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**Treatment of Irrigation Effluent Water to Reduce
Nitrogenous Contaminants and Plant Pathogens**

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ABSTRACT

Treatment of irrigation effluent water to reduce nitrogenous contaminants and plant pathogens.
J.D. MacDonald and A. Abeliovich.

The contamination of surface and subterranean drinking water supplies with nitrogen-laden agricultural wastewater is a problem of increasing concern in the U.S. and Israel. Through this research, we found that bacteria could utilize common organic wastes (e.g. paper, straw, cotton) as carbon sources under anaerobic conditions, and reduce nitrate concentrations in wastewater to safe levels. Two species of bacteria, *Cellulomonas uda* and a *Comamonas* sp., were required for denitrification. *Cellulomonas uda* degraded cellulose and reduced nitrate to nitrite. In addition, it excreted soluble organic carbon needed as a food source by the *Comamonas* sp. for completion of denitrification. We also found that recirculated irrigation water contains substantial amounts of fungal inoculum, and that irrigating healthy plants with such water leads to significant levels of root infection. Water can be disinfected with UV, but our experiments showed that Hg-vapor lamps do not possess sufficient energy to kill spores in wastewater containing dissolved organics. Excimer lasers and Xenon flashlamps do possess the needed power levels, but only the laser had a high enough repetition rate to reliably treat large volumes of water. Ozone was highly efficacious, but its use as a water treatment is probably best suited to moderate or low volume irrigation systems. This research provides critical data needed for the design of effective water denitrification and/or pathogen disinfection systems for different growing operations.

OBJECTIVES

The objectives of the original research proposal were to:

1. Develop an economically feasible method for reducing the nitrogen content of irrigation wastewater so that discharged effluents do not pose a significant pollution problem.
2. Determine the efficacy of selected water treatment methods against common plant pathogens and develop criteria for the successful application of treatment technologies to recirculated irrigation water.

Under Objective 1, we proposed to research the possibility of using common organic wastes (e.g. straw, sawdust, newspaper, etc.) as carbon sources for facultative anaerobic bacteria in denitrification reactors. Under Objective 2, we proposed to develop data regarding the efficacy of various water treatment methods, and quantify the effects of water quality on treatment efficacy. The overall goal of this research was to develop data that could be used by engineers in the design of water denitrification and/or pathogen disinfection systems for varied growing operations.

BACKGROUND

The contamination of surface and subterranean drinking water supplies with nitrogen-laden agricultural wastewater is a problem of increasing concern in the U.S. and Israel. In parts of the U.S., certain crop production systems (e.g. nurseries, or glasshouses) have been classified as "point sources" of nitrogen pollution and are being required to trap and recycle irrigation tailwater and crop effluents. Nitrogen contamination is a serious threat to drinking water (12, 33), but recycling introduces serious plant disease risks. Under this project, we have researched two key aspects of agricultural wastewater management: 1) methods for

denitrification of wastewater to minimize offsite pollution from controlled discharges and 2) methods for eliminating plant pathogens from recirculated irrigation water to minimize the disease risks associated with recycling.

Research toward the first objective was conducted in Israel under the direction of Dr. A. Abeliovich, while research toward the second objective was conducted in the U.S. under the direction of Dr. J. MacDonald.

Denitrification research focused on the design and operation of simple biological reactors in which cellulose degradation and denitrification were coupled. In the case of wastewater, the only economically feasible way to remove nitrates is through microbial denitrification, which consists of a series of enzymatic reactions that result in the reduction of nitrate to a gaseous product, usually N_2 . These reactions are carried out by facultative anaerobic bacteria that, under anaerobic conditions, use nitrate in place of oxygen as a terminal respiratory electron acceptor. The overall reaction in heterotrophic denitrification can be summarized as follows (11):

The reduction of nitrate to N_2 is a stepwise reaction mediated by the generation of nitrogen oxides in the following sequence (22):

The majority of microbial denitrification treatments rely on heterotrophic bacteria which require an organic carbon substrate. Usually, a simple carbon compound such as ethanol, methanol or acetate is used. However, these compounds are too expensive for treatment of agricultural wastewater. Less expensive and more abundant carbon sources (9), such as cellulose wastes from municipal and agricultural sources (paper, crop residues, etc.) could be used. Cellulose-dependent denitrification can be summarized as follows:

Cellulose is a less readily-available source of carbon for microbes. It consists of a linear glucose polymer with hydrogen bonding between hydroxyl groups of neighboring parallel chains. Native cellulose is organized in fibers and occurs in close association with lignin and hemicellulose. These characteristics confer resistance to enzymatic attack by microbes. However, specific bacterial groups do have the capacity to degrade cellulose. Even so, one or more forms of pretreatment (chemical, physical or physicochemical) are often applied to cellulose-rich materials to increase their susceptibility to degradation (13, 21, 36). The use of cellulose in the restoration of nitrate-contaminated ground water was described by Boussaid et al. (5), who packed denitrification reactors with a mixture of straw, sand, and maërl, or with straw and maërl, and then returned treated effluents to the aquifer.

An alternative means of controlling nitrate pollution is simply to prevent the release of nitrate-laden water into the environment. In parts of the US, this has been accomplished by requiring nurseries and greenhouses to trap and recycle irrigation runoff. This approach has the added benefit of conserving water resources, but also introduces disease risks since recirculated water may become contaminated with plant pathogens.

The spread of pathogens throughout crops via contaminated irrigation water is a well-documented problem (1, 6, 14, 16, 17, 18, 19, 25, 28, 29, 34). A small number of infected plants in a nursery crop can release large numbers of propagules into irrigation leachates which then drain into effluent holding ponds. In recent surveys of effluent ponds in California, we found large populations (up to 2,500 propagules liter⁻¹) of *Phytophthora* and *Pythium* species (18). Furthermore, we found that viable propagules of these fungi passed through filtration units and were detectable in the irrigation water applied to crops.

Although disinfecting irrigation effluents would appear to be a straightforward engineering problem, the efficacy of different treatment methods (e.g. UV, chlorination, ozonation, heat and microfiltration) against common plant pathogens has been largely unexplored. Furthermore, the efficacy of most disinfection methods in the presence of high levels of organic residues and colloidal solids typically found in agricultural wastewater also has been largely unexplored. Such contaminants can render UV treatments ineffective and have well-known detrimental effects on chlorine and ozone efficacy (10, 37).

The purpose of this project was to approach the problem of irrigation wastewater from two directions. We wanted to determine whether the wastewater could be denitrified and released back into the environment. We also wanted to determine whether the wastewater could be disinfected so that it could be reused on crops without risk of disease and the attendant use of fungicide treatments (which add further to environmental pollution). This report summarizes our accomplishments under these complimentary objectives.

RESEARCH METHODS

A. Denitrification of Wastewater

A.1. Laboratory Experiments. Most denitrification experiments utilized wheat straw or shredded newspaper as carbon sources. Experiments characterizing bacterial populations in the reactors, however, utilized a cotton substrate--a more pure form of cellulose.

Bench-top reactors were made of plastic cylinders: either perspex columns, 50 cm high and 8 cm dia, or PVC columns, 55 cm high and 10 cm dia. A thin glass wool layer was placed at each end of the column, and the reactors were packed with substrate. After packing, columns were inoculated with a small amount of material from an active denitrification column. The fresh columns were filled with nitrate- and phosphate-amended tap water and initially operated for 2-3 days in a recirculation mode. The columns then were switched to a flow-through mode and fed with tap water amended with nitrate (22.58 mg N l⁻¹) and phosphate (3 mg l⁻¹). Flow rates were regulated by peristaltic pumps and are presented as Darcy's flux ($q = Q/A$), in m day⁻¹ where Q is the measured flow rate (in m³ day⁻¹) and A is the cross section of the column (m²). A q value of 1 m day⁻¹ corresponded to a flow rate of 5.5 ml min⁻¹ in the 10-cm-dia PVC column and to 3.5 ml min⁻¹ in the 8-cm-dia perspex columns.

Unless otherwise indicated, ambient temperature was maintained at 24±1°C. Influent and effluent samples usually were collected twice a week and routinely assayed for nitrate, nitrite, ammonia, pH, and dissolved organic carbon (DOC). Nitrate was determined with a nitrate-selective electrode (Orion Research Inc., Boston, MA, USA) or by colorimetry (8). Nitrite and ammonia were assayed according to Standard Methods (2). DOC was determined by means of a high-temperature TOC analyzer (Dohrmann DC-190, Rosemount Analytical Inc., Santa Clara, CA, USA).

Bacterial populations in the reactors were determined by standard plating techniques on R2A agar (Difco Laboratories). Bacteria producing extracellular xylanase were detected in 1.5% (w/v) agarose plates containing mineral medium supplemented with 0.2% (w/v) remazol brilliant blue-xylan prepared as described by Biely et al. (3).

Wheat Straw: The chemical composition of straw was determined in samples ground to pass a 40-mesh screen with a Wiley intermediate mill (Thomas Scientific Apparatus, Swedesboro, NJ, USA). Extractable components, acid-detergent fiber, lignin and ash were determined by standard methods (7, 15). Inoculum used to seed columns was obtained as described by Volokita et al. (30, 31).

Newspaper: Newspaper was prepared for use by shredding into ribbons (0.4 cm width) using an office-type paper shredder. The chemical composition of newspaper was determined as described above for wheat straw.

In one series of experiments, paper was exposed to various treatments prior to packing in reactors to determine if pretreatment would facilitate cellulose degradation and enhance denitrification efficiency. Four pretreatments were tested: ball milling, treatment with 1% (w/v) NaOH, treatment with 1 N HCl and heat treatment in an autoclave. Ball-milling turned out to be unsuitable as a pretreatment, being too slow and costly (in terms of energy requirement) for large water treatment systems, and producing a finely-pulverized matrix that was not suitable as a solid support in a flow-through type reactor.

In the acidic, basic and heat treatments, the ratio of newspaper to liquid was 100 g/4 l. Treatments were followed by rinsing with tap water; the acid- and base-treated paper was washed until no change in pH was observed. The volume of water used was measured and an equal volume was used for rinsing the untreated control and the autoclaved paper. Four small columns then were packed with each of the following types of newspaper: untreated, autoclave-treated, acid-treated and base-treated. The columns were operated at identical flow rates

A.2. Microbial Characterization of Reactors. A denitrifying reactor based on a column filled with cellulosic material serving both as a matrix and a carbon source is expected to have a complex community structure. Understanding the metabolic and spatial interrelations among the major participants in the denitrification process is important for the evaluation of the potential and the operational limits of this process. Thus, experiments were done to determine the distribution of microorganisms in the cellulose column, the species composition, and the metabolic interrelations between major groups of bacteria. These experiments were done in reactors containing cotton as a substrate, so they consisted of a pure cellulose environment. Reactors were packed and inoculated as described above.

Determination of microbial biomass: The total mass of microbes accumulated in the reactor was estimated by measuring the concentration of protein on the cotton fibers. Samples were removed from reactors at 1, 3, 4.5 and 6 months and freeze-dried. One gram of freeze dried cotton was immersed in 5 ml of 0.5 N NaOH. The sample was sonicated for 10 minutes and then boiled for 60 min, vortexed vigorously and left overnight at room temperature. The supernatant then was centrifuged in a microfuge for 3 minutes at 14,000 RPM. Protein (as mg protein gram⁻¹ dried cotton) was determined by the standard Bio-Rad procedure with unused cotton as a control.

Isolation and identification of bacteria: One gram of wet cotton was taken from a column that had been actively denitrifying for 6 months. The sample was mixed in a small blender with 10 ml of 0.1% tween-80 and then sonicated in a cleaning bath for 2 min. The suspension was diluted and inoculated on minimal medium agar plates containing (w/v): 0.05% KNO₃, 0.1% K₂HPO₄, 0.01% KH₂PO₄, 0.05% KCl, 0.05% MgSO₄(7 H₂O), 1.5% agar. 0.5 ml/liter of filter sterilized micro elements solution was added after sterilization. Various carbon sources were tested: glucose (0.1%), methanol (0.1%), ethanol (0.1%), glycerol (0.5%), acetate (0.5%), malate (0.5%), lactate (0.5%) cellobiose (0.5%), low viscosity carboxy methyl cellulose (0.5%), avicel (0.5%), cellulose (0.5%), powdered filter-paper (0.5%). Nutrient agar (Difco) was also used.

Bacteria were isolated from the top, middle and bottom (water inlet) of the column-bed and the plates were incubated under aerobic and anaerobic conditions. Fifty isolates were selected for identification by the API and Biolog diagnostic kits after initial characterization to determine: (1) Shape and color of the colony on different media, (2) Shape of the bacteria, (3) Gram reaction, (4) Presence of cytochrome oxidase, (5) Endoglucanase activity, (6) Cellobiase activity, (7) Ability to degrade completely crystalline cellulose, and (8) The ability to accomplish partial (NO₃->NO₂) and/or complete (NO₃->N₂ and NO₂->N₂) denitrification under anaerobic conditions using glucose, malate, ethanol, carboxy methyl cellulose, or cotton as carbon sources. Bacteria producing extracellular xylanase were detected by the methods of Biely et al (3). The two most common isolates were identified by 16S RNA sequencing kindly performed by F. Rainey (24) and by fatty acids analysis (Dr. H. Lemmer).

A.3. Field Experiments. A small denitrification reactor was constructed on the property of Hines Nurseries, Inc., Irvine, California with the aid of G. Gutman (Director of Operations) and J. Feaster (Head of Pest Management). The reactor was constructed by excavating a ditch approximately 1 m wide, 1 m deep, and 5 m long. Brick baffles were installed, and the ditch was filled with oat and wheat straw (Fig. 1). The straw was inoculated by adding unsterilized compost, and the ditch was filled with water. Sheets of plywood, covered with black plastic film, were used to close the top of the ditch. The reactor was allowed to incubate for approximately 10 days.

Following incubation, the ditch was tested for its ability to denitrify irrigation runoff. Because the ditch was located in a low area of the nursery, irrigation runoff water could be diverted through it by gravity flow, eliminating the need for pumps. As nearby crops were irrigated, runoff water was channeled through the ditch. We collected inflow and outflow water samples at various times to assess whether denitrification was achieved.

B. Microbial Disinfection of Wastewater

B.1. Laboratory Experiments. For laboratory experiments, fungi originally recovered from irrigation water (*Phytophthora parasitica*, *Fusarium oxysporium*, and *Cylindrocladium* sp.) were grown on appropriate media to produce spores in quantity. Known amounts of fungal inoculum were added to pure water or filter-sterilized irrigation water, and then were exposed to various disinfection procedures to obtain quantitative dose-response data. In all cases, treatment efficacy was determined by quantitatively assaying viable propagules before and after treatment. The water disinfection treatments used in these experiments included:

UV radiation: Experiments with UV radiation employed three light sources: (1) a low-pressure Hg-vapor lamp mounted in a small, commercially-available water purifier (donated by Ultra-Dynamics Corp., Santa Monica, CA), (2) a KrF excimer laser (located at the UCD Crocker Nuclear Laboratory) that provided a monochromatic (248 nm) source of UV photons, and (3) a prototype Xenon flashlamp (loaned by Pulse Sciences, Inc., Hayward, CA) that provided high energy, broad-spectrum emissions (UV, visible, and IR).

The reactor vessel of the water purifier consisted of a 12-cm-dia. X 40-cm-long stainless steel cylinder in which the Hg-vapor lamp was mounted. Water entered and exited the closed cylinder through 3-cm-dia. pipe fittings attached to the sides, at opposite ends. In laboratory experiments, water was moved through the cylinder using a small centrifugal pump. The apparatus was fitted with appropriate valves and flowmeters to control and measure flow rates (exposure times) through the unit. To use the UV laser in flow-through experiments, we fabricated a treatment vessel from plexiglass and quartz (Fig. 2).

In experiments to precisely measure dose-response effects, known amounts of inoculum (suspended in distilled water or recycled nursery water) were placed in quartz cuvettes and exposed to different UV sources. For experiments with the Hg-vapor source, the tubular lamp was removed from the reactor vessel and mounted in a shielded benchtop enclosure. A quartz cuvette was placed 10 cm from the light source, and a shutter device was placed between the UV source and the cuvette. This shutter was opened for timed intervals, giving very precisely-controlled exposure doses in this fixed-geometry arrangement. A small joulemeter was used to precisely measure the UV energy entering the cuvette. Similar experiments were done with the laser (by placing quartz cuvettes in the laser beam path) and the Xenon flashlamp (by placing vessels 10-20 cm beneath the light source). The energy entering the spore suspensions from these two pulsed-power sources (usually delivered at a rate of 1-5 pulses per sec) was measured with a joulemeter.

Because irrigation wastewater water contains many impurities, a spectrophotometer was used to measure the UV "quenching" that occurred in individual effluent samples. Propagule survival was determined by taking small (0.5 ml) aliquots of the test solutions before and after treatment, and spreading them on agar media. Differences in the numbers of colonies derived from pre- and post-treated samples provided measures of treatment efficacy.

Chlorination and Ozonation: Many of these experiments employed generally similar methods. Fungi were grown on appropriate media and spores harvested into distilled water or nursery wastewater samples. Once spore concentrations were determined, chemical treatments were applied directly to the infested water samples. For chlorine experiments, a measured amount of NaOCl was added to the spore suspensions to achieve a desired Cl concentration. The amount of free and total Cl was measured using the Hach DPD test. As above, aliquots were removed at intervals to measure Cl concentration and the numbers of viable spores.

For experiments testing ozone, oxygen gas was passed through a commercial corona discharge ozonator (Aqua-Cleanser Corp.) and bubbled into water samples containing known numbers of viable spores. Ozone concentration in the spore suspensions was measured over time using a Hach Instruments DR 100 Colorimeter and the DPD reagent test. Aliquots of water were removed at intervals following initiation of ozone treatments to measure ozone concentration and the percent survival of fungal spores. This was accomplished by spreading measured aliquots of suspension onto agar media, and counting the number of colonies that resulted relative to the number expected (initial inoculum level).

Some experiments employed a flow-through device which we constructed to mimic commercial treatment equipment (see Fig. 3). A large volume (approximately 150 liters) of spore suspension was prepared and maintained with constant agitation. This solution was pumped from the storage reservoir into a 4-liter treatment vessel that was fitted with inflow and outflow tubes. Ozone was bubbled into the treatment vessel and the solution in the vessel was constantly stirred to assure thorough mixing of the dissolved gas. Spore suspension was pumped continuously through the vessel, and flow rates adjusted as necessary to give various residence times (volume turnover) in the ozonating vessel. Water samples were drawn off to measure ozone concentration, and flow rates were measured to determine average contact times. Treatment efficacy was determined by comparing viable inoculum in, to viable inoculum out of the treatment vessel.

Heat Treatment: For these experiments, fungi were grown on appropriate media and spores harvested into distilled water or nursery wastewater samples. Spores were exposed to heat by either of two methods. By one method, spore suspensions were dispensed into small (100 ml) flasks, which then were placed in temperature-controlled, heated water baths. The water baths were preheated and stabilized at desired temperatures prior to immersion of the inoculum-containing flasks. Thermocouples were used to measure solution temperatures within the flasks. In the second method, small flasks containing measured volumes of water were placed into water baths and allowed to stabilize to predetermined temperatures. Measured volumes of spores then were added to the preheated solutions, resulting in "instantaneous" heating of spores rather than a gradual heating. In both cases, aliquots of the heated spore suspension were removed at 5 min intervals and spread on agar media to count the number of viable propagules.

B.2. Field Experiments: Field experiments were initiated to test the efficacy of selected treatments in actual recirculated waters. Such waters typically contain numerically large, biologically-complex mixtures of organisms, as well as dissolved organic and suspended solid impurities. Portable treatment equipment was taken to and set up at several commercial nurseries. Equipment was plumbed into water recirculation systems and treatment efficacy was determined by quantitatively assaying viable propagules entering into and passing out of treatment systems. Only the Hg-vapor UV and Ozonation devices were portable enough to allow field testing.

UV radiation: The UV water purifier was plumbed into the water recirculation system of several different nursery locations at several different times of the year. This afforded a wide range of water qualities (in terms of dissolved and suspended materials) for testing purposes. For each test, a range of flow rates were employed to provide different transit (exposure) times in the treatment chamber. Samples of inflow and outflow water were collected and spread onto agar media (immediately after collection in the field) to obtain estimates of viable propagules.

Ozonation: Field trials with ozone utilized a small commercial prototype device that is schematically illustrated in Fig. 3. As with UV trials, the ozone device was plumbed into the recirculation systems of several different nursery locations at several different times of the year. Ozone concentration was controlled in this system by manipulating oxygen flow rate into the ozone generator, and water flow rate (turnover time) through the exposure tank. The O₃ concentration of outflow water was measured using the DPD reagent test and a Hach DR 100 Colorimeter. System flow rate also determined the duration of ozone exposure for fungal

propagules. Aliquots of water were collected from the inflow and outflow lines, and were spread on agar media to quantify viable propagules (via colony counts) before and after O₃ treatment.

B.3. Disease Risk Assessment: Regulations mandating recirculation of irrigation runoff have been enacted without benefit of data that defines the biological risk. To determine whether the inoculum present in recycled water can actually cause plant disease, we established a series of field and greenhouse experiments in several geographic locations.

Plant materials (hibiscus, azalea, mini-rose, and lilac) were propagated from cuttings under controlled. Rooted cuttings were established in 15-cm-dia plastic pots containing steam-sterilized media. All plants were initially free of fungal contamination. Fifteen of each plant species were placed in a greenhouse at Davis, CA, where they were irrigated as needed with distilled water. These plants formed a control group.

Another group of plants (15 of each species) was transported to a commercial nursery in Vacaville, CA (approximately 60 km from Davis). These plants were placed on low wooden platforms laid on the ground—the plant containers were never allowed to contact the ground. An irrigation valve was tapped into the recirculation plumbing so that plants were irrigated with the same water that was being applied to commercial crops. Irrigation water was applied to the experimental plants via drip emitters in each container to prevent any possibility of splashing or cross-contamination between pots.

Finally, three other groups of plants (each group consisted of 15 rooted cutting of each of the four species) were established in a greenhouse at the University of California's South Coast Research and Extension Center in Irvine, CA. One group of plants was irrigated with municipal water. The other groups were irrigated (by hand) with water collected daily from irrigation reservoirs at each of two nearby nurseries.

These trials ran a total of 6-10 months, during which time plants were observed for symptoms of root disease and soil samples (or irrigation leachates) were collected to test for the presence of fungal pathogens. At the termination of the trials, each plant was removed from the container. Root samples were cultured onto agar media and soil samples were placed in small plastic containers. The soil samples were flooded with distilled water, and hibiscus leaf disk baits were floated on the water surface to trap *Phytophthora* species. After 48 hr, the leaf disks were cultured onto agar media to detect fungi.

RESULTS AND DISCUSSION

A. Denitrification of Wastewater

A.1. Laboratory Experiments.

Newspaper: Newspapers are large component of the solid waste in landfills (3, 35) and a potential source of cellulose substrate. Fresh newspaper (dried at 105°C) contained: 25.49±0.15% extractives, 43.11±0.71% cellulose, 29.59±0.32% lignin, and 2.59±0.10% ash. The extractive fraction included protein, hemicellulose and other labile carbon fractions. The newspaper ink contains vegetable oil, mineral oil, resins and carbon black (lamp black) and probably accounts for a significant proportion of the extractable fraction.

Nitrate removal and water quality: The amount of N removed per day was as calculated as follows:

$$\text{N lost (mg day}^{-1}\text{)} = [\text{nitrate-N in} - (\text{nitrate-N out} + \text{nitrite-N out})] (\text{mg l}^{-1}) \times \text{Volume day}^{-1}$$

The performance of a column packed with 600 g of shredded newspaper and supplied with nitrate at a concentration of approximately 100 mg l^{-1} ($22.58 \text{ mg N l}^{-1}$) is shown in Fig. 4. The ambient temperature was not constant and the values presented are daily averages. Complete removal of nitrate without accumulation of nitrite was achieved after onset of flow (0.55 m day^{-1}). A temporary breakthrough of nitrate was observed after day 19; this may be due to a sharp drop in temperature ($6\text{-}10^{\circ}\text{C}$) during this period. Stable denitrification followed until the flow rate was raised to 0.77 m day^{-1} and later up to 0.95 m day^{-1} . These changes were accompanied by breakthrough of nitrate up to a maximum concentration of 54 mg l^{-1} ; nitrite also appeared although at much lower concentrations (the highest concentration measured was 8.4 mg l^{-1}). Upon decrease of flow rate to 0.4 m day^{-1} (day 148) nitrate and nitrite disappeared from the effluent. Ammonia was never detected. Washout of significant amounts of DOC was observed at the beginning of operation (up to day 12); the concentrations decreased rapidly and stabilized at $4\text{-}10 \text{ mg l}^{-1}$. The treated water never exhibited noticeable color or odor. The number of colony forming units was in the order of 10^5 ml^{-1} .

Effect of temperature: A column packed with 400 g of shredded newspaper was inoculated and left to stand for one week while the soluble fraction of DOC (the fraction eluted during the first week in Fig. 9b) was depleted. After flow initiation, the process was allowed to stabilize at 25°C for two weeks before the following temperatures were tested in sequence: 32 , 29 , 26 , 23 , 19 and 14°C , with two weeks per setting. As shown in Fig. 5, cellulose-dependent denitrification was markedly affected by temperature: denitrification rates at $25\text{-}32^{\circ}\text{C}$ were approximately three times higher than at 14°C .

Effect of flow rate: The effect of flow rate on denitrification was studied in a column packed with 400 g newspaper and kept at 25°C ; inlet nitrate concentration was 100 mg l^{-1} and the flow rate was varied between 0.5 and 1.5 m day^{-1} (Fig. 6). Complete removal of nitrate was achieved at flow rates up to 0.8 m day^{-1} . Breakthrough of nitrate occurred at higher flow rates, and a linear relationship ($R=0.96$) was observed between the degree of nitrate removal and q . At the highest flow rate tested, the concentration of nitrate decreased by 30% only (Fig. 6a). When the amount of nitrate removed per day was plotted (Fig. 6b) it became evident that the column operated below capacity at the lower fluxes, and that maximal efficiency was achieved at approximately 1.2 m day^{-1} . A very sharp decline was observed at higher flow rates, and this may be due to washout of bacteria or substrate.

Effect of ink: As considerable surface area of newspaper is covered by ink; its effect on colonization by bacteria was investigated with the aid of electron microscopy. In paper removed from active denitrification columns the printed areas appeared "flattened" and without attached bacteria. Thus ink apparently decreased the availability of free cellulose surfaces by occlusion and/or prevention of bacterial colonization. In order to assess the effect of ink in the performance of the denitrification system, the unprinted margins of newspapers were separated from the printed portions and two columns were packed with 400 g of printed or unprinted paper. The columns operated for three months with oversupply of nitrate. During

the first month of operation, the unprinted newspaper supported higher rates of denitrification (Fig. 7). This apparent advantage diminished with time and the cumulative N removal in the column packed with printed newspaper remained at about 75% of that in the column packed with unprinted newspaper. The decrease in denitrification rates observed in both columns on day 20 is, at least in part, due to the decline in average temperature; however, the original rates of denitrification were not recovered when temperatures were elevated again (see below).

Effect of newspaper pretreatment: The four small columns packed with untreated, autoclave-treated, acid-treated and base-treated newspaper were operated at identical flow rates (Fig. 8). Neither acid nor base pretreatment improved the denitrification capacity of the columns. On the contrary, a decline in the denitrification rate was observed after the first week of operation, with lower removal of nitrate and higher accumulation of nitrite. The high efficiency during the first week may be due to the available excess of soluble organic matter during this period (see Fig. 9b). The difference between control and autoclave-treated paper was not clear. A transient slight improvement of denitrification rates with lower nitrite accumulation may have occurred between days 13 and 27, but from then on both columns operated below capacity. To clarify this point, two fresh columns, with control or autoclave-treated paper, were operated at flow rates allowing breakthrough of nitrate; autoclave pretreatment did not improve nitrate removal (data not shown). Except for a decrease (approximately 5%) in the extractable fraction of the paper treated by alkali or acid, the pretreatments appeared to cause no change in the chemical composition of newspaper (data not shown). Print was faded and illegible suggesting that acid and alkali removed ink and this may explain the lower proportion of extractables.

Changes in newspaper during denitrification: Efficient nitrate removal in the newspaper-packed columns usually could be maintained up to 3-4 months. With time, however, an irreversible decrease in denitrifying capacity was observed. Addition of micronutrients or a fresh inoculum failed to improve the performance of "spent" columns. On the other hand, paper samples removed from these columns and transferred to batch experiments in an anaerobic chamber were capable of sustaining denitrification at rates 8-13 times higher than those measured in the columns at the time of sampling (an example is presented on Table 1). In an attempt to identify the cause(s) for this decline in denitrification capacity, changes in the composition, appearance, and weight of newspapers were investigated.

Table 1: Nitrogen removal in batch mode with fresh newspaper or with paper from a "spent" 10 month-old column, and removal of nitrogen by the column at the time of sampling (temperature, 30°C). Batches were maintained in an anaerobic chamber at 27°C; average daily rates during the second week are shown.

		Nitrogen removed (mg g ⁻¹ paper day ⁻¹)
Batch	Fresh paper + fresh inoculum	0.875
	Column paper	0.730
	Column paper + fresh inoculum	0.660
	Control (no paper, no inoculum)	0
Column		0.056

Weight loss: At the end of the operation, the packing paper was removed from the columns, dried and weighed. As expected, the extent of weight loss depended on the duration and regime of operation of the columns. During 6 months of continuous operation, a loss of 20% dry weight was observed; columns operated for 3-4 months showed lower weight losses (10-12%). Washout of paper fragments was never detected and the weight loss appeared to be due to microbial degradation.

Composition: On a dry weight basis, the microbial processes taking place in the columns should result in a decrease in the relative percentage of cellulose accompanied by an increase in the relative percentage of lignin. These changes, however, were apparently too small to be consistently detected by the methods employed: statistically significant differences were not always found between the chemical composition of fresh newspaper and that of newspaper removed from denitrification columns.

Appearance: The appearance of the paper was studied by scanning electron microscopy. Unless covered by ink, fresh newspaper had a loose appearance with thin flat structures at the surface. In contrast, the surface of ink-free spent newspaper appeared devoid of fine fibers and numerous bacteria were visible.

The contact times required for the complete removal of nitrogen (as $100 \text{ mg nitrate l}^{-1}$) were approximately 1.5 times (1.3-1.8) longer than those needed for the total removal of the same nitrogen concentration when the columns were packed with sand and supplied with a simple carbon source (sucrose, acetate or ethanol) at a C:N molecular ratio of 2 (26). The use of newspaper as sole physical matrix, in our view, contributes to the reliable performance of the system. Our previous denitrification studies, both in situ and in above ground sand-packed reactors, showed that entrapment in pore spaces of N_2 bubbles released during denitrification causes severe clogging (20, 26, 27). In a clogged reactor, water migrates at increased velocity along preferential canals and the efficiency of the system is impaired. In the experiments described free release of gas and no clogging was observed.

Wheat Straw: Because none of the pretreatments intended to improve the utilization of cellulose substrates as carbon source proved to be effective, no pretreatments were applied to the straw or the cotton.

A column packed with 103 g of wheat straw was inoculated and maintained in a recirculating mode for 2 days prior to start up (day 0) at q of 1.4 m d^{-1} . The column was fed continuously with medium containing $\text{Ca. } 22.6 \text{ mg nitrate-N l}^{-1}$ and operated for nine weeks (Fig. 9a). The flow rate was varied to achieve breakthrough of nitrate so that the capacity of the column to remove nitrate could be determined.

First effluent samples were collected after 24 h and by then most of the soluble and readily available fraction of DOC was eluted; thereafter, the concentration of DOC in the effluent was consistently below 10 mg l^{-1} (Fig. 9b). The highest rates of nitrogen removal were observed during the first days when q was increased to 2.06 m d^{-1} and little nitrate and nitrite washed out (Fig. 9a). By day 5 however, breakthrough of nitrate-N reached 6 mg l^{-1} and q was lowered to 1.65 m d^{-1} . From then on, decreasing rates of nitrogen removal were observed. Among possible reasons for the deterioration in the capacity of the column to remove nitrogen are quantitative and qualitative changes in the substrate. Indeed, by the end

of the experiment, the dry weight of straw was down to 60.8 g—over 40% of the original dry weight of the straw in the column was used during the experiment.

During the first week of operation of the column the effluent had a light yellow color which could be removed by passing the water through an activated carbon column. The number of colony forming bacteria in the effluent was in the order of 10^6 bacteria ml^{-1} . Ammonia seldom reached detection levels and its concentration never exceeded 0.3 mg l^{-1} .

The effect of q (flow rate) on the removal of nitrogen was studied in a column packed with 103 g straw and the feed water contained approximately $22.58 \text{ mg nitrate-N l}^{-1}$ (Fig. 10). The column was allowed to stabilize for 13 days at the lowest q that was still non-limiting the supply of nitrate. The flow rate was then further lowered for 5 days until complete removal of nitrogen was achieved prior to its gradual increase to 2.81 m d^{-1} (days 14 to 34).

At the beginning of this experiment breakthrough of nitrate and nitrite occurred at q 1.3 m d^{-1} with N concentrations in the effluent at Ca. 0.9 and 0.5 mg per liter respectively. Later, the concentrations of nitrate increased with the increase of q while those of nitrite leveled off at below 5 mg per liter (Figs 10 and 11). At the highest q tested (2.81 m d^{-1}), the effluent N-nitrate concentration was 12 mg per liter (Fig. 11).

The data presented in Fig. 10 were also used to calculate the correlation between flow rate and denitrification rate. Maximum rate of nitrogen removal was observed at q 1.29 when approximately a total of 134 mg of nitrogen were removed per column per day (Fig. 12). Decreasing amounts of nitrogen were removed as q was increased up to 2.21 m d^{-1} at which point the rate of nitrogen removal was just 78 mg N d^{-1} . Further increase of q up to 2.81 m d^{-1} had no marked effect on the total amount of nitrogen removed per day.

The data presented on Fig. 12 show that at q of 1.29 m d^{-1} removal of nitrogen was 60% of the maximum obtained. However, this decrease in efficiency was somewhat exaggerated by changes taking place in the substrate during the experiment. This became evident when on day 34 (Fig. 10) q was lowered to below 0.97 m d^{-1} and still breakthrough of nitrate and nitrite was observed, while on day 14, complete removal of nitrogen had been achieved at this flux (Fig. 10). By day 40 the dry weight of straw packed into the column was reduced by 42%. When the cumulative removal of nitrogen is calculated over the period of operation of the column (Fig. 13), three main linear denitrification rates can be drawn: 155 mg d^{-1} until day 7, 117 mg d^{-1} during days 7-26 and 82 mg d^{-1} between days 26-40.

The decline in denitrification rates observed at the high flow rates (Fig. 12) could be the outcome of several effects: wash out of bacteria that are not attached to the substrate, wash out of extracellular enzymes, wash out of solubilized substrate, or in a generalized form a discrepancy between the reaction rate(s) and the flow rate. To determine whether the marked decrease in nitrogen removal efficiency observed at q around 2.0 m d^{-1} (Fig. 12) was due to wash out of bacteria, a shift up experiment was carried in a column similar to that described in Fig. 11. In this experiment q was increased from 0.89 to 2.89 for 90 minutes, and then brought back to 0.89 for 210 minutes. During the first 90 minutes total bacteria were counted and nitrate and nitrite were determined every 5 minutes, and then again after the shift down of q at 210 minutes (Fig. 14a).

The total number of colony forming bacteria in the effluent increased 3-fold, to within $6 \times 10^6 \text{ ml}^{-1}$ during the first 5 minutes after shift up in the flow rate (Fig. 14a). The numbers

then dropped sharply, but gradually returned to the high level. 120 minutes after the shift down (90-210 min) the number of bacteria in the effluent declined almost to its original value. The volume of water in the column is 2.7 liters, and the flow rate at $q = 2.89$ is 10 ml per minute. Thus, after only 50 ml (1.85% of the void volume) passed through at the elevated q , the number of bacteria and concentration of nitrate in the effluent increased.

With such a small volume discharged, the bacteria probably came from the upper surface of the column, most likely the layer of fiberglass covering the straw. On it could settle non cellulolytic denitrifying bacteria (see below) that could be easily washed out at the increased flow rates. Later, there is a gradual increase in the number of bacteria washed out from the column itself. It is important to note that once the flow rate was dropped back to $q = 0.89$, the washout of bacteria also slowed. In Figure 14b, the fluctuations in the denitrification rates during the shift up and down are presented.

During the shift up of q , the nitrate concentration in the effluent was increased by a factor of 10, from Ca. 0.5 to 5.0, and that of nitrite by 100, from 0.01 to 1.06 mg/l. These values are much larger than could be expected if there was a simple stoichiometric relation between washout of bacteria and reduction in the rate of denitrification. It should be remembered that by the end of 90 minutes of shift down, only 1/3 of the void volume of the column was replaced at the high q . Therefore, the probable reason for the decline in denitrification rates with the increase in q is a result of the disturbance caused to the denitrifying biofilm that develops on the surface of the straw. Apparently, this biofilm is loosely attached to the straw, because it is at the same time both an attachment substratum for the bacteria and a carbon and energy growth substrate which is gradually utilized and therefore gradually disappears during the operation of the column.

A.2. Microbial Characterization

Microbial Biomass: After 6 months of operation, the protein concentration on the cotton fiber reached concentrations of up to 70 mg per gram cotton (Figure 15). At the time of the sampling, the cotton fibers were almost completely disintegrated, appearing slimy and gelatinous. The column was fed with fresh medium always from the bottom and it is obvious from the figure that the spatial distribution of bacteria along the column is not homogenous. This was also reflected in the different coloration of the center of the column, which was very dark.

The concentrations of protein on the cotton substrate were up to 10 times higher than those found in denitrifying columns prepared with newspaper as carbon source. The concentration of protein on the cotton increased with time: After 1 month: 1.5 mg/gram cotton, three months: 4.1-11.7, 4.5 months: 8.5-40.9 and after 6 months: 20-70 mg/gram.

Isolation and identification of bacteria: Two major groups of bacteria were identified among the 50 isolates obtained from the cotton column. One group formed yellow colonies on nutrient agar plates, and consisted of Gram positive, small rods. On minimal medium Oxidase +; Endoglucanase activity +; Cellobiase activity +; Ability to degrade completely crystalline cellulose (paper) +. These bacteria could use glucose, malate, acetate, carboxy ethyl cellulose, cellulose, filter paper, and cotton as carbon sources. They could not reduce nitrate beyond nitrite. These isolates (20 out of the 50 that were screened) were identified as *Cellulomonas uda*.

The second largest group of isolates were Gram negative, small to medium rods, forming brown-pink round smooth colonies. They were oxidase-, Endoglucanase-, Cellobiase-, and could not degrade crystalline cellulose. These bacteria could use malate, acetate, lactate, and β -hydroxy butyrate, as carbon sources, but not ethanol, methanol, glucose, cellobiose and CMC. This group of isolates could use both nitrate and nitrite as electron acceptors for respiration under anaerobic conditions using malate, acetate, lactate and as carbon sources.

This group of isolates was identified as *Comamonas* sp. Species is presently undetermined as it is apparently (according to the 16S R-RNA typing) a new species. Of 50 isolates screened, 8 belong to this group.

Eight other bacteria were present in the cotton column, among them *Kocuria rosea* (*Micrococcus roseus*), an unidentified *Enterobacter* sp., and an *Agromyces* sp. None of them had an ability to degrade cellulose and none of them was found to be a true denitrifier. We chose the 2 most abundant isolates, the *Cellulomonas uda* and the *Comamonas* sp. for further studies of the nutritional relationships between them, as already from the description of their nutritional requirements it seemed that the two species complemented each other in the cotton column.

As shown in Fig. 16, the *Cellulomonas* could carry the reduction of nitrate only to nitrite. Incubation in the presence of *Comamonas* alone left nitrate concentration unchanged, while in presence of both bacteria complete denitrification took place. This pattern of activity is also reflected in the pH of the incubation mixtures (Figure 17) and in the total organic carbon solubilized from cotton in the reaction mixture (Figure 18).

The pH rises only in the reaction vessel that contained both *Cellulomonas* and *Comamonas*, as a result of the elimination of both nitrate and nitrite. On the other hand, soluble organic carbon accumulated only with cotton in presence of *Cellulomonas uda*. From the data presented so far it is obvious that the species of bacteria that dominate the cellulose (cotton) column complement each other as the selection pressure forced them to cooperate in simultaneously carrying out cellulose degradation and denitrification. In all our selection and enrichment experiments we did not succeed in finding a single bacterium capable of both cellulose degradation and complete denitrification. The *Cellulomonas* degraded the cellulose and reduced nitrate to nitrite, and in addition excreted soluble organic carbon sufficient to feed a *Comamonas* sp. capable of complete denitrification. The problem with this arrangement is that while the *Cellulomonas* adheres to the matrix of cotton or straw, to bring into contact the cellulolytic enzymes with their substrate, the *Comamonas* can be easily washed out, as was described in the previous section.

To verify the observations above, that suggest symbiotic relationship between the bacteria found in the cotton column, an experiment was designed to test the ability of *Comamonas* to grow on spent medium of a culture *Cellulomonas uda*. The *Cellulomonas* was grown in 150 ml minimal medium (see methods) in 250 ml flasks without rotation at 27°C under anaerobic conditions and supplemented with medicinal cotton (0.25 g) as carbon source. The cultures were maintained for 2 months with regular replacements of spent medium by fresh minimal medium. The spent medium was stored frozen until enough was collected for the experiment. Then, all the spent aliquots were combined, centrifuged (15 min. 10,000 rpm) and filter-sterilized (Millipore NA 0.22 μ m).

The sterilized medium was divided into 4 batches that were inoculated with *Comamonas* cultures until all N (NO_3 and NO_2) was consumed. The results of this experiment (Fig. 19) clearly correlate bacterial growth (as increase in turbidity), denitrification and consumption of the soluble organic carbon released by the *Cellulomonas uda* grown on cotton.

We tried to identify the composition organic carbon that was released by the *Cellulomonas* by gas chromatography. Samples of medium from experiments were chromatographed against blanks and appropriate standards in a Carboxen column (SUPELCO 1-1889). Ethanol, acetate and another unidentified molecule were the most abundant species discharged by the *Cellulomonas*. The *Comamonas* utilized all available organic carbon except for the ethanol.

A.3. Field experiments:

Denitrification of irrigation wastewater in the field experiment was measured as the nitrate concentration in outflow water relative to inflow water (expressed as a fraction). The data (Fig. 20) appear quite erratic. However, there are a number of explanations for this effect. One important consideration is that there is no direct relationship between inflow and outflow nitrate concentrations at time = t . While we measured flow rate through the reactor in liters min^{-1} , we did not know the void volume of the reactor (total volume of the ditch minus the volume of baffle and straw solids). Hence, it was not possible to estimate the transit (or residence) time of any unit volume of water, so we could not predict a sampling interval that properly related inflow and outflow volumes.

Other complicating factors included sporadic operation of the reactor. There was no regular and constant inflow of water. Inflow consisted of excess water that drained from the irrigated fields, across the ground, and the reactor during each irrigation cycle. Irrigation schedules and volumes varied and, at the end of an irrigation cycle, water remained static in the reactor until the next irrigation cycle. Additionally, the nitrate levels in inflow water varied depending upon fertilization schedules. Laboratory experiments showed that denitrification was optimal when reactor temperature, flow rate and nitrate influx were optimized and consistent. Such conditions could not be provided in the field experiments.

In spite of these complications, the ditch did function, and removed nitrate from the irrigation runoff water to varying degrees. In 1995, in the six weeks following start-up, the facility removed about 30% of the nitrates from the water (Fig. 20a). Operation of the reactor was terminated with the onset of cold weather in November, but was re-started the following spring. When re-started, fresh straw was added to replenish the reactor. However, nitrate removal in 1996 was much less effective (Fig. 20b) than in the first weeks of operation (Fig. 20a). This may have been the result of a loss of reactor efficiency across time, similar to that described in Table 1. Note that in some cases (Fig 20a, 20b), the ratio of nitrate outflow:inflow exceeds 1.0. Since nitrate was not synthesized in the reactor, this is considered an artifact of the disconnection between outflow and inflow samples at given sampling times.

The data presented here were obtained through measurements using an Orion nitrate electrode. We also conducted measurements at times using a Hach field kit. The two pieces of equipment never gave the same answer, but in direct comparisons (Fig. 21), showed similar relative differences. Our measurements of Nitrate and Ammonia also varied considerably (Fig. 22), but generally showed lower levels in outflow water than in inflow water.

B. Microbial Disinfection of Wastewater

B.1. Laboratory Experiments.

U.V. radiation: The fixed-geometry experiments allowed quantitative comparisons between the Hg-vapor, the pulsed laser and pulsed xenon sources. In distilled water, 95-100% mortality was achieved with 40-50 mJoules of UV energy from the Hg lamp (Fig. 23). Accomplishing the same effect with the laser, on the other hand, required 150-200 mJoules energy (Fig. 23). The difference between the two devices is most likely due to differences in their emission spectra. The low-pressure Hg-vapor lamp emits photons with a range of energies, and a peak at 254 nm. This wavelength coincides with the maximum absorption of DNA molecules. The KrF laser, on the other hand, has a much narrower (nearly monochromatic) emission spectrum, which peaks at 248 nm. Because this wavelength does not correspond with the peak absorption for DNA, higher energies may be required for the same effect.

While the data presented in Figure 23 suggest that the Hg-vapor lamp is a superior source of UV energy, it is not. In these fixed-geometry experiments, the Hg lamp took 10-20 seconds to deliver the required 40-50 mJoules of energy. While the Hg lamp operates as a "continuous-emission" source of energy, the excimer laser is a "pulse power" source. The excimer source emits photons in 20 nanosec pulses. Because each pulse delivers 40-60 mJoules of UV energy, the excimer source delivered lethal doses in 3-4 pulses. The laser used in these experiments had a 5 Hz (5 pulses/sec) repetition rate, so it was capable of delivering a lethal dose in less than one second. More modern lasers have peak repetition rates that are 10-100 times greater than the laser used in our experiments. Hence, even though the photons emitted from 248 nm excimer sources are not optimally absorbed by DNA, these sources have far greater throughput capacity.

The differences in treatment times between the UV sources are even more pronounced in comparisons of spore kill in irrigation wastewater (Figs. 24-25). We used raw irrigation wastewater in these experiments, without sterilization. All data were recorded as the total number of viable propagules (all fungi) in treated samples relative to untreated water. Irrigation wastewater contains soluble organic materials that impart a yellow-brown pigment to the water. These contaminants are highly absorptive in the UV band and, therefore, severely degrade the efficacy of UV treatments. This effect is clearly illustrated by comparing Figures 23 and 24, where almost 10 times the amount of UV energy was required to kill the fungal spores suspended in irrigation water (Fig. 24) as compared to *Fusarium* spores in distilled water (Fig. 23). Approximately 1 Joule was required to kill 95-97% of the fungal spores suspended in irrigation water. While a modern excimer source can deliver this much energy in less than 1 sec, the Hg lamp did not deliver an effectively lethal dose after a total of 50 sec (Fig. 25).

Use of the flow-through treatment cell (Fig. 2) allowed us to conduct experiments with the UV laser that simulated anticipated commercial use. Treatment efficacy in these experiments was determined by measuring the total number of viable propagules in the outflow (treated) water relative to the inflow (untreated) water. When spores of *Phytophthora parasitica* or *Fusarium oxysporum* were suspended in distilled water, and pumped through the treatment cell at different velocities (to provide different exposure times), propagule mortality results (Figs. 26, 27) resembled those in the fixed geometry experiments (Fig. 23). We noted that *Fusarium* (Fig. 27) appeared somewhat more tolerant of UV radiation than *Phytophthora* (Fig. 26).

The adverse effect of water impurities on treatment efficacy were revealed again in experiments using irrigation wastewater in the flowcell apparatus. For these experiments, large volumes of irrigation wastewater (200 liters for each replicate experiment) were collected from a commercial nursery and sterilized by filtration through a 0.22 μm Milipore filter. Spores of *Fusarium* then were added to the filtered water to provide a known inoculum level. This water then was pumped through the treatment cell at different velocities to provide different exposure doses. We found that approximately 150 times more UV energy was required to kill *Fusarium* spores suspended in irrigation wastewater (Fig. 28) than in distilled water (Fig. 27).

In these experiments, we measured the amount of UV energy entering the flowcell by placing a joulemeter at the quartz window face (see Fig. 2). We also measured the absorbance of the water by means of a spectrophotometer. This allowed calculation of the UV "quenching" effect of impurities in the water. Thus, in the flowcell model, when a pulse of UV photons entered, those propagules immediately adjacent to the quartz window were exposed to energy levels nearly equal to the joulemeter measurements. However, because energy attenuated sharply with depth, that propagules more distant from the quartz window, received substantially less energy. We used the UV quenching value of the water, the flow rate through the cell, the laser energy output, and the laser repetition rate in formulas to calculate the actual UV energy "seen" by propagules as they passed through the cell. This data is summarized in Fig. 29a, where we plot survival as a function of "energy applied" (dark squares) and as a function of "energy absorbed" (gray diamonds). Expanding the scale to better view low energy levels (0-500 mJoules, Fig. 29b) shows that the amount of energy required to effect kill of propagules (appx. 250 mJoules) is similar to other experiments using distilled water (Figs. 23, 27). But, because so much energy is absorbed by the contaminants in wastewater, a very large amount of energy must be put into the system (Fig. 29a) to deliver a lethal dose to the propagules (Fig. 29b). In our experiments, only the laser was capable of delivering sufficient energy in the short time intervals needed to treat flowing water.

Most of our experiments utilized a Lamda-Physik 150 EMG laser with a maximum repetition rate of 5 Hz. Toward the end of this project, we gained access to a Lamda-Physik "Compex" laser, capable of a 200 Hz repetition rate. The newer laser provided more uniform energy levels per pulse than the older model. In flowcell experiments, this yielded a more consistent UV exposure, and resulted in greater propagule mortality at equivalent energy input levels (Fig. 30).

The xenon flashlamp used in our experiments emitted a broad band of electromagnetic radiation--from UV to far IR. Because they emit a broad spectrum of electromagnetic radiation Xenon lamps are inherently less efficient than Hg-vapor lamps or excimer lasers in converting electrical power into UV photons. However, this is an extremely powerful light source (500 mJoules cm^{-2} per pulse), so that the relatively small amount of UV radiation still is significant.

In some experiments, we isolated just the UV band by installing a filter assembly that blocked all wavelengths except a narrow band around 248 nm. The filtered flashlamp yielded a mortality curve (Fig. 31) which differed from that of the 248 nm excimer laser (Fig. 23). This may be due to the different geometry that had to be employed in these experiments, or differences in the kinetics of the pulses (photons from the laser were delivered in pulses of 20 nanosecond duration, while photons from the xenon source were delivered in pulses of 500 millisecond duration). When we tested the xenon flashlamp in an unfiltered state, it proved to be quite efficacious. Virtually 100% of the spores suspended in distilled water were killed with a single pulse from this lamp (Fig. 32). When spores were suspended in recycled irrigation

water, three pulses effected nearly complete kill (Fig. 32). This lamp has a pulse repetition rate of 5 Hz.

Chlorination and Ozonation: Our experiments with chlorine showed that virtually all *Fusarium* spores suspended in distilled water were killed following 30 min. exposure to 0.25 ppm NaOCl, or 10 min exposure to 0.85 ppm NaOCl (Fig. 33). When spores were suspended in nursery effluent water, however, treatment efficacy was reduced. Exposing spores to 0.25 ppm solutions for 30 min. had no effect on spore survival (Fig. 18). Spores still were killed after 10 min. in 1.2 ppm solutions, but more dilute solutions required longer exposure intervals (Fig. 34).

The efficacy of ozone also was reduced by materials in nursery effluents. When spores of *Fusarium* were suspended in distilled water, bubbling O₃ into the suspension caused 100% mortality in less than 5 minutes--the shortest practical treatment interval (Fig. 35). However, achieving 100% mortality of spores suspended in nursery effluent water, required almost 20 min of Ozonation (Fig. 35). This time interval varied between effluent samples, and appeared to be related to the time required to achieve an O₃ concentration of 0.4 ppm. In distilled water, that concentration was achieved in 1-2 minutes, but in effluent samples, O₃ accumulated much more slowly (Fig. 36). This is because ozone reacts with, and is rapidly depleted by, organic materials in the water. In nursery effluents, it again appears to be the soluble organics that are critical. Comparing O₃ accumulation in raw effluent, and the same effluent filtered through 0.22 µm filters to remove all solid contaminants, showed that simple filtration had no effect on O₃ accumulation (Fig. 37). Diluting the effluent 1:1 with distilled water, however, did increase O₃ accumulation significantly (Fig. 37). We accomplished a similar benefit by passing effluent samples through columns of activated charcoal to remove impurities (data not shown). Ozone accumulation in effluents appeared to be affected by the same materials that affected UV penetration. Prolonged ozonation visibly clarified effluent samples and increased their transmissibility in the UV wavelength.

While these results were informative, they probably overestimate the times or O₃ concentrations needed to effect spore kill. This is because as O₃ was bubbled into the spore suspensions, O₃ concentration increased gradually over time (Fig. 36). Thus, the times required for spore mortality (Figs. 35, 37) reflect the cumulative exposure low, moderate and high O₃ concentrations. This difficulty was overcome by use of our flow-through treatment device, wherein O₃ concentration in the reactor vessel could be held in steady-state conditions by adjusting ozone input and solution "turnover" time (average residence time) in the vessel. Using this apparatus, and holding O₃ concentration constant at 0.22 ppm, we achieved 90% mortality of propagules in 10 min (Fig. 38).

Heat Shock: Our experiments with heat shock were intended to explore the possibility of disinfecting water by heating to temperatures of 40-50°C. Heat has been used to disinfect water in some European countries, but temperatures of 90°C were employed. The energy costs of such treatments are very high, so we have sought to develop data utilizing lower temperatures. We have used water baths to expose a variety of fungal pathogens to elevated temperatures and to develop mortality curves. Fungal propagules differ in their sensitivity to heat. For example, zoospore cysts of *Phytophthora parasitica* were not killed as rapidly at 40°C (Fig. 39) as were spores of *Cylindrocladium* sp. (Fig. 40). However, propagules of both fungal species were quickly killed upon exposure to temperatures of 45 °C (Figs. 39, 40). This

suggests that heating of recycled water (by heat-exchanger, solar power, or a combination process) could be very effective.

B.2. Field Experiments.

In field experiments involving the UV water purifier, the unit was plumbed into the nursery irrigation pumping system. Flow rate through the unit was adjusted to yield specific exposure (transit) times. Water samples were collected at inflow and outflow points and plated onto agar media to count the numbers of viable propagules. Experiments were done at several times and at several nursery locations. In one representative experiment done early in the growing season, when water quality is good and fungal loads are moderate (1.5×10^5), 60-70 seconds was required for 95% reduction in viable propagules (Fig. 41). Later in the year, when recirculated water quality was degraded and fungal loads were higher (3.2×10^5), exposure times of nearly 90 seconds only yielded a 60% reduction in viable propagules (Fig. 42). In fact, at the slow flow rates required to provide such long exposure times, the mortality curve appears to flatten out (Fig. 42), leaving a high "residual" level of inoculum. This is probably due to the absence of water turbulence within the purification unit chamber. At very low velocities, laminar flow may predominate within the chamber, allowing some propagules to move through without ever passing near the UV lamp.

The ozonation unit used in our field experiments was a small demonstration unit owned by a company that manufactures water treatment equipment. The general design of the unit is illustrated in Fig. 3. We established constant flow rates through the treatment tank so that the average contact time (volumetric turnover time) was 12 min. With this factor constant, O_3 concentration was adjusted by metering the flow of O_2 through the ozone generator. The results from our field trials were very similar to the laboratory experiments: O_3 concentrations of 0.25-0.3 ppm caused nearly 100% mortality of fungal spores (Fig. 43).

B.3. Disease Risk Assessment: In these experiments, prolonged irrigation of plants with recirculated water led to significant root infection by Pythiaceous fungi (Fig. 44a-d). Additionally, baiting from soils indicated that a significant number of containers had become contaminated with Pythiaceous fungi. Differences were noted between plant species and nursery locations. This probably is due to differences in the species of fungi in the irrigation effluents. Previous research (not reported here) showed that effluents from the two southern California nurseries and the northern California nursery Nursery differed in populations of *Phytophthora* and *Pythium* species.

These experiments have provided the first clear evidence that use of recycled irrigation water presents a significant disease risk. It is important to note that throughout these experiments, we propagated our own plant materials in quarantine facilities to assure initial freedom from root infection, and to maintain the plants in a way that assures root infections can only develop as a result of contaminated irrigation water.

DESCRIPTION OF COOPERATION

Because of the distance between the research locations, Drs. MacDonald and Abeliovich have stayed in contact primarily by means of e-mail, and keep each other up to date on progress. Investigator Kabashima surveyed several commercial nurseries in California to obtain data relating to nitrogen concentrations of wastewater. This information was

summarized and forwarded to Investigator Abeliovich to allow denitrification experiments to be done with a view of the real problem. Investigator MacDonald was responsible for all water disinfection experiments, and Investigator Lagunas-Solar collaborated in the design and execution of all experiments involving laser treatments. To supervise the construction and operation of a field denitrification reactor, Investigator Abeliovich spent a week in California with Investigators MacDonald and Kabashima. MacDonald and Kabashima then assumed operational responsibility and provided data to Abeliovich via e-mail.

EVALUATION OF THE RESEARCH ACHIEVEMENT (with respect to the original research proposal and its objectives)

We believe that this project led to significant findings (see below) that have met the original objectives, and provide a core of information needed for commercial development.

CONCLUSIONS

Our research have shown that:

- ◆ Cellulose substrates (newspaper, straw and cotton) can effectively support denitrification of nitrate-contaminated water. The treated water is characterized by low DOC and no detectable odor and color.
- ◆ Pretreatment of substrates did not significantly improve their ability to support denitrification.
- ◆ Efficient nitrate removal in newspaper-packed columns usually could be maintained up to 3-4 months. With time, however, an irreversible decrease in denitrifying capacity was observed. Addition of micronutrients or a fresh inoculum failed to improve the performance of "spent" columns.
- ◆ *Cellulomonas* degraded cellulose and reduced nitrate to nitrite. In addition, it excreted soluble organic carbon sufficient to feed a *Comamonas* sp., which allowed complete denitrification.
- ◆ Irrigation wastewater in nurseries does contain significant numbers of plant pathogens. Healthy plants can become infected when irrigated with recycled wastewater.
- ◆ Hg-vapor lamps do not possess sufficient energy to disinfect nursery wastewater because of interfering dissolved organic matter. However, excimer lasers and Xenon flashlamps do possess the needed power levels. Of these, only the laser has a high enough repetition rate to reliably treat large volumes of water.
- ◆ Of the potential oxidizing agents (chlorine and ozone), O_3 poses the least risk of phytotoxicity (due to Cl-salt accumulation) and is highly efficacious in low-volume irrigation systems.
- ◆ In certain locations, heating of recycled water by means of a heat-exchanger, solar power, or a combination process, could be an effective means of treating smaller water volumes.

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Fig. 1. Schematic of the denitrification reactor used in field experiments in southern California. The reactor consisted of an excavated pit, 1 meter deep X 1 meter wide X 5 meters long. Brick baffles were installed and the entire pit was filled with straw. The pit was covered with sheets of plywood sheathed on the inner surface with black plastic film.

Fig. 2. Schematic of the flow-cell used in laser experiments. Fungal spores are added to water (distilled or nursery effluent) to provide known inoculum concentrations. Inoculum is pumped through the cell at controlled flow rates to vary spore exposure to UV radiation. Beam intensity and pulse rate are also controlled variables. In addition, aliquots of each water sample were placed in a spectrophotometer to measure absorbance at 254 nm, and to provide a means of calculating the depth of UV penetration into the cell. With distilled water, there was less than 50% attenuation across the full length of the flow cell. With effluent water, the beam only effectively penetrated 2-4 cm into the cell (depending upon water clarity). Samples of infested water were collected from the cell inlet and exit for culturing on agar media to quantify spore survival following treatment.

Fig. 3. Schematic of typical ozonation system. Raw water is pumped into the exposure tank at flow rate "x". Water is pumped out of the tank at an equivalent flow rate. The specific flow rate and volume of the holding tank determine the average exposure time (i.e., if flow rate is 1 liter/min and tank capacity is 15 liters, average "turnover" or exposure time is 15 min). Most commercial units extract oxygen from the air using a molecular sieve pump. The oxygen is passed through a corona discharge device to generate ozone. The ozone is pumped into the holding tank by means of an auxiliary pump with an aspirator-type injector that produces fine bubbles which maximize ozone dissolution in the water. The holding tank operates at atmospheric pressure, with excess ozone scrubbed via charcoal filter and vented to the atmosphere.

Figure 4: Concentrations of nitrate, nitrite, DOC and pH of water treated in a column packed with 600 grams of shredded newspaper and operated at room temperature (daily averages are shown).

Figure 5: Effect of temperature on the removal of nitrate in a column packed with 400 grams of shredded newspaper.

Figure 6: Effect of flow rate on the removal of N in a column packed with 400 g shredded newspaper and kept at 25°C. a, percentage of N lost; b, N lost per day.

Figure 7: Cumulative N removal in 3 columns packed with 400 grams of printed or unprinted newspaper.

Figure 8: Removal of nitrate and accumulation of nitrite in small columns packed with 154 grams of newspapers pretreated as described in Table 1 and kept at 250C.

Figure 9a: Concentration of nitrate-N in the influent, and pH and concentrations of nitrate-N, nitrite-N and DOC in the effluent of a column packed with 103 g of wheat straw and operated at the temperatures and flow rates indicated.

Figure 9b: Dissolved organic carbon and pH in the effluent of the denitrifying straw column. For details see fig 1a.

Figure 10: Effect of flow rate on the denitrification in the column. The column was packed with 103 g of wheat straw and operated at various flow rates and at temperature of 24°C.

Figure 11: The effect of flow rate on the removal of nitrogen in the column described on Fig. 2.

Figure 12: Nitrogen removed per day as a function of q .
Experimental data as in Fig 2.

Figure 13: Cumulative removal of nitrogen in the column described in Fig. 2.

Figure 14a: Breakthrough of bacteria upon shift up of q from 0.89 to 2.89 m d⁻¹ in day 37 of operation of a column similar to that described in Fig. 2. After 90 minutes q was lowered back to 0.89, and the 210 minute point represents the washout of bacteria at the reduced q . (a) and nitrate and nitrite (b).

Figure 14b: Breakthrough of nitrate and nitrite upon shift up of q from 0.89 to 2.89 m d⁻¹ in day 37 of operation of a column similar to that described in Fig. 2. After 90 minutes q was lowered back to 0.89, and the 210 minute point represents the concentrations of nitrate and nitrite at the reduced q .

Figure 15: Distribution of microbial biomass (presented as total protein) in a denitrifying cotton column. The column was fed with medium from the bottom. For details see methods.

Figure 16: Denitrification by *Cellulomonas uda* and *Comamonas* sp. in the presence of cotton as carbon source. Cultures were incubated anaerobically at 27°C in 250 ml flasks, in 120 ml minimal medium (see above) with 0.25 gram medicinal cotton as carbon source added 24 hours after inoculation.

Figure 17: pH values in the reaction mixtures of the experiment described in Fig. 10.

Figure 18: Dissolved organic carbon in reaction mixtures with cotton as carbon source. For details see Figure 7.

Figure 19: Growth of Comamonas sp. On spent medium Cellulomonas uda culture grown on cotton as sole carbon source. See text for experimental details.

Fig. 20 a&b. Relative nitrate levels (outflow concentration/inflow concentration) in irrigation wastewater after passage through the reactor illustrated in Fig. 1. A ratio of 1.0 indicates no difference between inflow and outflow samples.

Fig. 21. Comparison of nitrate readings using Hach and Orion instruments

Fig. 22. Relative concentration of Nitrate, Nitrite and Ammonia in inflow and outflow samples at various times after irrigation wastewater was directed into the reactor illustrated in Fig. 1.

Fig. 23. Exposure of *Fusarium* sp. in a 1-cm-wide quartz cuvette filled with distilled water. Energies were measured at the cuvette face nearest the light source. The laser delivered 150-200 mJoules of energy in 3-4 20 nsec pulses. The UV lamp delivered 50 mJoules of energy in 10-20 sec of continuous operation (measured at 10 cm from the light source).

Fig. 24. Survival of "Total fungi" in a sample of nursery irrigation water placed in a 1-cm-wide cuvette and exposed to UV from a pulsed power, 248 nm excimer source. The Avg. energy was 50 mJoules/ pulse. 20-30 pulses were required for the 1.0-1.5 Joules of energy needed to obtain 95-97% kill.

Fig. 25. Survival of "Total fungi" in a sample of nursery irrigation water placed in a 1-cm-wide cuvette and exposed to UV from a continuous-emission Hg-vapor source. Exposure times are indicated on the X-axis.

Fig. 26. Survival of *P. parasitica* zoospores in distilled water following exposure to a UV-emitting (248 nm) laser. Spores were exposed to the laser beam in a flow-cell apparatus (see Fig. 4). UV dose is the measured beam energy multiplied by duration of exposure (transit time through the treatment cell). Each point is the mean of four replicate treatments.

Fig. 27. Survival of *F. oxysporum* spores in distilled water following exposure to a UV-emitting (248 nm) laser. Spores were exposed to the laser beam in a flow-cell apparatus (see Fig. 4). UV dose is the measured beam energy multiplied by duration of exposure (transit time through the treatment cell). Each point is the mean of four replicate treatments.

Fig. 28. Survival of *F. oxysporum* spores in nursery effluent following exposure to a UV-emitting (248 nm) laser. Spores were exposed to the laser beam in a flow-cell apparatus (see Fig. 4). UV dose was estimated from the initial beam energy, the laser repetition rate, water flow rate, and depth of UV penetration to the 99% attenuation point (2-4 cm). Each data point is the the mean of four replicate treatments.

Fig. 29 a&b. Propagule survival in irrigation wastewater following exposure to various levels of UV radiation emitted by an excimer laser. Propagules were suspended in water and treated using the apparatus illustrated in Fig. 2. Black squares in Fig. 28A indicate the measured energy entering the flowcell. The gray diamonds indicate the calculated energy striking propagules. Fig. 28B shows those same data points, on an expanded X-axis scale for better resolution.

Fig. 30. Propagule survival in irrigation wastewater following exposure to various levels of UV radiation emitted by two different excimer lasers. Propagules were suspended in water and treated using the apparatus illustrated in Fig. 2. Black squares indicate propagules exposed to an older Lamda-Physik 150 EMG laser. Gray diamonds indicate propagules exposed to a newer Lamda-Physik "Compex" laser.

Fig. 31. Exposure of *Fusarium* sp. to UV radiation from a xenon source. Spores were placed in an open petri dish filled to a depth of 1 cm with distilled water. A filter was placed in the light path to eliminate all but a narrow band of the UV spectrum (around 248 nm). UV energies were measured beneath the dish.

Fig. 32. Exposure of *Fusarium* sp. to UV radiation from a xenon source. Spores were placed in an open petri dish filled to a depth of 1 cm with distilled water. Spores were exposed to unfiltered xenon spectrum. Total light energy was measured beneath the dish, although only a small fraction of it was in the UV band. Here, emissions are recorded as cumulative light pulses.

Fig. 33. Survival of *F. oxysporum* spores in distilled water to which differing levels of NaOCl were added. Spore suspensions were gently stirred throughout the experiment and samples were taken at intervals to measure cumulative residual Cl concentration and the numbers of surviving spores. Each data point is the mean of four replicate treatments.

Fig. 34. Survival of *F. oxysporum* spores in nursery effluent water to which differing levels of NaOCl were added. Spore suspensions were gently stirred throughout the experiment and samples were taken at intervals to measure cumulative residual Cl concentration and the numbers of surviving spores. Each data point is the mean of four replicate treatments.

Fig. 35. Survival of *F. oxysporum* spores in distilled water (dashed line) and nursery effluent water (solid line) during exposure to ozone. Spore suspensions were continuously ozonated and samples taken at intervals to measure cumulative ozone concentration and spore survival. Each data point is the mean of four replicate treatments.

Fig. 36. Ozone accumulation in nursery effluent water over time. Water was continuously ozonated and samples were taken at intervals to measure ozone concentration. Samples also were collected and placed in a spectrophotometer to measure percent transmission at 254 nm. Each data point is the mean of four replicate treatments.

Fig. 37. Survival of *F. oxysporum* spores in nursery effluent water (solid line), filtered effluent water (shaded squares) and effluent water diluted 1:1 with distilled water (dashed line). Water samples were continuously ozonated and samples were taken at intervals to measure ozone concentration and spore survival. Each data point is the mean of four replicate treatments.

Fig. 38. Survival of *Fusarium* spores in irrigation wastewater treated with ozone in a flow-through treatment system. Ozone concentration was held constant at 0.22 ppm, and flow rate was adjusted to yield different exposure intervals.

Fig. 39. Survival of *Phytophthora* spores in distilled water heated to various temperatures. Spores were added into preheated water and aliquots removed at timed intervals to assess survival. Each data point is the mean of four replicate treatments.

Fig. 40. Survival of *Cylindrocladium* spores in distilled water heated to 40 or 45 C. Spores were added into preheated water and aliquots removed at timed intervals to assess survival. Each data point is the mean of four replicate treatments.

Field data

Fig. 41. Disinfection of recirculating nursery irrigation water with a Hg-vapor UV lamp. Water was pumped through the disinfection unit at velocities that provided a range of exposure (average transit) times. This test was done in spring, and assays showed 154,000 viable propagules /liter entering the unit. The graph depicts propagule survival following passage through the unit.

Fig. 42. Disinfection of recirculating nursery irrigation water with a Hg-vapor UV lamp. Water was pumped through the disinfection unit at velocities that provided a range of exposure (average transit) times. This test was done in summer, and assays showed 316,000 viable propagules /liter entering the unit. The graph depicts propagule survival following passage through the unit.

Fig. 43. Survival of fungal spores in recirculated irrigation water treated with ozone. Experiment was done at a commercial nursery using a small demonstration device. Propagule mortality at all concentrations is based on average 12 min. exposures. Counts are of "total fung", and are shown relative to a base population of 700,000 propagules/liter.

Fig. 44 (A-D). Percent of root segments infected, and percent of bait tissues colonized by Pythiaceous fungi (*Pythium* and *Phytophthora* spp.) in plants irrigated over a 6-10 month period with recycled irrigation water. Water was collected from two nurseries in southern California and used to hand-water plants in a greenhouse. In the northern California nursery, plants were watered daily by means of an automated drip irrigation system. Check plants were maintained in a northern California greenhouse and a southern California greenhouse, and were watered with tap water. While splashing was kept to a minimum, some cross-contamination of check plants did occur.