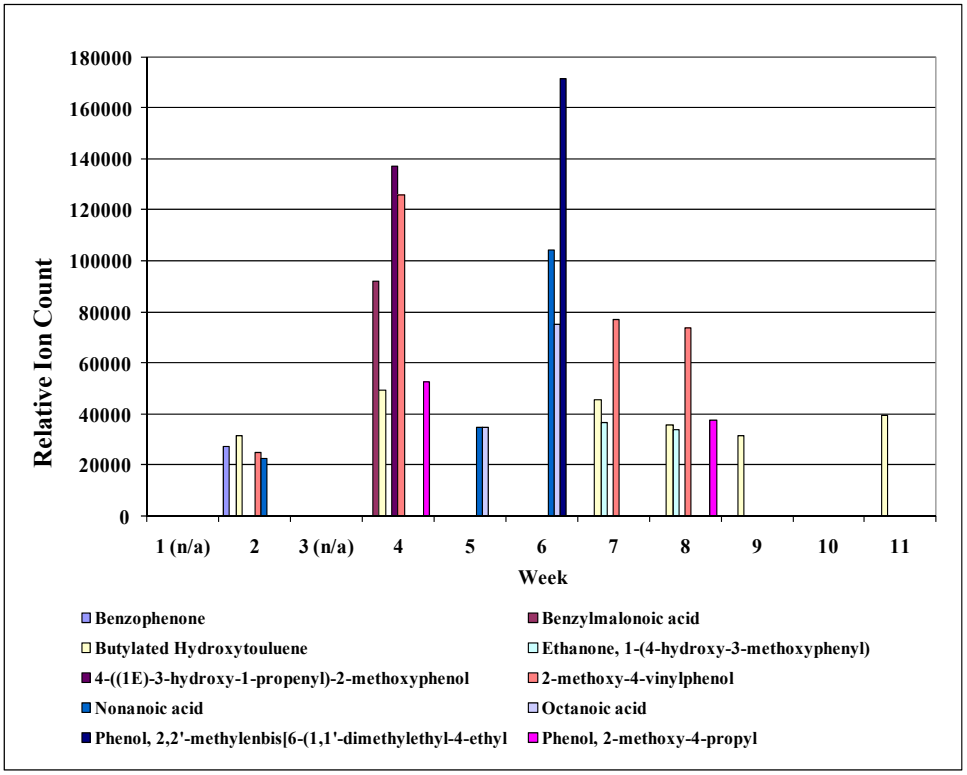

Fig. 20. Detected Compounds from Extract of 5.4 g/L Barley Straw (Batch Three)

4. Summary and Conclusions

This study found that:

1. Decomposing barley straw of 4 g/L was effective in inhibiting the growth of *Anabaena* and *Oscillatoria* in water at 22°C. By the end of the nine and a half weeks of the batch test, the chlorophyll *a* concentration of *Anabaena* in the "treatment" tank was 60 µg/L compared to 571 µg/L found in the "control" tank; the chlorophyll *a* concentration of *Oscillatoria* in the "treatment" tank was 80 µg/L compared to 589 µg/L found in the "control" tank.

2. Decomposing barley straw was also effective in inhibiting the growth of *Anabaena* in water at 27°C at both tested loading rates of 2 g/L and 4 g/L. No apparent differences were found between the two loading rates for their effects on reducing algal growth. It appears that the inhibitory effects became exhausted sooner at 27°C than at 22°C.

3. Each gram of decomposing barley straw in water at 22°C generated approximately 5.2 to 7.0 mg PO_4^{3-} and 1.4 mg NO_3^--N in nine weeks of decomposition. Decomposing barley straw also increased the amount of organics in the water.

4. Decomposing barley straw was also effective in inhibiting algal growth in wastewater at 22°C. A paired t-test revealed that, at a 90% confidence level, each of the tested loading rates (0.5, 1, 2 g/L) lowered algal growth. The effect of 2 g/L of barley straw was statistically more significant than that of 0.5 g/L or 1 g/L. There was no significant difference between 0.5 g/L and 1 g/L.

5. At the end of the ten-week wastewater batch test conducted at 27°C, live algae were still present in the "control" tank, though at very low levels. In contrast, no live algae (concluded from microscopic examination and not a recovery growth test) were found in the "treatment" tanks, which contained 2 g/L or 4 g/L of barley straw. However, due to the changes of algal species and the variability of chlorophyll *a* values in these tests, it was difficult to draw conclusions about the inhibitory effects of the barley straw on algae in wastewater at 27°C.

6. GC/MS/MS analysis was not able to detect 2,6-Dimethoxy-4-(2-propenyl)phenol and octanoic acid in water samples when 0.83 g/L of barley straw decomposed, but did identify several other different chemical compounds when 0.83 g/L or 5.4 g/L of barley straw was used. Butylated hydroxytouluene and 2-methoxy-4-vinylphenol appeared in many of the samples from the experiments with 5.4 g/L of barley straw and are good candidates for future investigation of their effects on algal growth.

This project produced new and valuable information about the effect of decomposing barley straw on algal growth and several water quality parameters. Several chemical compounds from decomposed barley straw were identified and could be further studied for their inhibitory effects. The results and conclusions discussed here are based on preliminary studies and should be considered with the knowledge that, because the available resources from this seed grant were limited, some experiments were not performed in replicates. This made data interpretation difficult at times.

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