FINAL REPORT
U.S. Department of Energy

BIOFILTRATION OF VOLATILE POLLUTANTS: Fundamental Mechanisms for Improved Design, Long-term Operation, Prediction, and Implementation

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Institution: **Oak Ridge National Laboratory**

Collaborators: John W. Barton, K. Thomas Klasson, ORNL

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Grant Project Officer: **Robert E. Price**
Project Duration: October 1, 1996 to August 31, 2000

2.0 Executive Summary

3.0 Executive Summary

This project's goal was to produce industrial- and EM-relevant scientific information for successful biofiltration operation, which was accomplished in the following three ways:

1. Enhancement of the biofiltration process was achieved through measurement and understanding of the underlying driving forces—measurement of kinetics, mass transfer, and solubility enhancements.
2. Fundamental methods were developed to make long-term operation of biofilter units possible via innovative applications of nutrient limitation and filter regeneration,
3. Biological and transport fundamentals were incorporated into a 2-dimensional mathematical model in order to successfully predict operating parameters and thus enable advanced design a priori.

Advances in these areas are particularly important for implementation and extended activity of biofilters in industrial settings and are directly relevant for high priority EM contaminants, including alkanes, aromatics, and chloro-organics. These tasks are part of the Remediation by Natural Processes focus area, which addresses the broad class of volatile organic contaminants with applications to hydrocarbons, chlorinated hydrocarbons such as TCE, and nitrates.

There are three key pieces of information generated by this project that can be used immediately to significantly enhance biological filtration processes. These include (1) methods for decoupling growth and degradation processes using nutrient limitation, (2) recognition of enhanced solubility effects due to the presence of biomass and how these effects could be quantified, and (3) access to easy-to-use software for enhanced design purposes. We have received interest from both academic and industrial sectors in this work, but have not linked directly with a DOE end user for site clean up or process enhancement.

Academic communities use the information primarily as published information available in peer-reviewed journal articles. We have received numerous requests for both electronic and hard copies of our papers, and have seen other researchers initiate studies that will further explore some of the work we have completed. Researchers at other national laboratories have expressed interest in both the published information and the Biofilter software. Various potential users have requested copies of the software for potential use on systems that they already have and want to enhance. In terms of application to a DOE focus area, we hope to locate end users (during the renewal phase of the project) who are willing to support pilot or larger scale implementation of the information gathered.

This project has been selected for Renewal, beginning in FY2001, under the title, *Biofiltration of Volatile Pollutants: Fundamental Measurements and Advanced Mathematical Modeling of Solubility Effects of Biological Material in Contaminant Zones*.

Gregory Clark Delozier received his Masters of Science in Biotechnology from University of Tennessee (December, 1998); his thesis work involved DNA-typing and isolation of the bacterial species responsible for degradation of VOCs in the columns used in the experiments on this project. His thesis may be found as Appendix A at the end of this document.

4.0 Research Objectives

Biofiltration systems can be used for treatment of volatile organic compounds (VOCs); however, the systems are poorly understood and are normally operated as "black boxes". Common operational problems associated with biofilters include fouling, deactivation, and overgrowth, all of which make them ineffective for continuous, long-term use. The objective of this investigation was to develop generic methods for long-term stable operation, in particular by using selective limitation of supplemental nutrients while maintaining high activity. As part of this effort, we have provided a deeper fundamental understanding of the important biological and transport mechanisms in biodestruction of sparingly soluble VOCs and have extended this approach and mathematical models to additional systems of high priority EM relevance - direct degradation and cometabolic degradation of priority pollutants such as BTEX and chlorinated organics.

Innovative aspects of this project included development of a user-friendly two-dimensional predictive model/program for MS Windows 95/98/2000 to elucidate mass transfer and kinetic limitations in these systems, isolation of a unique microorganism capable of using sparingly soluble organic and chloroorganic VOCs as its sole carbon and energy source, and making long-term growth possible by successfully decoupling growth and degradation metabolisms in operating trickle bed bioreactors.

5.0 Methods and Results

Experimental Methods

Microbial Consortium. The initial consortium was taken from a methanotrophic mixed culture used for cometabolic TCE studies. Several bacterial species were identified via Biolog™ and BBL Enterotube™ (Becton Dickinson Inc., Bedford, MA) assays in the mixed culture. These included *Citrobacter freundi, Pseudomonas putida, Alcaligenes paradoxus, Xanthomonas maltophilia*, and *Flavobacter indologenes*. Unidentified yeast species were also present. The culture had been fed n-pentane and isobutane and is self-maintained by using these VOCs as sole carbon sources (Davison and Thompson, 1993, 1994). The consortium no longer displays methanotrophic activity. The species responsible for pentane and butane uptake was isolated and identified as a part of this project as well.

Mineral Media. A well-defined mineral medium [NATE medium (Palumbo et al, 1991)] was used for all studies except those in which non-growth associated degradation was being studied. Nitrogen (as ammonium) was found to be the limiting supplemental nutrient in systems using NATE medium in batch experiments. The medium contained no carbon-containing compounds from which energy or carbon could be acquired.

Trickle-Bed Bioreactors. The trickle-bed reactors contained a structured polyethylene packing (Sulzer™ packing, Koch Industries, Houston, TX) to maximize surface area and minimize pressure drops. The structure also provided a stable support for a biofilm which developed on the surface. Humidified gas was fed at the top of the reactors containing 500 to 5000 ppm n-pentane and/or 500 to 5000 ppm isobutane; gas exited at the bottom for a single pass. The systems ran with gas flow rates of 5 to 500 mL/min, and a maximum degradation rate of approximately 60 g/h/m^3. Columns were 50 cm long with an internal diameter of 5.0 cm. Within each column were placed three set packing units, for which the surface area was known. The liquid media, which itself contained a substantial level of biomass, was recirculated (Masterflex peristaltic pumps) and distributed on the mesh packing at the top of the vertically-mounted column. The packing material was sterilized by soaking it in ethanol for 24 hr and the other components of the reactor were autoclaved for one hour at 121°C before use. The liquid medium fed to the reactor was also sterilized. Provision was made for a continuous supply of fresh sterile mineral nutrients to encourage growth and consumption. Strict aseptic conditions were not maintained after the bioreactor had developed a substantial level of VOC-degrading biomass for the following two reasons: (1) simulation of operating conditions likely to exist within an

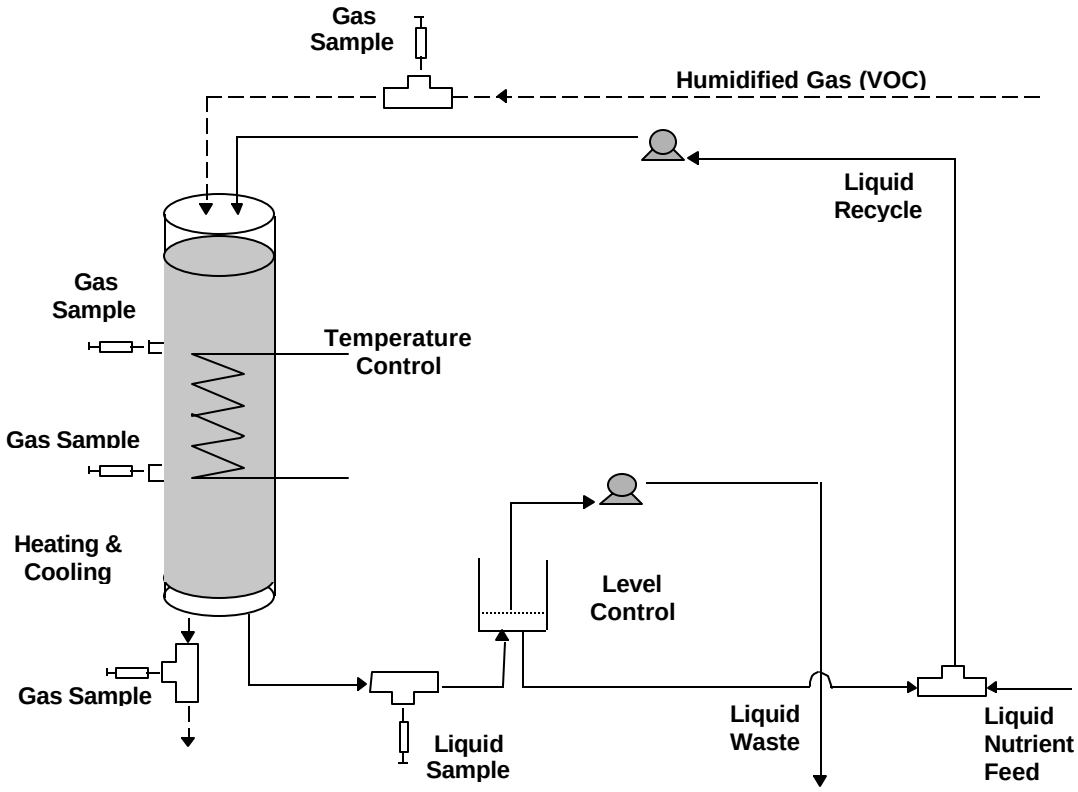

Figure 1. Schematic of trickle-bed bioreactor. Fresh humidified gas feed entered the top of the reactor and passed over a structured plastic packing which acted as a solid support for a biofilm Volatile organics were removed as they transferred into the liquid phase by the microbial consortium. Trickling mineral media (NATE), containing no added carbon and/or energy sources, recirculated through the reactor to keep the biomass moist. Gas and/or liquid samples were taken at various points to analyze for isobutane and/or n-pentane concentrations.

industrial setting was desired, and (2) the only carbon source available to for consumption by the biomass was the VOC or VOCs in the inlet stream, thus promoting selection of desired organisms. A schematic of the trickle-bed system is provided as Figure 1.

Sample Analysis. Gas chromatography was performed on a Varian Instruments (McFarland, WI) Model 3700 Gas Chromatograph with a Supelco (Bellefonte, PA) Model #2-17204 packed column equipped with flame ionization detection. Injector, oven, and detector temperatures were 160°C, 160°C, and 270°C respectively. Integration was performed by a Hewlett Packard (Greenville, NC) Model HP3396 Series II Integrator. Gas-phase concentrations of isobutane and n-pentane were monitored at inlet, outlet, and mid-length ducts of the column using 1.0 mL samples in gas-tight syringes (Hamilton Co., Reno, NV). Calibrations were performed using standardized gases purchased from Matheson Gases and Equipment (Morrow, GA). Liquid-phase concentrations were not detectable with the above analytical techniques; liquid phase concentrations are expected to be at most (using Henry's law assumption) about 300 ppb. The actual concentration, considering significant biological uptake, is suspected to be much lower.

Mathematical Model. The algorithm for calculating the column performance was programmed in Fortran and Microsoft Visual BASIC. The program is written as separated subroutines for different functions. Clarity is emphasized in the programming, with necessary lines being comments and definitions to aid the user. Microsoft Visual BASIC code is compiled into a 32-bit program that runs in Windows 95/98/2000 environments in the absence of external software packages. This particular type of coding is particularly useful, not only because of it's user-friendly graphical interface, but also because it can be run on most IBM-compatible computers.

Key Results

Three biofiltration units were assembled, inoculated with the VOC-degrading microbial consortium selected for use in this project, and operated continuously using selective nutrient limitation for 3 years. The units were approximately one meter long with 2-inch internal diameters and constructed of glass, Teflon, and stainless steel. Each unit was temperature-controlled and operated under a variety of continuous or recycling modes. This work represented a substantial breakthrough in filter operation by eliminating overgrowth of biomass in the column. Non-growth associated degradation kinetics were measured and do not incur a substantial decrease in removal capacity as compared with growth-associated removal. Prolonged starvation via elimination of supplemental nutrient(s) eventually resulted in decreased capacity but required at least six months before changes were noticeable.

Figure 2. ORNL Researcher Thomas Klasson working with one of four trickling biofilter reactors designed to remove dilute organics from contaminated air streams. Reactors were packed with structured packing units, woven out of polyethylene.

Measurements of mass transfer and kinetics were made for trickle-bed columns and indicated that kinetic limitations dominated all three systems under normal operating conditions (room temperature, moderate flow rates).

Quantification of biomass regeneration after prolonged starvation was accomplished. Biofiltration units operated under nutrient-limited conditions began losing removal capacity after approximately six months. Upon reintroduction of supplemental nitrogen, in the form of ammonium, rates rose rapidly as the biomass underwent regeneration. This procedure was found to be complete in less than 14 days, after which the columns operated at 100% of their previous removal efficiency. This was the first example of quantification/qualification of biomass regeneration after prolonged starvation in a trickle-bed system.

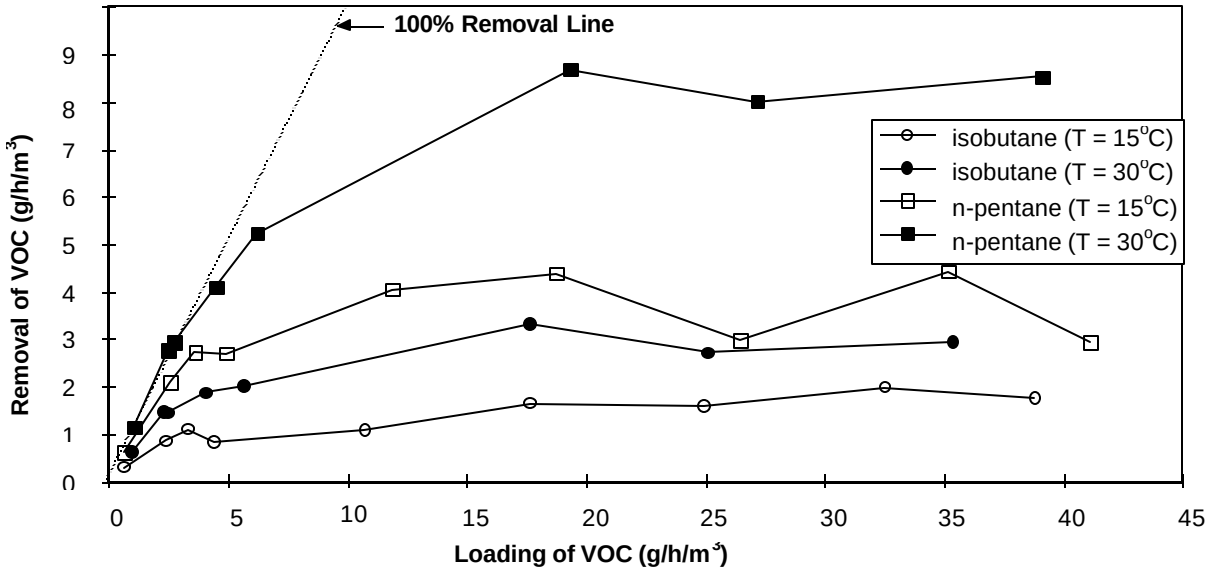

Figure 3. Removal Efficiencies in Trickle-bed Bioreactor. Removal efficiencies are highest as the residence time within the trickle-bed system increases. Of the two gases, n-pentane is preferentially removed. The solid line represents 100% removal. Data above were collected for low initial gas phase concentrations in the VOC feed. Higher removal rates are obtained when the initial gas phase concentration is raised.

Control of biofilm overgrowth, while sustaining high degradation rates, in operating trickle-bed bioreactors was accomplished by limiting selected nutrients. Optimal results were achieved by limiting ammonium, sulfate, or calcium in the liquid recycle stream. Each of these allowed for decoupling of growth and degradation such that long-term operation was possible. Other nutrients examined, including chlorine, magnesium, phosphate, and nitrate, were not effective and resulted in either continued growth or minimal degradation rates. *Significance:* EM has a number of dilute pollutant streams that could be potentially remediated using this technology. One of the problems traditionally associated with biofiltration is overgrowth of the biomass (a problem). The engineered approach taken for this accomplishment reduces such growth substantially while still allowing the biofilter to operate at near peak capacity.

We have measured fundamental solubilities of alkanes in biofilms and shown that they are four-fold higher that those reported and typically used for pure water. We have correlated these results in terms of partitioning between aqueous and biological material "phases". These results, some of which aresshown in Figure 4, will influence many degradative models. *Significance:* Several EM problems concern fate and transport of organic contaminants in environments where high biological material levels are present. The ideas and models developed for this accomplishment will impact such problems.

A comprehensive two-dimensional predictive model was developed to elucidate mass transfer and kinetic limitations in biofiltration systems. This model can be extended to a variety of columnar biofiltration systems by changing appropriate parameters. This user-friendly model/program (see Figure 5) can be installed and run independently on any IBM-compatible personal computer using Microsoft Windows 95/98. *Significance:* Dilute waste streams which are amenable can be treated with biofiltration. The easy-to-use software we have developed enables the user to design the biofilter a priori and is publicly available at **http://www.ct.ornl.gov/biofilter**

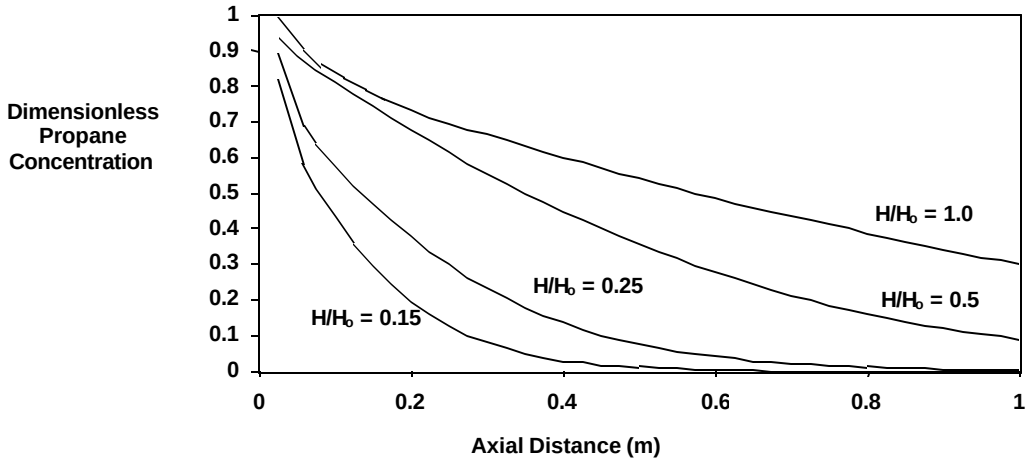

Figure 4. Example of how the value used for Henry's constant in a 2-D ORNL biofiltration mathematical model affects the concentration profile in a columnar remediation process. The top curve (H/H_o = 1.0) represents a predicted curve when the value for pure water is used. The curve for the case in which Henry's constant is halved (H/H_o = 0.5) suggests that outlet concentrations from a biofilter can be 50% lower than expected (as has been measured in actual systems with Henry's values in this range). We have also measured values of the Henry's constant in biomass which are only 15% of the pure aqueous value (meaning more soluble material). The bottommost curve (H/H_o = 0.15) demonstrates clearly the order-of-magnitude effect which the parameter has on the predicted outcome of a remediation effort.

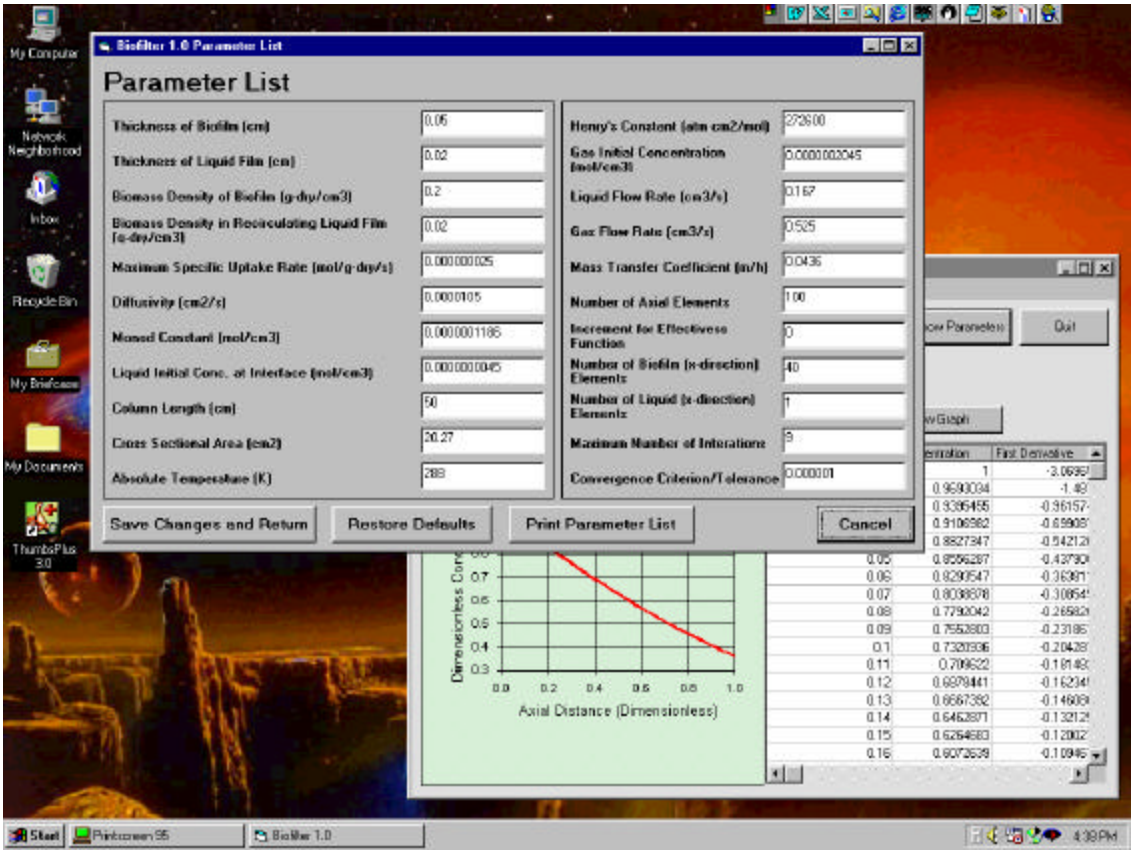

Figure 5. Biofilter 1.0 design software was developed for Windows 95/98™ to enable users to quickly and easily determine how various operating parameters will impact their biofilter designs a priori. The user interface is straightforward; data may be copied and then pasted into spreadsheets or presentation packages. Calculation times vary from 1 to 30 seconds on Pentium™-class processors.

Isolation and identification of the VOC-degrading microorganism present in a trickle bed consortium was accomplished. *Significance:* By isolating the organism responsible the bulk of the reactor's activity, startup time is minimized in new reactors. Cells can be grown up and added directly to the reactor at startup to quickly populate the biofilter.

Non-cometabolic degradation of a model chlorinated organic (1-chloropentane) was demonstrated in an operating trickle-bed system. This work is unusual because most dechlorination processes require the presence of an additional substrate before degradation of the target contaminant can be removed. *Significance:* EM has a number of chlorinated organic streams that could be remediated using a noncometabolic process.

Measurement of fundamental kinetics and mass transfer parameters for trickle bed bioreactors for removal of BTEX and chloro-organic contaminant treatment was completed. *Significance:* Kinetics and mass transfer parameters are difficult to measure in biofilters. We have a variety of techniques that can be employed to ascertain these operating parameters. They are necessary for deciding the best design option and operating conditions.

6.0 Relevance, Impact and Technology Transfer

How does this new scientific knowledge focus on critical DOE environmental management problems? The tasks performed were part of the Remediation by Natural Processes focus area, which addressed the broad class of volatile organic contaminants with applications to hydrocarbons, chlorinated hydrocarbons such as TCE, and nitrates. Specifically, this project examined the

"Emission-free destruction of organic wastes. Off-gas treatment that eliminates emissions in the environment that exceed Environmental Protection Agency requirements [including] bioremediation." (Notice 96-10, 1996).

The proposed work also directly supported EM Needs as listed in the EM-50 Rainbow series.

"Many DOE sites as well as private industrial sites have soil and/or groundwater that are contaminated with organic compounds. These sites will have to undergo remediation in the future. One method of remediation is vapor vacuum extraction, but air quality regulations require that the exhaust be free of organic vapors before they are vented to the atmosphere. Therefore, a method of organic vapor recovery or destruction will be required before vapor vacuum extraction can be used." (DOE/EM-0135P, 1994)

"Cost-effective methods are needed to remove VOCs in the gas stream. The cost of capturing VOCs by carbon adsorption is about $5-15/lb of VOC." and "This technique generates secondary waste that requires disposal or regeneration on a regular basis." (DOE/EM-0136P, 1994)

How will the new scientific knowledge that is generated by this project improve technologies and cleanup approaches to significantly reduce future costs, schedules, and risks and meet DOE compliance requirements?

VOC pollutants include alkanes, methanol, and chlorinated hydrocarbons including TCE. TCE is an example of a serious priority environmental problem throughout the U.S. Department of Energy (DOE) system. Other non-RCRA VOCs, such as the alkanes, are coming under tighter regulation, even at emission levels of less than 1%, due to both Clean Air Act regulation and compliance for Air Quality Non Attainment Areas. Alkanes and other solvents are present in mixed waste sites. Many site organic pollutants are volatile and can be stripped preferentially from soils into a dilute vapor phase. When VOCs are present in dilute concentrations, adsorption onto activated carbon followed by incineration can be used but is very expensive in both costs and energy; for this reason, we proposed biofiltration as a viable alternative. Not only can the proposed process treat gaseous streams, it has also been shown to be capable of removing contaminants from aqueous effluents before they have the opportunity to vaporize from the aqueous phase. Advantages include lower capital and operating costs, minimal maintenance, greater inherent safety, and no production of secondary pollution. Performing degradation on these VOCs in an ex-situ reactor can also be more efficient due to increased control over the operation and reactor environment conditions.

To what extent does the new scientific knowledge bridge the gap between broad fundamental research that has wide-ranging applications and the timeliness to meet needs-driven applied technology development?

There are three key pieces of information which can be used immediately to significantly enhance biological filtration processes. These include (1) decoupling growth and degradation processes

using nutrient limitation, (2) recognition of enhanced solubility effects due to the presence of biomass and how these effects could be quantified, and (3) access to easy-to-use software for enhanced design purposes. We have received interest from both academic and industrial sectors, but have not linked directly with a DOE end user for site clean up or process enhancement.

What is the project's impact on individuals, laboratories, departments, and institutions? Will results be used? If so, how will they be used, by whom, and when?

Academic communities are already using the information, primarily as published information available in peer-reviewed journal articles. We have received numerous requests for both electronic and hard copies of our papers, and have seen other researchers initiate studies that will further explore some of the work we have completed. Researchers at other national laboratories have expressed interest in both the published information and the Biofilter software. Various potential users have requested copies of the software for potential use on systems that they already use and want to enhance. In terms of application to a DOE focus area, we hope to locate end users during the renewal phase of the project who are willing to support pilot or larger scale implementation of the information gathered.

Are larger scale trials warranted? What difference has the project made? Now that the project is complete, what new capacity, equipment or expertise has been developed?

Deliverables for this project included primarily information and new experimental techniques that can be used to build better biofilters. A software tool was designed to also help builders design optimized systems.

How have the scientific capabilities of collaborating scientists been improved?

This project involved only scientists at the Oak Ridge National Laboratory. These scientists have continued to work together on this and other joint projects to take advantage of multidisciplinary strengths.

How has this research advanced our understanding in the area?

Although predictive mathematical models have been available for a wide variety of biological systems, development and verification studies of models for trickle-bed bioreactors remain sparse in the literature. Little consideration has been given thus far as to how the possible operating regimes associated with the processes of mass transfer and biological removal can be identified during bioreactor operation. Furthermore, the extent to which each plays a role in biofiltration is further clouded by the difficulty in measurement of the necessary parameters required to incorporate both processes into a successful mathematical model. We have attempted to provide a better understanding, both experimentally and through theoretical calculations, of biofilter operation and design.

What additional scientific or other hurdles must be overcome before the results of this project can be successfully applied to DOE Environmental Management problems?

Although pilot scale testing of biofilters on actual DOE waste streams would be most desirable, trials of this technology are essentially ready, but remain unfunded.

Have any other government agencies or private enterprises expressed interest in the project? Please provide contact information.

Excepting National Laboratories, no government agencies. Since biofilter methodology has applications to other industrial systems such as volatile chlorinated organic effluents produced during paper mill processing, off-gas emissions of alkanes from plastic blowing agents, and bioventing from contaminated sites, we have been contacted by a few individuals who have been interested in receiving data and software.

7.0 Project Productivity

Did the project accomplish all of the proposed goals? Yes

Was the project on schedule? Yes

Was the work plan revised? No

8.0 Personnel Supported

List professional personnel (Faculty, Post-Docs, Graduate Students, etc.) supported by and/or associated with the research effort

Brian H. Davison, Ph.D., ORNL, University of Tennessee
John W. Barton, Ph.D., ORNL
K. Thomas Klasson, Ph.D., ORNL
Gregory C. Delozier, Graduate Student to MS, University of Tennessee

9.0 Publications

Francisco, A.F., J.W. Barton, K.T. Klasson, and B.H. Davison. Nutrient Limitation Effects on a Microbial Consortium in Trickle-Bed Bioreactors. Submitted for publication.

Davison, B.H., J.W. Barton, K.T. Klasson, A. F. Francisco. Influence of High Biomass Concentrations on Alkane Solubilities. *Biotechnology and Bioengineering* 68:279-284, 2000.

Barton, J. W., B. H. Davison, K. T. Klasson, and C. C. Gable III. Estimation of Mass Transfer and Kinetics in Operating Trickle-Bed Bioreactors for Removal of VOCs. *Environ. Prog*. 18:1-5, 1999.

Klasson, K. T., J. W. Barton, and B. H. Davison. Performance of a Propane-degrading Bacterium. *Proceedings of the 92nd Annual Meeting of the Air and Waste Management Association, June 1999*.

Barton, J. W., X. S. Zhang, B. H. Davison, and K. T. Klasson. Predictive Mathematical Modeling of Trickling Bed Biofilters. *Proceedings of the 1998 USC-TRG Conference on Biofiltration,* October 22-23, Los Angeles, CA.

Barton, J. W., X. S. Zhang, K. T. Klasson, and B. H. Davison, Predictive Mathematical Modeling of Trickling Bed Biofilters for Elucidating Mass Transfer and Kinetic Effects, *Proceedings of the 91st Annual Meeting of the Air and Waste Management Association,* San Diego, CA, June 1998. Paper 98-WAA.13P.

Klasson, K. T., B. H. Davison, J W. Barton and J. E. Jacobs, Removal of Chlorinated and Nonchlorinated Alkanes in a Trickling Bed Biofilter, *Proceedings of the 91st Annual Meeting of the Air and Waste Management Association,* San Diego, CA, June 1998. Paper 98-WAA.06P.

Barton, J. W., S. Hartz, K. T. Klasson, and B. H. Davison, Microbial Removal of Alkanes from Dilute Gaseous Waste Streams: Mathematical Modeling of Advanced Bioreactor Systems," *J. Chem. Technol. Biotechnol.* **72**:93-98 (1998).

Barton, J.W., K.T. Klasson and B.H. Davison, "Extended Operation and Control of Biomass Overgrowth in Biofilters Designed for VOC Removal," *Proceedings of the;90th Annual Meeting & Exhibition of Air and Waste Management Association*, Toronto, Ontario, Canada, (1997).

Barton, J.W., K.T. Klasson, L.J. Koran Jr. and B.H. Davison, "Microbial Removal of Alkanes From Dilute Gaseous Waste Streams: Kinetics and Mass Transfer Considerations," *Biotechnology Progress,* 13, 814-821 (1997).

10. Interactions

Participation/presentations at meetings, workshops, conferences, seminars, etc. We have presented platform and poster presentations at several national conferences including Air and Waste Management Association, American Institute of Chemical Engineers, American Chemical Society, Symposium for Biotechnology on Fuels and Chemicals, USC-TRG Biofiltration Conference.

Collaborations. We collaborated with professors at the University of Tennessee, to fund and co-direct Master's level research for a student, Gregory Delozier, in the field of Biotechnology.

11. Transitions

We have had several requests from researchers and practitioners for access to our Biofilter software, primarily so they can apply it to the particular systems with which they work. Examples include Gero Leson of Berkeley, California, who is an independent contractor specializing in industrial biofilter applications and William Apel of the Idaho National Environmental and Engineering Laboratory, who conducts directed research in degradation of industrial VOCs.

12. Patents

None.

13. Future Work

This EMSP work showed that for two sparingly soluble organics that the presence of biological material can increase effective solubilities by an order of magnitude; therefore, the previous simple approaches are not valid and are extremely poor predictors of actual bio-influenced partitioning. We submitted a renewal proposal for this project with the express intention of investigating this phenomena, collecting the fundamental data and parameters, and incorporating this information into an updated version of the software generated through the first three years of the project. The abstract and title for the new project renewal, which begins in FY01, is as follows:

Biofiltration of Volatile Pollutants: Fundamental Measurements and Advanced Mathematical Modeling of Solubility Effects of Biological Material in Contaminant Zones

This project will investigate and collect fundamental partitioning data for a variety of sparingly soluble subsurface contaminants (e.g., TCE, etc.) between vapor, aqueous phase, and matrices containing substantial quantities of biomass and biomass components. Due to the difficulty of obtaining these measurements, environmental models have generally used solubility constants of chemicals in pure water or, in a few rare cases, simple linear models. Our prior EMSP work has

shown for two sparingly soluble organics that the presence of biological material can increase effective solubilities by an order of magnitude; therefore, the previous simple approaches are not valid and are extremely poor predictors of actual bio-influenced partitioning. It is likely that environmental contaminants will partition in a similar manner into high-biomass phases (e.g. biobarriers and plants) or humic soils. Biological material in the subsurface can include lipids, fatty acids, humic materials, as well as the lumped and difficult to estimate 'biomass'. Our measurements will include partition into these biological materials to allow better estimation. Fundamental data collected will be provided to mathematical models predicting transport and sorption in subsurface environments, with the impacts on bioremediation being evaluated based on this new information. Our 2-D Win95/98 software program, Biofilter 1.0, developed as a part of our prior EMSP efforts for describing biofiltration processes with consideration given to both kinetic and mass transfer factors, will be extended to incorporate and use this information.

This research will provide much-needed fundamental information regarding partitioning of priority contaminants in subsurface aquifers, vadose zones, and surface water impoundments where biological material is present. Accurate data measurements of phase distributions of sparingly soluble organics between biomass/water/air are needed to replace the current assumptions using pure water data. Beyond the detailed equilibrium measurements of a variety of contaminants and biomaterial types, we also will seek to characterize the materials in order to understand the fundamental properties that cause different biological materials to have different partition constants.

14. Literature Cited

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Davison, B. H.; Thompson, J. E. The Removal of Alkanes in a Liquid-Continuous Gas-Phase Bioreactor: Preliminary Considerations. *Appl. Biochem. Biotech.* **1994**, 45/46, 917-923.

Palumbo, A. V.; Eng, W.; Strandberg, G. W. 1991. in *Organic Substances and Sediments*, ed. R. Baker, Lewis Publishers, Chelsea, MI, **1991**, pp. 225-38.

15. Feedback

Proposal instruction complexity presented a challenge, and could have been much simpler. In addition, we would recommend a more rigorous review during the preposal process--as EMSP awards are recognized as being highly competitive, it may very well be that good proposals/ideas are never submitted because researchers feel that the time investment on full proposal development is not worth the probability of successful award.

16. Appendices

Master's Thesis, Gregory Clark Delozier

ISOLATION AND IDENTIFICATION OF VOC METABOLIZING MICROORGANISMS FROM AN ACTIVE BIOTRICKLING FILTER

Greg DeLozier

INTRODUCTION

As industrial production expands with each new technological advance or discovery, environmental concerns tend to increase at a proportional rate. Wastes generated by current industrial practices as well as the residual contaminants of past operations are the continuing focus of remediation efforts. Over the past several decades, regulations have been imposed on the unchecked release of deleterious substances into the environment. Conventional chemical and physical methods for treatment of these effluents prior to release may be costly, require constant maintenance and supervision, and have limited efficacy. In addition, many of these techniques simply transfer the contaminant between physical states or into another contaminant deemed less hazardous. Understandably, researchers have begun to exploit the incredible flexibility of microorganisms, employing them as agents of biodestruction.

Biological treatment of wastes and residual contamination is a widely accepted practice and has been extensively, and successfully, adapted for a spectrum of industrial and commercial applications. Solid and liquid-phase contaminants have received most of the attention to date and numerous alleviative bioprocedures have been developed in response. Early regulations merely addressing contaminants present in these states may have led to this bias. Groundwater and land contamination are much more accessible and easily measurable entities and therefore much easier to monitor for regulatory adherence. It was not until recently that toxic substances present in gaseous waste streams and their effects on health and the environment became more fully understood. The Clean Air Act Amendments (CAAA) of 1990 imposed strict regulations on gaseous emissions and left industries and public facilities scrambling for viable air pollution control (APC) technologies (Togna, Singh 1994).

Today, these regulations threaten to increase in stringency and have begun to limit the controlled release of even mild, dilute contaminants (e.g. Alkanes) into the atmosphere (Davison, Thompson 1993). In light of these impending changes, chemical, physical, and biological protocols have been designed to maintain airborne contaminant concentrations within defined, acceptable parameters. Chemical and physical methods of volatile organic compound (VOC) removal include incineration and adsorption onto

activated carbon (with subsequent incineration). Unfortunately, these techniques can be costly and tend to generate residual products which require additional treatment and disposal (Ergas, Schroeder, Chang, Scow 1994). Conversely, biofiltration efficiently oxidizes numerous VOCs into harmless CO_2, H_2O, and biomass at low operating cost and low energy input (Leson, Winer 1991).

BIOFILTRATION

Up to this point, the term "biofilter" has been used as an interchangeable, generic title for several current techniques concerned with the removal of contaminants from gaseous emissions. There are presently three fundamental types of biofiltration and all rely on the ability of a contained consortium of microorganisms to degrade a variety of VOCs into innocuous products and biomass. Biofilters, bioscrubbers, and biotrickling filters are widely accepted as competent, proven systems for the processing of airborne waste streams (Govind, Bishop 1993). Determination of the optimal system for use in a specific setting depends on a number of variables.

The key to successful remediation of an aerosolized contaminant is maximizing the area of interface and contact time between the pollutant and the microorganism. Microbial survival generally requires an aqueous environment and most VOCs are only sparingly soluble in water. To this end, natural matrices have been exploited as a means of increasing the level of interactions between microbe and contaminant. These "true" biofilters involve gaseous emissions forcefully pumped into and through a bed of soil, peat, compost, or any combination thereof. Some biofilters have more defined matrices such as activated carbon pellets or porous ceramic beads (Govind, Bishop 1993). Particle size within the bed determines the porosity and thus the extent of gas dispersion throughout. Microorganisms indigenous to the natural matrix or that have been introduced on the basis of VOC degradative capabilities, thrive within the cavities between and on the particles. These particular matrices are capable of providing an optimal environment for microbial proliferation and consequently, augment rates of degradation (Leson, Winer 1991). Passing vapors provide substrate VOC molecules that

are oxidized by the consortia as a primary means to obtain carbon for growth or as a cometabolic phenomenon.

Biofiltration units modeled in this fashion are the most common. Dynamic potentials of the system, cost-effectiveness, and a minimal level of energy input combine to make this form of bioremediation a popular one. However, disadvantages of biofiltration exist as well. Overgrowth of the microbial population within the bed may cause fouling of the gas inlet. Formation of preferential gas channels limits retention times within the bed. Certain wastes generate mineral acids upon degradation resulting in acidification and possible deactivation of the bed (Kamarthi, Willingham, 1994). Water must be sprayed intermittently on the bed and/or the gas must be humidified before entry to maintain a satisfactory level of moisture or risk drying out. Beds comprised of peat, compost, or wood chips provide the active consortia with a necessary source of nutrients and must be replenished to maintain activity. These are only a few of the complications that could lead to an incomplete conversion of the pollutant before release into ambient airways (Barton, Klasson, Koran, Davison, 1996).

Bioscrubbers provide a means of treating large loads of effluent gases at high rates of input. Oxidation of the VOC occurs as counter-current gas-liquid spray columns provide maximum interaction between the gaseous pollutant and the aerosolized microbes (Govind, Bishop 1993). Simple, fundamental bioscrubbing operations are merely retained suspensions of free-floating microbiota and the suspect gas is bubbled up through the liquid. Degradative rates are contingent on the duration of bubble migration through the medium as well as bubble size. An archetype bioscrubbing operation is currently in progress at a brewery in Golden, Colorado as ethanol emissions and exhaust from a neighboring cannery are treated with high-efficiency (Croonenberghs, Varani, Le Fevre 1994).

Like biofilters, bioscrubbers may experience shifts in pH as the oxidation of certain VOCs yields mineral acids. This necessitates the constant addition of chemical buffers such as lime. Again, overgrowth and fouling will decrease degradative rates of the system. An advantage of bioscrubbers is that a certain degree of control over

variables such as temperature and pH enables optimal conditions for degradation to be maintained. However, the intricate matrices of biofilters provide a greater reactive surface area between microorganisms and gas constituents than can be attained in a bioscrubber.

Biotrickling filter design and operation rests heavily on the ability to recognize and adjust parameters to encourage efficient VOC uptake by a consortium. A number of the dynamic variables that fluctuate within biofiltration and bioscrubbing procedures can be adjusted for optimal efficacy and then continue for the duration of the remediation process. Enhanced control over such intricacies as pH, temperature, biomass kinetics, and retention times makes biotrickling filtration an attractive option for waste treatment.

A model biotrickling filtration unit is comprised of a vertical column through which contaminated gas can flow in either direction. Within the column, a matrix, usually synthetic, provides a foundation to support the initiation and growth of biofilm. Generally, this packing media is structured which indicates an approximate understanding of passage-size and available surface area for microbial colonization throughout the column (Govind, Bishop 1993). Packing media should enable channel-free flow of the gas through the column, possess a high ratio of surface area to volume, and be exempt from microbial degradation (Barton, Klasson, Davison 1997). Structured packing resists the pressure drops and subsequent compaction of peat, compost, and most other materials employed in biofilters.

To encourage and maintain the biomass throughout the column, a nutrient solution must be readily available. This solution is "trickled" down the length of the bed, providing the critical moisture and primary nutrients (N, P, K, Mg, Ca, Fe, etc) without the benefit of a carbon-based substrate (Kamarthi, Willingham 1994). This compromised feedstock insures that the molecular carbon required for biomass growth can only be acquired from the waste-gas stream. Overgrowth and fouling of the system, complications associated with biofilters and bioscrubbers, may be avoided by limiting the availability of a choice nutrient within the solution. In a process called "selective-

limitation", this nutrient may be supplied to the feed at specific time intervals thus maintaining a level of biomass capable of maximum VOC utilization (Barton, Klasson, Davison 1997).

A unique characteristic of biotrickling filters is the ability to recycle this nutrient solution. Solution passes through and out of the bed into a collection reservoir from which it is pumped upwards and redistributed at the top of the bed. This minimizes waste from the filter as well as reapplies biomass to the column which may have been sheared or dislodged from the bed. Incidentally, this reintroduction of microbes to the top of the column may effect microbial community dynamics within the column and encourage uniform distribution of all species along the bed length (Stoffels, Amann, Ludwig, Hekmat, Schleifer 1997). Metabolic exudates produced at the bottom of the bed may act as substrates for consortia members existing within the upper levels of the bed upon recycle. A buffer present in the nutrient solution maintains an acceptable pH for biofilm survival.

Although biotrickling filters appear to maintain an advantage over biofilters and bioscrubbers in terms of their flexibility, they are not employed as often in industrial practice. Speculation attributes this to the fact that biotrickling filters require more maintenance and observation than biofilters and that the packing material of a biotrickling filter has less surface area than those of a biofilter (Togna, Singh 1994).

HISTORY

Biofiltration was recognized as a practical method to process gaseous emissions in the 1920's when H. Bach began to advocate the concept of biological removal of H_2S from the waste effluents of sewage treatment plants in Germany (Leson, Winer, 1991; Barton et al, 1995). Over the next thirty years, many European countries, most notably Germany and the Netherlands, would refine this technology and implement it in a spectrum of situations. Originally, odorous off gases were targeted as possible streams

for remediation as effective treatment was immediately obvious. Reduced sulfur compounds (H_2S and mercaptans) and nitrogen compounds (NH_x and NO_x) were efficiently cleared from emissions (Kamarthi, Willingham, 1994; Andrews, Noah, 1995). This included offensive vapors released from sewage treatment plants, water treatment plants, poultry and fish rendering plants, composting, food processing and farm animal facilities (Leson, Winer, 1991; Togna, Singh, 1994; Kamarthi, Willingham, 1994). These early ventures usually employed large soil beds as the biofiltration unit. Not until the 1950's did the first large-scale use of biofiltration for odor control come to the United States (Barton et al, 1995). In 1963, Pomeroy was granted the first patent for his soil bed biofiltration unit. H_2S biodegradation in "moist, loam soil" was assayed for industrial applicability and sewer gas treatment in 1966 by Carlson and Leiser (Govind, Bishop, 1993).

As biofiltration evolved through the years and engendered new processes such as bioscrubbing and biotrickling filtration, gaseous emissions from new industrial and commercial sources could be addressed. Detrimental effects of non-odorous, invisible gases began to be observed and consequentially, regulation was required. The modification of conventional biofilters allowed a number volatile organic compounds (VOCs) present in off gases to be oxidized prior to atmospheric release. Biofiltration is now employed in the coating, aerosol can filling, polystyrene foam, and petrochemical industries (Togna, Singh, 1994; Barton, Klasson, Davison, 1997; Andrews, Noah, 1995).

The Kraft lignin process of the pulp and paper industry which generates sulfur-bearing VOCs as a chemical cocktail is used to degrade lignin and liberate cellulose fibers. Volatile alkanes are generated from petroleum cracking operations. Even established remediation efforts such as bioventing in which O_2 is actively pumped into contaminated soil to promote *in situ* degradation by indigenous microorganisms tend to release VOCs (Barton et al, 1996; Barton et al, 1997; Govind, Bishop, 1993). In each of these cases, biofiltration advances a solution capable of promoting of satisfactory VOC uptake and removal.

Today, Germany and the Netherlands list biofiltration as the best air pollution control (APC) technology and successful facilities are in operation throughout Europe and Japan (Leson, Winer, 1991). Although the concept of biological removal of pollutants is highly accepted and developed within the international community, the progress of biofiltration in the US has been rather slow. This tentative approach exhibited by the US towards biofiltration can be attributed to the lack of available information on the subject. Many descriptions of development and instructions for proper performance of biofiltration units lack explanations in the English language. Until recently, an inadequacy of regulatory guidelines imposed on gaseous waste stream release kept the evolution of biofiltration as a low priority. Because the concept of biofiltration appeared superfluous when gases could simply be released into the environment, extremely limited financial support was given to research and development (Leson, Winer, 1991).

Photochemical air pollution is dependent on VOC concentration in the atmosphere. New APC regulations even require large breweries and bakeries to begin treatment of ethanol emissions. Industries producing waste gas contaminated with dilute, low concentrations of VOCs are discovering that biofiltration is possibly the best option for adherence to environmentally benign standards at relatively low costs.

CURRENT BIOTRICKLING FILTER

The biotrickling filter selected for use in this study has been in operation since January of 1997. After 18 months, the immobilized biomass continues to efficiently degrade VOCs present in dilute concentrations passing through the column (0.5% n-pentane/ 0.5% isobutane mixed gas). The column consists of a 50 cm glass tube with an internal diameter of 5.0 cm. Three cylindrical units of structured polyethylene packing (Sulzer™ Packing, Koch Industries, Houston, TX) rest end to end within the column and serve as the primary matrix for microbial colonization and subsequent biofilm development. Nutrient solution and humidified, substrate-bearing gas is pumped

downwards through the column under conditions favoring maximum retention time. *Figure 1* displays two current biotrickling filters.

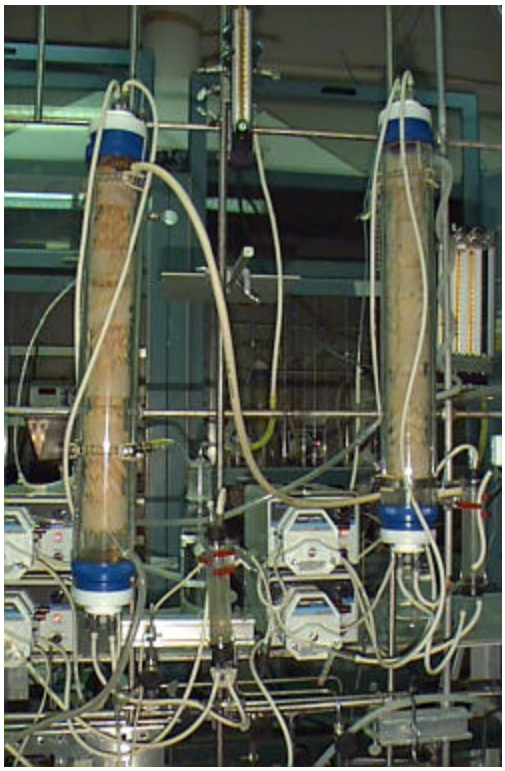


Figure 1. Two current biotrickling filters from which all biomass analyses were performed. (Photo courtesy

Overgrowth and fouling are avoided by using a nutrient-limited liquid recycle procedure in which available nitrogen is the provisional factor. As the biofilm begins to lose activity via nitrogen deprivation, ammonium is added to the solution in sufficient amounts to restore original rates removal.

Dr. Tony Palumbo of the Environmental Science Division of Oak Ridge National Laboratories donated the original inoculum used in the biofilter. Once cultivated as an entity capable of cometabolic degradation of chlorinated alkanes, this biomass contained methanotrophs imparting the biofilm a pink pigmentation. However, once applied to the current biotrickling filter and allowed to colonize the matrix under nonsterile conditions, the biomass soon lost the ability to effectively metabolize methane. The most obvious indicator of this change is the development of a characteristic yellow-orange color throughout the column. Assumption holds that the initial, dominant microbes were selected against or outcompeted by a microbe either present in minute concentrations within the original biomass or introduced from the immediate environment.

At this time, the biotrickling filter remains something of a "black box". Parameters have been established to promote optimal degradation rates, avoid system deactivation, and measure VOC input and output. In essence, the consortium is treated as a single entity with well-defined characteristics. Initial attempts to assign taxonomic identities to members were performed by BBL Enterotubes (Becton Dickinson Inc.,

Bedford, MA) and Biolog© Microbial Assay. Identified species included *Xanthomonas maltophilia*, *Flavobacter indologenes*, *Citrobacter freundi*, *Pseudomonas putida*, and *Alcaligenes paradoxus* (Barton, Klasson, Davison, 1997). Eukaryotic organisms such as yeasts, molds, and algae are also present within the biofilm.

OBJECTIVES

Considering the lack of information concerning the members of the consortium and their physiological contributions to the active biomass, the need to isolate and characterize the primary individual(s) capable of extracting carbon from VOCs is obvious. Once these initial harvesters fix carbon into compounds that are readily used as substrates by other organisms, an entire microfloral community can develop from the cascade of carbon-bearing metabolites. Some species have been determined to play essential roles within the biomass such as filamentous bacteria and mycohyphae providing additional support whereas others tend to exist as commensuals only. Therefore, an attempt to reduce the biomass into its fundamental components should begin with the isolation of microbes serving as primary producers within the column.

Once selected from the biomass based on degradative capability, the taxonomic identity of the microbe(s) is/are necessary for future reference. A VOC degradation rate comparison can be drawn between a pure culture of the competent organism and that of the consortium as a whole. Population and dynamics of this species throughout the column can be determined as well. This information can be applied to future biotrickling filter models to further enhance VOC degradation rates.

MATERIALS AND METHODS

ISOLATION

Aspiring to select only those species within the consortium capable of VOC degradation, the approach was straightforward. A sample was drawn from the nutrient

solution recycle reservoir near the bottom of the column. Assuming that shear forces generated within the matrix, dislodging, and normal microbial growth from the biofilm into the aqueous phase provide an inclusive suspension in the reservoir, all consortium members were present within the sample. This sample was subjected to serial dilutions and then plated on TSA (tryptic soy agar) and a minimal media composed of agarose only. These plates were stored at room temperature within bell jars (airtight vessels) "charged" with a saturating amount of n-pentane (2 mL n-pentane placed into open vial at the bottom of bell jar diffused freely).

After a period of 5 days, these plates were removed. Individual colonies exhibiting distinct morphology were picked and replated onto TSA and minimal media and returned to the "charged" bell jar for an additional 5 days. After this time, the plates were inspected for visual purity of the culture.

For each established isolate from the biomass, a small-scale bioreactor was created. A scale bioreactor consisted of a test tube (21 mL internal volume when capped) filled with 7 mL of a minimal nutrient solution. An identical solution was recycled throughout the actual biotrickling filter. Termed "NATE", the components included $MgSO_4$, $CaCl_2$, KNO_3, NH_4Cl, $FeCl_3$, KH_2PO_4, K_2HPO_4, and a trace element solution containing Cu, Mn, Zn, Co, and Mo. Therefore, this solution provided essential nutrients without the benefit of a carbon source. Each bioreactor was capped and crimped to create a headspace of ~14 mL. A model bioreactor is presented in *Figure 2*. To ensure sterility, all prepared bioreactors were autoclaved before further use.

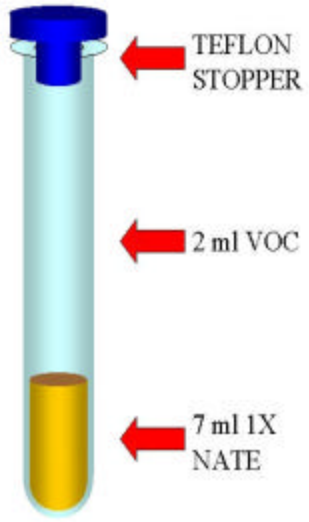


Figure 2. Scale

Subsequent to sterilization, the bioreactors were "charged" with 2 mL of pure, filtered propane to provide the carbon source. Colonies were selected from each pure

culture, resuspended in sterile NATE, and then injected into a bioreactor. These reactors were shaken at ~30°C (to increase the area of the gas/liquid interface and, thus, increase mass transfer). After 4 days, positive identification of competent organisms was possible via direct observations of turbidity in the medium. The bioreactors continued to shake in order that any slower growing microbes not be overlooked. Any species determined to utilize the propane will be selected and plated to verify purity.

TAXONOMIC CLASSIFICATION

A battery of techniques were applied in an attempt to classify of the competent microbes derived from the isolation experiment. Morphological characteristics of both the colony and individual cell levels were determined with light microscopy, Gram staining, and India ink analyses. The remaining techniques revolved around methods of rapid identification via simple biochemical assays and genetic fingerprinting. Enterotube® II (Becton Dickenson Microbiology Systems, Cockeysville, MD), Biolog© Microbial I.D., and 16s rRNA gene analyses were employed in an attempt to assign a name to the species.

VOC DEGRADATION RATE

To determine the rate at which a pure culture of a microbe removes a dilute VOC from a gas/air mixture, the test tube bioreactors were used again and prepared in the same manner as before (7 mL NATE) but involving a spectrum of VOCs. Propane, n-pentane, and isobutane were added to different bioreactors in 2 mL aliquots. Enough bioreactors were prepared for inoculation with a sample of the consortia, a sample or samples of a pure culture of VOC degrading microbes, and a negative control to check for contamination or gas leakage. Before injection into their respective bioreactors, the optical density of the consortia inoculum was equilibrated with that of the pure inoculum to ensure an approximately equal number of cells. The inoculae were then injected and the amount of target VOC within the headspace of each reactor was measured by a gas chromatograph (Hewlett Packard (Greenville, NC) Model HP5890 Series II, column (J & W Scientific, 30 meter, 0.53 I.D., THK 3.0 microns with injector, oven, and detector temperatures set at 175°C, 30°C, and 200°C respectively). Over the course of 7 days, daily measurements of remaining gas within the headspace was determined and plotted for direct comparisons of VOC uptake between the two samples.

POPULATION AND DYNAMICS

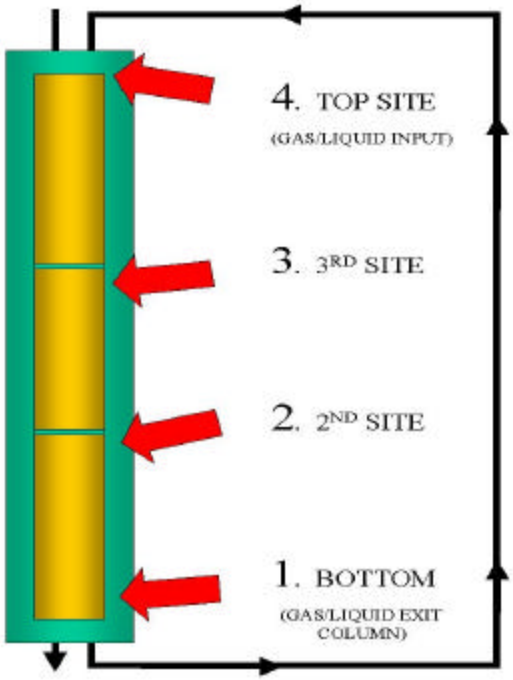


Figure 3. Simple diagram of the biotrickling filter showing sampling points for population and distribution assay

Future models for biotrickling filter construction will need to consider the dispersion and density of key species throughout the column. With this intuition, filters can be designed to maximize areas in which conditions favor an increased amount of pertinent microbes and possibly promote a greater rate of VOC degradation. When a particular species has been shown to metabolize VOCs, it will be necessary to gain insight into its population and dynamics within the biotrickling filter currently in use. 0.4 grams of wet biomass were taken from four points within the column: the top of the matrix, the top of the middle section of the matrix, the top of the bottom section of the matrix, and the bottom of the bottom matrix section (*Figure 3*). Biomass was readily scraped from the matrix, weighed, and suspended in 40 mL of NATE.

From the 4, 40 mL centrifuge tubes of biomass suspension (0.01 gm/mL of biomass) 0.1 ml was transferred to a 1.5 mL Eppendorf tube containing 0.9 mL NATE (now diluted to 0.001 gm/ml of biomass). Six serial dilutions were made. 0.1 mL from the last three dilutions (4, 5, and 6) was taken and plated. Selective media for VOC degrading microbes was comprised solely of agar reconstituted with NATE. The only available carbon source was introduced as a gas and any colony that formed was selected as one capable of VOC utilization. All plates were completed in triplicate to broaden sample size.

Special plastic bags were assembled to accommodate 12 petri plates. Teflon valves were inserted into the airtight bags for convenient sparging with gas. Each could hold up to 12 plates and the bags were then sealed and filled with the mixed gas. All bags created and charged in this manner were placed in an incubator at 35°C. Due to slow colony formation, three weeks were necessary as the duration between inoculation

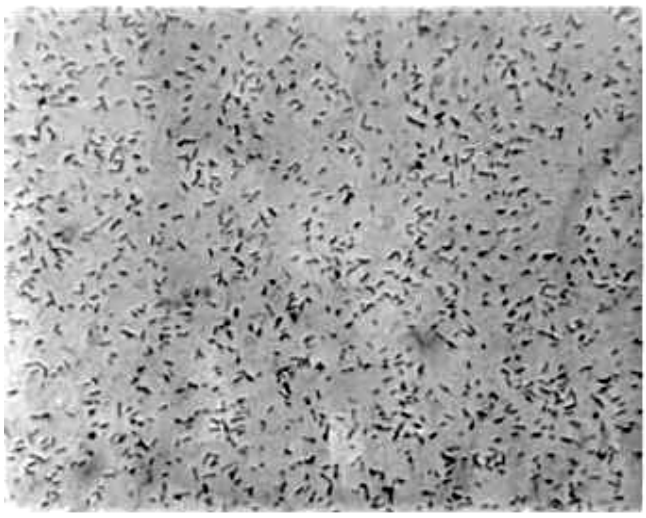
and plate reading. After this time, colony-forming units (CFU) were counted for each plate and the number of competent cells per gram of wet biomass were calculated for each location within the column. Thus, the population of the species as well as its dispersion throughout the column was determined.

RESULTS AND DISCUSSION

ISOLATION

After 5 days of incubation at room temperature within the "charged" bell jar, plates were removed and colonies were picked and isolated. Three distinct colonies were selected from the minimal media plates. A highly filamentous mold was present on a number of these plates, making final isolation of a pure culture difficult due to contamination. The TSA plates, providing an undefined media capable of supporting diverse growth, yielded 20 colonies of distinct morphology. Algae, mold, and yeast tended to cover large expanses of the plate quickly. To avoid contamination by these organisms, colonies were picked from the plate daily starting immediately after the 5 day period.

Once all colonies were decidedly pure, a colony from each plate was suspended in NATE and injected into a "charged" test tube bioreactor. After a week of continuous observation, the bioreactors remained clear, indicative of no growth and, hence, no isolated VOC-degrading agent. This entire experiment was repeated two more times before a VOC-degrading species was isolated.



Colony formation of a VOC-degrading species was noted on the

plates which remained within the bell jar for a period of 15 days. The plates remained within the bell jar for a period of 10 more days. These colonies were minute, translucent, and yellowish colonies were observed. What may have been easily overlooked as air bubbles or inert particles 5 days after inoculation were now colonies scattered in abundance across the plates. More interestingly, colony consistency seemed to remain the same whether on TSA or minimal media. These colonies were picked and cultured to purity. Upon inoculation of a "charged" bioreactor with this isolate, turbidity was observed after a period of 2 days. Complete saturation was noted after 5 days. This microbe was given the title 234-5 pending future taxonomic identification.

Gram staining and light microscopy of the isolate revealed a Gram positive, 2.0 X 1.0 μm rod (*Figure 4*). All bacterial cells existed singly or in V-shaped dyad formations and did not form slime or filaments. Numerous cells were observed in binary fission and appeared as two ovoid structures attached at one end. An India ink assay established the absence of an extra-cellular sheath, capsule, envelope, or polysaccharide layer. When grown in pure suspension, 234-5 does not form aggregate clumps, fibrils, or flocculate as does a suspension of the consortium grown under the same conditions. A test with 5% H_2O_2 revealed 234-5 expresses catalase, underscoring its aerobicity. The most striking visual characteristic of the bacteria is its pigmentation; a bright yellow-orange that explains the coloration of the filter matrix as a whole. This suggested a very dense population of 234-5 exists within the biofilm which was verified later.

TAXONOMIC CLASSIFICATION

After culturing 234-5 to absolute purity and confirming Gram stain and cellular morphology, the Enterotube® II rapid identification system was considered unsuitable. Although 234-5 is a rod, it is also Gram positive whereas this particular system is designed for Gram negative species. Biolog© Microbial Identification System provided disappointing results. 96 wells exist within the Biolog© microplate. In accordance with the Gram designation of a target bacterium, these plates are Gram specific as well (i.e. Gram positive plates contain 95 carbon-based substrates that can be oxidized by Gram positive bacteria). Tetrazolium redox dye present within each well serves as a positive marker to indicate active metabolism (respiration) of a substrate.

Upon reconstitution of the dehydrated substrate with a suspension of target bacteria, 24 hours must pass before the microplate can be "read" spectrophotometrically to identify wells in which oxidation occurred. Positive reactions can be compared to a Gram positive database to provide a table of possible species arranged in a decreasing order of certainty (certainty is assigned a value from 0.0 to 1.0 with 1.0 indicating a 100 % match). Closely related species are listed along with a table of biotype patterns.

Unfortunately, 234-5 was able to oxidize 39 of the compounds and 2 more were considered borderline. After 24 hours, of all Gram positive bacteria contained within the current database, none are capable of oxidizing an analogous spectrum of substrates. Therefore, the value of certainty was 0.19. This value is considered completely insignificant when attempting to

place the organism within a taxonomic compilation. Although this particular assay of 234-5 proved fruitless, the information gained concerning utilizable substrates was applied to a fundamental search of possible bacteria species as classified by Bergey's Manual of Determinative Bacteriology 9th Edition (Williams and Wilkins, 1994). Based on the known properties and metabolic capacity, 234-5 is tentatively labeled as a *Coryneform* bacterium. 234-5 is Gram (positive), aerobic, non-motile, slightly pleomorphic and tends to form V-shaped cell arrangements; all features of *Coryneform* bacteria. With a great deal of uncertainty, 234-5 may be accommodated within the genus *Arthrobacter* for which many morphological and physiological similarities exist but is not included within the current Gram positive Biolog© database.

Current procedures for rapid identification of an unknown bacterium bypass the battery of tedious biochemical assays and scrutinize unique genetic sequences. In this case, the gene encoding the 16s strand of rRNA was to be amplified, sequenced, and subjected to a gene database for species identification. The primer sequences recognizing universally conserved flanking segments of the bacterial 16s rRNA gene were prepared. All that was required was the extraction of genomic DNA from the bacteria, PCR amplification of the 16s rRNA gene, and a subsequent round of cycle sequencing to prepare the sample for automated sequencing. Unfortunately, the procedure could not be completed due to a simple physical hindrance; the cell wall was able to withstand all attempts of rupture and avoided release of nucleic acid.

Simple detergent treatments with SDS (sodium dodecyl sulfate) at 70°C for incubation times of 2 and 3 hours failed to disrupt the membrane. Extremes of heat and cold in which a cell pellet of 234-5 was quickly frozen to –70°C and then immediately heated to boiling in a microwave (3 consecutive times) were followed by another attempted detergent lysis. Subsequent gel electrophoresis (GE) and ethidium bromide staining revealed the absence of DNA in the "lysate". At this point, techniques for DNA extraction from Gram negative bacteria were abandoned in favor of procedures developed to rupture Gram positive cell walls.

Natural enzymatic degradation of transmembrane proteins appeared to present a feasible approach when working with 234-5. Egg white lysozyme (Sigma) was added to a 234-5 suspension and allowed to incubate at 37°C for one hour. A second lysozyme treatment incubated for 2 hours. This procedure was performed in manner analogous to one used to successfully extract genomic DNA from Gram positive, radiation resistant *Deinococcus radiodurans* (Narumi, Cherdchu, Kitayama, Watanabe, 1997). However, upon GE, no bands were detected and light microscopy was able to reveal intact cells.

Representatives from Promega and Qiagen recommended a lysis treatment with Lysostaphin© (Sigma, cat# L7386). This recombinant enzyme was developed for the efficient rupture of the thick cell walls of *Staphylococcus sp.* and is currently providing satisfactory yields when extracting DNA from *Lactobacillus sp.*. The Promega Genomic DNA Prep© for Gram positive bacteria was performed in triplicate to allow for different enzymatic incubation periods. Saturating amounts of Lysostaphin© and lysozyme were added to each reaction to avoid the possibility of rate limitation. However, the walls continued to resist breakage and no nucleic acid was extracted.

Physical methods were employed to simply fragment the cell without concern for organelle or nucleic acid recovery. A French Press subjected the cells to 2500 psi and then allowed immediate return to ambient atmospheric pressure. A treatment of this nature will

normally cause cells to burst upon the return to standard conditions but 234-5 remained intact and even demonstrated viability when plated onto TSA.

Sonication was performed as a last resort to merely fracture the cell wall, again without concern for cellular components. A treatment lasting for 30 minutes with 50 MHz pulses in an ice bath failed to open the cells but did impart a darkening of the suspension. The bacterial cells were suspended in NATE where the mineral constituents might react when heated and simultaneously submitted to high intensity waves thus providing the color change. But the cells remained intact (*Figure 5*). In the event that small pores or fissures, permitting the escape of cellular material, may have been formed in the wall during sonication, the cells were pelleted and replated. Although 234-5 did lose viability after sonication, gel electrophoresis and staining of the crude suspension confirmed a lack of DNA.

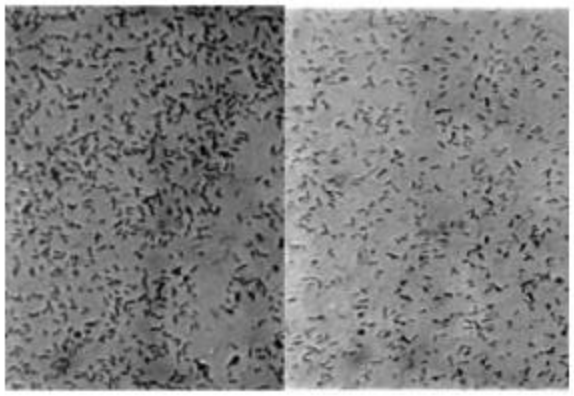


Figure 5. 234-5, 10^4 X, The left side shows 234-5 immediately prior to sonication. 30 minutes later, the cells remain intact (right

As bacteria complete the exponential portion of the growth curve and enter the stationary phase, certain processes such as reinforcing membranes, the addition of transmembrane proteins, and, in the case of Gram positive bacteria, thickening of the peptidoglycan layer increase wall resistance to rupture. To this end, younger cultures may provide less resistance to the extraction methods. However, 234-5 is a highly fastidious organism which exhibits an extremely slow growth rate. This fact tends to make estimations of optimal times of wall pliancy difficult. Dr. David Bemis of the Veterinary School at the University of Tennessee suggested an "exponential transfer" when attempting to amplify a satisfactory amount of cells within this crucial stage for DNA extraction. His procedure did result in an increased sample in terms of overall mass; however, when samples generated in this manner were submitted to the extraction processes outlined above, the results were equivalent.

An extreme test of endurance was performed on a pellet of 234-5 grown via "exponential transfer". This sample was exposed to the extremes of temperature, SDS detergent lysis, lysozyme and Lysostaphin© incubations, 2500 psi in the French Press, and one complete wash/dry cycle of laundry in a breast pocket. Light microscopy revealed intact cells (*Figure 6*). Viable CFUs (colony-forming units) were observed after streaking on TSA plates.

Classical methodology of bacteriological identification may need to be employed for classification of this extraordinary bacterium. Although tentative nomenclature has been provided by Bergey's Manual, direct genetic analysis is necessary to provide the identification of a genus or even a species with the highest degree of certainty.

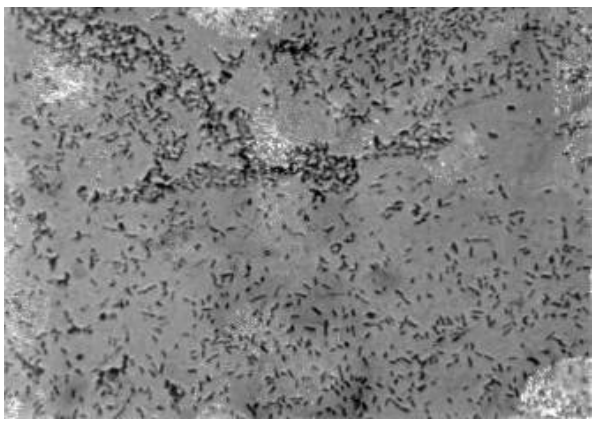


Figure 6. 10^4 X, 234-5 after the series of physical and chemical attempts to disrupt

VOC DEGRADATION

A pure culture of 234-5 and a culture of consortia suspension were analyzed for rates of VOC degradation. Measurements of VOC metabolism were considered inversely proportional to the volume of the VOC remaining in the headspace.

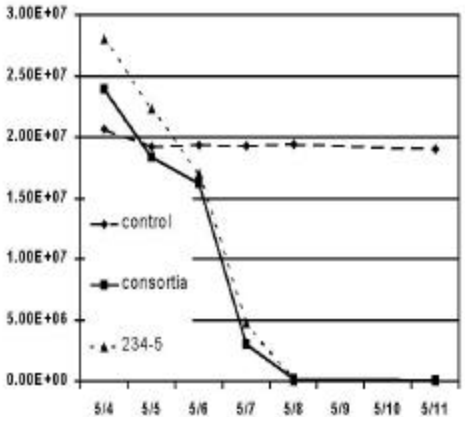


Figure 7. Propane degradation over 7 days.

The metabolism of propane by both the consortia and pure 234-5 occurs at a slightly higher rate than isobutane or n-pentane (*Figure 7*). Furthermore, within a matter of 5 days, the 2 ml of propane, added to the headspace of all bioreactors, was completely removed. Propane is not currently in use as a substrate for the biotrickling filter but would be assumed to have a positive effect on the overall growth of the biofilm. Rates of degradation between the two sample groups were not significantly different.

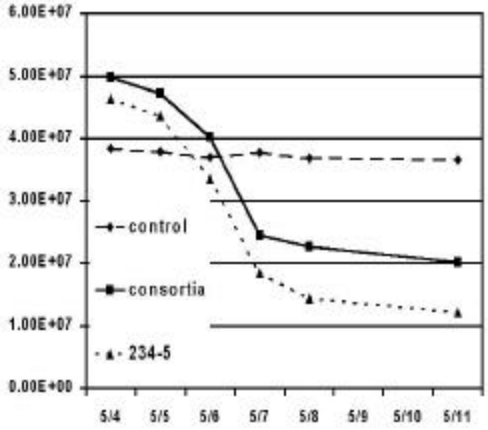


Figure 8. Isobutane degradation over 7 days.

Isobutane degradation rates are fairly consistent between the pure and whole cultures (*Figure 8*). Unlike propane, a fair amount of the VOC remains in all bioreactors after 7 days. Degradation begins to slow and will eventually stop as oxygen reserves are depleted and inhibitory metabolites begin to accumulate. Isobutane volume in the headspace of 234-5 was constantly lower than that of the consortium which can be attributed to the difference of volumes measured at time zero. Inconsistencies in gas/air mixtures when drawing from the cylinder often result in different amounts of VOC within the original 2 ml inoculum. Consequently, the difference between the rates of degradation between 234-5 and the consortia when presented with isobutane or propane is considered insignificant. Apparently, the density of 234-5 residing in the biomass is sufficient to remove certain VOCs at a rate comparable to that of a pure culture.

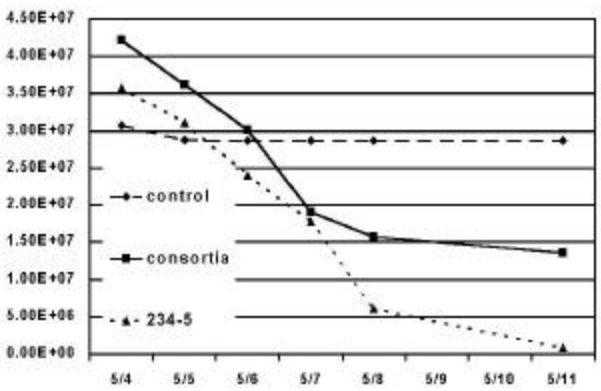


Figure 9. n-pentane degradation

Rates of degradation of n-pentane by the two sample groups vary to a greater extent than those determined for isobutane or propane (*Figure 9*). The pure culture, although originally charged with slightly less n-pentane than the whole culture, exhibited the capability to remove practically all of the VOC within 7 days. At the end of the same time frame, the

consortial degradation of n-pentane begins to flag. Certain members of the biomass tend to respire at higher rates than 234-5 and headspace oxygen becomes limited. By the same token, these symbiotic species also produce a greater spectrum of metabolites that may hinder degradative performance by increased volume and/or toxicity. Earlier assays of the removal of VOCs from a gas/air mixture concluded n-pentane as the substrate of choice (Barton et al, 1996). This preference is supported by comparing current bioreactor degradation of isobutane with that of n-pentane.

POPULATION AND DYNAMICS

Colony-forming units (CFU) per gram of wet biomass were determined along the length of the three-segment trickle-bed matrix. Results indicate that the distribution of 234-5 within the biofilm is fairly constant without regard to locale (*Figure 10*). In a biotrickling filter exhibiting 100% efficient VOC removal, speculation would suggest an increased density of the competent microbe in the areas of the column nearest the gas inlet. Greater gas volume entails more substrate for growth. Microbes that are content to survive on the metabolites of the primary VOC consumers should colonize positions furthest from the gas inlet. 234-5 should not be expected to exist in large numbers in these locations where VOC concentration is minimal after passing through the more active parts of the filter. A filter operating with 100 % efficiency will have a matrix characterized by species-specific regions throughout the column.

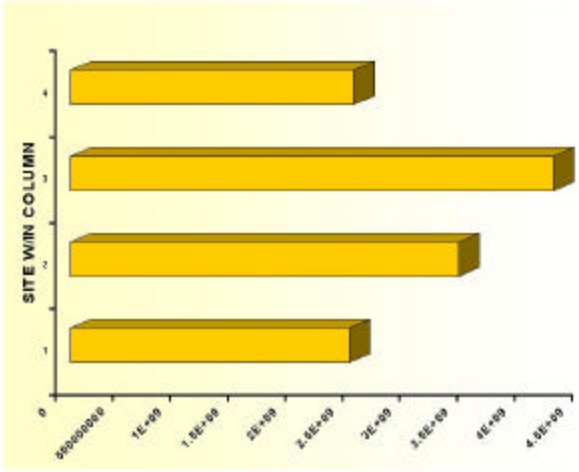


Figure 10. Population dynamics of 234-5 within the column. Values are CFU/ gm wet biomass. Position 4 represents the top of the matrix (closest to VOC input) and position 1 is

The yellow-orange pigmentation of 234-5 is consistent throughout the current biofilm. Because this biotrickling filter does not operate at a 100 % removal rate, residual VOCs are present throughout the column and pass, untreated, out of the filter. Thus, substrate is available at all locations along the column and 234-5 can flourish within, and lend its pigmentation to the entire biofilm.

CONCLUSIONS

234-5 has proven to be a unique bacterium in many respects. Although physical and chemical attempts to place this microbe within a well-defined taxonomic classification have not realized this final objective, much morphological and physiological information has been generated. Appropriate data of this nature may be applicable towards design of future biotrickling filter models.

Of all the objectives initially outlined, none were as crucial as the primary step; the isolation of VOC metabolizing microbe(s). From an active biomass, thriving for the past 18 months on minimal nutrients and dilute alkane gases, a solitary bacterium was determined to

serve as the primary harvester of carbon for the entire consortia. Techniques were developed for the rapid selection of this microbe from the whole biomass and subsequent amplification.

Experiments stemming from this isolation included the addition of a pure suspension of 234-5 to a sterile column matrix. This technique could ensure the bacterium of a colonization advantage over foreign microbes entering from an industrial environment. However, the results reveal a difference in rates of VOC uptake between pure 234-5 and the consortium as a whole that is barely significant. 234-5 is possibly the dominant microbe within the consortium at this time (the color of the biofilm is comparable to the pigmentation of the cell wall of 234-5).

The lack of aggregation or flocculation of 234-5 when grown in pure suspension indicates a possible dependence on other resources for adherence to the matrix. Symbionts capable of forming filaments, hyphae, and chains interact to form a meshwork foundation for colonization by other members, including 234-5. Therefore the development and treatment of the biofilm as a single entity is justified. 234-5 is capable of proportional rates of VOC uptake whether existing in pure form or as a member of the biofilm. However, matrix colonization potential is likely to be more significant when 234-5 is coupled with the array of other members.

Direct genetic analysis of 234-5 will facilitate identification in the future. Numerous methods of DNA extraction were addressed and ultimately exhausted during the course of this study. Suggested procedures, guaranteed protocol, and blind ventures would inevitably conclude with intact cells and a deficiency of successful DNA extract. Possible techniques to rupture the cell wall, for future reference, may lie within nebulization or sonication of greater duration and/or intensity.

This project was partially successful, achieving the majority of the primary objectives. Time, trial, and error will ultimately conceive a proficient technique for nucleic acid extraction from a species as unyielding as 234-5.

TECHNIQUES

- Large-scale, microbial isolations
- Development of bell jar incubation units
- Small-scale bioreactors
- Gas chromatography
- BBL Enterotube II©
- Biolog© Microbial Identification System
- Bergey's identification
- DNA extraction
    - Detergent lysis
    - Lysozyme treatment
    - Lysostaphin© treatment
- French Press
- Sonication
- Polymerase Chain Reaction
- Cycle sequencing
- Automated sequencing

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ISOLATION AND IDENTIFICATION OF VOC METABOLIZING MICROORGANISMS FROM AN ACTIVE BIOTRICKLING FILTER

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