PROCEEDINGS OF THE TENTH ANNUAL GENERAL MEETING OF BIOMINET

October 28, 1993
Mississauga, Ontario

Edited by W.D. Gould, L. Lortie and D. Rodrigue

COMPTE RENDU DE LA DIXIÈME RÉUNION GÉNÉRALE ANNUELLE DU BIOMINET

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Révisé par W. D. Gould, L. Lortie et D. Rodrigue

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PROCEEDINGS OF THE TENTH ANNUAL GENERAL MEETING OF BIOMINET

FOREWORD

BIOMINET presents in this volume the technical papers which were presented at the Tenth Annual General Meeting on October 28, 1993. The meeting was held at the Sheridan Park Conference Center in Mississauga, Ont. Four keynote addresses, seven technical presentations and a poster presentation were followed by a panel discussion on the future of **BIOMINET**.

We would like to thank Dr. V.I. Lakshmanan and Mr. P. Tackaberry of Ortech International, for hosting this meeting, Dr. A. J. Oliver for chairing the sessions and the speakers for their excellent presentations.

COMPTE RENDU DE LA DIXIÈME RÉUNION GÉNÉRALE ANNUELLE DU BIOMINET

AVANT-PROPOS

Dans ce volume sont publiés les articles techniques présentés lors de la dixième réunion générale annuelle du **BIOMINET** tenue le 28 octobre 1993, au Sheridan Park Conference Center à Mississauga, Ont. Quatre exposés principaux, sept présentations techniques et une présentation d'affiche furent suivies d'un forum sur l'avenir du **BIOMINET**.

Nous tenons à remercier MM. V.I. Lakshmanan et P. Tackaberry d'Ortech International, hôtes de la réunion, M. A.J. Oliver qui présidait les sessions techniques et les conférenciers pour leurs excellentes présentations.

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ABSTRACT

Biological oxidation for the treatment of refractory gold ores and concentrates has not been selected for commercial application in North America. The reasons why the domestic mining industry has not adopted this method of pre-oxidation cannot all be technical. Biooxidation is currently the process of choice of 20% of refractory gold treatment plants worldwide. If Canada and the US, where there are no plants, are excluded, the number of operations employing bacterial oxidation is nearly half. In addition, several pilot plant operations of significant size have been run in North America and have reported good metallurgical performance and successful scale-up from the laboratory. A review of the biooxidation process and the technical factors affecting the choice and operation of a biooxidation plant for refractory gold treatment is presented. The viability of the biooxidation approach to refractory gold treatment is affirmed through the presentation of the summary of the objectives and results of a recent and comprehensive pilot plant operation conducted in Canada. Reasons for the apparent reluctance of the domestic industry to adopt stirred-tank bioleaching for refractory gold treatment are discussed.

APPLICATION DE LA BIOLIXIVIATION EN RÉACTEUR À AGITATION CONTINUE POUR LA RÉCUPÉRATION ACCRUE DE L'OR DE MINERAIS RÉFRACTAIRES ET DE CONCENTRÉS

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RÉSUMÉ

L'utilisation commerciale de l'oxydation biologique pour le traitement de minerais d'or réfractaires et de concentrés n'a pas été choisie en Amérique du Nord. Les raisons pour lesquelles l'industrie minière domestique n'a pas opté pour cette méthode de pré-oxydation ne peuvent uniquement être techniques. La biooxydation est présentement le procédé utilisé par 25% des usines de traitement d'or réfractaire à travers le monde. A l'extérieur du Canada et des États-Unis, le nombre d'usines utilisant des bactéries est de bien plus que la moitié. De plus, plusieurs des opérations d'usine pilote d'échelle considérable ont été menées en Amérique du Nord et ont eu comme résultat une bonne performance métallurgique et un agrandissement avec succès à partir du laboratoire. Les raisons pour lesquelles l'industrie domestique a hésité à adopter la biolixiviation en réacteur avec agitation continue pour le traitement d'or réfractaire sont ici discutées. Une revue des facteurs techniques ayant une influence sur le choix et le fonctionnement d'une usine à biolixiviation pour le traitement d'or réfractaire est également présentée. De plus, un rappel sur la rentabilité de cette technologie est présentée à l'aide des résultats obtenus suite au fonctionnement de la plus récente et la plus étudiée des usines pilotes en opération au Canada.

INTRODUCTION

On the occasion of the 10th Annual Meeting of BIOMINET, it is appropriate to take a look at the progress made in the field of bioleaching as applied to refractory gold ores and concentrates, particularly over the past ten years. After all, the original purpose of BIOMINET, and the other Biotechnology Research Networks established in 1983, was to strengthen the Canadian biotechnological research capability and orient it towards areas of crucial importance to future Canadian economic development. For BIOMINET, organized and administered through the federal Ministry of Energy, Mines and Resources, the areas of responsibility were defined as mineral leaching and metal recovery. So, how have we done?

Bioleaching of refractory gold ores refers to a process in which certain bacteria are used to participate in the oxidation of gold-bearing sulfide minerals to liberate the contained gold through the solubilization of the sulfides. Gold can then be recovered by conventional metallurgical processing. Canada has been active in the development of microbiological leaching almost since the link between the oxidizing activity of the acidophilic bacterium *Thiobacillus ferrooxidans* and the formation of acidic drainage in coal mines was established (1). The subsequent advances made in the understanding of the biological and chemical mechanisms and factors affecting sulfide mineral oxidation and the application to the leaching of metals is well illustrated by the data in Figure 1, using copper as an example, which shows the increase in the rate of metal leaching over the period 1954 to 1985. Much of the pioneering work done to achieve these advances was carried out in Canada by organizations such as B.C. Research in Vancouver. Of particular note in this period of development was the demonstration of the potential for the biological treatment of flotation concentrates and not just ores using stirred-reactor technology (2).

The ability of certain bacteria to oxidize typical gold-bearing sulfides such as pyrite and arsenopyrite was, of course, well known before 1983. It is coincidental, however, that one of the first papers describing a serious evaluation to determine the commercial possibility of applying stirred-reactor bioleaching for refractory gold treatment appeared in Canada in BIOMINET's inaugural year (3). Following this work, many related investigations and evaluations at different scales were undertaken, with the industry no doubt encouraged to evaluate the exploitation of the significantly more complex refractory ores by the dramatic increase in the price of gold in the 1980's. Despite the obvious technical challenges and much higher processing costs, the refractory ores were seen as potentially viable sources of the precious metal. The rush was on to evaluate the best technical option. With subsequent developments, bioleaching graduated from being perceived as a low-level technology, with application apparently restricted to the scavenging of metals from low grade ores and wastes, to a technology on the shopping list of every metallurgist engaged in the process selection component of a feasibility analysis by the end of the 1980's.

Piloting of biological processes took place extensively in Canada, the US, and elsewhere in the world in the mid to late 1980's. Commercial implementation of biooxidation for refractory gold concentrates has occurred outside North America over the period since 1986. Some milestones in the development of refractory gold bioleaching in North America and the rest of the world are shown in Table 1. It is worth noting that many of the significant laboratory developments and evaluations in pilot plants shown in the table were achieved in Canada. Process parameters, control strategies and plant design criteria, understood and promoted by Canadian firms in the mid 1980's, are only now being published by commercial practitioners.

Process Selection for Refractory Ores and Concentrates

Refractory gold ores and concentrates do not respond well to conventional gold recovery processes such as cyanidation. This is because the gold in the ores is so finely disseminated within sulfide mineral assemblages that it is not liberated for recovery even by very fine grinding. Gold can be liberated for subsequent recovery only by oxidizing and solubilizing the sulfide host minerals. Selection of an oxidation process option depends on a large number of interrelated factors including; the size of ore reserves, ore mineralogy/gold occurrence, relationship between gold recovery and sulfide oxidation, operability and technical risk, value and market for by-products, environmental considerations, plant hygiene, and last but certainly not least, capital cost, operating cost and rate of return.

In the 1980's, several new refractory gold processes appeared and were evaluated by mining companies along with established technologies such as roasting. Table 2 shows a list of these oxidation processes, indicating the interest that the refractory ores received at this time. Of the new plants built and due to be commissioned since 1986, only roasting, pressure oxidation and biooxidation have been the processes of choice. The other options have not been developed to the commercial stage.

Table 3 shows the number of recent installations for refractory ore treatment, both in the world and North America. The list might not be complete, particularly with regard to roasting plants, some others of which might have been installed in China and elsewhere for which detailed records were not available. However, the data show the current trends and indicate that in the world, biooxidation has been the process of choice for 5 out of 26 installations. In North America, no commercial biooxidation plants have been built. Therefore, if we exclude North American operations, 5 out of 11 plants use biooxidation, or nearly half.

The Stirred-Reactor Biooxidation Process for Refractory Gold Ores and Concentrates

The biooxidation process for refractory ores and concentrates relies on the ability of acidophilic bacteria such as *Thiobacillus ferrooxidans* and moderately thermophilic bacteria to oxidize sulfides, typically pyrite and arsenopyrite, by direct mineral oxidation and, more significantly, through the generation of acidic ferric sulfate, a strong oxidizing agent.

A simplified schematic flowsheet representing the biooxidation process for concentrates is shown in Figure 2. The key components of the process involve concentration of a ground ore, usually by flotation, followed by oxidation of the gold-bearing sulfides to the required extent in a multi-stage reactor train. Oxidized solids discharged from the last reactor are separated from the bioleachate, washed and cyanide leached for gold extraction in a conventional CIP or other cyanidation circuit. The bioleachate containing, typically, soluble iron and arsenic, is treated by controlled lime neutralization to produce stable precipitates for disposal.

Oxidation of the sulfide host minerals in the bioleach reactors is dependent on bacterial growth and activity which are maintained at optimum process levels through control of temperature, pH, redox potential, oxygen and carbon dioxide concentrations in the gas and liquid phases, supply of other bacterial nutrients, soluble metal concentrations, and retention times in the reactors. The degree of sulfide oxidation, necessary to attain the target gold liberation for recovery in the subsequent cyanidation stage of the process, is a function of bacterial activity, retention time, reactor design, and reactor configuration. Reactor and agitator specifications are selected to ensure proper mixing conditions for solids suspension, gas mass transfer at the rates required to achieve target oxidation, and cooling for temperature control of the strongly exothermic reactions. The reactor configuration can be optimized using techniques such as logistic modeling to maximize reactor volume utilization (7). Selection of process parameters and reactor design, and the corresponding process economics, are strongly dependent on the quantity of sulfur to be oxidized per unit volume of reactor.

Exact process parameters and flowsheet configurations depend on ore mineralogy. Various flowsheet options might apply. For example, in the case of concentrates containing significant quantities of the reactive mineral pyrrhotite, FeS, provision for feed pretreatment, using recycled bioleachate, prior to the oxidation circuit, might be desirable to reduce acid consumption in the first stage of bioleaching and to minimize the reducing effect of the mineral on the system. Such a circuit was evaluated at the Dickenson Mine, Canada, pilot plant campaign (8) which will be discussed in more detail below.

An important feature of biooxidation as applied to refractory ores is the distinction that can be exploited between the relative reaction rates of the gold-bearing

sulfides. Unlike roasting and pressure oxidation which achieve rapid and total sulfide oxidation, biooxidation occurs more slowly. If the gold is predominantly contained in arsenopyrite, for example, in an ore which also contains pyrite in greater abundance, the ability in biooxidation to oxidize the arsenopyrite preferentially to the pyrite, thereby liberating gold for recovery without oxidizing all of the sulfur, can make the bacterial process attractive compared with the alternatives (9).

The disposal of iron and arsenic following precipitation as basic ferric arsenate in the solution treatment stage of the process has important environmental implications. Unlike pressure oxidation, where a high proportion of the precipitation reaction take place within the high-temperature conditions of the oxidation reactor, iron and arsenic remain in solution throughout the bioleach. If redox potentials are high (>760 mv SHE), both elements will be in their highest valency form. In these conditions, formation of stable precipitates can be achieved in a separate, controlled step, provided an adequate Fe:S ratio is present (3:1 molar ratio or higher).

To date, the biooxidation process has been applied commercially only to the treatment of concentrates. In these early days of commercial process application, this is largely due to the smaller size of plant which requires concentrate processing. The process is applicable, however, to the treatment of whole ore, and the economic feasibility of building large plants can be demonstrated. Table 4 summarizes some of the applications and advantages of whole ore versus concentrate biooxidation.

A detailed description of the bioleach process and a discussion of the many variables and flowsheet options is beyond the scope of this paper. The reader is referred to a recent review of the subject (10).

Commercial Viability of the Biooxidation Process

The commercial viability of biooxidation for the pretreatment of refractory ores and concentrates has been established as indicated by the data in Table 3. To date, four biooxidation plants have been built and are operating. A fifth plant, the largest, is nearing completion and is due to be commissioned in the second quarter of 1994. In all cases, the BIOX® technology of Biomin Technologies, South Africa, is used under license. Some details of these operations have been published in the literature (6, 11). The five commercial biooxidation plants are listed in Table 5.

The São Bento plant in Brazil is worthy of extra note since it combines a biooxidation plant and pressure leaching plant to carry out the sulfide oxidation. Figure 3 provides a simple flowsheet schematic showing that the refractory concentrate is first passed through a single stage biooxidation reactor. The partially oxidized residue, following solid-liquid separation, is then fully oxidized in a pressure autoclave. The biooxidation step was added to the circuit not only to increase plant

capacity but to improve the autoclave performance by oxidizing pyrrhotite which causes deleterious elemental sulfur formation in the autoclave, and to decompose siderite, $FeCO_3$, which forms CO_2 and thus lowers the O_2 partial pressure in the autoclave. These and other synergies in the 2-stage process will no doubt find other applications as operating experience and confidence is gained at São Bento.

In addition to the commercial plants, several pilot plant operations have demonstrated successful scale-up of microbiology and metallurgy from laboratory studies on a wide range of ores and concentrates. Table 6 provides a list of some of these pilot plant operations, many of which were conducted in Canada or elsewhere following Canadian research and development efforts.

Numerous studies to compare the technology and economics of biooxidation with the alternative technologies, roasting and pressure oxidation, have been carried out (18-23). Since process design and economics are site and ore dependent, such comparisons and general statements resulting therefrom are often misleading. However, consideration of other studies does facilitate the evaluation of process options for a given property. As a general statement, biooxidation has been shown by many to provide a viable alternative for a specific orebody.

The Dickenson Mines Biooxidation Pilot Plant Operation

A well documented biooxidation pilot plant campaign is used here to further provide evidence of the viability of the biooxidation process and to illustrate the advanced stage that process development had reached in Canada by the end of the 1980's.

The operation was conducted in 1990 at the Arthur White mine of Dickenson Mines Ltd. in Northern Ontario. Following extensive laboratory development work, a pilot plant was designed and constructed by Coastech Research Inc. of North Vancouver, British Columbia to treat the refractory concentrate produced by flotation of cyanidation tailings. The design of the plant allowed the treatment of a nominal 1 tonne per day of the Dickenson concentrate which assayed 27-32% S, 29-33% Fe, 7-11% As, and 19-27 g/t Au.

Figure 4 shows the general arrangement of the pilot plant. A feature of the plant and its operation was the provision of a feed pretreatment stage in which concentrate was contacted with recycle bioleachate to reduce pyrrhotite and remove readily soluble arsenic from the feed prior to the biooxidation stage. Further detailed description of the plant and its operation is given here. This has been provided elsewhere (8). However, it is worth summarizing and stressing the objectives and principle findings and successes of the campaign to illustrate process viability as follows:

Pilot Plant Objectives

- To demonstrate the process to the mining company and the industry at large.
- To obtain steady state operating data for process evaluation and commercial plant design.
- To evaluate reactor design performance.
- To evaluate solution management strategies for improved biooxidation performance.
- To obtain an operators perspective on plant operation.
- To evaluate plant safety and hygiene aspects of biooxidation plant operation.
- To conduct a commercial feasibility analysis.

Plant Operability

- Inoculation of the pilot plant with a bacterial culture developed at the Coastech laboratory and development of the biomass to allow start-up of continuous feed operation proceeded on schedule.
- Steady state continuous feed operation was readily established and target feed rates obtained and maintained.
- Variations in feed composition were handled without process interruptions.
- A 10 hour power interruption at the mine site did not cause a significant process interruption, although return to target process performance was somewhat sluggish.

Metallurgical Performance

- A first stage retention time of 65 h allowed target sulfide oxidation to be achieved while operating at a feed solids concentration of 14% (w/w).
- Oxygen uptake rates calculated from off-gas analysis correlated well with rates calculated from solid residue analyses and corresponded to the stoichiometry of both pyrite and arsenopyrite oxidation.
- Incorporation of a high rate thickener for solution exchange in the first biooxidation stage increased oxygen uptake rates to up to 1 kg O_2 $h^{-1}m^{-3}$ while operating at 14 % feed solids concentration.
- The critical dissolved oxygen concentration for the chemical oxidation of sulfides was established. The relationship between CO_2 consumption and the dissolved oxygen concentration indicated that the critical DO_2 value for bacterial sustenance is lower than for chemical oxidation. Controlled biooxidation under chemical oxidation rate-limiting conditions is therefore possible and might be desirable in specific operations.
- Laboratory process development had indicated that over 90% gold recovery could be achieved in cyanidation following 65-70% sulfide oxidation. This was achieved in the pilot plant operation, with 90% or greater gold extractions obtained from residues over 40 days of continuous feed operation.

- A high Fe:As ratio in solution was maintained at high redox potentials throughout the campaign. Precipitation of a ferric arsenic precipitate which passed the Ontario MOE Regulation 309 leach test was achieved using a non-optimized single-stage precipitation reactor.

Mechanical Performance

- Plant feed systems and reactor peripherals performed well over the duration of the campaign.
- A simply constructed high-rate thickener performed well as a solution exchange system for solution composition control and improved metallurgical performance.
- The mechanical performance of a variety of agitators, pumps and other equipment was assessed and provided useful information for specifying equipment in future operations.

Bioleach Circuit Optimization

The bioleach circuit was optimized and the oxidation profiles as a function of reactor stage determined through carrying out the following tasks:

- Determination of sulfide oxidation and oxidation rates for each reactor.
- O_2 and CO_2 uptake rates were determined by measuring inlet and off-gas concentrations.
- Gold extraction profiles for the reactor train were determined for each steady state condition (retention time profiles).
- Logistics modeling was applied to determine optimum reactor configuration for process feasibility.

Reactor Optimization

Detailed studies were carried out on one of the primary stage bioreactors to determine essential design parameters for process scale-up. The following parameters and relationships were determined:

- Calculated and measured power draw.
- Mass transfer performance at varying superficial gas velocities ($m.s^{-1}$), varying specific agitation ($W.m^{-3}$), and k_la values calculated.
- Gas hold up at varying superficial gas velocity and specific agitation values, and the volume of gas hold up.
- Solids suspension by determining particle size profiles under varying reactor conditions.

Plant Health and Safety

- All air samples taken in the confined space of the pilot plant building were below allowable limits for all substances including sulfuric acid, hydrogen sulfide, arsine, carbon monoxide, carbon disulfide, and airborne arsenic.
- Arsenic in urine was below limits for all operators.
- No unusual plant safety precautions were required.

Operators Perspective

Several of the pilot plant operators had conventional milling experience. Their observations are pertinent for commercial bioleach operations.

- Training for biooxidation plant operation was accomplished in a short time period. Similar skill levels are required as for other milling operations.
- Level of control required not sophisticated compared with other oxidation pretreatment options. Accurate feed control essential as well as continuous pH, redox potential measurement and continuous control of temperature and dissolved O_2.
- Biomass maintenance easily achieved without direct biomass measurement. Simple on-stream process measurements provide adequate indicators of biomass performance. Off-gas analysis provides instantaneous measure of bacterial activity.
- Good working conditions, with health and safety considerations similar to a cyanidation circuit.
- Labour requirements would be small for commercial operation.
- Maintenance is relatively easy. Preventative maintenance required due to corrosive bioleachate.
- The process was seen to be relatively forgiving when encountering changing feed characteristics, mechanical variations or down-time.

Why Are There No Biooxidation Plants in North America?

The commercial success of biooxidation as a pretreatment for refractory gold concentrates and the results of pilot plant operations such as that carried out at the Dickenson Mine described here, leaves little room for doubt that the technology is viable. Yet, the fact remains that no plants are operating in Canada or the United States.

The previously discussed data in Table 3 shows clearly that pressure oxidation has been the process of choice in North America (9 recent installations) followed by roasting (6 installations). In a recent review of methods for precious metal recovery in North America (24), it was perhaps whimsically observed that it is difficult to believe that the potential success of biooxidation is a phenomenon associated only with the

southern hemisphere. The same authors, however, suggest that power requirements associated with whole ore biooxidation make this option more costly than pressure oxidation. However, an informal survey of some members of the industry indicated that it is perhaps the perceptions of technical and economic matters, and the unsuitability of refractory orebodies exploited to date in North America, that are the main reasons for the lack of commercial application. Following are some possible reasons for the non-acceptance of biooxidation in North America.

Perception of Greater Technical and Environmental Complexity and Risk

Despite the success of commercial biooxidation in South Africa, Australia and Brazil, there is a perception in some quarters of the domestic industry that biooxidation is an unproven technology. Certainly in the 1980's, biooxidation had more to prove than did the competing technology of pressure oxidation for which full scale operating experience had already been gained. Then as now, biooxidation was often seen to be more complex than pressure oxidation which could be easily defined. It is interesting to note that one of the criteria for process selection at the new Ashanti biooxidation plant was that the process should be simple for African operation. Another perception was that big problems would be encountered in plant scale-up particularly with regard to aeration and agitation. Again, the Ashanti plant and the single stage 580 m^3 reactor at São Bento has helped to dispel such beliefs. GENMIN (Biomin) wisdom suggests that 880 m^3 is the maximum reactor size to allow confident design of agitation and aeration systems (6). Larger tanks might be possible but even at 880 m^3, a large concentrate or ore oxidation plant, utilizing parallel reactor trains can be envisaged.

Another perception of biooxidation is that it provides greater environmental risk than pressure oxidation. This specifically refers to the requirement for disposal of leached iron and arsenic in an environmentally stable form. The formation of stable ferric arsenate from pressure oxidation can be confidently predicted, mainly due to the formation of stable crystalline precipitates such as scorodite, $FeAsO_4.2H_2O$, under pressure oxidation conditions. The formation of ferric arsenates under the lower temperatures of biooxidation, however, gave rise to concern about long term stability. Much of this misgiving arose from a misunderstanding of biooxidation chemistry. As previously discussed, iron and arsenic remain largely in solution during the oxidation stage, precipitation in the reactors is minimized, and stable ferric arsenate can be formed under controlled conditions in a separate process stage. Several studies over the past few years have confirmed the environmental acceptability of precipitates.

Conservative and Risk-Averse Industry in North America

The mining industry is by necessity conservative and risk-averse. For new technologies such as biooxidation, novelty is an additional factor which, even if

technical and economic parity or superiority with other technologies can be demonstrated, can weigh against its selection as the process of choice for large scale application. Whichever pretreatment process is selected, the oxidation step will often represent only a small proportion of the cost of the overall design from mining of the ore to the production of gold bullion, especially for large tonnage operations. It could be anticipated, therefore, that until biooxidation had proven itself as a viable commercial process, it would be initially ignored or considered only for small scale application where the oxidation step has a more significant impact on overall project cash flow. Larger operations would more likely employ more proven processes due to lower inherent risk factors.

However, perhaps the domestic industry is more conservative than its counterparts in South Africa and Australia. Not that this is a criticism, for there are good reasons for it to be so. Development of new technologies in the mining industry in South Africa, where political isolation has required self-sufficiency and where the industry is dominated by very large and long-lived companies which have invested heavily in long-term R and D, has been encouraged in a way we do not generally see here. Similarly, investment in new technologies in North America has over the past few years been tempered by financial restraint in a way not apparently evident in Australia, for example, where capital for new ventures seems to be more easily raised on its stock exchanges.

Lack of Support and Incentives

Financing of the development of commercial biooxidation in North America has been carried out to a large degree by small companies established specifically for the purpose. In large measure, much of the leading edge work done in Canada was done on small budgets, financed out of cash flow from other enterprises. Once a leader in the number of companies developing biooxidation for refractory gold ores, Canada can now boast only one or two companies who can offer realistic and practical laboratory test programs for process development.

Specific mining companies have invested money and resources in supporting specific projects but, in general, the industry has not supported development of the technology in a manner that can be seen in other countries. Certainly less money is spent in Canada on R and D than in most other industrialized nations. Have the entrepreneurial and mining companies been at a disadvantage in taking financial risks, making commitments to industrial R and D, and in being able to maintain successful programs due to a lack of tax incentives and science policy? In Canada, government has supported the development of biotechnology through the efforts of organizations such as BIOMINET but again, investment and support in a technology in which Canada was a clear leader in the 1980's, has been disappointing.

Poor Promotion and Publicity

As stated above, the development of biooxidation was largely fronted by small entrepreneurial companies. The initial strategies of these companies was to derive royalties, equity positions, or other types of business arrangements in return for improving gold recoveries at a particular mine. Such strategies proved to be the demise of some companies as the industry was not impressed by the promise of such arrangements and clearly biooxidation suffered an acceptability problem. To some degree this is related to the nature of the industry here which in a few cases would rather spend years itself climbing the learning curve rather than rely on the expertise of specialist groups (the Not-Invented-Here syndrome). This go-it-alone philosophy resulted in several attempts at unsuccessful biooxidation application leading to rejection of the technology as unsuitable. A notable example of this was the full scale plant built at the Tonkin Springs Mine in Nevada. This plant was apparently constructed without prior piloting and without proper engineering of the critical biooxidation stage. Industry held its breath during construction and the conspicuous plant's failure to perform was a bad blow for the technology. Other promoters of the technology similarly did it a disservice by prematurely claiming commercial readiness.

Lack of Applicable Orebodies in North America

Probably every refractory orebody in North America which has been evaluated for possible development, whether a positive production decision was made or not, was tested for its biooxidation potential in the period 1983 to the present time. Since biooxidation was not selected in any case that went commercial, it is interesting to look at those for which roasting and pressure oxidation was selected to determine if any might have been suitable for a bacterial oxidation process. Table 7 lists the recent commercial roasting and pressure oxidation installations in North America and the rest of the world.

Inspection of the list indicates that for the roasting plants of North America, biooxidation would not have been a suitable process for these orebodies. In Canada, the Giant Yellowknife operation predates the development of biooxidation and at Golden Bear, the clayey nature of the ore makes hydrometallurgical processing very difficult. The U.S. orebodies processed by roasting are all carbonaceous, for which biooxidation is largely ineffective if significant quantities of gold are carbon-associated.

For pressure oxidation, the Canadian orebodies would be unsuitable for biooxidation. At Campbell Red Lake, the timeline requirements for a replacement to the roaster for the treatment of a difficult concentrate did not allow sufficient biooxidation process development lead time at a time when commercial experience was lacking. Eskay Creek is a large and extremely complex sulfide orebody for which

pressure oxidation is more ideally suited. The processing of arsenic materials at Nerco Con is a special case requiring pressure oxidation treatment.

Of the US orebodies, the McLaughlin plant predates biooxidation development and Barrick Mercur is an alkaline oxidation leach. Barrick Goldstrike ore is very amenable to biooxidation but the large size of the operation coupled with the lack of commercial biooxidation of ores made pressure oxidation the logical choice. Similarly, the Getchell and Lone Tree ores are amenable to biooxidation. The former ore is however complex with respect to the arsenic mineralogy and biooxidation was realistically perceived to be a technical risk at the time of the production decision. The decision to use pressure oxidation at Lone Tree was no doubt based on the lack of whole ore biooxidation experience and the success of pressure oxidation in Nevada.

In summary, the claim that there have been no suitable ores or concentrates for biooxidation application in North America, based on recent commercial installations appears to be quite well founded. There are however, numerous smaller potential applications involving concentrates (and ores) which have been evaluated and in some cases piloted successfully, but for which no production decision has been made. These decisions are likely influenced by one or more of the factors discussed above.

CONCLUSIONS

The viability of biooxidation as a technology for the pretreatment of refractory gold concentrates has been established through the installation of five commercial plants and the successful demonstration of process scale-up from laboratory to pilot plant at a number of mine sites around the world. This paper has described the process and the factors important in its selection and application. An example of the leading position in the engineering knowledge and application of the technology once enjoyed by Canada has been provided through a summary of pilot plant operations conducted at a Canadian mine site. Much of the momentum in process development has been lost due to non-application in North America. Technology has been transferred to overseas interests through collaborative arrangements or involvement of foreign firms in North American biooxidation development projects. All commercial applications of the technology are offshore.

The North American mining industry has not adopted the technology, preferring to select either pressure oxidation or roasting for commercial application at 15 recent projects in Canada and the US. An examination of the reasons for the apparent reluctance of the industry to apply biooxidation reveals that a combination of factors probably apply. For most of the 15 projects, biooxidation was not appropriate due either to ore mineralogy or to the size of plant required at a time when concentrate processing experience and confidence was still lacking. For whole ore

processing, biooxidation experience is still limited to pilot plant scale, and the choice of pressure oxidation or roasting has been understandable in past process selections, notwithstanding the perhaps more conservative nature of the domestic mining industry relative to other parts of the world and a relative lack of support and incentives.

There remain, however, potential operations for which biooxidation would be an ideal candidate. In many of these cases, non-application of biooxidation is likely due to the fact that the technology has suffered from a perception of greater technical and environmental risk. In addition it has received poor publicity due both to marketing strategies that were disliked by the industry, and to premature and ill-conceived attempts at commercialization. The success of the commercial biooxidation plants elsewhere in the world will help to dispel the technical and environmental perceptions and image problems in North America. As operating experience and confidence in the technology is gained at these operations, application in North America for suitable orebodies will no doubt follow. It is hoped that the significant body of biohydrometallurgical and engineering expertise in Canada will play a part in future developments.

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REFERENCES

1. **Colmer, A.R., M.E. Hinkle.** 1947. The role of microorganisms in acid mine drainage: A preliminary report. Science, **106**:252-56.

2. **Bruynesteyn, A., D.W. Duncan.** 1970. Microbiological leaching of sulfide concentrates. Canadian Metallurgical Quarterly **10** (1):57-53.

3. **Lawrence, R.W., A. Bruynesteyn.** 1983. Biological preoxidation to enhance gold and silver recovery from refractory pyritic ores and concentrates. CIM Bulletin, **76** (857):107.

4. **Pinches, A.** 1975. Bacterial leaching of an arsenic-bearing sulfide concentrate. In: Leaching and Reduction in Hydrometallurgy. A.R. Burkin (Ed.), Inst. Min. Met., London, pp.28-34.

5. **Marchant, P.B., R.W. Lawrence.** 1986. Flowsheet design, process control and operating strategies in the biooxidation of refractory gold ores. In: Proc. 3rd Annual Meeting of BIOMINET. R.G.L McCready (Ed.), CANMET Special Pub. SP86-9, Ottawa, pp.39-51.

6. **Van Aswegen, P.C.** 1993. Commissioning and operation of biooxidation plants for the treatment of refractory gold ores. In: Hydrometallurgy - Fundamentals, Technology and Innovation, J.B. Hiskey and G.W. Warren (Eds). Soc. Min. Met. and Exploration, AIME, pp.709-725.

7. **Pinches, A., J.T. Chapman, W.A.M. Te Riele and M. Van Staden.** 1988. The performance of bacterial leaching for the preoxidation of refractory gold bearing sulfide concentrates. In: Proc. Intl. Conf. on Biohydrometallurgy, Warwick, 1987. P.R. Norris and D.P. Kelly (Eds). Science and Technology Letters, New, England, pp.329-344.

8. **Chapman, J.T., P.B. Marchant, R.W. Lawrence and R. Knopp.** 1993. Biooxidation of a refractory gold-bearing high arsenic sulphide concentrate: A pilot study. FEMS Microbiological Reviews **11**:243-252.

9. **Lawrence, R.W., P.B. Marchant.** 1988. Biochemical pretreatment in arsenical gold ore processing. In: Arsenic Metallurgy - Fundamentals and Applications, R. Reddy, J.L. Hendrix and P.B. Queneau (Eds). TMS-AIME, Warrendale, PA, pp.199-211.

10. **Lawrence, R.W.** 1990. Biotreatment of gold ores. In: Mineral Microbial Recovery. H.L. Ehrlich and C.L. Brierley (Eds), McGraw-Hill Publishing Co., New York, pp.127-148.

11. **Nicholson, H.** 1993. Selection of a refractory gold treatment process for the Sansu Project. Biomine 93, Australian Mineral Foundation, Glenside, South Australia, pp. 20.1-20.11

12. **Marchant, P.B.** 1986. Commercial piloting and the economic feasibility of plant scale continuous biological tank leaching at Equity Silver Mines Limited. In: Fundamental and Applied Biohydrometallurgy. R.W. Lawrence, R.M.R. Branion and H.E. Ebner, (Eds), Elsevier Science Publishing, New York, pp.53-76.

13. **Hackl, R.P., F.R. Wright.** 1989. Scale-up experiences in biooxidation of refractory gold ores and concentrates. Proc. Randol Gold Conference, Phase IV, Sacramento, November 9-11, pp.123-127.

14. **Moore, D.C.** 1989. Development of a biooxidation leach process at Asamera Minerals (U.S.) Cannon Mine. In: Biotechnology in Minerals and Metal Processing,. B.J. Scheiner, F.M. Doyle and S.K. Kawatra (Eds), Society of Mining Engineers, AIME, Littleton, Colorado, pp.123-130.

15. **Marchant, P.B., R.W. Lawrence, J.T. Chapman, W. Brooks and J. Kuipers.** 1989. Bioleaching at Austin Gold Venture: Project summary. 118th Annual Meeting of AIME, Las Vegas, Nevada, February 27 - March 2.

16. **Emmett, R.C., R.P. Klepper, L.W. Luinstra, L.T. O'Connor and J.T. Sitarski.** 1989. Experience with a large scale bioleaching pilot plant on a refractory gold concentrate. Proc. Biohydrometallurgy 89 Symposium, Jackson Hole, Wyoming, August 13-18.

17. **Neale, J.W., A. Pinches, H.H. Muller, N.H. Hannweg and P. Dempsey.** 1991. Long term bacterial oxidation pilot plant operation at Mintek and Vaal Reefs. Proc. Colloquium on Bacterial Oxidation, SAIMM, Johannesburg.

18. **Renner, C.W., M.T. Errington, D.J. King and F.D. Pooley.** 1984. Economics of bacterial leaching, 23rd Conf. Metallurgists, CIM, Quebec, August p. 19-22.

19. **Haines, A.K.** 1986. Factors influencing the choice of technology for the recovery of gold from refractory arsenical ores. In: Gold 100. Proc. Intl. Conf. on Gold. Vol. **2**. Extractive Metallurgy of Gold, SAIMM, Johannesburg, pp.227-33.

20. **Randol,** 1987. Gold and Silver Recovery Innovation Phase III. Randol International Ltd., 21578 Mountsfield Drive, Golden, CO 80401, USA. Vol. **3**, p. 1420.

21. **Gilbert, S.R., C.O. Bounds and R.R. Ice.** 1988. Comparative economics of bacterial oxidation and roasting as a pre-treatment step for gold recovery from an auriferous pyrite concentrate. CIM Bulletin, **81** (910):89-94.

22. **Litz, J.E., W. Carter and C.W. Kenney.** 1990. Refractory gold treatment options. Economics rule the way. Proc. Randol Gold Forum,Squaw Valley, NV, September, pp.39-44.

23. **Carter, A.J.** 1991. Economic comparison of the alternative methods for the recovery of gold from refractory gold ores. Proc. Colloquium on Bacterial Oxidation, South African Inst. Min. and Met., Johannesburg.

24. **Marsden, J.O., J.G. Mansanti and S.A. Sass.** 1993. Innovative methods for precious metal recovery in North America. Mining Engineering, September, pp.1144-1151.

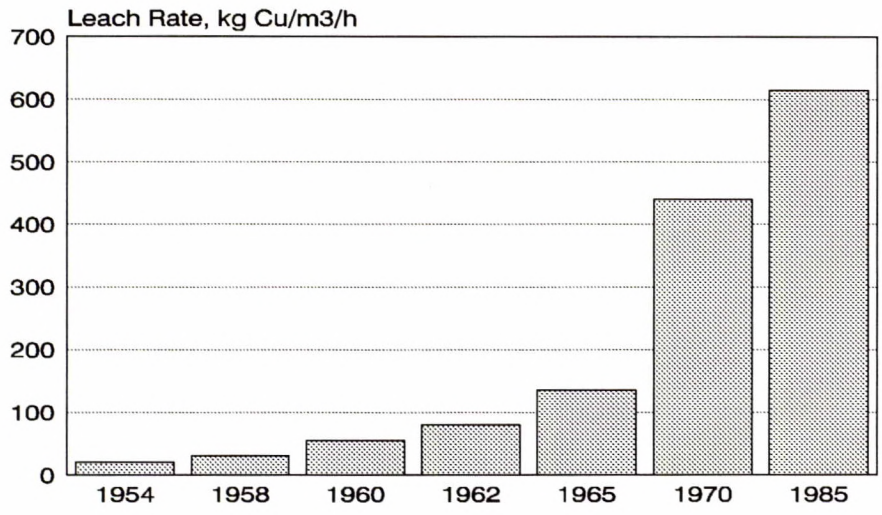


Figure1. Progress in the development of bioleaching showing the increase in copper leaching rates over the period 1954 to 1985

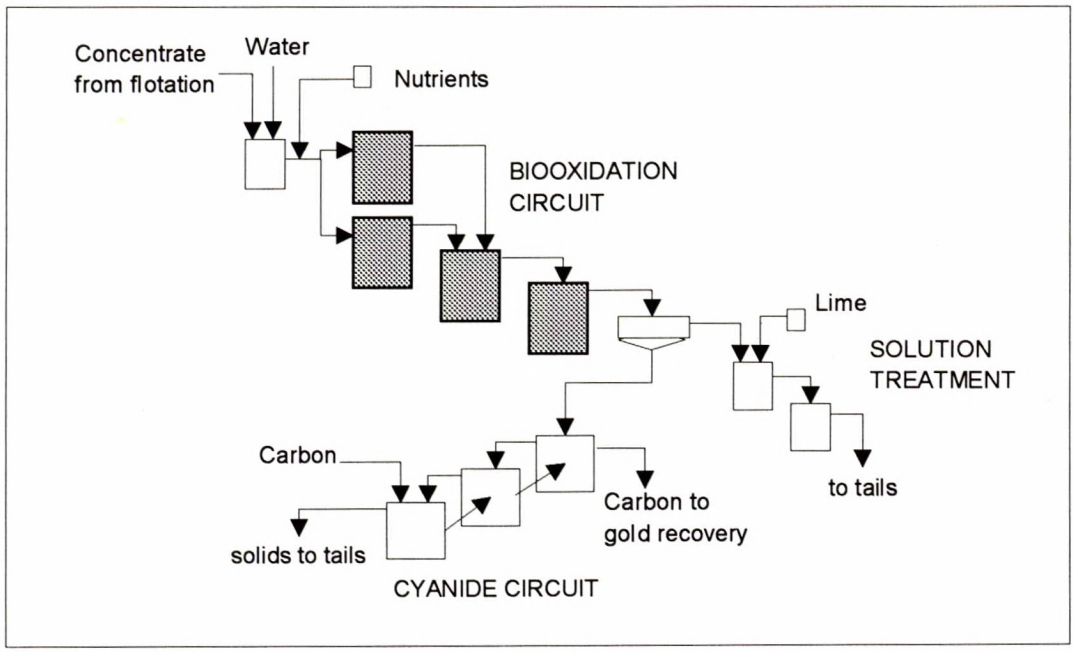


Figure 2. Simplified flowsheet of the biooxidation process

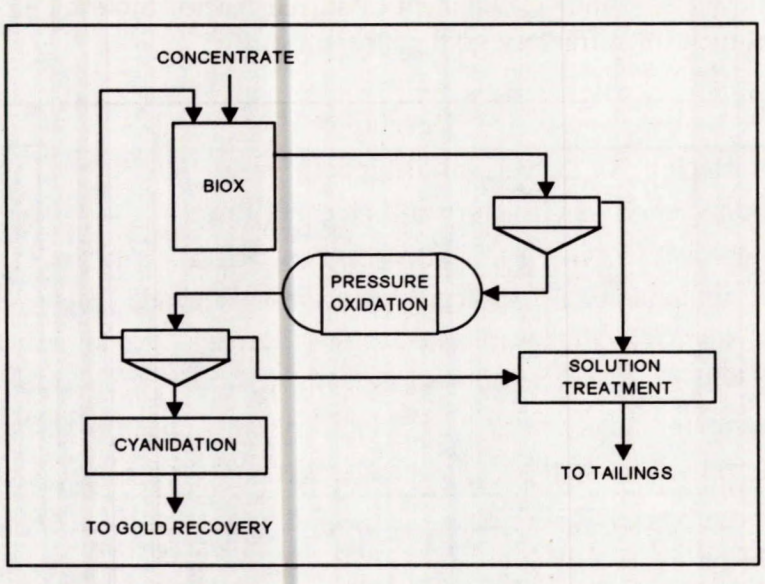
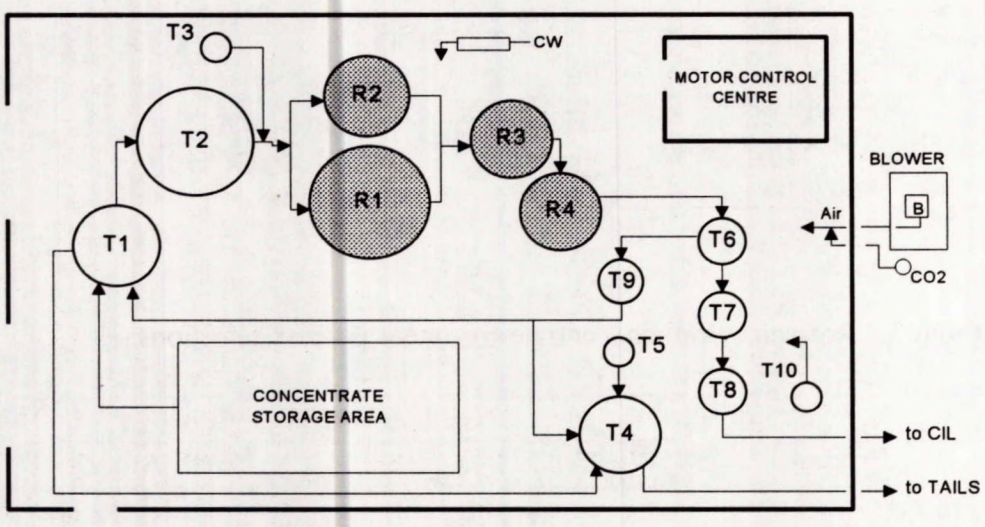


Figure 3. Combination of biooxidation and pressure oxidation for refractory concentrate treatment



T1	FEED PREPARATION TANK	T6 - T8	CCD THICKENERS
T2	FEED HOLDING TANK	R1 - R4	BIOOXIDATION REACTORS
T3	NUTRIENT STOCK TANK	T9	ACID HOLDING TANK
T4	NEUTRALIZATION REACTOR	T10	FLOCCULANT STOCK TANK
T5	LIME STOCK TANK	CW	COOLING WATER SUPPLY

Figure 4. Dickenson pilot plant general arrangement

Table 1. Milestones in the development of stirred-reactor bioleaching for the treatment of refractory gold concentrates

Year	Development	Ref
1975	Bioleaching of arsenic sulfide concentrate	4
1983	Biooxidation of refractory sulfide concentrate	3
1985-90	Biooxidation pilot plants in North America	see text
1986	Design and control strategies published in Canada	5
1986	Fairview plant commissioned in South Africa	6
1991	Saõ Bento plant commissioned in Brazil	6
1992-93	Harbour Lights and Wiluna plants commissioned in Australia	6
1994	Ashanti plant start-up scheduled in Africa	6

Table 2. Refractory gold ore/concentrate oxidation treatment options in the 1980's

Oxidation Processes
Fluo-Solids Roasting
Pressure Oxidation
Biooxidation
Arseno (or Redox) Process
Nitrox Process
Chlorination

Table 3. Refractory ore/concentrate treatment process selections

Process	Number Of Installations	
	World (incl N. America)	North America
Roasting	11 (1 in construction)	6 (1 in construction)
Pressure Oxidation	10 (2 in construction)	9 (2 in construction)
Biooxidation	5 (1 in construction)	0
Other	0	0

Table 4. Comparison of the biooxidation of ore and concentrate

Ore	Concentrate
Applications: Poor flotation recovery Low concentration ratios Low acid consumption Low clay content	**Applications:** High acid consuming ore Low sulfur ore High flotation recovery Low weight recovery
Advantages: Higher pulp density Reduced cooling requirements Reduced retention times Reduced solution chemistry problems	**Advantages:** Smaller plant size Improved solid/liquid separations

Table 5. Commercial biooxidation plants

Plant	Sulfur (%)	Arsenic (%)	Capacity (tonnes/day)
Fairview, South Africa	23.0	4.5	40
Harbour Lights, Australia	18.0	8.0	40
Wiluna, Australia	24.0	12.0	115
São Bento, Brazil	18	15	150
Ashanti, Ghana			760

Table 6. Biooxidation pilot plants (1985 - 1992)

Pilot Plant Operation	Country	Ref
Equity Silver Mine	Canada	12
Congress Mine	Canada	13
Salmita Yellowknife Mine	Canada	13
Campbell Red Lake Mine	Canada	na
Dickenson Mine	Canada	8
Cannon Mine	US	14
McLaughlin Mine	US	na
Austin Gold Venture Mine	US	15
Riverlea Mine	Zimbabwe	16
Luleå Technical University	Sweden	na
Vaal Reefs, No.8 Shaft Mine	South Africa	17
Mintek	South Africa	17

na Published information not available

Table 7. Recent roasting and pressure oxidation installations (world)

Roasting		Pressure Oxidation	
Golden Bear	Canada	Campbell Red Lake	Canada
Giant Yellowknife	Canada	Nerco Con	Canada
Cortez	US	Eskay Creek (in design)	Canada
Jerritt Canyon	US	Barrick Mercur	US
Big Springs	US	Barrick Goldstrike	US
Gold Quarry (in constr.)	US	McLaughlin	US
New Consort	South Africa	Getchell	US
Syama	Mali	Lone Tree (in constr.)	US
Billiton-Bogusu	Ghana	São Bento	Brazil
Kalgoorlie Cons	Australia	Porgera	PNG
North Kalguri	Australia		

THE NEED FOR *THIOBACILLUS FERROOXIDANS* STRAIN SELECTION IN APPLICATIONS OF BIOLEACHING

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ABSTRACT

As bioleaching systems differ considerably with respect to their physical and chemical characteristics, it is important to select a strain of *Thiobacillus ferrooxidans* which is suited to the conditions, especially as the extent of variation among strains of this bacterium can be significant. In this communication, we show strain variability with respect to temperature, resistance to heavy metals, and plasmid DNA content.

With respect to temperature, ten strains showed different rates of growth at test temperatures between 2 and 35 °C, and some did not grow at the lowest temperatures. Moreover, strains isolated from a particular mining environment had different temperature growth profiles. Surprisingly, this was also true when the temperature of the environment was low and supposedly a selective pressure. Survival to exposure to moderately-high temperatures was also strain dependent, as was sensitivity to freezing. With respect to susceptibility to certain metals common to mining environments, the strains showed different levels of resistance to copper, nickel, uranium, and thorium. As for their genetic makeup, the strains exhibited different plasmid profiles.

Such variability suggests that the strains most efficient with respect to a particular leaching process are likely to be found *in situ.*

NÉCESSITÉ DE SÉLECTIONNER LES SOUCHES DE *THIOBACILLUS FERROOXIDANS* POUR DES APPLICATIONS DE LIXIVIATION BIOLOGIQUE

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RÉSUMÉ

Étant donné les grandes différences observées entre les caractéristiques physiques et chimiques des divers systèmes de lixiviation biologique, il est important de sélectionner une souche de *Thiobacillus ferrooxidans* qui convienne, d'autant plus que des variations importantes peuvent être observées entre les diverses souches de cette bactérie. Notre rapport fait état de la variabilité des souches relativement à la température, la résistance aux métaux lourds et la teneur en ADN plasmidique.

En ce qui concerne la température, on a observé des taux de croissance différents pour dix souches à des températures comprises entre 2 et 35 °C, certaines souches ne montrant aucune croissance aux températures les plus basses. De plus, on a observé des profils différents de croissance avec la température pour les souches isolées d'un milieu minier particulier. Chose curieuse, ce phénomène a également été observé lorsque la température du milieu était basse et qu'elle était censée exercer une pression de sélection. La survie en cas d'exposition à des températures moyennement élevées dépend également des souches, de même que la sensibilité au gel. Pour ce qui est de la sensibilité à certains métaux couramment présents dans l'environnement minier, on a observé des niveaux différents de résistance des souches au cuivre, au nickel, à l'aluminium et au thorium. Quant à la composition génétique des souches, différents profils plasmidiques ont été observés.

Une telle variabilité suggère que les souches les plus efficaces pour un procédé particulier de lixiviation se retrouvent possiblement *in situ*.

INTRODUCTION

The chemolithotrophic bacterium *Thiobacillus ferrooxidans* is the major microorganism of bioleaching operations (1). It dominates mineral leaching sites because it has the ability to oxidize both iron and reduced sulfur compounds and, shows unusually high tolerance to the low acidity typical of such environments (2, 3). The significance of *T. ferrooxidans* in bioleaching is a result of its mode of metabolism. Metals can be released from sulfidic ores by a direct or an indirect mechanism. In other words, the bacterium can solubilize metals either directly, by oxidative attack on the mineral sulfide, or indirectly, by an interaction involving the production of a reagent that causes solubilization to occur. In field conditions, direct and indirect oxidation are undoubtedly occurring in concert (1) . With regard to direct oxidation, several different minerals can be acted upon by the cells of *T. ferrooxidans*. These include the sulfides of copper, nickel, lead, iron, gallium, cobalt, and zinc (4, 6), and the oxides of uranium (1).

Therefore, the various ecological factors that affect the activity of *T. ferrooxidans* also affect the efficiency of the bioleaching process. The major factors are temperature, pH, iron supply, oxygen, the availability of other nutrients required for growth, and the presence of toxic substances in the environment. These must be optimal for the process of interest to occur at optimal rates.

The advent of gene cloning techniques in microorganisms has raised exciting possibilities for the microbiologist to produce new strains of *T. ferrooxidans* having traits which would increase the efficiency of the bioleaching process. However, the bacterium's unusual physiology creates special problems in genetic research (7) . In addition, federal and provincial regulations prohibit the release of genetically-engineered microorganisms unless extensive laboratory studies have been previously conducted.

Another approach which may be used to increase the efficiency of the bioleaching process is strain selection. It is well known that native populations of *T. ferrooxidans* show considerable genomic and phenotypic diversity. In fact, Harrison (8) concluded that *T. ferrooxidans* shows a wide genomic diversity encompassing five DNA homology groups. It is assumed that such genetic diversity leads to phenotypic diversity. Furthermore, the reports by Yates and Holmes (9) , Holmes *et al.* (10) , and Schrader and Holmes (11) show that high-frequency mutations in *T. ferrooxidans* are common. It has been suggested that the commonly-observed ability of *T. ferrooxidans* to adapt to specific laboratory culture conditions such as pH and resistance to metals may be explained by the wide-spread occurrence of such mutants (7).

It is in this light that we report the results of a study which shows strain variability in native populations of *T. ferrooxidans* with respect to temperature, resistance to heavy metals, and plasmid DNA content. An understanding of this variability is important as it underscores the need for strain selection in applications of bioleaching.

MATERIALS AND METHODS

Temperature Studies

Growth Rates

A total of ten strains of *T. ferrooxidans* were characterized with respect to temperature: isolates D2, D6 and D7 were isolated from the depths of a uranium mine (Denison Mines Ltd., Elliot Lake, Ontario); isolates F1 and N1 were indigenous to tailings areas from Falconbridge Nickel Mines Ltd., Falconbridge, Ontario and Noranda Mines Ltd., Noranda, Québec, respectively; isolate R1 was collected from Rio Algom Mines Ltd., Elliot Lake, Ontario; isolates N2, W1 and A1 were gifts from Dr. Marvin Silver (Dalhousie University, Halifax, Nova Scotia); and, isolate S2 (ATCC 33020) was purchased from the American Type Culture Collection (Rockville, Maryland, U.S.A.).

All bacteria were enriched in the iron salts broth (TK) of Tuovinen and Kelly (12), pH 2.1, and plated on a modification of Manning's (13) iron-salt purified (ISP) agarose medium. After three single-colony isolations, the native isolates were identified as *T. ferrooxidans* by microscopic examination.

For each isolate, triplicate shake-flasks containing 100 mL of fresh TK broth (pH 2.1) were inoculated with 5.0 mL of stationary phase cells and incubated at the appropriate temperature (2, 5, 10, 15, 20, 25, 30 and 35 °C, respectively) in a gyratory shaker at 150 rpm. For the test temperatures in the range 2-35 °C, the rate of oxidation of ferrous iron was determined titrimetrically over the course of the growth cycle, using 0.01 N $KMnO_4$. Residual ferrous iron concentrations were transformed to ferric iron concentrations so that growth curves (natural logarithm of the ferric iron concentration versus time) could be constructed from the data.

The period of linearity representing an exponential rate of ferrous iron oxidation was obvious by visual inspection of the plots. Therefore, a line of best fit was computer-generated by a simple linear regression analysis of the data, and the equation of the line was used to calculate the growth parameter generation time (g) using the following relationship between the slope of the line (m) and g: $g = 0.693/m$. Mean generation time (± the standard error) was determined for each set of triplicates for each isolate. It is assumed that an exponential rate of iron oxidation reflects an exponential rate of cell proliferation.

Temperature Growth Profiles

Temperature growth profiles were determined for isolates of *T. ferrooxidans* native to uranium mines. Mine-water samples were collected from active bioleaching areas at Denison Mine and Stanleigh Mine in Elliot Lake, Ontario. Flood leaching and spray

leaching areas were sampled at both mines. The temperature of the leachate was recorded at the time of sampling, and all samples were refrigerated until processing, which was usually within 24 h.

Samples were serially diluted in the range 10^{-1} to 10^{-4} using TK broth supplemented with 0.05% Tween 80 as diluent. Tween 80 aided in the spreading of the inoculum onto the surface of the agarose-solidified medium. For each sample, the dilutions were plated in triplicate onto ISP agarose using a 0.2 mL inoculum. To minimize the loss of psychrophiles and psychrotrophs, uninoculated plates were equilibrated at 18 °C prior to inoculation, and two bent glass rods were used alternately for spreading the inocula to allow a sufficient cooling-off period.

Following an incubation at 18 °C for two weeks, colonies were counted at 7 to 10 day intervals until the counts were the same for two consecutive determinations. Only those colonies having an orange-rust color, indicative of iron oxidation, were counted. Subsequent to the 18 °C incubation, approximately 20 colonies from a representative plate were randomly selected for temperature characterization. The entire colony was lifted from the plate and placed into 6 mL of TK broth contained in a culture tube, for another incubation at 18 °C to provide inocula for subsequent incubations. For each isolate, an aliquot (0.3 mL) of the 18 °C broth culture was incubated into four tubes containing 6 mL of TK broth and incubated at the test temperatures: 4, 18, 21, and 37 °C, respectively. The development of an orange-rust color and turbidity were indicative of growth.

Temperature-Dependent Survival

Both mesophilic (F1, N1, and S2) and psychrotrophic (A1 and D6) strains of *T. ferrooxidans* were examined for their ability to survive exposure to a temperature above their Tmax, the maximum temperature for growth, and to freezing.

For the supermaximum temperature studies, Tmax was determined by growing each isolate in TK broth at approximately 2.5 °C increments from 35 °C. Growth was indicated by a significant degree of iron oxidation when compared to an uninoculated control. The supermaximum temperature chosen for each isolate was 2 to 4 °C above its Tmax. Each isolate was grown at 25 °C to the onset of stationary phase in TK broth. Growth was monitored by determining the decrease in ferrous iron concentration in the medium as described by Herrera *et al.* (14) . At the onset of maximum stationary phase, an aliquot of the culture was removed, serially-diluted, and enumerated using the plate count method on ISP agarose. The culture flasks were then transferred to a water-bath shaker set at the supermaximum temperature. At various time intervals, aliquots of culture were removed and plated out to determine the number of colony-forming units/mL and thus the extent of survival.

For the freezing temperature studies, the five strains were grown, in duplicate, to the onset of maximum stationary phase and 1.5 to 2.0 mL aliquots were frozen (-15 °C)

in 2.0 mL cryovials by a slow freeze. The slow freeze consisted of placing the cryovials in a freezer set at -15 °C. At intervals, vials were removed from the freezer, thawed, serially-diluted, and plated out, and the number of colony-forming units/mL was determined.

Resistance to Heavy Metals

All ten strains of *T. ferrooxidans* listed earlier were examined for their ability to resist the heavy metals copper, nickel, uranium, and thorium.

Inhibitory concentrations (ICs) of the heavy metals, defined as the concentration which showed a significant ($p < 0.01$) decrease in the percentage of ferrous iron oxidized when compared to an untreated control, were determined in triplicate for each strain of *T. ferrooxidans* by transferring 1.0 mL of log-phase cells in TK broth into 50 mL of fresh TK broth containing requisite amounts of the metals of interest. Cultures were incubated at room temperature on a gyratory shaker for approximately 100 h and ferrous iron concentrations were determined by titrating 2.0 mL samples of culture with 0.01 N $KMnO_4$ in 0.4 N H_2SO_4. Ferrous iron oxidized was expressed as a percent relative to an untreated control.

Plasmid Profiles

Plasmids were extracted from all strains of *T. ferrooxidans* mentioned above by a combined adaptation of the procedures of Birnboim and Doly (15) , Kado and Liu (16) , and Casse *et al.* (17) .

Cells for plasmid extraction were grown in 1 L of TK medium at pH 1.8 to minimize the formation of iron precipitates. Cultures were incubated at 25 °C up to maximum stationary phase (approximately 72 h) in a gyratory shaker at 150 rpm. When necessary, iron precipitates in the form of jarosite were removed by filtration through a Whatman No. 1 filter and Buchner funnel apparatus or by low speed centrifugation at 400 x g for 10 min at 4 °C in a Beckman™ J2-21 centrifuge. Cells were collected by centrifugation at 26,000 x g for 10 min at 4°C in the above centrifuge and suspended in 25 µL of TE buffer (50 mM Tris-HCl, pH 8.0; 20 mM EDTA). Cells which were not used immediately were suspended in 1.0 mL TE buffer, frozen in 1.8 mL capacity sterile cryovials in liquid nitrogen for 20 sec, and stored in a freezer at -20 °C. Cells were lysed by the addition of 400 µL of lysis buffer (1.5% sodium dodecyl sulfate (SDS) in TE buffer, pH 12.35). The tube contents were inverted six times and incubated for 30 min at room temperature. Cell lysates were neutralized by the addition of 150 µL of 3 M sodium acetate (pH 4.8). The SDS-protein complexes and most chromosomal DNA were allowed to precipitate by placing the mixture on ice for 60 min, and removed by centrifugation at 12,700 x g for 15 min at room temperature in an IEC Micro MB™ microcentrifuge (model EM218). The supernatant, containing plasmid DNA, was extracted with 500 µL Tris-HCl-saturated phenol by inverting the tubes several times.

The emulsion was centrifuged at 12,700 x g for 10 min in a microcentrifuge and the upper aqueous phase was saved. The DNA in the upper aqueous phase was precipitated by gentle mixing with two volumes of cold absolute ethanol and refrigeration at -70 oC for 30 min or at -20 °C overnight. Subsequently, the precipitate was collected by centrifugation at 12,700 x g for 10 min at room temperature in a microcentrifuge. The pellet was air dried and suspended in 575 µL of TE buffer by trituration with a pipette. RNA was removed by treating the cleared lysate with 5.75 µL of RNase A stock (1 mg/mL in 5 mM Tris-HCl, pH 8.0). The lysate was mixed gently and incubated for 15 min at 37 °C. The lysate was extracted once more with 500 µL Tris-HCl-saturated phenol as described above. The emulsion was centrifuged at 12,700 x g for 10 min in a microcentrifuge and the upper aqueous phase was saved. To remove residual phenol, the aqueous phase was extracted once with an equal volume of reagent-grade ether and the upper ether phase was discarded. The lower aqueous phase, containing the plasmid DNA, was allowed to sit at room temperature for approximately 15 min to allow residual ether to evaporate. The DNA was precipitated using two volumes of cold absolute ethanol and concentrated by centrifugation as described earlier. The air-dried DNA pellet was solubilized in 20 µL TE buffer and mixed with 20 µL of a 1:6 dilution of loading buffer (40% (wt/vol) sucrose, 0.25% (wt/vol) bromophenol blue). Samples (6 µL aliquots) were applied to a horizontal 0.6% (wt/vol) agarose gel prepared and run with a 1:10 dilution of 10X TEA buffer (400 mM Tris base, 200 mM sodium acetate, 18 mM EDTA (pH 7.8)). Electrophoresis was carried at 5 V/cm for 2.5 h at room temperature. The gels were stained with ethidium bromide (1.0 µg/mL) for 10 min and destained in distilled water for the same period of time prior to visualization at 312 nm using a Spectroline™ (model TX-213A) transilluminator. Gels were photographed using a DS34 Polaroid™ direct screen instant camera with type 667 film and a Wratten No. 2E gelatin filter.

RESULTS AND DISCUSSION

Table 1 shows the mean generation times for ten isolates of *T. ferrooxidans*. It is clear that the rate of oxidation of ferrous iron mediated by this bacterium was highly temperature dependent. Specifically, the mean generation time ranged from a minimum of 5.9 h for isolate D6 at 30 °C to a maximum of 420 h for isolate A1 at 2 °C. All isolates except W1 oxidized iron over the range 2 to 35 °C. The data also reveal the existence of two distinct thermal groups. One group, mesophiles, consisted of those isolates (F1, N1, N2, R1, S2, and W1) which oxidized iron exponentially between 10 and 35 °C. Another group, psychrotrophs, comprised those isolates (A1, D2, D6, and D7) which were capable of oxidizing iron exponentially over the entire range of temperatures (2 to 35 °C). A clear differentiation of the isolates into mesophiles and psychrotrophs is interesting in that, it may be the result of a selection for strains which are better adapted to oxidize ferrous iron at the temperature of their environment. This observation is congruent with those reported by Ferroni *et al.* (18) .

Fig. 1 depicts the categorization of 559 iron-oxidizing bacteria native to an uranium mining environment into four distinct thermal groups. Those isolates capable of growth at the two temperature extremes (4 and 37 °C) were denoted as "broader temperature range psychrotroph" and accounted for 23% of the population studied. A second thermal group, representing 66% of the isolates and designated as "narrower temperature range psychrotrophs", was established for those isolates capable of growth at 4 and 21 but not at 37 °C. Interestingly, only 1% of all isolates tested were mesophilic in that they grew at 18, 21, and 37 °C. Finally, 10% of the isolates studied were incapable of growth at either of the temperature extremes and, thus, were categorized as "intermediate".

In a related study, 46 iron-oxidizing bacteria, native to a tailings effluent with *in situ* temperatures in the range 0.5 to 5 °C, were categorized into the same thermal groups (Fig. 2). In this case, the sample temperature was important as far as the recovery of the different thermal groups was concerned. Although sample temperature did not considerably influence the percentage of psychrotrophs recovered, it was a factor with respect to growth temperature ranges of the psychrotrophs recovered, and the recovery of mesophiles. To be more precise, 4% "broader temperature range psychrotrophs" and 91% "narrower temperature range psychrotrophs" were recovered from this study, compared to 23% and 66%, respectively, for the earlier investigation. The fact that the lower-temperature samples did not yield mesophilic iron-oxidizing bacteria is not surprising as their growth would be retarded or even arrested at the lower environmental temperatures. On the other hand, the shift from "broader temperature range psychrotrophs" to "narrower temperature range psychrotrophs" in the lower-temperature samples is more difficult to explain as the selective pressure of low temperature should not favor one group over the other. The observation that psychrophilic representatives were not recovered even though the sample temperatures were near 0 °C is noteworthy. Although the data suggest the absence of psychrophilic thiobacilli from this environment, it is possible that they have maximum temperatures for growth lower than 18 °C, the initial isolation temperature.

It is clear from these studies that different thermal types of *T. ferrooxidans* exist, and that the population of iron-oxidizing bacteria (most of which were *T. ferrooxidans*) indigenous to the uranium mining environment examined is heterogeneous with respect to temperature growth profiles, and that psychrotrophic members outnumber all other thermal groups. The numerical predominance of psychrotrophic *T. ferrooxidans* is evidence of a population that can grow, and therefore, leach metals, at appreciable rates throughout the seasonally-based temperature cycle. The existence of this population is critical to *in situ* bioleaching processes in Canadian environments.

Figure 3 shows the survival of different strains of *T. ferrooxidans* at supermaximum temperatures. It is evident that the viable counts decreased as a function of time and that the rates of decline were strain-dependent. Of the five strains examined, two of which were psychrotrophic and three of which were mesophilic, there were no

thermoduric types, and death occurred fairly rapidly at temperatures a few degrees above their maximum temperatures for growth.

Strains S2, A1, F1, D6, and N1 were subjected to a slow freeze to -15 °C and then survival was examined after 24 h (Table 2). The strains of *T. ferrooxidans* studied did not survive well the process of freezing as the viable counts decreased substantially, with percent survivals ranging from 0.0006% (D6) to 0.0155% (A1). It is important to note that, in addition to the freezing effect, there was also a storage effect. Specifically, cell viability decreased with increasing storage time in the freezer. The explanation for this observation is not immediately obvious, and would require additional experimentation.

Again, it is evident from these data that the survival of *T. ferrooxidans* to extremes in temperature is strain-dependent in that some strains were more resistant than others at both ends of the temperature range examined. Besides showing strain variability with respect to another aspect of temperature, these data shed light on the persistence of *T. ferrooxidans* in the environment.

Table 3 shows the inhibitory concentrations (ICs) for ferrous iron oxidation by the metals copper, nickel, uranium and thorium for ten isolates of *T. ferrooxidans*. The data reveal that uranium and thorium were much more toxic to the bacterium than either copper or nickel. Specifically, only two of ten strains were resistant to the second highest concentration of uranium tested (4.0 mM) and, one of ten strains was resistant to the second highest concentration of thorium tested (2.0 mM). In contrast, four of ten strains were resistant to the second highest concentration and one was resistant to the highest concentration of both copper and nickel. In other words, uranium and thorium were 20-40 times more toxic to ferrous iron oxidation than either copper or nickel. It is evident, therefore, that different strains showed different sensitivities to the metals. This might have been due to an acquired tolerance to the heavy metal as a result of natural selection during the leaching process (19) .

Table 4 shows the plasmid DNA profiles for ten strains of *T. ferrooxidans* native to mining environments. It is clear that the strains examined exhibited different plasmid profiles. Specifically, eight of the strains examined contained plasmid DNA, and some of these (D2, A1) harbored more than one plasmid. Although two strains (F1 and N2) did not yield plasmid DNA, it is possible that the protocol used to isolate plasmids from these strains was ineffective. This is particularly important when dealing with large plasmids. It is clear from these plasmid profiles that significant genomic variability exists in *T. ferrooxidans*. This phenomenon is not uncommon for this species as others have reported on it (8, 11) .

Therefore, it seems reasonable to expect that the *T. ferrooxidans* strain variability that exists for temperature and heavy metal resistance also exists for other characteristics, especially as the plasmid DNA content of strains is also variable. Therefore, in applications of bioleaching where an inoculum of *T. ferrooxidans* is employed, attention

to the individual characteristics of the strain is essential. Moreover, it is likely that appropriate strains are to be found in the environment, and that genetically-engineered strains might not be needed.

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REFERENCES

1. **Lundgren, D.G., M. Silver**. 1980. Ore leaching by bacteria. Ann. Rev. Microbiol. **34**: 262-283.

2. **Ehrlich, H.L.** 1963. Bacterial action on orpiment. Econ. Geol. **58**: 991-994.

3. **Tuovinen, O.H., D.P. Kelly**. 1972. Biology of *Thiobacillus ferrooxidans* in relation to the microbiological leaching of sulphide ores. Z. Allg. Mikrobiol. **12**: 311-346.

4. **Silverman, M.P., H.L. Ehrlich.** 1964. Microbial formation and degradation of minerals. Adv. Appl. Microbiol. **6**: 153-206.

5. **Torma, A.E.** 1971. Microbiological oxidation of synthetic cobalt, nickel and zinc sulfides by *Thiobacillus ferrooxidans*. Rev. Can. Biol. **30**: 209-216.

6. **Torma, A.E.** 1978. Oxidation of gallium sulfides by *Thiobacillus ferrooxidans*. Can. J. Microbiol. **24**: 888-891.

7. **Holmes, D.S., J.R. Yates**. 1990. Basic principles of genetic manipulation of *Thiobacillus ferrooxidans* for biohydrometallurgical applications. In: Microbial Mineral Recovery, H.L. Ehrlich and C.L. Brierley (Eds), McGraw-Hill, New York. pp. 29-54.

8. **Harrison, A.P., Jr.** 1986. The phylogeny of iron-oxidizing bacteria. Biotechnol. Bioeng. Symp. **16**: 311-317.

9. **Yates, J.R., D.S. Holmes.** 1987. Two families of repeated DNA sequences in *Thiobacillus ferrooxidans*. J. Bacteriol. **169**: 1861-1870.

10. **Holmes, D.S., J.R. Yates, and J. Schrader.** 1988. Mobile repeated DNA sequences in *Thiobacillus ferrooxidans* and their significance for biomining. In: Biohydrometallurgy, P.R. Norris and D.P. Kelly (Eds), Science and Technology Letters, Kew Surrey, U.K., pp. 153-160.

11. **Schrader, J.A., D.S. Holmes.** 1988. Phenotypic switching of *Thiobacillus ferrooxidans.* J. Bacteriol. **170**: 3915-3923.

12. **Tuovinen, O.H., D.P. Kelly.** 1973. Studies on the growth of *Thiobacillus ferrooxidans* I. Use of membrane filters and ferrous iron agar to determine viable numbers, and comparison with $^{14}CO_2$-fixation and iron oxidation as measures of growth. Arch. Microbiol. **88**: 285-298.

13. **Manning, H.L.** 1975. New medium for isolating iron-oxidizing and heterotrophic acidophilic bacteria from acid mine drainage. Appl. Microbiol. **30**: 1010-1016.

14. **Herrera, L., P. Ruiz, J.C. Aguillon, and A. Fehrmann.** 1989. A new spectrophotometric method for the determination of ferrous iron in the presence of ferric iron. J. Chem. Tech. Biotechnol. **44**: 171-181.

15. **Birnboim, H.C., G. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**: 1513-1523.

16. **Kado, C.I., S.T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**: 1365-1373.

17. **Casse, F.C., C. Boucher, J.S. Julliot, M. Michel, and J. Dénairié.** 1979. Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. J. Gen. Microbiol. **113**: 229-242.

18. **Ferroni, G.D., L.G. Leduc, and M. Todd.** 1986. Isolation and temperature characterization of psychrotrophic strains of *Thiobacillus ferrooxidans* from the environment of a uranium mine. J. Gen. Appl. Microbiol. **32**: 169-175.

19. **Tuovinen, O.H., D.P. Kelly.** 1974. Studies on the growth of *Thiobacillus ferrooxidans* IV. Influence of monovalent metal cations on ferrous oxidation and uranium toxicity in growing cultures. Arch. Microbiol. **98**: 167-174.

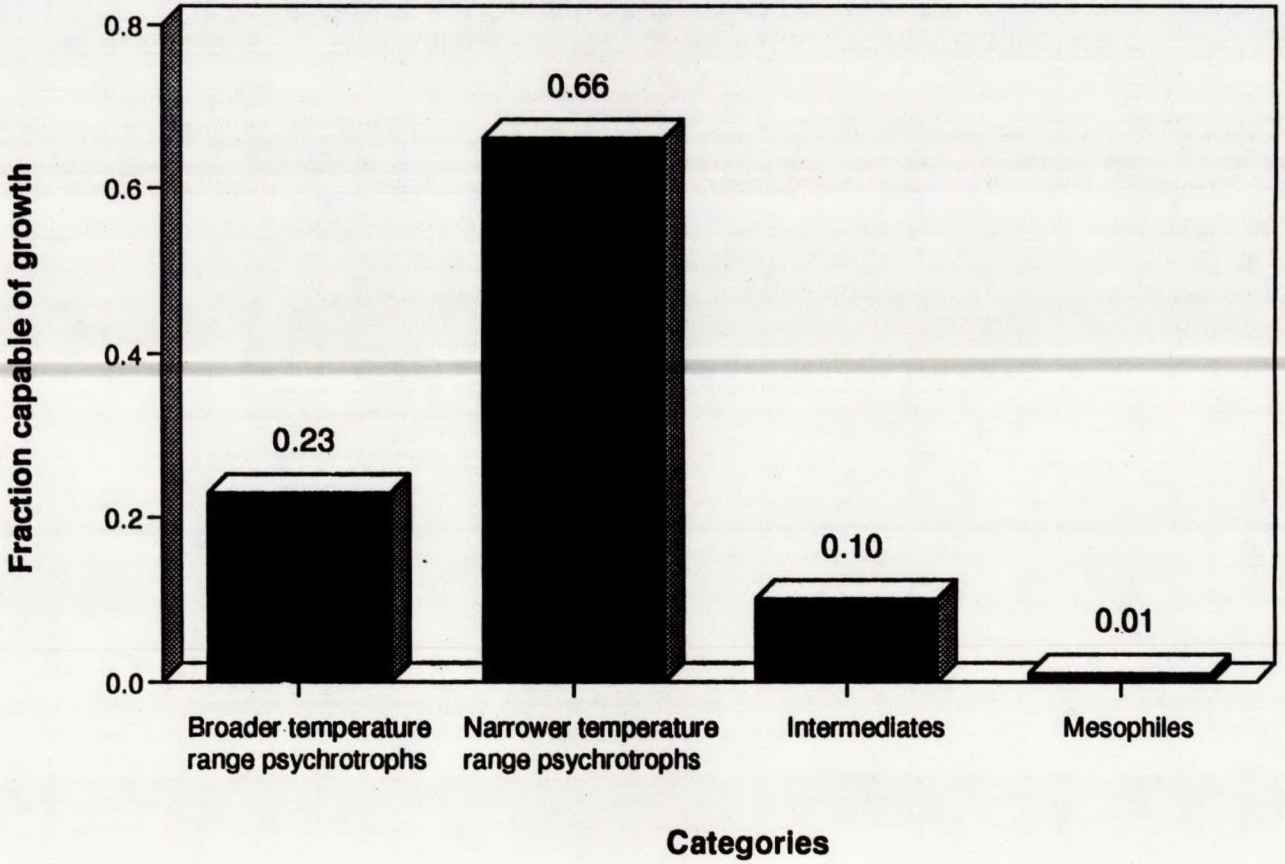

Figure 1. Thermal categories of iron-oxidizing bacteria isolated from underground uranium mine bioleachates.

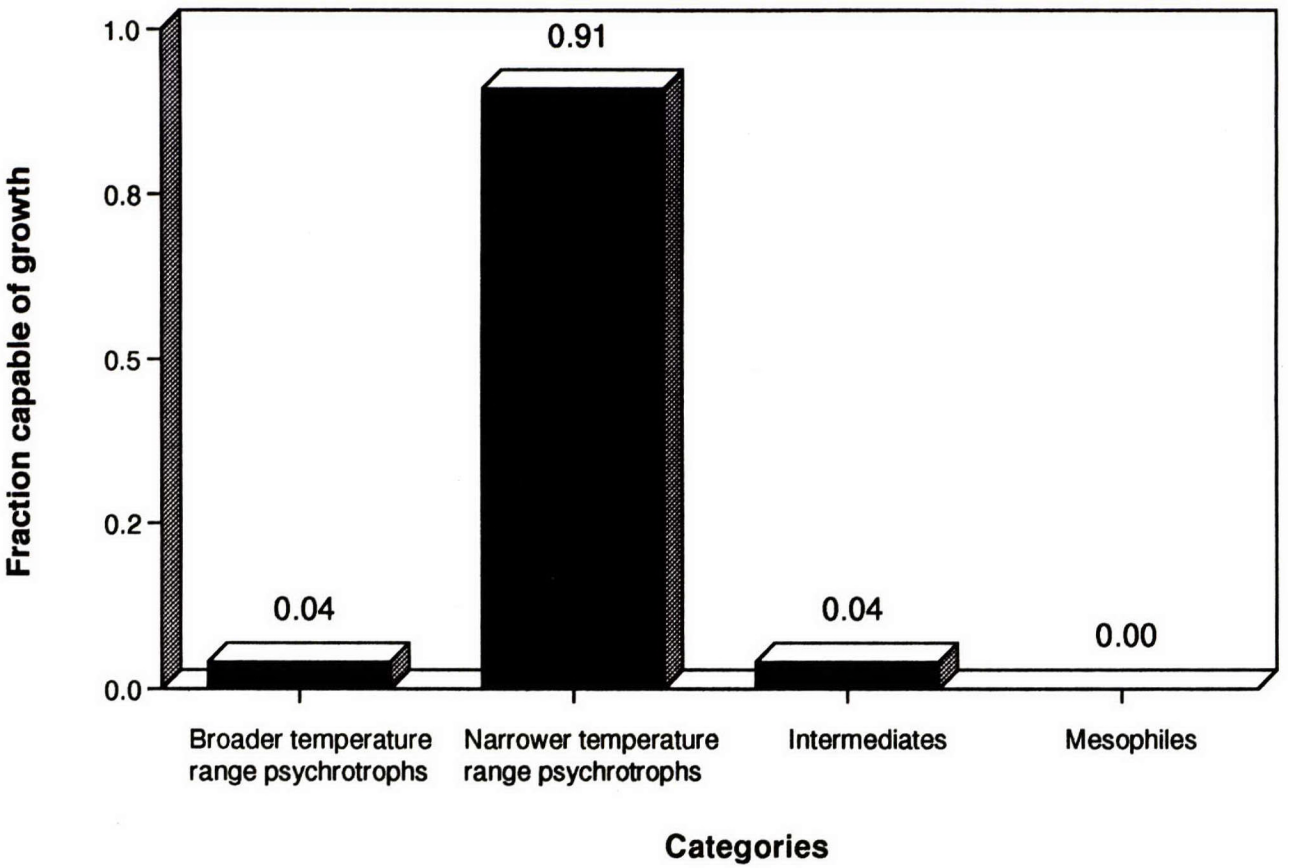

Figure 2. Thermal categories of iron-oxidizing bacteria isolated from cold, uranium tailings effluents

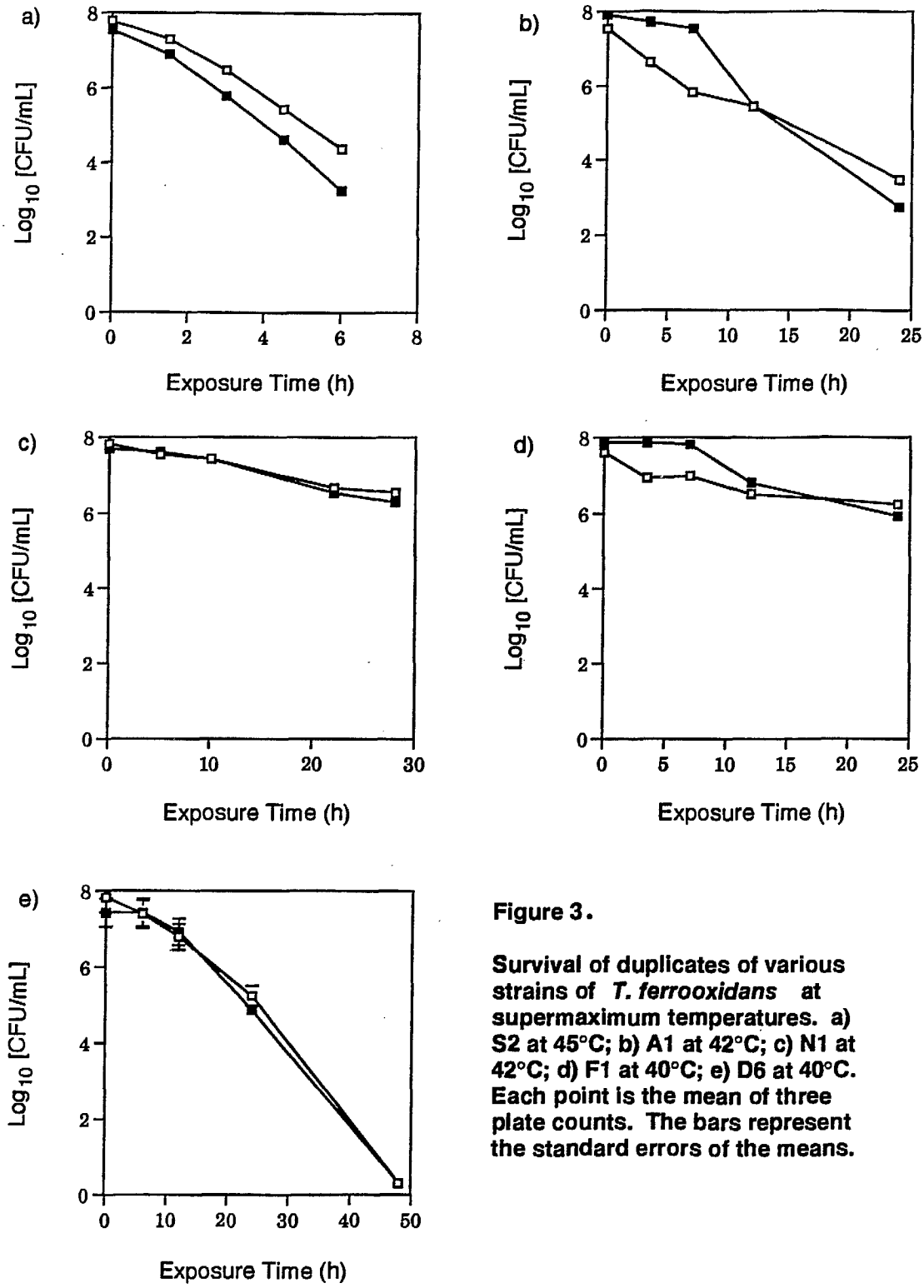

Figure 3.

Survival of duplicates of various strains of *T. ferrooxidans* at supermaximum temperatures. a) S2 at 45°C; b) A1 at 42°C; c) N1 at 42°C; d) F1 at 40°C; e) D6 at 40°C. Each point is the mean of three plate counts. The bars represent the standard errors of the means.

Table 1. **Mean generation times for triplicates of ten different isolates of *T. ferrooxidans* at 2, 5, 10, 15, 20, 25, 30 and 35°C, respectively**

Isolate	Mean Generation Time (h)							
	2°C	5°C	10°C	15°C	20°C	25°C	30°C	35°C
A1	420.0[a]	201.3 ± 5.1	49.4 ± 8.1	24.0 ± 1.1	12.3 ± 0.7	11.8 ± 1.1	13.0 ± 2.2	9.3 ± 0.7
D2	268.8[a]	147.8 ± 7.5	41.3 ± 5.0	22.3 ± 1.2	13.6 ± 0.2	9.9 ± 0.4	8.5 ± 0.8	11.0 ± 0.4
D6	253.5 ± 16.0	149.9 ± 19.0	32.7 ± 4.1	18.5 ± 1.3	12.4 ± 0.5	9.3 ± 0.6	5.9 ± 0.3	12.6 ± 1.0
D7	306.4 ± 36.8	185.5 ± 21.8	39.6 ± 1.1	31.7 ± 6.1	15.1 ± 2.4	11.6 ± 0.3	6.7 ± 0.2	8.5 ± 0.6
F1	A.O.	A.O.	50.1 ± 3.2	32.8 ± 0.2	9.4 ± 1.9	7.6 ± 0.4	7.6 ± 0.4	6.3 ± 0.7
N1	A.O.	A.O.	53.6 ± 2.3	28.7 ± 1.4	13.2 ± 1.1	10.5 ± 0.7	10.6 ± 0.2	7.3 ± 0.5
N2	A.O.	A.O.	40.3 ± 2.6	38.0 ± 2.6	15.4 ± 1.5	13.2 ± 0.3	17.8 ± 0.8	9.5 ± 0.4
R1	A.O.	A.O.	54.9 ± 1.8	23.0[a]	14.8 ± 2.0	12.4 ± 0.7	10.1 ± 0.3	10.9 ± 2.0
S2	A.O.	A.O.	78.7 ± 4.6	28.4 ± 3.4	16.6 ± 3.8	11.2 ± 0.8	10.4 ± 0.1	8.5 ± 0.4
W1	N.S.O.	A.O.	55.6 ± 7.7	24.1 ± 2.7	15.5 ± 1.0	11.0 ± 0.1	11.3 ± 0.5	14.0 ± 1.4

A.O. – arithmetic oxidation
N.S.O. – no significant oxidation
[a] – single value determination
± standard error

Table 2. Survival of various strains of *T. ferrooxidans* after 24 h at -15°C

Exposure Time (h)	CFU/mL[a]				
	Strain				
	S2	A1	F1	D6	N1
0	4.45×10^7	2.65×10^7	7.45×10^7	4.55×10^7	6.0×10^7
24	1.35×10^3	4.1×10^3	5.65×10^3	2.55×10^2	9.9×10^2
% survival	0.0030	0.0155	0.0076	0.0006	0.0016

[a] Each value is the average of two, triplicate counts.

Table 3. Inhibitory concentration[a] (IC) for ferrous iron oxidation by copper, nickel, uranium, and thorium

Isolate	IC (mM)			
	Cu^{2+}	Ni^{2+}	UO_2^{2+}	Th^{2+}
A1	40.0	40.0	2.0	4.0
D2	160.0	10.0	8.0	2.0
D6	160.0	160.0	4.0	2.0
D7	10.0	20.0	8.0	2.0
F1	160.0	40.0	2.0	1.0
N1	160.0	160.0	4.0	1.0
N2	10.0	40.0	2.0	2.0
R1	40.0	160.0	2.0	2.0
S2	160.0	160.0	2.0	1.0
W1	40.0	160.0	1.0	2.0

[a] defined as the concentration which shows a significant ($p < 0.01$) decrease in the percentage of ferrous iron oxidized when compared to a control.

Table 4. The plasmid DNA content of ten different strains of *T. ferrooxidans*

Strain	Number of Plasmids
A1	1
D2	2
D6	1
D7	1
F1	0
N1	0
N2	1
R1	1
S2	1
W1	1

All plasmids had a size in excess of the pBR322 standard (4.3 kb).

APPLICATIONS OF BIOTECHNOLOGY IN METALS RECOVERY (TECHNICAL REVIEW)

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ABSTRACT

The history of biotechnology in the mining industry as well as current applications are technically reviewed. Underground heap leaching in the uranium industry was developed into a fully functional economic process. The copper industry, however, remains the main area where extensive application of the technology is practiced. In the gold industry, tank bioreactors are being used increasingly to oxidize sulfide concentrates prior to gold recovery. There is also increasing interest in applying heap bioleaching to low grade refractory gold ores. In the treatment of effluent from metals production facilities, biotechnology is also coming into its own. A commercial scale sulfate reduction plant is operating in the Netherlands treating contaminated groundwater at the Budelco zinc refinery. Wetlands treatment of effluent is also receiving a great deal of attention. Biotechnology is beginning to be widely used in the metals industry and will doubtless continue to expand as more knowledge is accumulated about the organisms and processes involved.

LES APPLICATIONS DE LA BIOTECHNOLOGIE POUR LA RÉCUPÉRATION DES MÉTAUX (REVUE TECHNIQUE)

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RÉSUMÉ

Dans cet article, on passe en revue l'histoire de la biotechnologie dans l'industrie minière, ainsi que les applications courantes de cette technologie. On a réussi à transformer la lixiviation souterraine en tas, employée dans l'industrie de l'uranium, en un procédé exploitable avec une rentabilité complète. Toutefois, l'industrie du cuivre reste le principal domaine d'application à grande échelle de cette technologie. Dans l'industrie de l'or, on emploie de plus en plus des bioréacteurs de type réservoirs pour oxyder les concentrés sulfurés avant de procéder à la récupération de l'or. On s'intéresse également de plus en plus à l'application de la biolixiviation en tas aux minerais aurifères réfractaires de basse teneur. Pour le traitement des effluents produits par les installations productrices de métaux, la biotechnologie gagne aussi du terrain. Une usine de réduction des sulfates, exploitée à l'échelle commerciale, fonctionne aux Pays-Bas et traite les eaux souterraines contaminées dans l'affinerie de zinc de Budelco. Le traitement des effluents dans les secteurs de terres humides est également examiné avec attention. L'industrie des métaux commence à utiliser à grande échelle la biotechnologie, laquelle continuera sans doute à prendre de l'importance au fur et à mesure de l'augmentation de nos connaissances sur les organismes et sur les réactions entrant en jeu.

INTRODUCTION

The use of biotechnology in the mining industry has been practiced for many years, often without a realization of the processes involved. It is only relatively recently that an increased level of knowledge in the field has led to significant engineering applications. The gold industry is developing an increasing number of facilities utilizing the BIOX process for bacterially oxidizing refractory gold concentrates. The copper industry, particularly in South America, is becoming increasingly sophisticated in the use of biological leaching of primary sulfides in a heap situation. In the environmental field the technology is also becoming more widely accepted. Groundwater contamination at the Budelco zinc refinery in the Netherlands is being remediated using a sulfate-reducing bacteria process to remove sulfates and heavy metals from the groundwater. Cyanide, thiocyanate and heavy metals are being successfully removed from tailings effluent at the Homestake operation in Lead, S.D.

There are numerous areas of biotechnology where development is currently proceeding in the hope of developing new processes for use in the mineral industry. Biosorption of metals from process and effluent streams is an area with considerable potential. In-heap degradation of cyanide and fixing of metal contaminants is also being pursued at various locations. Microbial capping of heaps and tailings dumps may have significant potential for reduction of the acid mine drainage (AMD) problem. Finally, wetlands treatment of seepage from tailings ponds continues to develop as a method of AMD treatment. The range of bacterial cultures which are proving useful in the metals industry is wide and continues to grow.

This paper provides a general overview of the various activities in progress in the metals recovery industry which use biotechnology in some manner. The commercialized processes are briefly described and the potential developments in the field are discussed.

Biologically Assisted Leaching

Gold

The BIOX process developed by Genmin S.A. has been the most successful of the competing technologies in treating refractory gold concentrates. Successful operating plants at Fairview (South Africa), Sao Bento (Brazil), and Harbour Lights (Australia) have helped to gain acceptance of the BIOX process as an economical, environmentally acceptable method of treating gold-bearing sulfides. New, larger operations are under construction or in the commissioning process at Wiluna in Australia and at Ashanti in Ghana. In low tonnage operations such as sulfide concentrate processing, tank bioleaching is significantly less expensive than autoclave treatment. It also does not have the environmental challenges associated with roasting plants. In remote areas, it is simpler and less costly to operate, plant operation can be handled by a relatively untrained

work force and access to high technology industries is much less important than with the competing processes.

Development of a low cost, heap leach technology for pretreatment of low grade refractory gold ores is currently in progress at several locations. At Mount Leyshon in Australia, the Normandy Poseidon Group has developed a process for bioleaching ore to remove copper prior to gold recovery. In this case the bioleach is not exposing gold but simply removing copper to reduce cyanide consumption. Many of the physical design constraints are similar, however, to the preoxidation process in development at the two major companies recovering gold from the Carlin Trend in Nevada.

Barrick Goldstrike Mines Inc. operates the largest single gold mining operation in North America. The company produced in excess of 1 million ounces of gold from the operation for the first time in 1992. Production continues to grow and is expected to reach the 2 million ounce mark in 1995. Gold production comes almost entirely from autoclave treatment of sulfide ore. Since autoclave treatment, while efficient and environmentally friendly, is relatively high cost, there are significant reserves of low grade material for which the method is less attractive. A heap leach method is therefore under development which will use traditional bacterial leaching methods to preoxidize the ore. The material will then be transferred to a conventional cyanide leaching facility for recovery of the gold.

Newmont Gold Company is in the process of developing a bioleach method for treatment of their low grade ores. Their method may also be used to treat Barrick low grade ores under an existing Joint Venture Agreement. Newmont is presently operating a 27,000 ton pilot heap which is providing encouraging results.

Barrick is presently operating column simulations of the process and will be developing a 100,000 ton demonstration facility in 1994. The results from Barrick's bioleach program so far indicate that the finely disseminated sulfides which host the gold oxidize very rapidly under optimum conditions and it should be possible to reach the target oxidation within a very short period of time.

Copper

The use of bacteria to aid in copper extraction is probably the oldest application of the technology, "natural" leaching of low grade copper ores has been practiced for centuries. Dump leaching was practiced at first by accident, with waste heaps producing acidic, cupric solutions from which copper was recovered by cementation with scrap iron. More recently, with the advent of cheap, reliable solvent extraction technology for concentration of the dilute solutions, dump and heap leaching of both oxide and sulfide ores has become a very attractive and widely practiced method. Copper heap leaching was originally applied to mainly oxide materials. It was found however, that many leach dumps were of mixed sulfide and oxide composition and that bacterial leaching was

playing an important part in copper recovery. Most operations now have significant sulfide heaps practicing bacterial leaching.

There are a significant number of new installations in Australia and in North and South America where sulfide heap operations are being developed at this time. In North America, over 10% of copper production comes from heap leaching, with the majority of the operations located in the Southwest, in Arizona. The Phelps-Dodge, Morenci operation has seen copper recovery from the area for over 100 years and is presently the largest copper producer in North America. The milling operation process 130,000 tons per day of sulfide ore to feed the smelter. The heaps process both oxide and sulfide material at separate locations. A total of 500,000 tons per day of new material is added to the heaps, primarily as sulfide ore. The heap leach, SX/EW operation produces high purity cathode copper for less than half the world price.

In Chile, there are a number of plants in operation or under development utilizing the Bacterial Thin Layer Leaching method. This is essentially standard heap leach technology applied to lower lifts of finely crushed, higher grade ore. Sociedad Minera Pudahuel Ltda. has been applying the technology at its Lo Aguirre Plant since 1988. This was a copper oxide heap operation which began to encounter mixed oxide-sulfide material in its deposit and adapted the method to accommodate the change. The facility is now treating entirely chalcocite-bornite sulfide ore, producing 14,000 tons of high purity cathode copper annually. Two other operations are currently under construction which will utilize the method; Quebrada Blanca and Cerro Colorado. Cerro Colorado is the smaller of the two treating 10,000 tons per day of ore at approximately 1.4 % copper to produce 40,000 tons per year. The Quebrada Blanca plant will treat 15,000 tons per day to produce 75,000 tons per year. These plants will recover between 80 and 90% of the copper from sulfide ore over a relatively short leach cycle.

Uranium

In uranium, the first application of the technology took place in Portugal. It was observed that surface ore stockpiles exposed to rainwater suffered a significant decline in grade. This led to the creation of a commercial scale heap leaching operation in 1952. The role of bacteria in this operation was not, however, recognized until much later. In the Elliot Lake camp in Northern Ontario, bacterial leaching was practiced on a small scale shortly after development of the camp in the late 1950's. At Denison Mines, bacterial growth was well established in the underground workings three years after the mine opening and all mine water was acidic. Over the succeeding 20 years small quantities of uranium were produced from this source but little effort was made to increase the amount.

In 1982, development of a full-scale production technique was initiated with laboratory testwork at BC Research. The development program was successful and bacterially assisted, underground heap leaching became a significant producer of uranium

at Denison. By 1987, 20% of production came from this source, in excess of 840,000 pounds per year.

The operation broke new ground in a number of areas and, as well as providing an economic production source for Denison, provided a wealth of information and experience to all involved in the work. Significant basic research was performed in the areas of strain isolation, nutrient optimization, microbial metal tolerance and oxygen consumption requirements. All of this research was performed as part of the overall development project. The success of the project also made Denison more willing to experiment in other areas of microbiology, leading to involvement in research projects in biosorption, microbial plugging of tailings piles and wetlands remediation of acidic seepage from tailings facilities.

The process at Denison consisted of taking underground openings which had been developed by removal of the mill grade ore and converting them to large leaching vessels. The leach grade ore lay directly above the higher grade material and was accessed by drilling up from the mined out stopes. The ore was blasted down into the stopes over pre-installed air lines. The stopes sloped southward and a bulkhead was installed at the bottom entrance to allow flooding of the ore. The stopes were generally connected in pairs, creating a leach vessel holding 40,000 - 80,000 tons of ore.

To initiate the leaching process, the stopes were flooded with mine water and then immediately drained. All of the mine water at Denison was acidic and contained large populations of bacteria. The flooding inoculated the ore and started the dissolution of the uranium. After the stopes were emptied, air was blown through the lines buried under the heap to provide oxygen for the bacteria in the now closed environment. The bacteria began to oxidize the pyrite in the ore, producing the acid necessary to dissolve uranium and the ferric ion necessary to oxidize the insoluble tetravalent uranium to the acid soluble hexavalent state. This proceeded until the interstitial moisture reached an uranium concentration which was toxic to the bacteria, approximately three weeks time. The stopes were then reflooded and drained to recover the dissolved uranium and reinitiate the leach process. This process was repeated for approximately 18 months, providing 70% recovery of uranium from the low grade ore.

Environmental Applications

Sulfate-Reducing Bacteria: Process Applications

Shell Research in England and Paques Environmental in the Netherlands recently developed a commercial scale process for anaerobic reduction of sulfates in solution to remove both the sulfates and heavy metal contamination. A plant capable of treating 1500 gallons/minute of contaminated groundwater using the technology went into operation in May 1992. The Budelco BV zinc refinery, one of the worlds largest, has been in operation at its present location for over 100 years. Over this period of time, a

variety of pyrometallurgical and hydrometallurgical processes have been practiced to refine the zinc. The waste streams from these processes have accumulated at the property and, over an extended period, minor amounts of contamination have escaped from the storage facilities. This has resulted in contamination of the groundwater under the site with sulfates and heavy metals. To prevent contamination of nearby drinking water aquifers, a geohydrological control system and groundwater treatment plant were installed.

The process uses a fluidized bed of sludge containing the reducing bacteria in a reactor providing 4 hours retention time for the groundwater. The purified groundwater overflows from the reactor saturated with sulfides and is sent to a second reactor where different organisms oxidize the sulfide to elemental sulfur. The water is then filtered and released to the environment. Table 1 contains results of the pilot stage research showing that the system is effective in removing contaminants to very low levels.

Sulfate-Reducing Bacteria; Wetlands Applications

The process described above is an engineering adaptation of the process which occurs naturally in swamps and other natural wetlands. A considerable effort has taken place in North America over recent years to design and build wetlands which will reliably treat contaminated effluent streams. While there has been considerable success in this pursuit, the technology has not developed to the point where any and all solutions can be treated. Where the flows are lightly contaminated, as in the Budelco case, the technology will probably be effective. There are many cases, however, particularly the acid mine drainage associated with old sulfide tailings dumps, where the level of contamination is too high. This will continue to be a challenge for these systems which will need considerable ingenuity to solve.

Cyanide Biodegradation; Process Applications

The Homestake Mine in Lead, S.D. has been successfully operating a biological treatment plant for purification of the effluent from their gold property since 1984. The treated discharge from the plant makes up 60% of the water flow in a cold water trout fishery. The stream, which had been barren of life for over 100 years due to mine drainage, is now an established trout fishery.

The process consists of two trains of rotary biological contactors operating in series. In the first train, bacteria, principally *Pseudomonas* sp., degrade the cyanide and thiocyanate in the effluent to produce ammonia. Metal contaminants in solution are also adsorbed onto the biofilm in the first train. The second train of reactors contain nitrifying bacteria which convert the ammonia to nitrate. The plant has continued to operate successfully for many years now and has continually improved in efficiency. Table 2 shows typical solution treatment results from the operation.

Cyanide Biodegradation; Heap Leach Applications

Several groups have been involved over the years in attempts to use mixed bacterial cultures, predominantly *Pseudomonas* sp., to detoxify gold leach heaps prior to decommissioning. The Unites States Bureau of Mines, Salt Lake City Research Center has developed one method, which was used in a large scale field trial at USMX's Green Springs gold operation near Ely, Nevada. The method involves growing a biofilm on the carbon particles in the gold adsorption columns. In the field trial, the recycle process solution was pumped over the carbon columns containing the biofilm to degrade the cyanide. Over a 15 week trial, the weak acid dissociable (WAD) cyanide was lowered from 20 ppm to 8.5 ppm.

The Yellow Pine Mine in Idaho, belonging to the Hecla Mining Company used a different approach to detoxifying the heap. Large volume, high density cultures were generated in the leach ponds and pumped over the heap. The approach used far more nutrients than the Green Springs trial but grew the biofilm within the heap itself. This allowed for destruction of the more stable cyanide complexes such as iron and gold, thereby more completely detoxifying the heap. The effluent from the heap was lowered to 1 ppm WAD cyanide in a single summer. Pintail Systems Inc. designed the system for performing this trial.

Biosorption

During the Denison Mines development program a good deal of research was performed using process solutions to investigate the potential for using immobilized biomass as an adsorbing agent for metals. The research, performed primarily by Dr. M. Tsezos at McMaster University, used immobilized *Rhizopus* organisms, to adsorb uranium from bioleach process solutions. The process suffered from high cost and lack of selectivity. Essentially the biomass pellets were not much less costly than conventional ion exchange resins and did not have the desired selectivity. Typically, the material would load iron at the expense of any other metals. This is a field with considerable potential, particularly in environmental management. It deserves continued study at a basic research level.

CONCLUSION

The interest of the minerals industry in biotechnology has had many ups and downs. In the mid 1980's, when Denison was successfully demonstrating the technology, interest was high, however this was followed by a period where interest was concentrated on environmental aspects. With successful applications in gold and copper, the interest is probably higher now than it has ever been. Interestingly, it is the countries outside

North America such as Chile, Australia and South Africa, where the technology is being successfully developed as a competitive production technique. This should change in the coming years as Barrick, Newmont and other companies successfully implement the technology in the U.S. There is also some indication that interest is increasing again in the Canadian industry, particularly in the gold tank bioleach process. The technology is here to stay, and with a continuing increase in experience and expertise among everyday mining operators, new developments and applications are bound to come.

SUGGESTED READINGS

van Aswegen, P. C., M.W. Godfrey, D.M. Miller and A.K. Haines. 1991. Developments and Innovations in Bacterial Oxidation of Refractory Ores. Minerals & Metallurgical Processing, pp. 188-191.

Brierley J.A., C. L. Brierley. 1993. Reflections on and Considerations for Biotechnology in the Metals Extraction Industry. Hydrometallurgy, Fundamentals, Technology and Innovation , Chapter 40, pp. 647-660.

Hammer, D.A., R. K. Bastian. 1989. Wetlands Ecosystems: Natural Water Purifiers? Constructed Wetlands for Wastewater Treatment, Municipal, Industrial and Agricultural, Lewis Publishers, Chapter 2, pp. 5-20.

Marchbank, A. 1986. Underground Biological In-Place Leaching of Uranium Ores. Denison Mines Company Brochure.

Monteleagre, R. ***et al.*** 1993. Application of Bacterial Thin Layer Leaching Process to Quebrada Blanca Ore. International Biohydrometallurgy Symposium '93, Jackson, Wyoming, Vol.**1,** pp.1 - 14.

Scheeren, P.J.H., R.O. Koch, C.J.N. Buisman, L.J. Barnes, and J.H. Versteegh. 1991. New Biological Treatment Plant for Heavy Metal Contaminated Groundwater, EMC '91: Non-Ferrous Metallurgy - Present and Future, Elsevier, New York, pp. 403-416.

Whitlock, J. L. 1990. Biological Detoxification of Precious Metal Processing Wastewaters. Geomicrobiology Journal, Vol. **8**, pp.241-249.

Table 1: Budelco SRB Plant Results

Component	Influent (ppm)	Effluent (ppm)
Zinc	250	< .050
Cadmium	1.100	< .001
Iron	33	.030
Lead	13	< .020
Copper	2.200	< .020
Sulfates	1400	120

Table 2: Homestake Biological Treatment Plant Results

Component	Influent (ppm)	Effluent (ppm)
Thiocyanate	62	< .5
Total Cyanide	4.10	.06
WAD Cyanide	2.30	< .02
Copper	0.56	.07
Ammonia (N)*	5.60	< .5

* Ammonia peaks at 25 ppm within the plant as a cyanide degradation by-product

APPLICATIONS OF BIOTECHNOLOGY IN THE FOSSIL FUEL SECTOR

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ABSTRACT

For more than fifty years, petroleum microbiology has been a recognized need and activity in the oil and gas sector. Effort has focused on both the problems generated by undesirable microbial activities as well as opportunities envisaged for their application. Traditional problem areas related to corrosion, fouling and reservoir fouling continue to attract attention. A history of development initially focused on microbially enhanced oil recovery, oil diagenesis and prospecting has been extended first to oil spill clean-up and more recently to the application of bioremediation technologies for site clean-up and emissions control. This overview will attempt to highlight key areas of commercially important activity and promise in the hydrocarbon production, transportation and processing industries today.

APPLICATIONS DE LA BIOTECHNOLOGIE AU SECTEUR DES COMBUSTIBLES FOSSILES

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RÉSUMÉ

Depuis plus de cinquante ans, la microbiologie du pétrole est une activité nécessaire et reconnue du secteur de l'exploitation du pétrole et du gaz. On a concentré les efforts sur les problèmes que pose l'activité des microbes indésirables, et aussi sur les possibilités d'application de la microbiologie. Les problèmes classiques associés à la corrosion, aux salissures et à l'acidification des réservoirs continuent à retenir notre attention. Les efforts de développement qui ont initialement porté sur la récupération du pétrole assistée par l'activité microbienne, sur la diagenèse et sur la prospection du pétrole, ont été étendus au nettoyage des lieux de déversement de pétrole, et plus récemment à l'application des technologies de la remise en état biologique des sites contaminés et du contrôle des émissions. Dans cette vue d'ensemble, on met en relief les secteurs-clés d'activité commerciale importante et les secteurs prometteurs pour l'industrie actuelle de la production, du transport et du traitement des hydrocarbures.

INTRODUCTION

Biotechnology in the Hydrocarbon Industries

Industrial sectors can be defined by their market, their technology base, geographic or political factors, size, or any number of other criteria. Because biotechnology is so dependent on the nature of the basic material involved, it is useful for the purpose of this overview to define the industrial sector considered here as all industries based on the production, transportation, refining, processing and marketing of hydrocarbon materials. This definition spans both the oil and gas sector and petrochemicals industry.

Biotechnology is defined broadly as any technology based on or required by the activities of microorganisms in the hydrocarbon based industries. These activities can be both beneficial and detrimental. Traditionally, detrimental microbial activities have received more attention than potential opportunities but in the last two decades, the revolution in biotechnology has fostered significant effort to exploit microorganisms and their products commercially. Some direct and indirect commercial successes are already apparent. An even larger number of technologies have been demonstrated at various levels of feasibility and practicality and await economic opportunity and an appropriate champion for their commercial application.

Hydrocarbon Metabolism

Petroleum microbiology as an identifiable area of scientific enquiry began in the 1940's focused on the study of the interactions of microorganisms with fossil fuels. Fostered by the oil industry initially and more recently by environmental concerns a considerable body of knowledge now exists on hydrocarbon metabolism. Metabolic pathways for the aerobic breakdown of alkanes, naphthenes and aromatics (1) are now being complemented by the discovery and elucidation of anaerobic pathways for the biotransformation and destruction of various classes of hydrocarbons (2).

In general it remains true that aerobic degradation is faster than anaerobic metabolism and that smaller simpler molecules are more easily degraded than larger branched or polycyclic ones. It is also apparent that significant amounts of nitrogen and phosphorus are required for hydrocarbon metabolism under any circumstances and that emulsifying agents can have potent effects on the process.

Detrimental Activities

Biodegradation of Petroleum Products

Biodegradation of fuels and lubricants produced through the refining of crude oil is a significant commercial problem and has spawned specifications and control practices.

The problem appeared in the 1950's in the aviation industry. Where fuels were contaminated with excess water, growth of organisms resulted with corrosive consequences for aircraft fuel systems and plugging problems for engine fuel filters (3). The problem is not limited to the aviation industry. Fuel systems in general are at risk and are now protected by strict limits on the water content of fuels, construction of drainage systems to remove fortuitous water from tankage and in some cases by use of oil soluble biocides.

Special detection and monitoring kits have been developed for specific applications. For example, specific kits are available to monitor bacterial and fungal numbers in the crackcase oil of marine diesel engines in ocean going ships. Here, water entering the crankcase from the combustion cylinders is removed from the oil by a centrifuge circuit. Where this removal system is inadequate, microbial growth occurs in the crankcase oil with further emulsification of water and degradation of lubricating ability. This can ultimately lead to engine failure at great cost and risk unless caught in time (4).

Similar concerns arise in the use of cutting oil emulsions in machine shops. Here oil and water are emulsified to produce a recirculating lubricant to facilitate the machining of metals. Development of hydrocarbon degrading organisms in the cutting oil emulsions compromises their performance and leads to health concerns for exposed workers (4). Again detection and control are the technological issues.

Biodegradation of Petrochemical Products

Coatings and plastics derived from hydrocarbon feedstocks are susceptible to biological degradation. Polyvinyl chloride products for example require the inclusion of relatively low molecular weight plasticizers usually phthalates to attain performance requirements in most applications. While the polymer itself is protected by its large size from microbial attack, the plasticizer is easily leached and degraded. This can lead to failure of the plastic in a given application. Pipelines coated with PVC tapes in the 1960's have lost their protection and PVC liners installed extensively in irrigation canal projects in Alberta in the late 1970's have doubtless lost their integrity.

More subtle performance issues can also be important. For example, polyethylene tape coatings used as a protective coating for many Canadian gas pipelines are inert to microbial attack but most of the adhesives used to cement the polyethylene to the pipe surface are not. Subsequent disbondment of the polyethylene exposes underlying steel to serious corrosion and stress corrosion cracking scenario's.

Other examples of materials performance problems with hydrocarbon based products abound yet selection protocols which include susceptibility to biodegradation under in service conditions are rare. In some cases, coating suppliers now claim biocidal properties for their products.

Corrosion

Microbially influenced corrosion (MIC) has been the subject of much study and controversy over the last seventy years since Von Wolzogen Kuhr first attributed the external corrosion of steel pipe in black wet soil to the action of sulfate reducing bacteria (5). His basic mechanism survives today in a slightly elaborated form as an explanation of pipeline corrosion problems prevalent in wet anaerobic clay soils (6). In this corrosion scenario, sulfate reducing bacteria reduce groundwater sulfate to sulfide which precipitates with ferrous ions released by corrosion of the steel surface to form insoluble iron sulfides. Iron sulfides form a galvanic corrosion couple with steel draining electrons from the corrosion process in a battery like action. Reducing power possibly in the form of hydrogen is then taken off the sulfide matrix by sulfate reducing bacteria using it in the metabolic reduction of sulfate to continue and extend the process. A similar mechanism can be invoked in oilfield brine handling systems (7) and inside oil pipelines where water has accumulated at low spots such as river crossings.

An extensive contract research program undertaken by the Gas Research Institute in Chicago followed up earlier Canadian reports of microbial involvement in external pipeline corrosion problems (8, 9). This work identified corrosion scenario's in which bacteria other than sulfate reducers were active. In particular, microbial production of organic acids was felt to be important (10, 11).

Research at the University of Alberta identified a new group of bacteria present in oil pipelines. These organisms are able to reduce iron (III) to iron (II) under anaerobic conditions. This activity has implications for internal corrosion of liquid carrying pipelines (12).

Mechanistic work of this sort has been the basis of the development of new commercially available assays for the kinds of bacteria implicated. For example, Dupont has produced a colour test kit for APS-reductase an essential enzyme in sulfate reducing bacteria through a Conoco subsidiary. Caproco has commercialized a hydrogenase kit developed at the University of Calgary. Several gene probes for various groups of sulfate reducing bacteria and growth media for enumerating so called "Acid Producing Bacteria" have appeared. Most recently a new approach to DNA probe assays, the Reverse Sample Genome Probe has been described as a technique which allows the quantitative identification of specific sulfate reducing bacteria in field samples in a single test (13). This is a very active area of research and commercialization. A round robin comparison of several assay kits appeared recently in a National Association of Corrosion Engineers publication with the authorship and involvement of Canadian groups (14).

Control procedures including the use of biocides in liquid handling facilities have evolved considerably over the last decade. Largely due to efforts at the University of Calgary the importance of surface bound biofilms in the corrosion process has become fully appreciated and has spawned the invention of new sampling devices and monitoring

methods. Specific biofilm sampling devices including devices for high pressure systems are now offered commercially by Caproco.

The discovery by the University of Calgary that small applied electric fields can greatly assist the action of biocides has opened up new opportunities for controlling undesirable biological activity (15).

The control of undesirable microorganisms in industrial settings has been reviewed more fully elsewhere (16).

Souring

Corrosion is only one of the problems caused by sulfate reducing bacteria (17). A related issue is the problem of the release of noxious quantities of hydrogen sulfide. This gas poses a serious health risk and causes a variety of corrosion and cracking problems. Hydrogen sulfide above trace concentrations requires special design considerations and materials selection for "sour service".

Reservoir souring can occur over the life of an oilfield especially during secondary production in which water is injected to displace oil from underground. Hydrogen sulfide in this case arises from promotion of *in situ* populations of sulfate reducing bacteria. Depending on the reservoir mineralogy and water characteristics, hydrogen sulfide production can be sufficient to require a complete refit of production and handling facilities to meet sour service specifications at a cost of millions of dollars. This problem is now appearing in major oil reservoirs on the North Slope of Alaska and is even feared in hot North Sea reservoirs as seawater floods mature.

Because of the dollar value of the consequences and the threat to human life, there is considerable interest in modelling the microbial souring process (18) and in devising control procedures (19). Use of microorganisms to promote oil recovery as described in the next section must avoid souring problems. Injection of nitrate and downhole growth of *Thiobacillus denitrificans* have been proposed as control strategies to prevent hydrogen sulfide formation and release (20).

Beneficial Activities

Microbially Enhanced Oil Recovery

The idea that bacteria could be used as beneficial agents in the recovery of oil was suggested as early as 1926. Serious lab work began in the late 1940's with field trials and patent activity following in the 1950's in the USA, eastern Europe and the USSR (3). Since the late 1970's, biannual international workshops sponsored by the U.S. Department of Energy have tracked progress in eleven or more countries (21). Reviews are available (22, 23). A summary of some key areas of activity follows.

- Exploration

Oil and gas deposits often release methane and ethane at appreciable rates. These gases seep to the surface through intervening geological formations and support development of methane and ethane oxidizing bacteria in surface soils where atmospheric oxygen is available to support growth. Mapping the presence of the ethane oxidizing bacteria has been used as an exploration tool to identify the presence of underlying reservoirs (3). The technique is inexpensive and can help prioritize drilling targets identified by sophisticated seismic and geophysical methods.

- Drilling and Fracturing

Projects at the University of Calgary have looked at the use of bacterial systems to plug off thief zones encountered during drilling. Thief zones are geological formations of sufficient permeability to allow easy entry and loss of the recirculating fluid or "drilling mud" used to lubricate the bit and carry back rock chips in the drilling process. It was found that slime forming bacterial cultures injected in conjunction with particulates can substantially plug off such zones so that drilling can proceed.

Fracturing is a process in which hydraulic pressure is used to fracture the geological formation about an oil well to improve fluid or gas flow. In fracturing operations, the key is to prevent the fracture from resealing on release of the applied hydraulic pressure. Bacterial cultures have been used in at least one field test to etch out fractures in limestone through the *in situ* production of organic acids (24).

- Stimulated Production

Most MEOR field applications have injected bacteria with a nutrient package containing a large volume of a cheap fermentable substrate such as molasses. Fermentation downhole leads to formation of acids, solvents, gas and surfactants as agents of stimulated oil release (25). Acids are able to etch limestone formations to improve fluid flow. Gas pressure provides a drive and surfactants and solvents facilitate oil release. Such systems have been used to clean the well bore region around choked wells in eastern Europe and to extend the life of depleted waterfloods in the southern United States. Field tests by the National Institute for Petroleum and Energy Research at the Mink Unit in Oklahoma (26) showed that this approach was cheap enough to prolong marginal oil recovery operations and maintain access to reservoirs that would be lost with their closure.

Russian workers have concentrated on the injection of air into low sulfate reservoirs to stimulate the direct attack of indigenous organisms on the oil. Agents resulting from this microbial activity can apparently increase oil production rates (27).

Another approach tried with commercial success in Australia and more recently

in the North Sea is based on providing indigenous communities with selected missing nutrients. In this case, the bacterial cells themselves are implicated in process of oil release (28).

- Selective Plugging

As early as 1958, Van Heiningen *et al.* recognized that aqueous bacterial systems injected into a geological formation selectively enter the most permeable zones for water flow (3). Subsequent growth and metabolism can potentially plug the pores in such a zone lowering its permeability and reducing fluid flow. This can serve two purposes.

In waterflood operations, injected water is used to displace oil from underground but the injected water takes the path of least resistance through the reservoir sweeping out the most susceptible zones. Overall oil recovery can be improved by selectively plugging these swept zones and diverting subsequent water injection into new zones still rich in oil.

In heavy oil reservoirs common to the Lloydminster region of western Canada, viscous oil is produced in competition with water. Because of its lower viscosity, water from adjacent aquifers is preferentially pulled in causing production wells to "water out". Selectively plugging water channels in such cases can potentially restore oil flow.

A number of patented systems have been developed.

One approach is based on the fermentation of injected nutrients to form insoluble polymers and/or biomass. In the "bug plug" system developed by NOVA Husky Research Corporation (now NOVACOR Research & Technology Corporation) and Husky Oil, *Leuconostoc* species produce chunks of insoluble dextran which clog pore throats in sand formations typical of heavy oil deposits in the Lloydminster region. A field test demonstrated the practicality of the approach (29).

An alternate approach uses bacteria to shift the pH of the reservoir system to promote deposition of minerals *in situ.* The "bug rock" system again developed by NOVACOR Research & Technology Corporation injects urea in a saturated solution of calcium bicarbonate. Hydrolysis of the urea by a previously injected bacterial community shifts the pH to more alkaline values precipitating calcite in water permeable streaks and fractures. This technology offers the chance to control water incursion into producing fields, cement off fractures and consolidate loose sand normally produced in heavy oil recovery operations (30).

Controlled starvation of bacteria leads to the formation of very small resting cells called ultra-microbacteria (UMB). The idea of using UMB to carry out microbial processes in very tight reservoirs where small pore throats preclude easy injection of full size cells has been patented by Costerton *et al.* (31) at the University of Calgary. In such

tight formations, bacterial biomass and associated slime are sufficient to plug off fluid flow.

Microbial plugging is currently under test in the Burbank field in California by Phillips Petroleum (32).

- Dewaxing

Perhaps the most successful commercial process to date in this area has been the use of microbial products in cleaning applications.

Microbial products have shown a good success rate in removing precipitated waxes which can restrict oil flow in wells producing paraffinic crudes. Prior to introduction of this technology, wax build up was removed periodically by the circulation of hot oil. This was relatively costly and resulted in lost production during frequent treatments. The microbial process introduces live organisms as well as surfactants into the target well to achieve prolonged effect. In fields which respond to this approach, dramatic economic returns have been documented (33). Similar formulations have also been successful at resuspending precipitated waxes and asphaltic solids which collect in tanks in production and pipelining operations. Shut down and mechanical cleaning in such cases can cost hundreds of thousands of dollars for large tanks with serious sludge deposits.

- Biopolymers

Biopolymers such as xanthan and scleroglucan act as shear thinning viscosifying agents in oilfield applications.

In recirculating drilling muds, xanthan assists in removal of rock fines by suspending particulates under the low shear conditions found in the vertical casing but thins in the high shear region around the drill bit to allow efficient drilling. Its durability is a marked improvement over chemical polymers such as polyacrylamide which are degraded by high shear.

Shear thinning biopolymers can also improve the sweep efficiency of waterflood operations by thickening the water to match oil viscosities. This improves oil recovery by allowing the water to push oil through the formation without fingering through it. While the effectiveness of polymer floods is well established the incremental cost of the polymer precludes economic use of the technique at present oil prices.

- Transportation

Microbial surfactants and emulsifying agents have an extraordinary ability to form oil in water emulsions. This makes them candidates for facilitating the production and pipelining of heavy oil.

Western Canadian heavy oils are sufficiently viscous that they cannot be pipelined to asphalt markets in the northern United States with being substantially diluted with lighter hydrocarbons like gas condensates. Oil in water emulsions in which water forms the continuous phase offer an alternative. Chemical surfactants are adequate for the task but biological surfactants may offer environmental advantages and greater ease of emulsion separation at the delivery point. Preparation of oil and water emulsions could be done in the production well where pump shear can invert the high viscosity water in oil emulsion normally produced. Improved pumping and field collection could result.

This scenario was evaluated in a joint industry study organized by the Alberta Oil Sands and Research Authority several years ago (34) and was deemed to be a reasonable alternative to diluent pipelining. Implementation would require a complete acceptance of the new approach by all shippers since different shipments are batched through major pipelines and mixing diluted oil batches and emulsions would not be desirable. The technology awaits as an option should shortages of diluent or other mark et factors dictate its use.

In carrying out related research it was discovered that forming oil in water emulsions for heavy fuel oils actually improves their combustion efficiency. Rapid vapourization of the continuous water phase departing a combustion nozzle results in efficient atomization and combustion of the oil droplets contained in the parent emulsion. British Petroleum now markets products of this sort although it is unclear whether biological emulsifiers are used in their formulations.

- Upgrading

As early as 1926, the use of microorganisms to upgrade oil was suggested (3). The target suggested was viscosity reduction of asphaltic heavy crude to a yield a more valuable conventional crude better suited to supply products such as gasoline. Ironically this unlikely prospect continues to be proposed sporadically as an innovative concept even today.

A more promising opportunity is the removal of sulfur from fossil fuels. This is desirable because sulfur content is regulated in most fuels to avoid undue production and release of sulfur oxides from the combustion process.

Work at the University of Alberta is focusing on removal of sulfur from heterocyclic components of Canadian oils (35). The venue for commercial application remains undefined but implications may exist for bioremediation of sites contaminated with sulfur rich crude. As the partially oxidized intermediates produced in the desulfurization process become better understood, the risk of their formation and release in clean-up operations can also be evaluated.

Internationally, removal of organic sulfur from coal continues to be an active topic

(36) but is perhaps of less interest to Canada with its abundant supply of low sulfur coal in Western Canada.

- Biodegradable Products

Consumer concerns over the environmental impact of fossil fuel based products has forced development of new "green" options. These products are not hydrocarbon derivatives necessarily but require a knowledge of biodegradation in their design. A good example is the development of biodegradable polymers to replace standard chemical products such as polystyrene.

One of the first products on the market in North America was a blend of chemical polymer and starch for use in disposal garbage bags. Biodegradation was limited to the starch component and was found to occur to only a limited extent in most landfills where extraordinary effort is made to prevent biological degradation. Introduction of this product caused a considerable reexamination of objectives.

Truly biodegradable polymers based on polyhydroxybutyrate and valerate have been marketed by ICI for some years. They are restricted to specialty markets because of production costs and without major improvements in all facets of the fermentation and product purification sequence are unlikely to invade commodity plastics markets.

Polylactide made by a semi-synthetic route will enter the U.S. market shortly from a new Cargill plant. Its cost and markets will be proven with time but it potentially could displace petrochemical polymers from certain applications.

Biodegradable lubricants and other products are also appearing but their ultimate market impact is not clear. In many cases including PHB/V, petrochemicals are still used as a feedstock in production. The most successful products are likely to result from the marriage of biotechnology with traditional petrochemical market access and expertise. This could be an area of significant future interest for the Canadian chemical industry if production costs can be reduced to competitive levels.

- Remediation

Use of biotechnology in the clean-up of the environment is a separate topic in its own right but its suitability for application in the hydrocarbon based industries justifies some comments here on potential targets.

- Methane Seeps and Air Streams

Methane is a potent global warming gas. Methane seeps and leaks in the oilfield are of concern both in the overall context of global warming and because of the local impact on vegetation. Where seepage is sufficient to cause the root zone to become

anaerobic, plant life dies off. The extent of this problem is currently being addressed. Oil well abandonment designs which promote microbial methane oxidation in the subsurface soil without a loss of vegetation could mitigate this problem.

Numerous designs are appearing for the biofiltration of hydrocarbon contaminated air streams. In these units, microbial metabolism converts the hydrocarbon to carbon dioxide and water. Reduction of fugitive emissions especially from low pressure sources is a stated industry objective. Robust practical all weather systems able to provide reliable continuous operation must be demonstrated.

- Site Remediation

Numerous historical spills on various operating sites have left the hydrocarbon based industries a legacy of contaminated soils but site remediation methods developed for oil spills are not always appropriate.

Petrochemical sites in particular usually produce specific chemicals often of significant volatility. Where excavation is impossible or where excavation would release unacceptable volatiles to the atmosphere, *in situ* remediation methods such as bioventing are needed to remediate spills. Unfortunately, many sites in Western Canada are on glacial tills of extremely low hydraulic conductivity. A challenge exists to develop effective *in situ* technologies for such sites.

It seems likely that future clean-up criteria will be based on risk assessments rather than arbitrary target clean-up levels. As part of risk assessment, accurate estimates and methods of measurement of naturally occurring biodegradation rates are needed for the vadose zone and the water saturated zones below it. While oil spills sport a myriad of hydrocarbon compounds, petrochemical spills feature significant concentrations of single chemicals. In the biodegradation of mixed hydrocarbons, the opportunity for cometabolism exists. In pure chemical spills, this is not so. Work is needed to develop ways to biodegrade specific hydrocarbons to vanishingly small residual levels. One example is dicyclopentadiene, a very smelly difficult to degrade hydrocarbon produced and shipped in significant quantities around North America. This challenge is more akin to work presently in progress to remediate PCB's than to earlier work on oil spills.

Research is needed to develop ways to achieve extended biodegradation perhaps by the addition of appropriate nutrients to foster and sustain an appropriate microbial population until all the target compound has been destroyed. These methods must also perform in cold climate applications where low soil temperatures frustrate sustained activity.

- "Slop Oil" and Oil Spills

Refineries and other operations produce unbreakable emulsions generally referred to as "slop oil". Traditional methods of disposal such as road spreading for dust control

or landfarming are disappearing practices. Use of traditional stirred tank reactors is expensive, requires dilution of the slop oil and creates waste streams more numerous and voluminous than the original problem.

Adsorption of slop oil onto an oleophilic peat product provides a matrix for water recirculation in a novel bioreactor patented by Novacor Research & Technology Corporation (37). In this invention, the oil stays absorbed in the peat throughout the degradation process. Volatile hydrocarbons are preferentially destroyed and water leachables collected in a closed water system. The approach is surprisingly suited to the rapid destruction of asphaltic heavy oil emulsions (38). It provides a "missing" technology for the biodegradation of modest volume, high oil content wastes and may be used in conjunction with reclamation technologies such as filter presses to handle larger volume problems.

Oil spill clean-up by bioremediation remains an issue even after the expensive trials organized by the U.S. E.P.A. on the Exxon Valdez spill. Suggestions for research areas were made in an expert report to the Canadian Department of Transport in 1991 (39).

CONCLUSION

Because of the inherent susceptibility of hydrocarbons to biotransformation and their ability to support the growth and activity of microorganisms it is likely that biotechnology will continue to develop around the hydrocarbon based industries. Immediate gains are being made in the area of diagnostic kits, monitoring devices and control procedures for detrimental activities. Microbially enhanced oil recovery technologies have reached field trial with success including commercial success in several cases. Exploitation will be by operators wishing to extend reservoir life with inexpensive technology. Well and tank cleaning operations based on microbial formulations are a commercial success. Biological products such as biopolymers have captured niches in drilling mud applications for example but await higher oil prices to achieve broad application in oil recovery. A number of other biotechnologies exist as feasible options in some stage of development awaiting market opportunity. These include bioemulsifiers for oil production and transport, microbial upgrading technologies for sulfur rich oils and biodegradable products including thermoplastic polymers. The "hottest" area of application for the 1990's will undoubtedly be the biological remediation of hydrocarbon contaminated sites and emissions. Demonstration of reliable technologies adapted to specific target applications in the oil and gas and petrochemical sectors remains a hurdle to widespread use.

REFERENCES

1. **Gibson, D. T.** 1984. Microbial Degradation of Organic Compounds, Marcel Dekker, Inc., New York.

2. **Kamely, D., A. Chakrabarty, and G. S. Omenn.** 1990. Biotechnology and Biodegradation, Gulf Publishing Co., Houston.

3. **Davis, J. B.** 1967. Petroleum Microbiology, Elsevier, New York.

4. **Hill, E. C.** In: Biodegradation of Hydrocarbon Based Products in Industrial Use, Vol. **1**, Applied Science Publishers Ltd, London. pp. 201-225.

5. **Von Wolzogen Kuhr, C. A. H. and I. S. Van der Klugt.** 1934. De Grafiteering van Gietzer als Electrobiochemisch Proces in Anerobe Groden, Water (the Hague), **18**: 147-165.

6. **Jack, T. R., R. G. Worthingham, and F. G. Ferris.** 1991. External Corrosion of Line Pipe Under Disbonded Coatings; Field Observations and Mechanistic Studies. In: Microbially Influenced Corrosion and Biodeterioration. N. J. Dowling, M. W. Mittelman and J. C. Danko (Eds). University of Tennessee, Knoxville, pp. 4.19 - 4.24.

7. **Ferris, F. G., T. R. Jack, and B. J. Bramhill.** 1992. Corrosion Products Associated with Attached Bacteria at an Oil Field Water Injection Plant. Canadian J. Microbiol. **38**: 1320-1324.

8. **Worthingham, R. G., T. R. Jack, and V. Ward.** 1986. External Corrosion of Line Pipe, Part I: Identification of Bacterial Corrosion in the Field. Biologically Induced Corrosion. S. C. Dexter (Ed.), National Association of Corrosion Engineers, Houston, pp. 330-338.

9. **Jack, T. R., M. McD Francis, and R.G. Worthingham.** External Corrosion of Line Pipe Part II: Laboratory Study of Cathodic Protection in the Presence of Sulfate- Reducing Bacteria, pp. 339-350 *in ibid.*

10. GRI Field Guide I. 1990. Microbiologically Influenced Corrosion (MIC): Methods of Detection in the Field. Gas Research Institute, Chicago.

11. GRI Field Guide II. Microbiologically Influenced Corrosion (MIC): Investigation of Internal MIC and Testing Mitigation Procedures. Gas Research Institute, Chicago.

12. **Obuekwe, C. O., D. W. S. Westlake, F. D. Cook, and J. W. C. Costerton.** 1981. Surface Changes in Mild Steel Coupons from the Action of Corrosion Causing Bacteria. Appl. Environ. Microbiol., **41**: 766-774.

13. **Voordouw, G., J. K. Voordouw, T. R. Jack, J. Foght, P. M. Fedorak, and D. W. S. Westlake.** 1992. Application of Reverse Sample Genome Probing to the Identification of Sulfate-Reducing Bacteria. Proceedings of the ASTM. International Symposium on Microbiologically Influenced Corrosion. November 16-17, Miami, Florida. ASTM, Washington, in press.

14. **Scott, P. J. B., M. Davies.** 1992. Survey of Field Kits for Sulfate-Reducing Bacteria. Material Performance, pp. 64-68.

15. **Blenkinsopp, S. A., A. E. Khoury and J. W. C. Costerton**. 1992. Electrical Enhancement of Biocide Efficacity Against *Pseudomonas aeruginosa* Biofilms. Appl. and Environ. Microbiol. **58**(11): 3770-3773.

16. **Jack, T. R., D. W. S. Westlake**. Control in Industrial Settings. In: Biotechnology Handbooks: Sulfate-Reducing Bacteria, L L. Barton (Ed.), Plenum Publishing, in press.

17. **Postgate, J. R.** 1979. The Sulphate-Reducing Bacteria, Cambridge University Press, London.

18. **Ligthelm, D. J., R. B. de Boer, and J. F. Brint**. 1992. Reservoir Souring: An Analytical Model for H_2S Generation and Transportat on in an Oil Reservoir Owing to Bacterial Activity. SPE 23141, Society of Petroleum Engineers, Richardson, Texas.

19. **Lizama, H. M., B. M. Sankey**. 1993. On the Use of Bleach Soaks to Control Bacteria-Mediated Formation Souring. J. Petroleum Science and Engineering, **9**:145-153.

20. **McInerney, M. J., V. K. Bhupathiraju, K. Vishvesh and K. L. Sublette**. 1992. Evaluation of a Microbial Method to Reduce Hydrogen Sulfide Levels in a Porous Rock Biofilm. J. Ind. Microbiol. **11**(1): 53-58.

21. Microbial Enhancement of Oil Recovery - Recent Advances. 1993. Proceedings of the 1992 International Conference on Microbial Enhanced Oil Recovery. E. T. Premuzic and A. Woodhead (Eds). Elsevier, New York.

22. **Jack, T. R.** M.O.R.E. to M.E.O.R.: An Overview of Microbially Enhanced Oil Recovery. pp. 7 -16 *in ibid.*

23. **Moses, V., D. G. Springham.** 1982. Bacteria and the Enhancement of Oil Recovery. Applied Science Publishers, London.

24. **Moses, V., M. J. Brown, C. C. Burton, D. S. Gralla and C. Cornelius.** 1993. Microbial Hydraulic Fracturing. In: Microbial Enhancement of Oil Recovery - Recent Advances. Proceedings of the 1992 International Conference on Microbial Enhanced Oil Recovery. E. T. Premuzic and A. Woodhead (Eds). Elsevier, New York, pp. 207-229.

25. **Jack, T. R.** 1983. Enhanced Oil Recovery by Microbial Action. In: Chemical and Geochemical Aspects of Fossil Energy Extraction. T. F. Yen, F. K. Kawahara and R. Hertzberg (Eds). Ann Arbor Science, Ann Arbor, Michigan, pp. 13-22.

26. **Bryant, R. S., A. K. Stepp, K. M. Bertus, T. E. Burchfield, and M. Dennis.** 1993. Microbial Enhanced Waterflooding Field Pilots. In: Microbial Enhancement of Oil Recovery - Recent Advances. Proceedings of the 1992 International Conference on Microbial Enhanced Oil Recovery. E. T. Premuzic and A. Woodhead (Eds). Elsevier, New York, pp. 289-306.

27. **Ivanov, M. V., S. S. Belyaev, I. A. Borzenkov, I. F. Glumov and R. R. Ibatulin.** 1993. Additional Oil Production During Field Trials in Russia. In: Microbial Enhancement of Oil Recovery - Recent Advances. Proceedings of the 1992 International Conference on Microbial Enhanced Oil Recovery. E. T. Premuzic and A. Woodhead (Eds). Elsevier, New York, pp. 373-382.

28. **Sheehy, A. J.** 1990. Field Studies of Microbial EOR.SPE/DOE 20254. Proceedings of the 7th Symposium on Enhanced Oil Recovery, Tulsa, Ok., April 22-25.

29. **Stehmeier, L. G., T. R. Jack, B. A Blakely and J. M. Campbel.** 1990. Field Test of a Microbial Plugging System at Standard Hill, Saskatchewan to Inhibit Oilfield Water Encroachment. In: Biohydrometallurgy, Proceedings of the International Symposium, Jackson's Hole, Wyoming, August 13-18, 1989. CANMET, EMR, Ottawa, pp. 699-712.

30. **Jack, T. R., F. G. Ferris, L. G. Stehmeier, A. Kantzas and D. F. Marentette.** 1993. Bug Rock: Bacteriogenic Mineral Precipitation Systems for Oil Patch Use. In: Microbial Enhancement of Oil Recovery - Recent Advances. Proceedings of the 1992 International Conference on Microbial Enhanced Oil Recovery. E. T. Premuzic and A. Woodhead (Eds). Elsevier, New York, pp.27-36.

31. **Cusack, F., A. F. MacLeod, J. W. Costerton, and H. M. Lappin-Scott.** 1990. Formation and Utilization of Ultramicrobacteria to Enhance Oil Recovery by Selective Plugging. In: Gas, Oil and Coal Biotechnology, I. C. Akin, J Smith (Eds). Institute of Gas Technology, Chicago, pp. 343-354.

32. **Jenneman, G. E., J. B. Clark, and P. D. Moffitt.** 1993. A Nutrient Control Process for Microbially Enhanced Oil Recovery Applications. In: Microbial Enhancement of Oil Recovery - Recent Advances. Proceedings of the 1992. International Conference on Microbial Enhanced Oil Recovery. E. T. Premuzic and A. Woodhead (Eds), Elsevier, New York, pp. 319-334.

33. **Nelson, L. and D. R. Schneider**. 1993. Six Years of Paraffin Control and Enhanced Oil Recovery with the Microbial Product, Para-BacTM. In: Microbial Enhancement of Oil Recovery - Recent Advances. Proceedings of the 1992 International Conference on Microbial Enhanced Oil Recovery. E. T. Premuzic and A. Woodhead (Eds), Elsevier, New York, pp. 355-362.

34. AOSTRA/Industry. 1986. Bitumen Water Transportation Study. Phase I-Organization and Evaluation. Final Report. Alberta Oil Sands Technology and Research Authority, Edmonton.

35. **Fedorak, P. M., J. D. Payzani, D. S. Montgomery, and D. W. S. Westlake.** 1988. Microbial Degradation of N-alkyltetrahydrothiophenes Found in Petroleum. Appl. Environ. Microbiol., **54**:1243-1248.

36. Fourth International Conference on the Biological Processing of Fossil Fuels, Sardinia, Italy, September 19 - 23, 1993. Proceedings in preparation.

37. **Francis, M. McD., T. R. Jack**. 1991. Biodegradation of Oily Waste. Canadian Patent Application, 2 032 610 - 7.

38. **Francis, M. McD**. 1992. Biotreatment of Waste Oil and Sludges. In: Proceedings of the Eighth Annual Meeting of BIOMINET, October 2, 1991. Calgary, Alberta. W. D. Gould and S. Lord (Eds), CANMET MSL, EMR, Ottawa, pp. 55-74.

39. **Gutnik, D. L.** 1991. Bioremediation in Marine Oil Pollution. TP 11261E. Transportation Development Centre, Transport Canada.

A PROCESS FOR THE BIOLOGICAL OXIDATION OF HYDROGEN SULFIDE

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ABSTRACT

A process is described whereby H_2S in a gaseous stream is converted to sulfur and sulfate by the bacterium *Thiobacillus thiooxidans*. Gas-bacteria contact was achieved by flowing a stream of H_2S in air up through a bed of glass beads, countercurrent to a liquid medium percolated down from the top. Initial conversion to sulphur was much faster than subsequent oxidation to sulphate, allowing for removal of elemental sulphur. The rate of H_2S removal was proportional to the available surface area in the column bed, and increased very slowly with time, placing great importance on the initial concentration of bacteria in the system.

A range of key variables has been addressed including the effect of H_2S concentration and ultimate inhibitory effects, size of column packing and gas phase residence time.

UN PROCESSUS D'OXYDATION BIOLOGIQUE DU SULFURE D'HYDROGÈNE DANS UN COURANT GAZEUX

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RÉSUMÉ

On décrit un processus au cours duquel le H_2S présent dans un courant gazeux est converti en soufre élémentaire et sulfate par la bactérie *Thiobacillus thiooxidans*. On a réussi à établir un contact entre le gaz et les bactéries en faisant circuler un courant ascendant du H_2S et d'air dans un lit de billes de verre, à contre-courant d'un liquide percolant à partir du sommet du lit. Il y avait quelques bactéries dans la phase liquide; la majorité étaient fixées à la garniture de la colonne. La conversion initiale en soufre a été beaucoup plus rapide que l'oxydation ultérieure en sulfates, ce qui a permis d'enlever le soufre élémentaire. La vitesse d'enlèvement du H_2S était proportionnelle à la surface disponible dans le lit de la colonne et augmentait très lentement en fonction du temps, ce qui indique la grande importance de la concentration initiale des bactéries dans le système.

On a établi une gamme de variables fondamentales, notamment l'effet de la concentration du H_2S et des effets inhibiteurs ultimes, la dimension de la garniture de la colonne et le temps de séjour de la phase gazeuse. On a recueilli et analysé les données cinétiques. Étant donné l'expérience fournie par les études en laboratoire, on a pu examiner les problèmes potentiels de mise à l'échelle de ce nouveau procédé.

INTRODUCTION

Hydrogen sulfide is widely encountered in the petroleum industry, originating in produced gases, refinery hydrotreating processes or various small sources such as storage tanks. In view of the toxicity of H_2S, and controls applied to emissions of its combustion product, sulfur dioxide, its concentration in natural gas or fuel gas must be reduced to very low levels prior to marketing or combustion. Once separated, the H_2S must be converted to non-toxic, manageable products. Commonly, this is achieved using a Claus plant which oxidizes part of the H_2S to SO_2, then uses the SO_2 to oxidize the bulk of the H_2S to elemental, saleable, sulfur. However, this is economic only on a large scale. For smaller scale situations, when H_2S levels are low but still unacceptable, alternative technology is required. Adsorption with zinc oxide is effective for small volumes of H_2S but disposal of the spent adsorbent limits scope of this approach.

Biological H_2S treatment with *Thiobacillus denitrificans* has been studied by Sublette (1); however, contamination at the neutral pH utilized represents a potential scale-up problem.

Another approach has been the chemical oxidation of H_2S to elemental sulfur by a ferric iron solution:

$$H_2S + 2Fe^{3+} \text{ ----> } S^\circ + 2Fe^{2+} + 2H^+$$

and the regeneration of ferric iron from the resulting ferrous iron by iron-oxidizing bacteria (2).

The bacterium *Thiobacillus thiooxidans* is a chemolithotrophic acidophile which oxidizes sulfur or sulfide for its sole source of metabolic energy and meets all its carbon requirements through fixation of CO_2 from the atmosphere (3). It oxidizes sulfide through the reaction:

$$S^{2-} + 2O_2 \text{ ----> } SO_4^{2-}$$

and plays a role in the leaching of metals from sulfide ores (4). *T. thiooxidans* appeared very suitable for the oxidation of H_2S in an industrial process; since CO_2 fixation is a high energy-requiring process, it must oxidize large amounts of sulfide for growth, resulting in a relatively low rate of increase in biomass. As an acidophile, *T. thiooxidans* is capable of biological activity at pH's lower than 1.0 (3), a particularly desirable trait since sulfuric acid is a by-product of H_2S oxidation.

The objective of this study was to provide a relatively low cost, low energy route for the conversion of H_2S into environmentally acceptable products namely sulfur or water-soluble sulfate.

MATERIALS AND METHODS

Apparatus

Several versions of the apparatus shown in Figure 1 were employed. The column contactors were made of plexiglass and had dimensions of 91 cm in height by 2.5 cm (inside) diameter. The packing comprised glass beads (3 or 5 mm diameter) and was supported by a perforated plate below which was a space for the collection of liquid. Total bed volume was 340 mL. The gas stream consisting of H_2S in air was injected into the bottom of the column below the perforated plate, exiting from the top, above the packing. For some experiments, two columns were connected in series.

Liquid medium was recirculated with an Ismatec peristaltic pump; typical values for total volume and flow rate were 70 mL and 63 mL/min respectively. In the gas recycle mode, the air/H_2S mixture was also circulated by the peristaltic pump from one or two 10 L volume-adjustable plexiglass reservoirs. A gas flow rate of 100 mL/min was used. In the gas flowthrough mode, air and H_2S were metered from separate cylinders, then mixed to give a mixture of the desired composition.

Media and Microbiological Growth Conditions

The liquid medium used throughout these experiments was HP medium which consisted of 0.1 g K_2HPO_4; 0.4 g $(NH_4)_2SO_4$,; and 0.4 g $MgSO_4.7H_2O$ per litre and adjusted to pH 2.3 with H_2SO_4 (5). *T. thiooxidans* strain SM-7 was kindly supplied to us by Dr. I. Suzuki from the University of Manitoba. The bacterium was grown in 250 mL erlenmeyer flasks containing 100 mL of HP medium including a 10% inoculum. After inoculation, powdered elemental sulfur was spread evenly over the surface of the liquid and the culture was incubated in stationary mode at room temperature for 5-6 days. In order to prepare sample for the column, 50 mL of full-grown culture was filtered through Whatman No. 1 filter paper to remove elemental sulfur, and combined with 20 mL of HP medium.

Analyses

Samples of the recirculating liquid stream were taken at different times using a syringe (2-3 mL). The sample was then analyzed by ion chromatography with Na_2CO_3 and $NaHCO_3$ as a carrier/eluent and weak H_2SO_4 as a suppressor (6). To determine dissolved H_2S levels at the end of some experiments, column effluent was quickly mixed

with 0.1 N iodine and back-titrated with $Na_2S_2O_3$ using a starch indicator.

Analysis for gaseous H_2S was carried out using a Gastec detector tube either directly or by first taking a sample into a gas sampling bag. In the gas recycle mode experiments, the gas and liquid were allowed to run for 20 min prior to taking a zero-time H_2S sample. In the gas flowthrough modes the gas flow was monitored and kept constant for at least 30 min prior to taking a sample.

Analysis of sulfur deposit on glass bead surfaces was carried out by energy dispersive X-ray analysis in a scanning electron microscope chamber. The sample was mounted on carbon studs and coated with carbon.

RESULTS

Gas Recycle Experiments

As *T. thiooxidans* removed H_2S from the recirculating gas stream, soluble sulfate and acidity increased as expected from the oxidation of sulfide to sulfate. At the end of each experiment, bacterial viability was tested by culturing an effluent sample in sulfur medium. However, from the stoichiometry observed, it was apparent that not all the removed H_2S was being oxidized completely to sulfate. Table 1 shows differences ranging from 11% to 34% in unaccounted for sulfur. It was not possible to use pH to compare H_2S removal in the various experiments because of the buffering action of sulfate and phosphate ions in the liquid medium.

It became apparent that removal of H_2S was much more rapid than the formation of sulfate. Both sulfate and H_2S were determined throughout the duration of the experiments. As seen in Figure 2, the overall bacterial H_2S removal rate was 0.068 mmoles/h in a 10 L system whereas the formation of sulfate was much slower at 0.017 mmoles/h. Thus, the rate of H_2S removal was 4 times that of sulfate formation, indicating the accumulation of elemental sulfur as an intermediate. Figure 2 also shows a control gas-recycle experiment for comparison. In the absence of bacteria, no sulfate formed and no increase in acidity occurred. It was subsequently discovered that the H_2S removal observed in the control experiments was due to reaction with steel fittings; replacement with inert material provided a control showing no significant H_2S loss.

With time, the beads in the contactor became covered with yellowish particulate matter. This material was shown by energy dispersive X-ray analysis to be elemental sulfur. This sulfur was crystalline in appearance and could be easily trapped by a glass wool filter placed in the liquid recycle line.

Gas Flowthrough Experiments

In some early experiments, pressure imbalances at the base of the column interfered with uniform flow of the liquid, causing buildup in the level. To explore the effect of bead size on pressure drop, a second column was set up, with a packing comprising larger glass beads: 5 mm diameter as opposed to 3 mm. The columns were run in parallel, with a continuous once-through gas stream at various flow rates but using the same source of gas mixture, the same volume of liquid, and the same liquid flow rates. With equal bed volumes of about 340 mL, the 3mm and 5mm bead sizes in the two columns provided total surface areas of 4092 cm^2 and 2325 cm^2, respectively. Although the columns were run for several days, measurements of H_2S removal were carried out within 9 h to minimize any effect of activity change over time.

The mean rate of H_2S removal increased with gas flow rate as shown in Figure 3. Control experiments with no bacteria showed no H_2S removal activity. The increases in activity were linear for the conditions used, with column 1 activity increasing at about twice the rate of column 2. This correlated with the differences in bead surface area for the columns. The dependence of reaction rate on gas flow rate indicates that a mass transfer limitation exists, such that increased gas rate enhances the transfer of H_2S from gas to liquid, partially via increased concentration driving force, since the higher flow rates actually resulted in higher H_2S concentrations in the exit gas stream.

Series Column Operation

Runs were carried out with the two columns connected in series, with respect to gas flow. Liquid medium for each column was recycled independently. With inlet H_2S concentration of 2100 ppm, final gas out of the second column contained no detectable H_2S, as shown in Table 2. Most of the H_2S was removed in the first column, since its concentration was highest there, but it was not possible to remove all the H_2S in a single column at practical gas flow rates. In a separate experiment, the positions of the columns were reversed so that the gas passed through column 2 first (5 mm beads) and then column 1 (3 mm beads). Results were essentially similar to those in Table 2 in that most of the H_2S was again removed in the first column.

Change of Bacterial Performance with Time

The influence of time on H_2S removal activity was examined by running two columns in parallel with a continuous gas stream at constant flow rates for several days. As shown in Figure 4, the mean H_2S conversion rates increased linearly with time, with column 1 showing a higher rate of increase than column 2. The rates of activity increases were very small, suggesting that the majority of active microorganisms in the columns came from the original inoculum and not from proliferation of the culture.

Inhibitory Effect of H_2S

During the course of the study, H_2S levels were increased in an effort to determine the ultimate limits to conversion rate. Results are presented in Figure 5 which plots conversion rate (ordinate) against H_2S concentration expressed both as volume fraction or ppm, with curves for different gas flow rates. H_2S was clearly inhibitory to the cells when present above a certain concentration which depended on gas flow rate.

Effect of Methane

In view of potential applications related to petroleum streams, the effect of hydrocarbons on activity of the bacteria was considered. In addition to the air and H_2S gas inlet streams, a third gas stream was metered into the columns, comprising methane. The effect was complex, as illustrated in Figure 6, where $G_v\Delta[H_2S]_g$ represents the mean H_2S oxidation rate. CH_4 inhibition was nonexistent at low H_2S concentrations but became significant with increasing H_2S content.

DISCUSSION

In both the gas recycle and flowthrough modes of operation, *T. thiooxidans* bacteria were able to remove H_2S from a gas stream in a countercurrent contacter. During initial control experiments, some H_2S reduction was observed with gas recycle but this could be accounted for by absorption into the water and, in one case, by reaction with metal surfaces. Abiological H_2S removal was not observed in the gas flowthrough mode since aqueous H_2S in the recirculating liquid builds up to saturation, impeding further removal via this route. Chemical oxidation of aqueous H_2S to sulfur is likely to be very slow at the low pH used.

The H_2S removed by bacteria was eventually oxidized all the way to sulfate. The overall rate of sulfate formation, however, was only one fourth the rate of H_2S removal, with the result that elemental sulfur accumulates in the system. This is a concern, since the generated sulfur must be removed to prevent plugging. It was possible to remove a large portion of the accumulated sulfur by installing a glass-wool filter in the liquid recycle line. There was little concern of filtering out active bacteria since a pore size of 2 μm or less would be required.

Bacterial activity increased with increasing available surface area in the column bed, which is in turn related to the size of the glass beads in the column. Although the smaller beads did give higher conversion rates, there is a practical lower limit to their size because of pressure drop effects; the 3 mm beads used gave pressure drop problems whereas the 5 mm beads did not. With time, the use of 3 mm beads was discontinued in favor of 5 mm beads.

The H_2S removal capability of the system increases with time, as seen in the flowthrough experiments. This improvement in performance could be due to adaptation or very slow growth of the bacterial population in the column. This places importance on the inoculum used since it is the main source of active bacteria.

H_2S was inhibitory to its own oxidation by *T. thiooxidans* under the conditions used, being significant at concentrations above 10% and lethal to the organism above 25%. Being a sulfide oxidizer, it is not surprising that *T. thiooxidans* is only weakly inhibited by moderate concentrations of H_2S. As an aerobic respirer, however, *T. thiooxidans* can still be overwhelmed by it since H_2S binds reversibly to cytochrome oxidase and reduces it (7, 8).

The inhibitory effect of methane, observed at inhibitory levels of H_2S, can be explained by an ordered inhibitor binding mechanism (9) where CH_4 does not bind to free cells or the "cell-H_2S complex" but does bind to the H_2S-inhibited cell complex. Thus, CH_4 has no effect on conversion rate at low H_2S concentrations but at high [H_2S] it is more inhibitory than H_2S alone.

CONCLUSIONS

1. The system comprising the bacterium *Thiobacillus thiooxidans* in an aqueous medium, arranged to contact gas in countercurrent flow, is effective for converting H_2S present in a gas stream containing excess air. The presence of bacteria was confirmed by observation in scanning electron micrographs.

2. H_2S removed by bacteria could be eventually oxidized all the way to sulfate. However, initial oxidation to elemental sulfur occurred at a rate four times faster than the subsequent oxidation of sulfur to sulfate, with the result that sulfur accumulates in the system. It was possible to remove much of this sulfur by installing a filter in the recycle liquid stream.

3. Capability of the system to convert H_2S increases slowly with time. This improvement in performance could include adaptation but is mostly due to growth of the bacterial population in the column. The multiplication of bacteria in the column is very slow. Generation times (time required for the population to double in size) are in the order of 200 h, as opposed to 6-10 h when the bacteria are grown in sulfur media. This places extreme importance on the inoculum used since it is the main source of active bacteria.

4. High concentrations of H_2S in air inhibit the bacterial process. Dilution with air should be a feasible means of managing this constraint.

5. Issues which need to be addressed for demonstration of this process on a larger scale are: improvement of conversion rates by enhancing the rate-limiting diffusion steps, removal of elemental sulfur to avoid buildup and plugging of the contactor, and definition of appropriate materials to handle the acidic medium.

REFERENCES

1. **Sublette, K.L.** 1988. Production of microbial biomass protein from autotrophic fermentation of hydrogen sulfide. Biotechnol. Bioeng. **32**: 408-409.

2. **Satoh, H., J. Yoshizawa, and S. Kametani.** 1988. Bacteria help desulfurize gas. Hydrocarbon Processing. **67**(5): 76D-76F.

3. **Harrison A.P.** 1984. The acidophillic thiobacilli and other acidophillic bacteria that share their habitat. Annu. Rev. Microbiol. **38**: 265-292.

4. **Lundgren, D.G, M. Silver.** 1980. Ore leaching by bacteria. Annu. Rev. Microbiol. **34**: 263-283.

5. **Lizama, H.M., I. Suzuki.** 1988. Bacterial leaching of a sulfide ore by *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*: 1. Shake flask leaching experiments. Biotechnol. Bioeng. **32**: 110-116.

6. **Weiss, J.** 1986. Handbook of ion chromatography, Dionex Corp., Sunnyvale, pp. 23-35.

7. **Sawhney, V., D.J.D. Nicholas.** 1978. Sulfide-linked Nitrite Reductase from *Thiobacillus denitrificans* with Cytochrome Oxidase Activity Purification and Properties. J. Gen. Microbiol. **106:** 119-128.

8. **Smith, L., H. Kruszyna and R.P. Smith.** 1977. The Effect of met Hemoglobin on the Inhibition of Cytochrome-*c* Oxidase by Cyanide Sulfide or Azide. Biochem. Pharmacol. **26:** 2247-2250.

9. **Segel, I.H.** 1975. Enzyme Kinetics, New York, NY: John Wiley & Sons Inc., pp. 487-488.

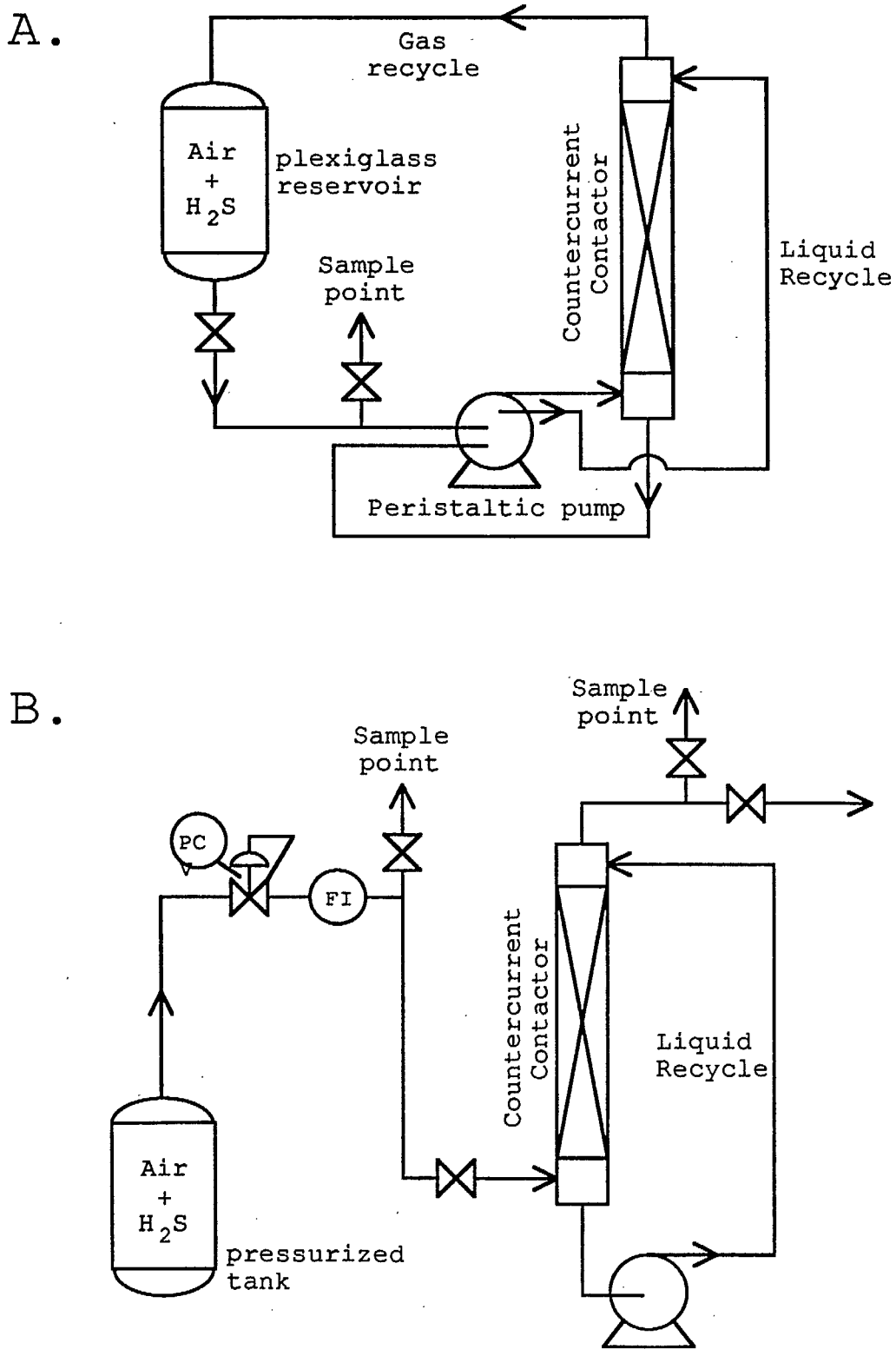

FIgure 1. Continuous laboratory unit configurations: A Gas recycle mode; B Gas flowthrough mode

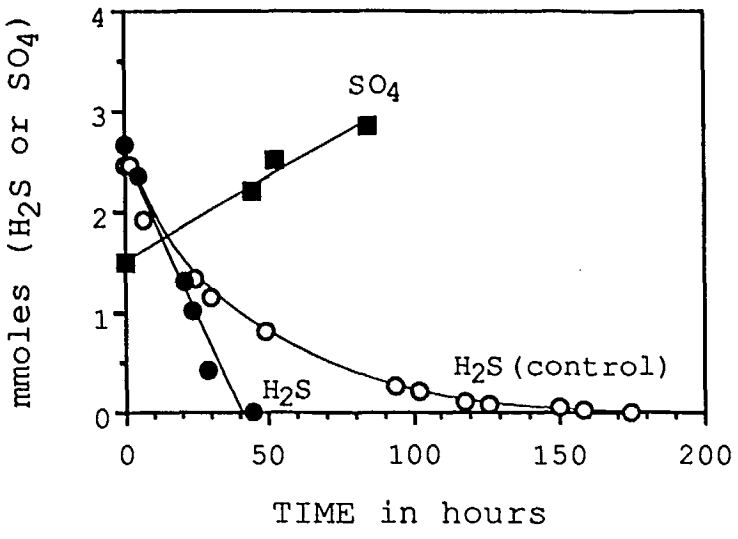

Figure 2. H_2S removal and SO4^{--} formation with time during bacterial action.

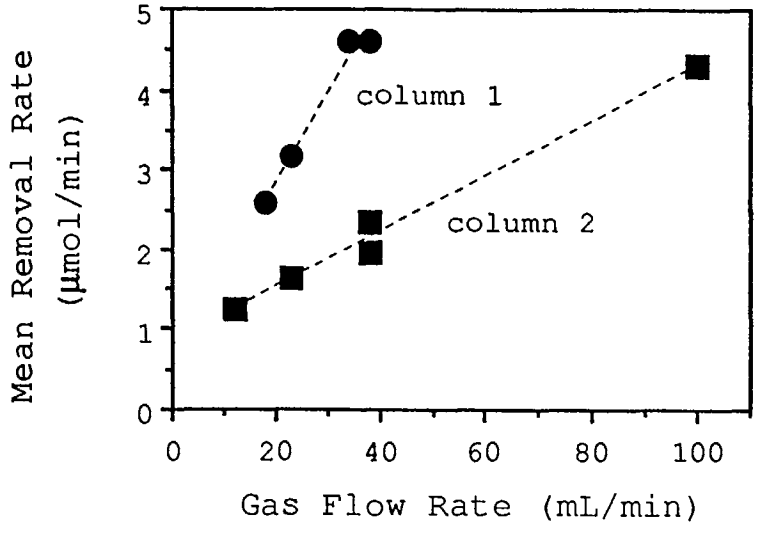

Figure 3. Plot of mean H2S removal rate versus gas flow rate. Column No. 1 had 3 mm bead packing while column No. 2 had 5 mm beads. H_2S concentration in feed gas = 3500 ppm (in air).

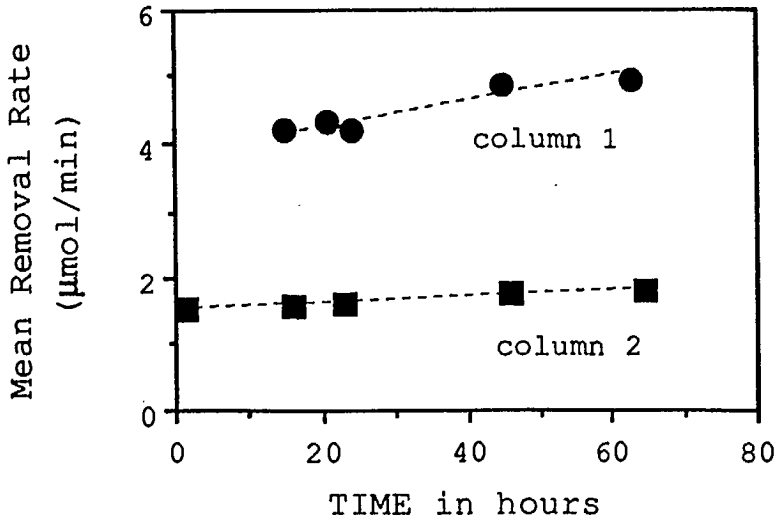

Figure 4. Change in mean H2S removal rate with time for both columns. Column 1 (3 mm beads) had a gas flow rate of 34 ml/min while rate for column 2 (5 mm beads) was 12 ml/min. H2S concentration in feed gas was 3750 ppm.

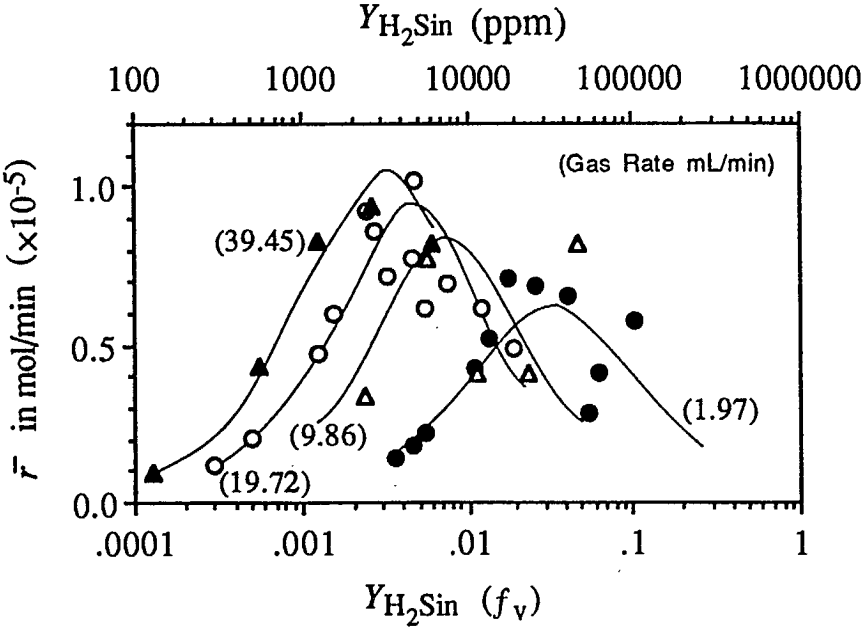

Figure 5. Effect of H2S Concentration on its Conversion Rate

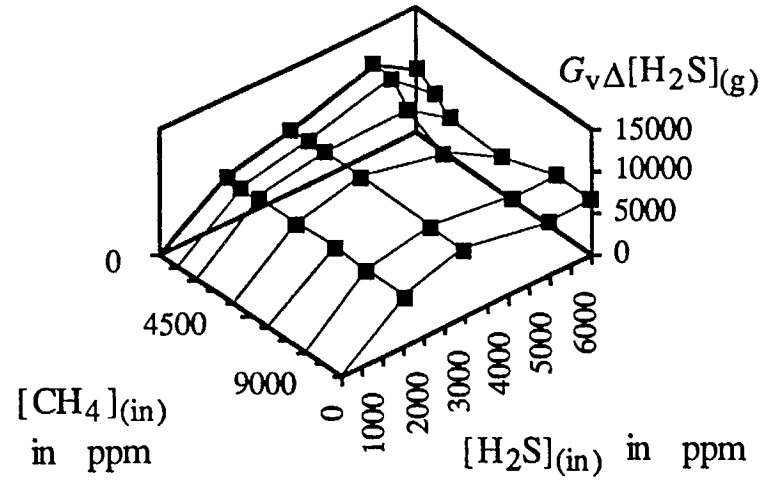

Figure 6. Plot showing the effect of methane on H2S conversion, at different levels of H2S in feed gas.

Table 1. Reaction balances from bacterial H2S oxidation

Run #	H2S Consumed (mmoles)	SO4-- Generated (mmoles)	Change in pH (ΔpH)	Sulfur (%)
1	1.94	1.49	-0.23	89
2	1.94	1.38	-0.26	86
3	4.91	2.69	-0.26	66
4	4.2	2.78	-0.69	78
5	5.12	3.41	-0.43	74

These experiments involved gas recycle using 10 l gas reservoirs. The sulfur recovery data take into account initial and final aqueous H2S and sulfate concentrations not shown in the table.

Table 2. Total removal of H2S by two columns in series

	Column 1			Column 2
Duration hrs	H2S In ppm	Gas Flow ml/min	H2S Out ppm	H2S Out ppm
0	3000	23	17	0
25	2100	58	330	0
96	2100	58	550	0

MICROBIOLOGICAL STUDY OF THE DEGRADATION OF PHENOL UNDER METHANOGENIC CONDITIONS

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ABSTRACT

An anaerobic consortium that carboxylated phenol to benzoate under methanogenic conditions was studied. This transformation was accomplished by co-metabolism in presence of protease peptone. Yeast extract or tryptophan and lysine, but not glucose, glycerol, pyruvate, volatile fatty acids and sodium carbonate could replace protease peptone. p-Hydroxybenzoate is an intermediate in the transformation of phenol to benzoate. Carboxylation occurs in the para position relative to the phenolic hydroxyl group. The transformation of phenol to benzoate is induced by phenol. The carboxylation activity of the consortium is not restricted to phenol but efficiently transforms phenols with ortho substitutions to meta substituted benzoic acids. However, meta and para substituted phenols were not transformed. The carboxylating microorganisms are probably nonsyntrophic since inhibition of methanogenesis and the presence of hydrogen in the gaseous phase did not prevent the carboxylation of phenol. Facultative anaerobic microorganisms were not detected in the consortium. Sporulated bacteria identified as *Clostridium* were shown to be implicated in the transformation of phenol. With a specific petrochemical effluent containing 120 mg/L of phenol, it has been shown that 95% of the phenol could be degraded by the consortium after a hydraulic residence time of only 4 h at 37 °C using a fixed film reactor. A better understanding of the microbiological aspects of the anaerobic degradation of pollutants should lead to the development of a more efficient process.

ÉTUDE MICROBIOLOGIQUE DE LA DÉGRADATION DU PHÉNOL DANS DES CONDITIONS FAVORABLES À LA MÉTHANOGÉNÈSE

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RÉSUMÉ

On a étudié un ensemble expérimental anaérobie, qui par carboxylation a transformé le phénol en benzoate dans des conditions favorables à la méthanogenèse. On a accompli cette transformation par cométabolisme en présence de protéose-peptone. Un extrait de levure ou de tryptophane et de lysine, mais pas le glucose, le glycérol, le pyruvate, les acides gras volatils ni le bicarbonate de sodium ne pourraient remplacer la protéose-peptone. Le p-hydroxybenzoate est un produit intermédiaire lors de la transformation du phénol en benzoate. La carboxylation a lieu en position para par rapport au groupe hydroxyle du phénol. La transformation du phénol en benzoate est induite par le phénol. L'activité de carboxylation de l'ensemble expérimental ne se limite pas au phénol, mais transforme efficacement les phénols substitués en ortho en acides benzoïques substitués en méta. Toutefois, les phénols substitués en méta et para n'ont pas subi de transformations. Les microorganismes carboxylants ne sont probablement pas syntrophes puisque l'inhibition de la méthanogénèse et la présence d'hydrogène dans la phase gazeuse n'ont pas empêché la carboxylation du phénol. On n'a pas décelé de microorganismes anaérobies facultatifs dans l'ensemble expérimental. On a démontré que des bactéries sporulées identifiées comme appartenant au groupe *Clostridium* participaient à la transformation du phénol. Avec un effluent pétrochimique spécifique contenant 120 mg/L de phénol, on a démontré que 95 % du phénol pouvait être dégradé par l'ensemble expérimental après une durée de séjour hydraulique de seulement 4 h à 37 °C; un réacteur à film fixe a été employé. On parviendra sans doute à mettre au point un procédé plus efficace, lorsqu'on aura réussi à mieux comprendre les détails microbiologiques de la dégradation anaérobie des polluants.

INTRODUCTION

In addition to the biological sources of aromatic compounds, agricultural and industrial wastes introduce a great variety of xenobiotic aromatic compounds into the biosphere. Many of these aromatic compounds are important pollutants because they are produced in large quantities, toxic, persistent and accumulating in sediments. Some of these compounds are mutagenic, teratogenic and/or carcinogenic. Our research program is to acquire more knowledge on the anaerobic microorganisms and their degradation mechanisms of aromatic compounds. Phenol is the aromatic compound we have mainly studied up to now.

Phenol is one of the most widely used aromatic compounds in the world. Annual production of phenol is approximately 1.25 billion kg (1). Phenol is the basic structural unit for a variety of synthetic organic compounds, including many agricultural chemicals. Phenol and substituted phenols are common transformation products of several pesticides. Many substituted phenols, including cresols and chlorophenols, have been designated as priority pollutants by the U.S. Environmental Protection Agency. As a group of compounds, phenols are highly toxic.

The degradation of aromatic compounds under aerobic conditions has been widely studied. In contrast, the metabolism of these compounds by anaerobic bacterial populations is less well documented. However, the ability of anaerobic microorganisms to degrade aromatic compounds has been demonstrated (5, 17, 18). Anaerobic treatment offers several advantages which are mainly economic, over aerobic treatment. It does not need energy-intensive aeration equipment, requires lower nutrient additions, produces less biological solids and generates methane which may be used as an energy source.

Phenol and substituted phenols have been shown to be biodegraded to methane and carbon dioxide in batch anaerobic cultures (9, 10, 11, 33). In addition, application of anaerobic microbiological processes for the removal of phenols from industrial wastewaters has been proposed and demonstrated on a laboratory-scale (12, 14, 15, 30, 31, 32).

Kobayashi *et al.* (23) have reviewed the metabolic pathways proposed for phenol degradation under methanogenic conditions. Two main metabolic routes have been observed. In one of these, phenol is first carboxylated to benzoate before reduction and cleavage of the aromatic ring (22, 24, 35). The carboxylation activity involved in the transformation is not well known but was shown to occur at the para position relative to the phenolic hydroxyl group (16, 26, 34). These authors determined that their consortium was composed of three to five different microorganisms. Up to now, they have not succeeded in isolating the microorganisms responsible for the carboxylation of phenol to benzoate. Also, none of them have tested the activity of their consortium on an industrial effluent.

Since anaerobic digestion is an alternative treatment method for phenolic compounds, a better understanding of the carboxylating activity is important. In the present paper, some metabolic microbiological aspects of the consortium carboxylating phenol are presented. A better understanding of the carboxylation of phenol to benzoate is necessary to evaluate the feasibility of such treatment on contaminated effluents and soils.

MATERIAL AND METHODS

Consortium of Anaerobic Bacteria

The acclimation culture was carried out in a 250 mL bottle containing 150 mL of a mixture composed of an equal volume of swamp water, activated sewage sludge, swine waste, 2-3 g of soil sample and 1 mM of phenol (3). The medium was flushed with 10% H_2 - 10% CO_2 - 80% N_2 gas and maintained stationary in the dark under anaerobic conditions at room temperature.

The enrichment culture were carried out in serum bottles (100 mL) sealed with a butyl rubber stopper and capped with an aluminum crimp seal. The mineral salts medium of Boyd *et al.* (10) was used and its pH was adjusted to 7.2 with $NaHCO_3$. Oxygen was removed from the medium by boiling and flushing with oxygen-free gas composed of 10% H_2 - 10% CO_2 - 80% N_2 followed by the addition of sodium sulfide. Phenol was added to a final concentration of 1.6 mM. The cultures were incubated at 29 °C and maintained by periodic transfer in fresh medium.

Metabolic Pathway

The metabolic pathway used by the consortium was determined by identifying the possible degradation intermediates of phenol (1.6 mM) in the culture medium (4). Also, to confirm this pathway, the consortium was grown in media where phenol was replaced by potential degradation intermediates (1.6 mM) (6).

The substrates used and their metabolites were detected with a Hewlett Packard gas chromatography (GC) (model 5890A) with a 5% phenyl-methyl-silicon capillary column (25 m) with nitrogen as the carrier gas. The chromatograph was equipped with a flame-ionization detector. Samples of the culture fluid were extracted as follows: 1 mL of the sample was first acidified by adding 0.2 mL of 9 M H_2SO_4 then 0.4 g NaCl and 1 mL ethyl ether were added. The extract was evaporated and derivatized with N, o-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) (Pierce Chemicals, Rockford, Il.) using 0.5 mL BSTFA-acetonitrile (1:4 v/v) containing 150 mg/L of m-cresol as an internal standard and the mixture was incubated at 70 °C for 30 min. Two microliters of this solution was

injected into the GC. The initial column temperature was 50°C and was increased to 230°C at a rate of 11°C/min. The injection temperature was 250°C and the detector temperature was 300°C. Standards were run and used for identification and quantification of the unknown compounds.

GC-mass spectrometry (MS) was also performed using a similar GC column and an Ion Trap 800 (Finnigan) mass spectrometer. The initial column temperature of 70°C was increased to 120°C at a rate of 10°C/min., held for 2 min., increased to 230°C at a rate of 5°C/min., and increased to 300°C at a rate of 20°C/min. The mass spectra were recorded in the repetitive scanning mode from 70 to 400 atomic mass units. Identifications were made by comparison of the retention times and the mass spectra of the extracted products with those of authentic standards.

Carboxylation Ability

The carboxylating ability of the consortium was determined towards phenols with ortho substitutions and the three isomers of cresol, fluorophenol and chlorophenol. Phenol (1 mM) and the test compound (1 mM) were both added to the culture medium. In the course of the experiment, when phenol was depleted from the medium, fresh phenol together with 0.05% (w/v) proteose peptone was supplied to the culture to a final concentration similar to the residual concentration of the test compound. The type of reaction occurring was assessed by identifying the metabolites appearing in the culture medium with the GC and GC-MS. Appropriate controls were used.

Position of Carboxylation

The transient intermediate observed in the transformation of o-cresol was identified to determine the position where carboxylation occurs (7). In order to do so, 4-hydroxy-3-methylbenzoic acid was synthesized from o-cresol as described by Komiyama and Hirai (24). This substance was used as a substrate in some cultures and as a standard for GC and GC-MS analysis.

o-Cresol-6_d (1.0 - 1.1 mM) was used to confirm the carboxylation position on the aromatic ring. Phenol was substituted by this compound in the culture medium. o-Cresol-6_d was synthesized in three steps : 2-bromo-6-methylphenol was converted to 2-bromo-6-methyl-phenol-t-butyldimethylsilyl ether which was then transformed to 2-methyl-6_d-phenol-t-butyldimethylsilyl ether and to 2-methyl-6_d-phenol. o-Cresol-6_d and its metabolites in the cultures were followed by GC and GC-MS analysis. o-Cresol free controls were also used.

Microbiological Aspects

The morphology of the cells was observed with a Leitz microscope after Gram staining. Observations were also made with an electron microscope (model EM300,

Philips Electronic Instrument, Mont Vermont, N.Y.) after fixation of the microorganisms with 1-2% glutaraldehyde and negative staining with 2% phosphotungstate.

The number of phenol-carboxylating microorganisms for a culture where most of the phenol had been transformed to benzoate was estimated. Twelve serial dilutions of the culture were made by using 12.5% (v/v) inoculum each time and these cultures were incubated at 29°C for eight weeks. The culture that had received the most diluted inoculum and where most of the phenol was degraded was used for our estimation.

A concentration of 5 mM of 2-bromoethanosulfonic acid (BESA) (Sigma Chemicals, St-Louis, MO), a specific inhibitor of methanogenesis, was added to the culture medium to determine if the phenol-carboxylating microorganisms were syntrophic. Also, the effect of H_2 in the gaseous phase on the carboxylation of phenol was determined. The culture media were sparged with one of the following gas mixtures: 10% H_2 - 10% CO_2 - 80% N_2; 80% H_2 - 20% CO_2; 20% CO_2 - 80% N_2; and each bottle was filled to a pressure of 2 atm with the gas mixture used for sparging the medium.

Proteose peptone in the culture medium was replaced by amino acids to reduce the number of stains present in the consortium. The following mixture of amino acids (0.05% w/v each) was used: isoleucine, threonine, valine, serine, phenylalanine, alanine, proline, ornithine, lysine, tyrosine, tryptophan and cysteine. From this mixture, the minimal combination of amino acids supporting the carboxylation of phenol to benzoate was determined. The culture able to carboxylate phenol in the less complex medium was observed with the electron microscope as described previously.

In order to show the implication of the sporulated bacteria in the carboxylation of phenol to benzoate, the consortium was heated to 80°C for 15 min and inoculated in the minimal medium of Boyds *et al.* (10) containing 0.05% (w/v) proteose peptone No 3 (Difco) and 1.6 mM phenol. Phenol and benzoate were followed in these cultures by GC. Preliminary identification of the sporulated bacteria isolated on solid medium was achieved with API 20A system (API Analytab Product, N.Y. USA).

Fixed Film Anaerobic Bioreactor

An upflow tubular anaerobic bioreactor was used. The glass column was 45 cm high with an internal diameter of 3 cm. Total volume of the reactor was 200 mL and the effective volume (liquid phase) was 60 mL. Rocks of 0.7 - 1 cm diameter were used as support for the development of the biofilm. The reactor was maintained 37°C.

RESULTS AND DISCUSSION

An acclimation period of five weeks was necessary before anaerobic degradation of phenol was observed (Fig. 1) (3). Complete removal of phenol (1 mM) was obtained after eight weeks. If a similar concentration of phenol was added to this culture, only six days were now required for complete removal of phenol. Thus, phenol degrading microorganisms were present in at least one of the sample taken but their number was probably low as suggested by the long period of acclimation required. The phenol degrading population grew and became more effective.

The acclimated culture was enriched by periodic transfer in a mineral salts medium containing phenol as the sole source of carbon. After many months of enrichment in this medium, some cultures lost degradative activity (3). In order to maintain the activity of the microbial strains involved in the degradation, some other sources of carbon were added to the mineral salts medium containing phenol. The activity of the culture was maintained in the presence of 0.05% (w/v) proteose peptone but not in the presence of glucose (0.05% w/v), acetate (0.5% w/v) or nitrate (0.2% w/v). These results suggest that the degradation of phenol is accomplished by co-metabolism. In the consortium, it is probably proteose peptone or one of its degradation intermediates that is the carbon and energy source for the phenol degrading microorganisms. Subsequent cultures were carried out in mineral salts medium containing 0.05% (w/v) proteose peptone (Difco) and phenol. The production of methane gas by the culture and the presence of several morphological bacterial types suggest that the degradation of phenol is carried out via methanogenic fermentation by a bacterial consortium.

Proteose peptone could be replaced by yeast extract (0.05% w/v) or the mixture of amino acids described previously without affecting the degradation of phenol (6). However, it could not be effectively replaced by glycerol (0.5% w/v), pyruvate (0.05% w/v), volatile fatty acids (acetic, propionic, butyric, 0.05% w/v each), or sodium bicarbonate (0.015% w/v). Further experiments showed that only lysine and tryptophan of the amino acids mixture were required for the degradation of phenol. This combination of amino acids needed for the degradation of phenol might indicate that a *Clostridium* species carrying out a Stickland reaction is involved (29).

Figure 2 shows the concentrations of phenol and its degradation intermediates found in the methanogenic culture as a function of time of incubation (4). A compound, identified as benzoate by GC and GC-MS, accumulated as phenol disappeared. Prolonging the incubation of the culture resulted in the degradation of the accumulated benzoate. Such a metabolic pathway where phenol is first carboxylated to benzoate has also been obtained by others (22, 23, 26, 35). However, much remains to be discovered about the carboxylation reactions and the microorganisms involved. Acetate was obtained when all benzoate had disappeared and it was thereafter degraded rapidly. Gas was

produced during benzoate degradation and the maximum volume was obtained when acetate was completely degraded. Methane was found in all gas samples analyzed.

The following potential intermediates (1.6 mM) of benzoate degradation were completely metabolized by the culture after 25 days of incubation of the consortium at 29°C : 1-cyclohexene carboxylate, heptanoate and caproate (6). Only a certain percentage of isocaproate and adipate was metabolized by the culture while cyclohexanol, cyclohexanone, cyclohexane carboxylate, 2-methylcyclohexanone and pimelate were not metabolized. These results confirmed that the anaerobic consortium degrading phenol under methanogenic conditions does not use the reductive pathway described by Evans (13). On the basis of the results obtained, 1-cyclohexene carboxylate and heptanoate are probably two probable intermediates of benzoate degradation by our consortium. However, these intermediates were never observed (GC), probably because they do not accumulate in sufficiently high concentration. During the degradation of 1-cyclohexene carboxylate by the consortium, a substance accumulated in the culture that was identified by GC-MS as cyclohexane carboxylate. Thus, part of 1-cyclohexene carboxylate was apparently transformed to a dead-end product. In contrast with our findings, Keith *et al.* (21) and Kobayashi *et al.* (23) have shown that cyclohexane carboxylate was degraded by their consortium.

The potential of the consortium for transforming a number of phenolic and related compounds in the presence of phenol is shown in Table 1. Phenols with ortho substitutions (chloro-, fluoro-, bromo-, hydroxyl-, amino-, or carboxyl-) were transformed to meta substituted benzoic acids. Of the resulting products, only benzoic acid, 3-hydroxybenzoic acid, and isophthalic acid were further metabolized, probably to methane. The other transformation products accumulated in the culture medium and were not further metabolized. Anisole and 2-methoxyphenol were o-dimethylated by the consortium to phenol and catechol respectively, and 2-hydroxybenzyl alcohol was oxidized to 2-hydroxybenzoic acid. None of the transformation products were found in the controls free of test compounds. The meta and para isomers of cresol, fluorophenol, and chlorophenol were not transformed after 63 days, although the phenol in these cultures was metabolized. The importance of the substitution position was also observed by Sharak Genthner *et al.* (27) by means of fluorophenols. The nontransformation of para substituted phenolic compounds is not surprising, since it has been shown with our consortium (6) and with others (16, 27, 34) that carboxylation occurs para to the phenolic hydroxyl group.

The kinetics of transformation of o-cresol by the anaerobic consortium under methanogenic conditions is presented in Fig. 3 (7). After 59 days of incubation, o-cresol was nearly completely transformed to 3-methylbenzoic acid which accumulated in the culture medium. The transient metabolite 4-hydroxy-3-methylbenzoic acid was detected after o-cresol metabolism began and reached a maximum concentration when about half of the o-cresol had been transformed. Thereafter, the concentration of this metabolite in the culture decreased, suggesting that it was an intermediate in o-cresol metabolism. The

stoichiometric transformation of o-cresol into 3-methylbenzoic acid indicated that carboxylation occurred at either the ortho or para position to the hydroxyl group of o-cresol. The identification of the transient metabolite as 4-hydroxy-3-methylbenzoic acid suggested that the consortium introduces a carboxyl function para to the phenolic hydroxyl group of o-cresol. This hypothesis was further confirmed using o-cresol-6_d in which the hydrogen atom in the ortho position is replaced by a deuterium. The accumulated and transient metabolites resulting from the transformation of this compound were both deuterated. This eliminates the possibility of carboxylation occurring at the ortho position which would generate unlabelled degradation products. Sharak Genthner *et al.* (27) and Zhang *et al.* (34) have found a similar para carboxylation with their consortium using fluorophenols and ^{13} C-1-labelled phenol.

The bacterial consortium grown in the presence of phenol and proteose peptone contains seven different morphological types as observed with the electron microscope (6). Gram-negative rods were dominant. Replacement of proteose peptone with tryptophan and lysine reduced the morphological types to six; a crescent-shaped bacteria was no longer observed. When the consortium was grown with BESA, the long chains of large rods were diluted out. They were tentatively identified as *Methanothrix* on the basis of their morphological features (19). Only five types remained in the consortium and they were cocci, two types of short rods, long rods and pointed-ends coccobacilli. Spores were also observed. The resulting consortium can transform phenol to benzoate without subsequent degradation of benzoate. Knoll and Winter (22) have shown that their phenol carboxylating consortium is composed of 3 to 5 morphological types. The microorganisms responsible for carboxylation have not yet been isolated and identified.

Since the carboxylation of phenol occurred in a culture receiving an inoculum diluted as much as 8×10^{-9} and did not occur with an inoculum diluted to 8×10^{-11} the microorganisms responsible for this reaction were estimated to be present at a concentration of between 1×10^{8} and 8×10^{9} (4). These values suggest that the carboxylating bacteria are probably part of the dominant microorganisms in the consortium. If this is the case, the isolation of these microorganisms should be facilitated.

Similar rates of phenol transformation by the consortium were obtained in the presence and absence of the methanogenesis inhibitor (4). The addition of 5 mM BESA to the culture medium completely inhibited the production of methane. In agreement with our results, Knoll and Winter (22) and Jeannin (20) have shown that inhibition of the methanogens did not influence the carboxylation of phenol. These results suggest that the carboxylating microorganisms are nonsynthrophic.

The effect of three different gas mixtures on the carboxylation of phenol was determined (4). In the presence of H_2, even at high concentration, the carboxylation of phenol occurred. Kobayashi *et al.* (22) have obtained similar results with their consortium. These results also suggest that the carboxylating microorganisms are nonsyntrophic.

The heated consortium (80°C, 15 min) was able to carboxylate phenol to benzoate after 30 days of incubation but was unable to further degrade benzoate. Successive replication of this consortium in fresh medium reduced to 15 days the time required for complete transformation of phenol. A stable consortium was obtained which was constituted of sporulated bacteria identified as *Clostridium*. To our knowledge, sporulated bacteria have never been implicated in the carboxylation of phenol. Jeannin (20) has observed *Clostridium* in her phenol carboxylating consortium when she had glucose to the medium but their role is unknown. Zhang and Wiegel (35) have isolated a sporulated bacteria ressembling to a *Clostridium* that can decarboxylate p-hydroxybenzoate to phenol but was not able to carboxylate phenol.

The performance of the fixed film anaerobic reactor is presented in Table 2. The concentration of phenol, the main phenolic compounds of the effluent, was reduced by 98% for a hydraulic residence time of only 6 hours. This represents a degradation rate of 578 mg/L/day. Knoll and Winter (22) have obtained twice this rate under different experimental conditions. They used adapted sludge to a petrochemical effluent to which high concentrations of phenol was added with a hydraulic residence time of 10 days. As expected, only the concentration of the isomer o-cresol was significatively reduced by the treatment (8). The concentration of the phenolic compounds was reduced by 83% by the treatment. This value is comparable to the one obtained by Parker and Farguhar (25) using a mixture of specific petrochemical effluents and a hydraulic residence time of three days. The toxicity of the effluent determined by the Microtox technique was reduced by 44% by the treatment in the reactor. The consortium of anaerobic bacteria can thus be efficiently used to treat a petrochemical effluent containing a high concentration of phenol with a fixed film reactor.

Experiments are now being carried out in our laboratory to isolate the carboxylating microorganisms and to optimize the performance of the fixed film reactor. A better understanding of such a consortium may lead to improvements in the anaerobic treatment of aromatic compounds.

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REFERENCES

1. **Anonymous**. 1980. Key Chemical and Polymers. In: Chemical and engineering news, 34th ed., vol. **58**, American Chemical Society, Washington, D.C.

2. **Battersby, N.S., V. Wilson**. 1989. Survey of the Anaerobic Biodegradation Potential of Organic Chemicals in Digesting Sludge. Appl. Environ. Microbiol. **55**:433-439.

3. **Beaudet, R., J.-G. Bisaillon, M. Ishaque and M. Sylvestre**. 1986. Isolation of an Anaerobic Bacterial Consortium Degrading Phenolic Compounds - Assay in Swine Waste". Agric. Wastes **17**:131-140.

4. **Béchard, G., J.-G. Bisaillon and R. Beaudet**. 1990. Degradation of Phenol by a Bacterial Consortium under Methanogenic Conditions. Can. J. Microbiol. **36**:573-578.

5. **Berry, D.F., A.J. Francis and J.-M. Bollag**. 1987. Microbial Metabolism of Homocyclic and Heterocyclic Aromatic Compounds under Anaerobic Conditions. Microbiol. Rev. **51**:43-59.

6. **Bisaillon, J.-G., F. Lépine and R. Beaudet**. 1991. Study of the Methanogenic Degradation of Phenol via Carboxylation to Benzoate. Can. J. Microbiol. **37**:573-576.

7. **Bisaillon, J.-G., F. Lépine, R. Beaudet and M. Sylvestre**. 1991. Carboxylation of o-Cresol by an Anaerobic Consortium under Methanogenic Conditions. Appl.Environ. Microbiol. **57**:2131-2134.

8. **Bisaillon, J.-G., F. Lépine, R. Beaudet and M. Sylvestre.** 1993. Carboxylation Potential of an Anaerobic Consortium against Phenolic and Related Compounds under Methanogenic Conditions. Can. J. Microbiol. **39**:642-648.

9. **Boyd, S.A., D.R. Shelton**. 1984. Anaerobic Biodegradation of Chlorophenols in Fresh and Acclimated Sludge. Appl. Environ. Microbiol. **47**:272-277.

10. **Boyd, S.A., D.R. Shelton, D. Berry and J.M. Tiedje**. 1983. Anaerobic Biodegradation of Phenolic Compounds in Digested Sludge. Appl. Environ. Microbiol. **46**:50-54.

11. **Chmielowski, J., A. Grossman and S. Labuzek.** 1965. Biochemical Degradation of Some Phenols During Methane Fermentation. Zesz. Nauk. Politech. Slask. Ing. Sanit. **8**:97-122.

12. **Cross, W.H., E.S.K. Chian, F.G. Pohland, S. Harper, S. Kharkar and F. Lu.** 1982. Anaerobic Biological Treatment of Coal Gasifier Effluent. Biotechnol. Bioengng. Symp. No. 12, pp. 349-363.

13. **Evans, W.C.** 1977. Biochemistry of the Bacterial Catabolism of Aromatic Compounds in Anaerobic Environments. Nature **270**:17-22.

14. **Fedorak, P.M., S.E. Hrudey.** 1986. Anaerobic Treatment of Phenolic Coal Conversion Wastewater in Semi-continuous Cultures. Water Res. **20**:113-122.

15. **Fox, P., M.T. Suidan and J.T. Pfeffer.** 1988. Anaerobic Treatment of a Biologically Inhibitory Wastewater. J. Water Pollut. Control Fed. **60**:86-92.

16. **Gallert, C., G. Knoll and J. Winter.** 1991. Anaerobic Carboxylation of Phenol to Benzoate. Use of Deuterated Phenols Revealed Carboxylation Exclusively in the C_4 Position. Appl. Microbiol. Biotechnol. **36**:124-129.

17. **Hollinger, C., A.J.M. Stams and A.J.B. Zehnder.** 1988. Anaerobic Degradation of Recalcitrant Compounds. In: Anaerobic Digestion 1988, E.R. Hall et P.N. Hobson (Eds). Pergamon Press, 211-224.

18. **Horowitz, A., D.R. Shelton, C.P. Corwell and J.M. Tiedje.** 1982. Anaerobic Degradation of Aromatic Compounds in Sediments and Digested Sludge. Dev. Ind. Microbiol. **23**:435-444.

19. **Huser, B.A., K. Wuhrmann and A.J.B. Zehnder.** 1982. *Methanothrix soechngenii* gen. nov. sp. nov,, a new Acetotrophic Nonhydrogen-Oxidizing Methane Bacterium. Arch. Microbiol. **132**:1-94.

20. **Jeannin, P.** 1986. Dégradation du Phénol en Conditions Méthanogènes. Aspects Microbiologiques et Biochimiques. Thèse de Doctorat, Université Claude Bernard, Lyon, France.

21. **Keith, C.L., R.L. Bridges, L.R. Fina, K.L. Iverson and J.A. Cloran.** 1978. The Anaerobic Decomposition of Benzoic Acid During Methane Fermentation. Arch. Microbiol. **118**:173-176.

22. **Knoll, G., J. Winter**. 1988. Degradation of Phenol via Carboxylation to Benzoate by a Define Obligate Syntrophic Consortium of Anaerobic Bacteria. Appl. Microbiol. Biotechnol. **30**:318-324.

23. **Kobayashi, T., T. Hashinaga, E. Mikami and T. Suzuki**. 1989. Methanogenic Degradation of Phenol and Benzoate in Acclimated Sludges. Water Sci. Technol. **21**:55-65.

24. **Komihama, M., H. Hirai.** 1984. Selective Synthesis using Cyclodextrins as Catalysts. II . Para-Oriented Carboxylation of Phenols. J. Am. Chem. Soc. **106**: 174-178.

25. **Parker, W., G.J. Farguhar.** 1989. Treatment of a Petrochemical Wastewater in an Anaerobic Packed Bed Reactor. Water Poll. Res. J. of Canada **24**:195-205.

26. **Sharak Genthner, B.R., W.A. Price and P.H. Pritchard**. Anaerobic Degradation of Chloroaromatic Compounds in Aquatic Sediments under a Variety of Enrichment Conditions. Appl. Environ. Microbiol. **55**:1466-1471.

27. **Sharack Genthner, B.R., G.T. Townsend and P.J. Chapman**. 1989. Anaerobic Transformation of Phenol to Benzoate via Para Carboxylation: Use of Fluorinated Analogues to Elucidate the Mechanism of Transformation. Biochem. Biophys. Res. Comm. **162**:945-951.

28. **Sharack Genthner, B.R., G.T. Townsend and P.J. Chapman**. 1990. Characterization of an Anaerobic Consortium which Converts Phenol to Benzoate Before Degradation. Abstract Annual Meeting American Society Microbiology. K-179, p. 274.

29. **Stickland, L.H**. 1934. The Chemical Reactions by which *Clostridium sporogenes* obtains its Energy. Biochem. J. **28**:1746-1759.

30. **Suidan, M.T., P. Fox and J.T. Pfeffer**. 1987. Anaerobic Treatment of Coal Gasification Wastewater. Water Sci. Technol. **19**:229-236.

31. **Suidan, M.T., C.E. Struber, S.W. Kao and J.T. Pfeffer**. 1983. Treatment of Coal Gasification Wastewater with Anaerobic Filter Technology. J. Water Pollut. Control Fed. **55**:1263-1270.

32. **Vogel, P., J. Winter**. 1988. Anaerobic Degradation of Phenol and Cresol in Petrochemical Wastewater. In: Anaerobic Digestion 1988, E.R. Hall et P.N. Hobson (Eds). Pergamon Press, pp. 689-694.

33. **Young, L.Y., M.D. Rivera.** 1985. Methanogenic Degradation of Four Phenolic Compounds. Water Res. **19**:1325-1332.

34. **Zhang, X., T.V. Morgan and J. Wiegel**. 1990. Conversion of ^{13}C-1 Phenol to ^{13}C-4 Benzoate, an Intermediate Step in the Anaerobic Degradation of Chlorophenols. Fems Microbiol. Lett. **67**:63-66.

35. **Zhang, X., J. Wiegel.**. 1990. Sequential Anaerobic Degradation of 2,4-Dichlorophenol in Freshwater Sediments. Appl. Environ. Microbiol. **56**:1119-1127.

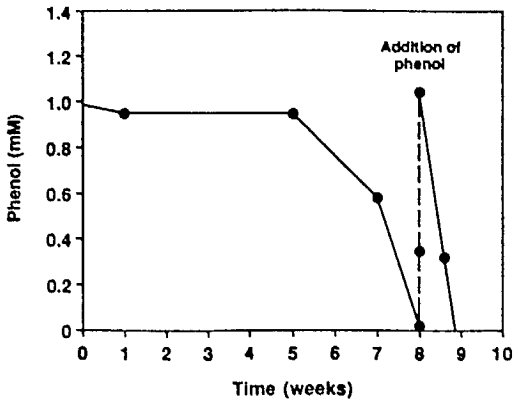

Figure 1: Degradation of phenol under anaerobic conditions by the adapted culture which is comprised of a mixture of: swamp and sewage water; swine waste and soil.

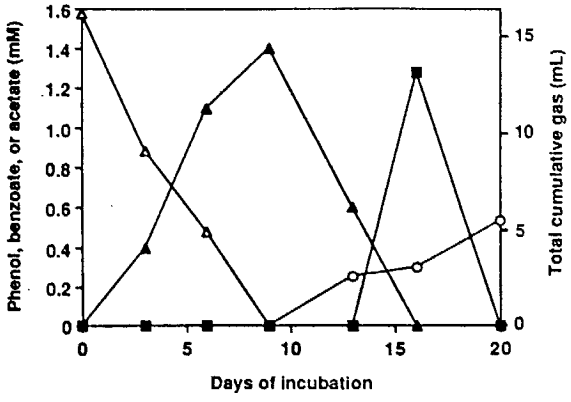

Figure 2: Degradation of phenol by the anaerobic consortium at 29°C. Values presented are those in excess of that in the phenol-free control. Phenol (△), benzoate (▼), acetate (■), gas(○).

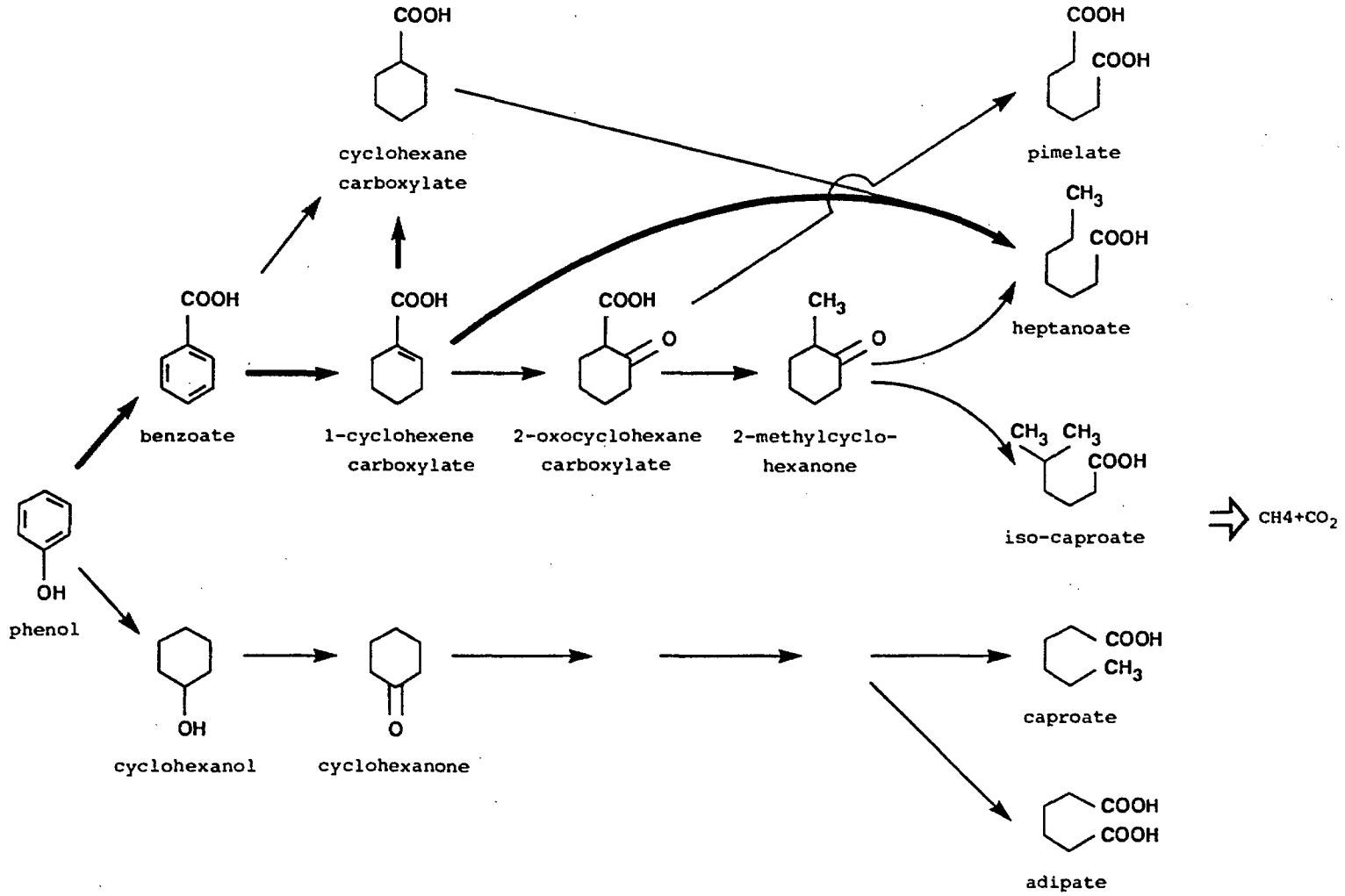

Figure 3: Proposed pathways in the degradation of phenol. Bold lines indicate the pathway proposed for the consortium isolated by the present authors.

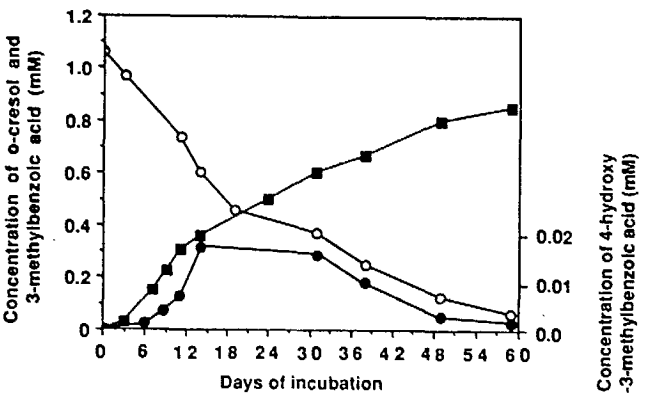

Figure 4: Kinetics of o-cresol transformation under methanogenic conditions. Samples of culture supernatants were analyzed by GC-MS. o-Cresol (o), 3-methylbenzoic acid (■), 4-hydroxy-3-methylbenzoic acid (●).

Table 1. Transformation potential of the anaerobic consortium against a variety of phenolic and related compounds in the presence of phenol.

Added to the culture medium	Detected in the culture medium
Phenol	Benzoic acid
2-Cresol	3-Methylbenzoic acid
Catechol	3-Hydroxybenzoic acid
2-Fluorophenol	3-Fluorophenol acid
2-Chlorophenol	3-Chlorobenzoic acid
2-Bromophenol	3-Bromobenzoic acid
2-Aminophenol	3-Aminobenzoic acid
2-Hydroxybenzoic acid	Isophthalic acid
Anisole	Phenol
2-Methoxyphenol	Catechol
2-Hydroxybenzyl alcohol	2-Hydroxybenzoic acid

Table 2. Performance of the fixed film anaerobic reactor.

Parameters	Specific petrochemical effluent		% of reduction
	Before treatment	After treatment	
pH	7,5[a]	7,7	--
Phenol (mg/L)	148,19	3,56	98
o-cresol (mg/L)	26,63	5,6	79
m-cresol (mg/L)	39,97	37,6	6
p-cresol (mg/L)	20,33	17,87	12
Phenolic compounds (mg/L)	167,97	29,11	83
Toxicity EC50 (%)	2,9	5,2	44

[a] HRT = 6 hours

PERFORMANCE PARAMETERS FOR *EX SITU* BIOREMEDIATION SYSTEMS

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ABSTRACT

The use of biological principles for the remediation of nuisance and toxic organic compounds has received extensive attention recently. The potential of this technology to significantly reduce the concentration of undesirable hydrocarbons, *i.e.* gasoline and diesel fuel is extremely attractive to industry, government and the public. The technology's apparent benign nature and potentially low cost has also attracted attention.

Bioremediation is not a panacea. It is, when properly applied, a useful tool in the battle to reduce the concentrations of unwanted hydrocarbons. Like any technology, when good industrial practices are used in the design, construction, and administration of the bioremediation system, the performance of the technology can be predicted and monitored. The paper will examine some of the principles behind the design, construction, and operation of the *ex situ* bioremediation systems and facilities. It will also demystify some of the fiction and magic that has become associated with this powerful technology.

PARAMÈTRE DE PERFORMANCE POUR DES SYSTÈMES *EX SITU* DE BIODÉCONTAMINATION

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RÉSUMÉ

L'emploi des principes biologiques pour remédier aux nuisances et aux composés organiques toxiques fait depuis peu l'objet d'une grande attention. Cette technologie pourrait aider à réduire significativement les concentrations d'hydrocarbures indésirables, tels que l'essence, le diesel, et les produits organiques halogénés, et ainsi est très intéressante pour l'industrie, le gouvernement et le public. Le caractère apparemment inoffensif de cette technologie et son faible coût ont également attiré l ' attention.

La biodécontamination n'est pas une panacée. Correctement mise en pratique, elle constitue toutefois un bon outil pour aider à réduire les concentrations d'hydrocarbures indésirables. Comme avec toute technologie, si l'on emploie de bonnes pratiques de gestion industrielle pour la conception, la construction et l'administration du système de biodécontamination, on peut prévoir et surveiller la performance de la technologie. Dans cet article, on examine quelques-uns des principes régissant la conception, la construction et l'exploitation des installations finales de biodécontamination. On pourra aussi démystifier en partie cette puissante technologie auprès du public.

INTRODUCTION

In the simplest terms, bioremediation is the use of bacteria with the framework to promote the removal of complex hydrocarbons from the soil. Bacteria use the complex hydrocarbons as a source of energy and building blocks for the creation of more cells. To accomplish this, the bacteria require certain nutrients (*e.g.* nitrogen and phosphorus) and physicochemical conditions (*e.g.* temperature, oxygen and water). The challenge for the remediation facility operator is to get all these ingredients to the bacteria at the optimum concentration and at the correct time.

In North America, significant experience has been gained over the last decade in the development and optimization of remediation systems that utilize bacteria for the management of hydrocarbon impacted soil. As with all developing technologies, significant growing pains must be overcome before mainstream adoption of the technology is achieved. Acceptance of the technology is therefore based on rates of success. Much of the attention must therefore be directed towards precise monitoring and documentation of controls that effect the process. This will permit for modifications of the controls to enhance the performance and ultimate success rate of the bioremediation process. In general, the more control an operator has over the bacteria, the more predictable the performance of the bacteria in the soil will be. The key to the successful selection of a bioremediation that meets the final remediation criteria should include a detailed evaluation of the cost efficiency and design of the proposed technology as well as the level of control over the performance factors (biological, physical and chemical) effecting the system during operation. This paper addresses general performance factors effecting *ex situ* bioremediation systems, possible constraints to the successful operation of an *ex situ* bioremediation system, and in some cases, cost and benefits associated with the adoption of this technology.

The optimum remedial performance is dependent on the metabolic well being of the bacteria. Unlike purely chemical or physical processes, the biological process can not be controlled in absolute terms. The metabolic mechanism used by bacteria to collect and degrade complex hydrocarbons, the interrelationships between different groups of bacteria, and the ability of living organisms to adapt to changes in the environment contribute to the complexity of the biological process. Despite these complexities, studies have shown that the collection of empirical information does allow for a description of the bioremediation process in sufficient detail to develop strategies to optimize the performance of the biodegrading bacteria in the soil (Severn and Adams, 1992).

Bioremediation is becoming one of the most widely accepted and used technologies for the clean-up of sites contaminated with petroleum hydrocarbon wastes. Unfortunately, bioremediation has been exploited as a simple and easily applied remediation technology by individuals with little knowledge of its biological, chemical and physical processes. A complete understanding of performance constraints is

necessary to ensure the success of bioremediation. Improper implementation of safeguards could lead to the introduction of contaminants or further complicate the recovery/distribution of existing contaminants. It is the purpose of this paper to educate the project scientist and engineer on the bioremediation process as it applies to hydrocarbon effected soil.

DISCUSSION AND OBSERVATIONS

The successful implementation, operation and closure of an *ex situ* bioremediation system will take into consideration numerous performance parameters. To achieve optimum results, the engineer and scientist will want to monitor the biological, chemical and physical factors that effect the performance of the system. As such, each of these factors are addressed with consideration given to practical implementation of a frequently used *ex situ* bioremediation facility (biopile).

Biological Considerations

Metabolic Bio-Process

The net nutritional value of the petroleum hydrocarbons will influence the metabolic effort that will be spent by a bacteria to acquire and degrade the hydrocarbon. This process is easily depicted as a cost/benefit analysis. Bacteria spend the least amount of energy possible when collecting the resources required for growth. Therefore, petroleum hydrocarbons that cost the least amount of metabolic energy to obtain, but provide a significant return will be attacked first. Unfortunately, the hydrocarbons that are of the greatest concern (*e.g.* benzene, ethylbenzene, toluene, xylene) tend to be those with the lowest net nutritional value to the bacteria and are likely to be attacked last. Therefore, the most effective way to promote degradation of the recalcitrant hydrocarbons is to create an environment in the soil that encourages the resident bacteria population to acquire and utilize what nutritional value these hydrocarbons provide. This assumes the resident population of bacteria have the metabolic framework to use the more complex petroleum hydrocarbons.

There are two strategies that can be used to achieve this goal. In soils with a high available natural organic content (*e.g.* top-soil and loams), the indigenous population of bacteria can be encouraged to acquire and degrade the recalcitrant hydrocarbons through the application of inorganic nutrients. The source of the inorganic nutrients can be as simple as agricultural fertilizer. The soil is then aerated and watered following fertilizer application. If applied properly, the fertilizer will encourage the resident population of bacteria to increase in number and make use of the readily available sources of carbon provided by the high organic content of the soil. As these readily available carbon sources begin to run out, the resident population of bacteria is forced to adapt to

the more recalcitrant hydrocarbons or die out. Often, once the initial source of carbon is used up, the original population will decline in number, however, certain bacterial strains are able to obtain metabolic energy from the more complex recalcitrant petroleum hydrocarbons, and in-turn grow to dominate the bacterial community.

The second approach is more appropriate for soils with a lower level of natural organic content (*e.g.* gravels and sands). The indigenous population of bacteria in these soils is likely smaller than in the previous example. To achieve optimum remediation performance, the soil can be fertilized with a mixture of organics, nitrogen and phosphorus. In this process, the resident population of bacteria is enhanced through the introduction of an added source of carbon. It is important that the soil be well aerated and watered for the bacteria to adapt. The introduced and readily available source of carbon will then encourage the growth of bacteria in the soil. As the added source of carbon is depleted by the flourishing bacteria, the oil degrading population can then begin to attack the more recalcitrant hydrocarbons. This approach will often increase the biodegradation rate enough to compensate for the initial loss in performance. This process is often more costly to implement due to the added expense of the inoculant and additional time required to monitor the bacteria performance.

Bacteria Selection

Biodegradation, as it applies to bioremediation, is functionally defined as the degradation of complex hydrocarbons by microorganisms. The process of biodegradation is typically carried out by a complex microbial community. Therefore approaches that promote the addition of a single species of bacteria are often not as successful as those that take advantage of the natural degrading ability of the microorganisms already present in the soil. In many cases, the addition of a non-resident or commercially available bacteria for the remediation of hydrocarbon contaminated soil is unnecessary but may be required under certain circumstances. This may be the case when the resident population is either too small to degrade the target contaminant in a reasonable period of time, or does not contain the necessary genetic material to produce the enzymes required to degrade the hydrocarbons of interest. It then becomes necessary to amplify the native population or to enhance the resident population with new strains of bacteria that carry the requisite genetic information. The process of bacteria introduction can be expensive, but depending upon project time constraints, may be more efficient.

Process By-Products

Bacteria are used to reduce the concentration of petroleum hydrocarbons in soil because they have the ability to reduce hydrocarbons (oil) to more benign molecules (carbon dioxide and water). The energy and mass produced during the process are captured as energy and cell biomass.

During this process the bacteria create other chemicals that include fatty acids,

lipids and proteins that participate in the reduction of the complex hydrocarbon to carbon dioxide. Some of these by-products can be used to increase the performance of the bacteria (*e.g.* natural emulsifying agents formed by the bacteria). Unfortunately, some of the reactions are not completely efficient and result in partially degraded hydrocarbons. This typically happens when the nutrient or oxygen concentrations are too low or become absent.

As a normal component of bacterial growth and development, bacteria often produce oily substances. Unfortunately, the natural products of bacterial growth are not easily distinguished from hydrocarbon contaminants with commonly used analytical techniques (using infrared spectrophotometry). This problem often arises when the soil is approaching the target level of decontamination required for closure (removal of the soil from the bio-processing facility). The apparent concentration of oil in the soil near the end of the monitoring program may dramatically increase. This is typically due to the production of natural oils by the resident bacteria. The problem is most effectively dealt with by using analytical procedures that distinguish mineral-based hydrocarbons from bacteria-produced hydrocarbons (such as gas chromatography).

Given the nature of the contaminant, the bacteria have the ability to break down complex hydrocarbon chains into less complex compounds such as benzene, toluene or xylene through the process of biotransformation. Often, towards the end of the operation of a bioremediation program, these volatile components will appear in the soil at a higher concentration than expected. This can be deceiving for the operator, however, as the biotransformation process continues, concentrations of these volatile compounds can be expected to decrease. Depending upon the time constraints of the project, tilling to encourage removal of the volatile components may be required.

Hydrogen sulphide gas may accumulate in the soil, if oxygen is depleted within the remediation facility, thus allowing the anaerobic bacteria in the soil to become active. Sulphur-reducing bacteria can transform sulphate within the facility to sulphide and promote the formation of hydrogen sulphide gas. The maintenance of appropriate moisture and oxygen conditions in the soil will inhibit this type of condition within the remediation facility.

Occasionally, the presence of certain metals, *e.g.* mercury, can cause problems during the bio-processing of the hydrocarbon contaminants. A number of species of bacteria have the ability to methylate the mercury, causing the mercury to become more biologically available thus increasing the potential toxicity of the soil.

Another potential problem that the remediation system operator may encounter is the build-up of nutrients within the facility leachate resulting from over fertilization. This can be a significant problem if the water escapes the processing facility. It can also create a problem to the mechanical operation of a remediation facility through the fouling of the collection and re-circulation piping throughout the system.

Chemical Considerations

Nutrient Augmentation

The processing of hydrocarbons in soils using biodegradation should be thought of as the use of a complex set of biochemical catalysts to transform the soil contaminants into an acceptable form. The process requires certain inputs; *e.g.* hydrocarbon contaminated soil, nitrogen, phosphorus, oxygen and water in the correct ratio for the reaction to take place. If these ingredients are not present or are not present in the correct ratios, then the reaction does not take place or does so inefficiently.

Like any processing operation, the more that is known about the inputs, the better the control and more efficient the process. In the case of contaminated soil, this means that the better characterized the soils are prior to beginning the bio-processing, the better defined the overall processing will be. In general terms, the concentrations and type of hydrocarbons, pH, soil type, concentration of major inorganic nutrients (nitrogen, phosphorous, potassium, sulphur, calcium, and magnesium), total organic carbon, total soluble organic carbon and concentrations and types of heavy metals should be known prior to determining the application concentration of any additional nutrients. An evaluation procedure referred to as a bio-treatability study can be performed to optimize the remediation performance standards by providing us with the appropriate concentrations of the above parameters prior to the application of nutrients.

Through hands-on experience we have found that there are several rules of thumb that can be used for very small volumes of soil requiring treatment when the economics of performing and treatment study become prohibitive. The nutrient concentration that is generally thought of as maximizing biodegradation is a ration of: 100 available petroleum hydrocarbons: 10 available nitrogen: 1 available phosphorus: 0.1 available potassium.

These ratios assume that a well developed soil (*e.g.* able to sustain plant growth) has been contaminated and therefore contains many of the micronutrients that bacteria require to achieve optimal levels of biodegradation. However, soils of poor quality may require additional nutrient enhancement to achieve optimal levels of biodegradation.

There are several commercially available nutrient packages available. All of the commercial products contain the basic nutrients required to promote the growth of the biodegrading bacteria. The cost of the commercial products vary widely and some manufacturers have prepared formulations designed to address intermediate levels of soil contamination.

The operators may also choose to prepare their own supplement. This method is more time consuming but significant cost reductions are potentially available. The simplest approach (and we have found most cost-effective) is to purchase a quantity of the nutrient supplement formulated for chickens. Chicken-gut flora require a stable

population of bacteria for digestion. The chicken supplement is then diluted and applied to the soil in the appropriate amounts.

Once nutrient levels have been established that promote growth, it then becomes a matter of replacing nutrients lost during the operational period. The continuous addition of nutrients to the soil will yield the greatest growth of bacteria, however, this is not often practical. The best alternative is to periodically dose the soil with a concentrated solution of nutrients. Unfortunately, nutrients (*e.g.* nitrogen and phosphorus) if added in a too high concentration become toxic to the bacteria. Therefore, it is critical to achieve a balance between adding enough of the required nutrients but not adding too much. Determining in absolute terms what is enough is highly dependant on site specific conditions and is determined most effectively from a bio-treatability study.

Oxygen Availability

The supply of oxygen available to bacteria in a biopile has a direct relationship to the amount of soil pore space available, the way that the pore space is interconnected in the soil mass and the degree to which that pore space is filled with water and/or petroleum hydrocarbons. Water is commonly used as a transfer medium for oxygen to enhance the process of bioremediation. It has been suggested that biodegradation of most organic contaminants requires approximately two parts of oxygen to completely metabolize one part of organic compound (Wilson *et al.*,1986). However, if the soil is too saturated with water (more than 80% of water holding capacity), the available oxygen will be used up by the resident microbial population faster than it can be replaced and anaerobic conditions can persist.

Maintaining oxygen content in the soil is a key factor when evaluating how a system should be designed and operated to increase the performance of the bioremediation process. Sparging of air into the bioremediation system has proven to be an economical method of increasing available oxygen. This can take the form of air injection points and vapour extraction outlets dispersed throughout the soil profile or may involve sparging of the leachate prior to re-percolation in a non-passive system. Other methods of enhancing the availability of oxygen to the bioremediation process may include injection of oxygen enriched air, pure oxygen or hydrogen peroxide to the soil profile. These methods are more expensive than sparging but may have added benefits (*i.e.* hydrogen peroxide may assist in controlling the fouling of system piping and pumps caused by an explosion of bacteria growth).

Surfactants

Gasolines, oils and diesel fuels are mixtures of complex hydrocarbons. Each hydrocarbon component in the mixture has a different level of volatility, solubility in water and net metabolic value to the bacteria and these vary with temperature and pressure. Highly volatile hydrocarbons (*e.g.* benzene) are lost from the soil relatively quickly by tilling or venting the soil. Bacteria can contribute to the reduction of the volatile hydrocarbons in the soil by mobilizing the volatile components with the production of surfactants during degradation. Addition or production of surfactants can also increase the apparent solubility of hydrocarbons making them more available for volatilization.

Biodegradation of complex hydrocarbons is further complicated by the chemical nature of petroleum hydrocarbons and the physical environment of the soil. Petroleum hydrocarbons tend to be hydrophobic (do not dissolve readily into water) and tend to concentrate in the hydrophobic zones or patches in the soil.

The chemicals in the membranes that surround bacteria prefer to be in contact with an hydrophillic (water loving) environment. Therefore, the oil degrading bacteria must overcome the hydrophobic boundary imposed by the nature of the petroleum hydrocarbons. Bacteria do this by producing chemicals (surfactants and/or dispersants) that increase the apparent solubility of hydrophobic chemicals in water.

Surfactants and dispersants are available commercially. The commercial products are complex hydrocarbons that contain additional ionic groups and mimic the activity of the bacteria-produced surfactants (also referred to as emulsans).

The use of low toxicity surfactants or bacteria-produced emulsans can be a useful addition to soils that have been contaminated with long chain hydrocarbons, *e.g.* C25 and greater. The surfactant increases the homogeneity of the petroleum hydrocarbons in the soil and the growth of the bacteria. As the bacteria remove the short-chain hydrocarbons, the surfactant assists the bacteria in contacting the long chain hydrocarbons. Surfactants may mobilize free-phase contaminants by altering the surface tensions at the soil pore interfaces. This is attractive in *ex situ* biopiles, as the free-phase hydrocarbon may be collected and removed from the liquid stream, however, this effect may not be desirable for an *in situ* remediation system, especially while working in the vadose zone.

Physical Considerations

Soil Textures and Structures

A major factor in considering the approach to a bio-processing facility is the nature of the soil. Sands, gravels and loose loamy soils are the best candidates for bio-processing of contaminants. The type of soil structure will affect the mobility of

microorganisms through the soil profile. The composition of soil influences infiltration rate, permeability, water-holding capacity, adsorption capacity and microbial selection, thus effecting the ability of microorganisms to biodegrade petroleum hydrocarbons and the ability of the microorganisms to metabolize the compounds (Riser-Roberts, 1992).

Soils that have a large percentage of clay or silt require more thought in establishing a program for bio-processing. Over compaction of these soils may result in ponding of water in sections of the biopile, reduced pore space for water holding capacity (reducing the available oxygen supply and nutrient transport to the microorganisms), extended "turn-around-time" or percolation periods for nutrient flushing, and the uneven distribution of adsorption capacity for the petroleum hydrocarbons. Compaction may also result in the release of concentrated petroleum hydrocarbons causing a toxic effect to the recipient bacteria population.

Fluffed soils will encourage more effective bacterial growth and ultimately enhance the degradation of the hydrocarbons. This can be achieved by screening of the soil prior to placement into the biopile and/or the addition of bulking agents. Physical manipulation of the soil will enable a wider distribution of bacteria, promote nutrients and oxygen distribution in the soil and enhance the rate of degradation. It is important to gain as much information on the physical and chemical characteristics as possible regarding the optimum soil conditions for bioremediation prior to selecting a bulking agent as the introduced material may result in a significant change to the physical attributes of the mixture. Alternative sources of oxygen to the bio-process may not react as planned if the texture and structure of the soil is altered.

Soil Temperatures

Soil temperature is a major consideration to the overall performance of a bioremediation system especially in more northern climates. It has been suggested that the rate of degradation of organic compounds in soil may double with each increase of 10°C between the range of -2°C to 70°C (Riser-Roberts,1992). Consequently bioremediation would be significantly affected by cooler temperatures experienced throughout Canada and the Northern U.S.A. during the fall, spring and winter months. Controlling soil temperature during the cooler periods can be achieved by a variety of methods including the addition of an insulating cover, the addition of bulking agents, or the insertion of a external source of heat.

The addition of a cover to a biopile is probably the most cost effective method of increasing the net solar energy within the pile. It also can be added to control the amount of moisture within the remediation facility and reduce the odours generated during operation of the facility. The addition of heat generating bulking agents may also be considered, however, it is extremely important to ensure that the bacteria that are non-hydrocarbon degraders within the bulking agent do not over compete with the hydrocarbon reducing bacteria. Maintaining a healthy microbiological environment will

increase the performance of the remediation process. A more recent application is the use of external heating agents. Electrical heat strips and gas powered heaters have been successfully used to control the temperature of the remediation facility during colder months. An increase in volatilization is likely as well as an increase in the loss of soil moisture. This may effect the overall performance and cost of the remediation facility and should be closely monitored by the facility operator.

Moisture Content

The movement of water through the soil is an important factor in the delivery and movement of nutrients, bacteria and soil gasses through the soil pore spaces. In addition, the movement of water assists in the removal of waste products, *e.g.* organics, acids, and carbon dioxide from the soil.

There is considerable debate as to "how much water" should be added to the soil. Bacteria require some water to carry out bioremediation. Our experience has been that the amount of water necessary for bacteria to thrive depends on the following factors:

1. Evaporation rates.
2. Rainfall.
3. Rate of metabolic activity (biodegradation rate) of the bacteria.
4. Water holding capacity of the soil.

Another important factor is the oxygenated state of the water. Bioremediation facilities are designed to deal with excess water in the form of leachate. If the leachate is well aerated, it is not unreasonable to operate the facility with high levels of water moving through the soil.

The water supply should also be free of other potential contaminants. This is especially true of metals (*e.g.* mercury, lead, zinc and copper) that may result in the additional contamination of the soil.

Some care should also be taken to ensure the water will not add to the petroleum hydrocarbon contamination in the soil. However, it should be noted that the biopile can be turned into a "trickling filter" and could, in some instances, be used to remediate contaminated groundwater (Severn and Adams, 1992).

City water is usually chlorinated and should be avoided. However, if this is the only source, then sparging the water for periods of not less than two hours should reduce the chlorine concentration sufficiently to be a useful water source.

Bulking Agents

Bulking agents, *e.g.* straw, hay, wood, chips, gravel and/or sand are sometimes necessary, especially in soils with significant clay content to increase the potential for air flow. In general, the use of hay, straw and wood chips can help increase the air

permeability of the soil. However, these materials may result in significant changes to the physical structure the soil and may limit the ultimate use of the soil, such as future disposal or use as structural fill. They also contribute additional populations of bacteria to the soil that will compete with the hydrocarbon degrading organisms for nutrients, water and oxygen. If remediation does not result in success with the added bulking agents, then future remedial actions will have to be re-established by undertaking bio-feasibility studies. This may increase the overall remediation operation costs due to increased volume of materials and duplication of efforts.

When bulking agents are required, ingredients such as sand and gravel may be more suitable. Gravels and sands may be available from other contaminated feedstock or from previously processed materials. Therefore, there may be no further cost to the operator. Sands and gravels can also be added to improve the value of the remediated material for its final use.

CONCLUSION

The successful implementation of bioremediation technology for the remediation of petroleum hydrocarbon contaminated soil is dependent on observing good scientific practices. The successful implementation of this potentially powerful technology requires consideration of biological, chemical and physical parameters. The level of control that the operator has over the remediation process will directly effect the level of performance and the capital costs required to obtain closure of the facility.

Our experience has shown that indigenous microbes will usually be sufficient to implement bioremediation of petroleum hydrocarbons if encouraged through the application of fertilizers. However, the introduction of additional carbon sources may be considered if rapid bioremediation rates are desired or if soil conditions are poor. Bacteria will produce certain waste products that are innocuous but may interfere with commonly used analytical techniques. It therefore becomes important to correlate your analytical test results to the stage of the degradation process. Bacteria require certain inorganic nutrients (N, P), oxygen and proper physical conditions (*e.g.* moisture and temperature) to be effective. Petroleum hydrocarbon-bacterial interactions can vary significantly in different soil types. Adjustments to an *ex situ* bioremediation system can be made to enhance the performance of the bacterial community by introducing bulking agents and external temperature sources. Additionally, surfactants may be helpful in promoting bacteria-hydrocarbon contact and may be particularly useful for the mobilization of free-phase hydrocarbons.

SUGGESTED READINGS

Severn, S.R.T., R.H. Adams. 1992. Bioremediation: The fact and fiction. In: Environmental Issues and Waste Management in Energy and Minerals Production. Singhal *et al.*

Fox, C.A. 1991. Status and trends in bioremediation treatment technology. Remediations Summer 1991.

Kuhn, E.D., J. M. Sulfita. 1989. Dehalogenation of pesticides by anaerobic microorganisms in soils and groundwater - A review. In: B.L. Sawhney and K. Brown (Eds). Reactions and movement of organic chemicals in soil. SSSA Special Publication No. 22, Soil Society of America and American Society of Agronomy, Madison, Wis.

McCarty, P.L. 1988. Bioengineering issues related to *in situ* remediation of chlorinated soils and groundwater. In: Environmental Biotechnology. G.S. Omenn (Ed.). Plenum Press, New York.

Murphy, V.G., P.J. Unkefer, M.R. Stranger, K. Gorman-Bates and M.A. Alvares. 1991. Effects of cosubstrate selection in the biodegradation of trinitrotoluene. Proceedings of the 1991 Institute of Gas Technology Symposium of Biotechnology. Dec. 9 - 11, Colorado Springs, Colorado.

Wilson, J. T., L. E. Leach, M. Henson, and J. N. Jones. 1986. *In situ* biorestoration as a groundwater remediation technique. Groundwater Man. Rev. 56-64.

Riser-Roberts, Eve. 1992. Bioremediation of Petroleum Contaminated Site Remediation.

REMOVAL OF ORGANIC CARBON AND SULPHUR COMPOUNDS FROM PROCESS AND FUGITIVE EMISSIONS: PHASE I, RESULTS FROM LABORATORY STUDIES

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ABSTRACT

Lab-scale biofilters were constructed from black polypropylene plastic tubing with internal dimensions of 5 x 100 cm. A biofilter matrix was provided using coarse sphagnum peat which was washed with water and inoculated with enriched cultures of appropriate microorganisms. Compressed air was applied to the biofilters and various compounds were added to the air stream at appropriate concentrations. Included in these compounds were hydrogen sulphide and organic compounds such as n-hexane, cyclo-hexane, propionic acid and butyric acid. Removal efficacy was evaluated for each compound resulting in greater than 99% hydrogen sulphide removal, whereas removal levels for n-hexane, cyclo-hexane and the organic acids were lower. Numbers of microorganisms were assessed and their identity determined throughout the biofilter matrix. A mass balance for sulphur was determined throughout the biofilter system and will also be reported.

ÉLIMINATION DU CARBONE ORGANIQUE ET DES COMPOSÉS DU SOUFRE, DES ÉMISSIONS PRODUITES PAR LES INSTALLATIONS DE TRAITEMENT ET DES ÉMISSIONS FUGITIVES: PHASE I, RÉSULTATS OBTENUS D'ÉTUDES EN LABORATOIRE

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Vegreville, Alberta.

RÉSUMÉ

On a construit quatre filtres biologiques à l'échelle du laboratoire, avec des tubes en polypropylène noir de dimensions intérieures de 5 x 100 cm. On a utilisé comme matrice du biofiltre de la tourbe de sphaigne grossière lavée à l'eau, que l'on a inoculée avec des cultures enrichies en microorganismes appropriés. On a introduit de l'air comprimé dans les biofiltres et ajouté divers composés au courant d'air, en concentrations convenables. On a inclus dans ces composés l'hydrogène sulfuré, le n-hexane, le cyclohexane, l'acide propionique et l'acide butyrique. On a évalué l'efficacité de l'enlèvement de chaque composé, et obtenu un taux supérieur à 99 % dans le cas de l'hydrogène sulfuré, mais un taux inférieur dans le cas du n-hexane, du cyclohexane, et des acides organiques. On a dénombré et identifié les microorganismes dans l'ensemble de la matrice du biofiltre et déterminé un bilan de masse du soufre dans tout le système de biofiltre.

INTRODUCTION

The removal of sulphur and carbon containing compounds from process and fugitive gaseous emissions may be achieved using bacteria and fungi resident within biofilters. In Europe the use of biofilters has been exploited more vigorously than has been done in North America (1, 2, 3, 4, 5.). As on-line, real-time process tools these biofilters are used to remove airborne pollutants in a wide variety of applications including; sewage treatment off-gases and odours, meat packing waste odours and miscellaneous volatile organic carbon (alkanes, alcohols, etc.) and treatment of industrial emissions containing organic sulphur containing compounds (mercaptans, sulphides, etc.) and hydrogen sulphide.

Reported here are the specification, design, construction and physical testing results of lab-scale biofilters and a discussion of the biological removal of hydrogen sulphide and certain organic compounds (*e.g.* hexane).

MATERIALS AND METHODS

Microbiological

Microbiological procedures used for total heterotrophic counts employed R2A medium and detailed methods may be found in the Alberta Environmental Centre (AEC) Microbiological Methods Manual (6) and methods used for isolating sulphur-oxidizing bacteria are outlined in Coleman and Gaudet (7). Identification of microbial isolates were performed using the procedures documented in the (AEC) Microbiological Methods Manual (6) for heterotrophs and in Coleman and Gaudet (7) for sulphur oxidizing bacteria.

Sample Dry Weight

Biofilter matrix material was removed as a core and triplicate sub-samples of approximately 1 g were weighed into tared aluminum pans, dried at 105 °C for 24 h and cooled over anhydrous $CaSO_4$ and re-weighed. The difference determined the moisture content and the microbial counts were adjusted accordingly to obtain the numbers of microorganisms (colony forming units) per g dry weight of peat.

Microbial Enrichment and Selection

A microbial population was selected from soil to oxidize reduced sulphur compounds by providing 1% elemental sulphur to a 10% soil in water mixture. The selection mixture was shaken at 25 °C in air on a rotary shaker (eccentricity was 2.5 cm).

Transfers to fresh medium were made every 14 days. Specific cultures of *Thiobacillus thioparus* and *T. thiooxidans* were grown on S6 medium (7) in 2 litre quantities. The soil derived bacteria and the cultures were seeded to the biofilters as an inoculum source.

Provision of Nutrients

All biofilters were provided with 5 mL of an appropriate trace element solution (8) and 10 mL of 2 % (w/v) NH_4Cl solution, twice weekly and provided water continuously at a flow rate of 10 mL/h.

Lab-Scale Biofilter Construction

Biofilters were fabricated from a 5 cm x 1 m section of black polypropylene plastic tube with unions attached at both ends and held vertically in a large frame support structure (Figure 1). Coarse Alberta peat (2.1 L or 196 g) that had been rejected, as too large, from an agricultural grade peat bagging operation provided the matrix and was held in-place using exclusion mesh placed at the bottom of the vertical section of the black polypropylene plastic tube. Water was provided to maintain the matrix in a moist condition and to remove soluble bacterial metabolic products. All excess water was collected below the biofilter in a side arm flask connected to a U-tube containing water to a level of approximately 15 inches of water. A 5 cm x 0.65 m black polypropylene plastic tube was connected at the bottom of and at a right angle to the biofilter to serve as a mixing tube. To the top of the biofilter another 5 cm x 0.65 m black polypropylene plastic tube was connected at right angle and used as a downstream sampling area and as a connection to a disposal manifold. The mixing tube, biofilter section and downstream sampling area had rubber septa sampling fittings installed for needle and syringe sampling. Air was supplied at a rate of 8.5 L/min to the mixing tube into which was inserted a port to deliver the chemical of concern. The air delivery rate represented a nominal residence time in the biofilter matrix of approximately 20 sec. Below the base of the biofilter matrix a port was inserted and connected to the Magnahelic® gauge that measured the pressure drop across the biofilter matrix. The gauge range was 0-10 inches of water.

Scanning Electron Microscopy (SEM)

After the biofilter matrix material was completely removed as a core (Figure 2), samples were removed from various portions of the core and allowed to air dry for two days. Selected sub-samples were mounted on a SEM stub using silver paste. Mounted samples were gold coated using a sputter coater (Edwards model #S150B). Coated samples were stored in a plastic storage box and then examined using a scanning electron microscope (Hitachi model S-510).

Energy Dispersive X-ray Microanalysis (EDX)

Biofilter matrix samples were examined using a scanning-transmission electron microscope (Hitachi model H600). All samples were air dried followed by two preparation methods (A and B below). Method A involved selecting sub-samples in the 1 x 0.5 mm size range and then placing them in a folding grid. Method B involved breaking the sample into small pieces in 70% ethanol. Broken samples were then placed on formvar carbon coated copper grids and allowed to air dry. Grids from preparation methods A and B were analyzed by exposing them to X-rays at three locations each by focusing a beam spot of 0.5 to 1.0 nm in diameter on the matrix material and emission spectra collected for 100 sec at 75 kV in the EDX mode. All samples yielded an emission spectra that contained copper resulting from the use of copper grids and not from the biofilter sample material. The copper emission served as a qualitative reference standard.

Wet Chemical Methods

Sulphate was analyzed using the barium chloride turbidimetric method number 126 C (9). Determination of pH was accomplished using a Fisher pH meter (Accumet pH meter 925, Fisher Scientific, Ottawa, ON).

Gas Chromatography

Sulphur Containing Compounds:

A gas chromatograph (Shimadzu GC-9A, Japan) was equipped with 1/8" OD teflon chromatographic column packed with Chromosil 330 and a Flame Ionization Detector (FID) in which the FID combustion products were sampled by a Sulphur Chemiluminescence Detector (SCD) (Sievers SCD-350, Sievers Research, CO, USA). Gas samples were injected onto the Chromosil 330 column using either a 500 μL or 20μL gas sample loop connected to a gas chromatograph with an FID detector coupled to the SCD detector. Gas chromatograph operating conditions were nitrogen carrier gas, 20 mL/min; air, 400 mL/min and hydrogen, 140 mL/min. Temperatures were set as follows; oven temperature at 80 °C and injector/detector temperature at 30 °C. SCD operating conditions were dry compressed oxygen supplied at 8 psi and the vacuum pump in continuous operation providing vacuum at 21 - 25 Torr.

Organic Acids:

A gas chromatograph (model 5840A, Hewlett-Packard, U.S.A.) equipped with a Nukol fused silica wide bore capillary column was used (30 m, 0.53 mm ID) (Supelco, Inc., Bellefonte, PA, U.S.A.) and FID. Instrument operating conditions were; oven temperatures were 125 °C for 5 min., temperature programmed at 15 °C/min until 200 °C which was held for 20 min; carrier helium flow, 10 mL/min; FID make up flow, 20

mL/min; hydrogen, 50 mL/min and air, 190 mL/min. The standards used were n-propionic and n-butyric acids. Gases were sampled using two methods. The first method involved the passing of 10 L of gas through 1.25 g activated silica, the silica gel was placed in a vial which was then sealed with a septum. A volume of 4 mL of distilled-deionized water was added through the septum and the vial and contents shaken on a wrist-action shaker for 1 h. The vial was allowed to stand vertical and 1 mL of clear liquid was removed and placed in another vial and to which was added 10 µL of 10 % pentanol in water as an internal standard. A 1 µL sample was injected into the GC for analysis. The second method involved the direct injection of gas removed from the biofilter with no downstream processing. Retention times were the same as for the previous method.

Hexane and Cyclo-Hexane:

The instrumentation used was a gas chromatograph (Shimadzu GC-9A, Japan) equipped with a 1/8" OD teflon chromatographic column packed with Chromosil 330 and a FID. Gas was injected onto the Chromosil 330 column using either a 500 µL or 20 µL gas sample loop and analyzed. Operating conditions were; oven temperature, 30 °C; injector/detector temperature, 50 °C; nitrogen carrier gas flow, 20 mL/min; air flow, 400 mL/min and hydrogen flow, 48 mL/min.

RESULTS AND DISCUSSION

Microbiological

Observations of the matrix in biofilter 1 treating H_2S at approximately 10 ppm yielded various populations and levels throughout the biofilter matrix (Table 1). High numbers of heterotrophs and low numbers of autotrophs were observed throughout the matrix. The biofilter effluent numbers for both microbial groups were very low. Biofilter 2, treating H_2S at approximately 10 ppm yielded slightly lower numbers of heterotrophs throughout the matrix compared with biofilter 1, but yielded no reproducible numbers for autotrophs. As in the case of biofilter 2 the water effluent numbers were very low. The low numbers in the effluent are primarily due to low effluent pH and in this case not to matrix dryness (which could reduce numbers) since the numbers at the lower end of the matrix approximated those of the mid region. The mid region did appear to contain (grossly) large quantities of fungal mycelium which may have impacted the enumeration techniques and provided variable results for autotrophs on S6 medium. Although not specifically investigated, another explanation could be the presence of substantial mixotrophic bacteria less able to grow on S6 medium.

Little difference in microbial numbers was observed in a biofilter challenged with a mixture of H_2S, n-hexane and thiophene when compared to H_2S alone . Although the

degradation of thiophene could not be demonstrated, H_2S and n-hexane were degraded.

Electron Microscopy

Scanning electron microscopy results for biofilter 1 (H_2S) are shown in Figure 4. The lower micrograph was taken from a sample of matrix at the entry or lowest part of the biofilter. At this location a moderate number of bacterial cells of various shapes are present. Some of the cells are rod shaped typical of *Thiobacillus* spp. The middle micrograph is that of a mid-biofilter region and the upper micrograph was taken immediately before the biofilter matrix terminated. Progressing through the biofilter yielded gradually increasing microbial populations that increase in diversity at the same time. The mid-biofilter region appeared to indicate that many kinds of bacterial forms as well as possibly fungal mycelium were present. The upper biofilter matrix contains similar levels of microbial flora with increased diversity compared to that of the lower portions of the biofilter.

Scanning electron microscopy results for biofilter 2 (H_2S) are shown in Figure 5. The lower micrograph was taken from a sample of matrix at the entry or lowest part of the biofilter, the middle micrograph taken from material from the mid-point of the biofilter and the upper micrograph taken from the immediately before the biofilter matrix terminated. Similar changes occurred in this biofilter as occurred in biofilter 1.

Scanning electron microscopy results for biofilter 3 (n-hexane, H_2S and thiophene) are shown in Figure 6. The lower micrograph was taken from a sample of matrix at the entry or lowest part of the biofilter, the middle micrograph taken from material from the mid-point of the biofilter and the upper micrograph taken from the immediately before termination of the biofilter matrix. Crystals were seen in the lower portion of the matrix and their nature was unknown. Again, as in the other biofilters, there were varied morphological types and changes in appearance through the biofilter matrix.

Removal of Added Chemicals

Hydrogen Sulphide:

Biofilter 1 was packed with 2.1 L (196 g) coarse peat matrix and the air and H_2S flow initiated on Dec. 9/92 to provide a concentration of approximately 11 ppm (vol.). Water flow was then provided on Dec. 15 but the matrix was not inoculated with a competent microbial population or provided with nitrogen or mineral solution until Feb. 5/93 (Table 2). During the time at which H_2S and air was flowing little if any H_2S was removed by the biofilter matrix, however, following the introduction of water (Dec. 15) approximately 32 % or more of added H_2S was removed (apparently abiotically). Within approximately 14 days following inoculation with an enriched sulphur oxidizing population H_2S removal was $\geq$99.8% of that added. Upon increasing the H_2S concentration up to approximately 39 ppm the microbial population was able to remove

≥98.7% of the H_2S added. Hydrogen sulphide flow was shut down due to an activated safety sensor detecting high H_2S levels in the biofilter effluent. Water delivery system failure caused the shut down effecting reduced microbial H_2S oxidation capacity.

Hydrogen sulphide flow initiated in biofilter 2 to provide a concentration of approximately 5 ppm (vol.). Water flow was provided but the matrix was not immediately inoculated with a competent microbial population or provided with nitrogen or mineral solution. During this time 16 - 40 % of added H_2S was removed (Table 3; Figure 8). Within 4 weeks following inoculation with a competent bacterial population 95 % of added H_2S was removed. The H_2S level was increased over the next 4 weeks from approximately 10 ppm (vol.) to approximately 69 ppm (vol.) with little impact on the biofilter effluent H_2S concentration. Increasing the influent H_2S level to over 125 ppm (vol.) demonstrated that H_2S break through occurred, however within a short time period effluent levels lowered. An example of the high removal capacity was when influent H_2S levels were at 117.55 ppm (vol.) effluent levels of 0.88 ppm (vol.) were observed, equivalent to a removal efficiency of 99.993%. In comparing biofilters 1 and 2, biofilter 2 was more capable of rapidly oxidizing H_2S than biofilter 1 (Tables 2 & 3).

Sulphate, pH and biofilter pressure (pressure drop across the biofilter matrix due to resistance imparted by the porosity of the matrix) data are presented in Table 4 for biofilter 2. In general, the sulphate production rate moved from low initial rates of around <75 mg/day to rates in excess of 3,000 mg/day, following inoculation with sulphur oxidizing bacteria. pH levels over the same time period started at approximately 4.7 and during high sulphate production dropped to <2.0. The resistance (pressure drop) of the matrix to the passage of gases remained in the range of 0.2 - 2.6 in (0.51 - 6.6 cm) of water.

Accounting for, or the mass balance of, sulphur throughout the process is presented in Table 5. The amount of sulphur unaccounted for was 3.5 %. Overall, 92.8% of H_2S was oxidized but peak levels yielded removal levels of 99.9998% The decrease in overall removal effectiveness was impacted by poor removal efficiency which occurred during start-up when the matrix tended to become water saturated causing air and H_2S to channel past the matrix and not through it. This became less of a problem as the biofilter aged.

The removal of n-hexane is revealed in Table 7. Although technical difficulties caused the premature termination of the experiment, approximately 40 % of the added n-hexane was removed during a single 20 second residence time. A graphic representation can be seen in Figure 9. Presently further experiments are in progress.

When biofilter 2 was disassembled and arranged as a core, whitish material was present at the influent end of the matrix (Figure 2). Using energy dispersive X-ray analysis (EDX) and scanning for sulphur a larger proportion of sulphur appeared at the influent end of the matrix as compared to the effluent end. When the counts from the

emission spectra were evaluated approximately 40 times more sulphur appeared at the influent end compared to the effluent end (Table 6). The spectra for the influent, mid-point and effluent aspects of the biofilter matrix are shown in Figures 7a and 7b.
If the peak heights are compared to the relatively stable copper emission spectrum (due to the copper grids used for sample mounting) the relative amounts are much higher at the influent (labelled bottom) end when compared to the effluent (labelled top) end.

CONCLUSION

Analytical methods were selected, tested and implemented for use in the assessment of biofilter processing of high volume low strength gas emissions. Lab-scale biofilters were successfully designed, assembled and tested to treat such emissions. Compounds including H_2S, n-hexane and thiophene were tested. Hydrogen sulphide was effectively removed from gas emissions at concentrations up to 125 ppm (vol.), n-hexane was demonstrated to be removed, whereas, thiophene has not been demonstrated as being removed.

During the treatment of H_2S, microbial cells were shown to be present and when water was discontinued the metabolic processes appeared to slow resulting in reduced processing of H_2S. All effluents from the building in which the biofilters are housed are evaluated by H_2S sensors set at 10 ppm (vol.). When water flow was stopped (technical difficulties) the effluent H_2S concentration exceeded this threshold, the sensor responded resulting in automatic shut down of the building H_2S supply system.

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REFERENCES

1. **Kampbell, D. H., J. T. Wilson, H. W. Reed and T. T. Stocksdale**. 1987. Removal of volatile aliphatic hydrocarbons in a soil bioreactor. J. Air Poll. Cont. Assoc. **37**:1236-1240.

2. **Ottengraf, S. P. P.** 1987. Biological systems for waste gas elimination. TIBTECH **5**:132-136.

3. **Bohn, H. L.** 1990. Control of VOC emissions from waste management facilities.T. Environ. Eng. **115**:1002-1004

4. **Ottengraf, S. P. P., R. Diks**. 1990. A biological purification of waste gases. Chim. Oggi. **8**:41-45.

5. **Diks, R. M. M., S. P. P. Ottengraf.** 1991. A biological treatment system for the purification of waste gases containing xenobiotic compounds. In: On-site bioreclamation. Processes for xenobiotic and hydrocarbon treatment. R.E. Hinchee and R.F. Olfenbuttel (Eds.). Butterworth-Heinemann. U.S.A. pp. 452-463.

6. **Coleman, R. N.** (ed.). 1990. Microbiological methods manual. Alberta Environmental Centre, Vegreville, AB, Canada. AECV90-M2. 437 p.

7. **Coleman, R. N. and I. D. Gaudet**. 1993. *Thiobacillus neopolitanus* implicated in the degradation of concrete tanks used for potable water storage. Wat. Res. **27**:413-418.

8. **Krieg, N. R.** (ed.). 1984. Bergey's manual of systematic bacteriology. Vol.**1**. Williams & Wilkins, Baltimore, London. p. 675.

9. A.P.H.A. 1992. Standard methods for the examination of water and waste water. 18th Edition. American Public Health Association, Washington, DC.

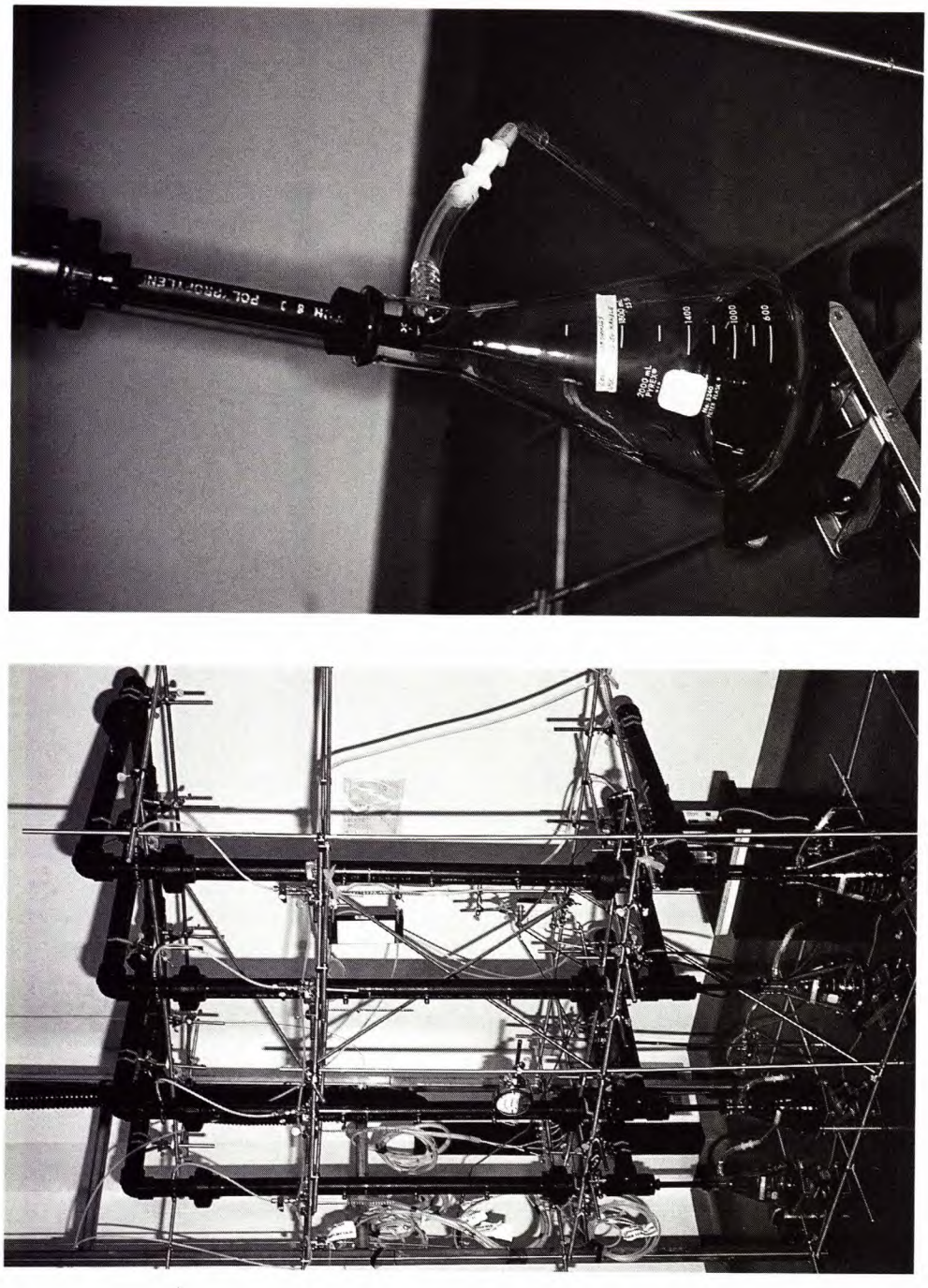


Figure 1: Lab-scale biofilter

Figure 2: Lab-scale biofilter core[1]

[1] Treatment of hydrogen sulphide.

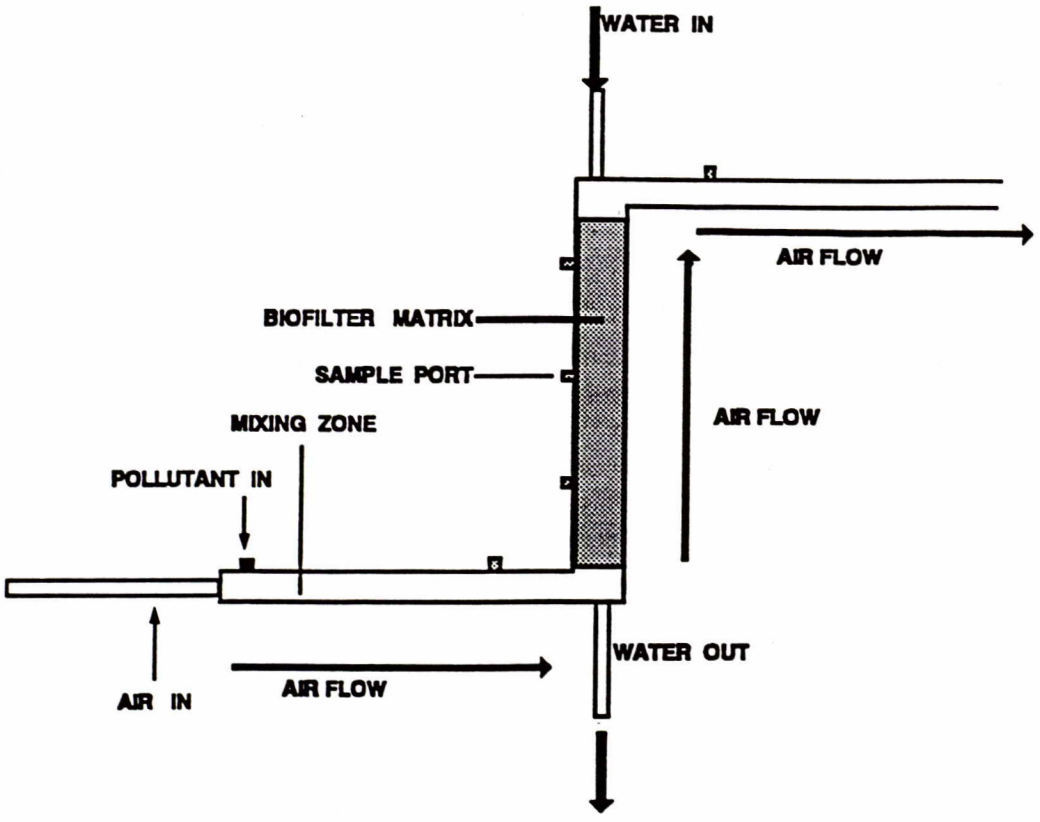


Figure 3: Biofilter schematic

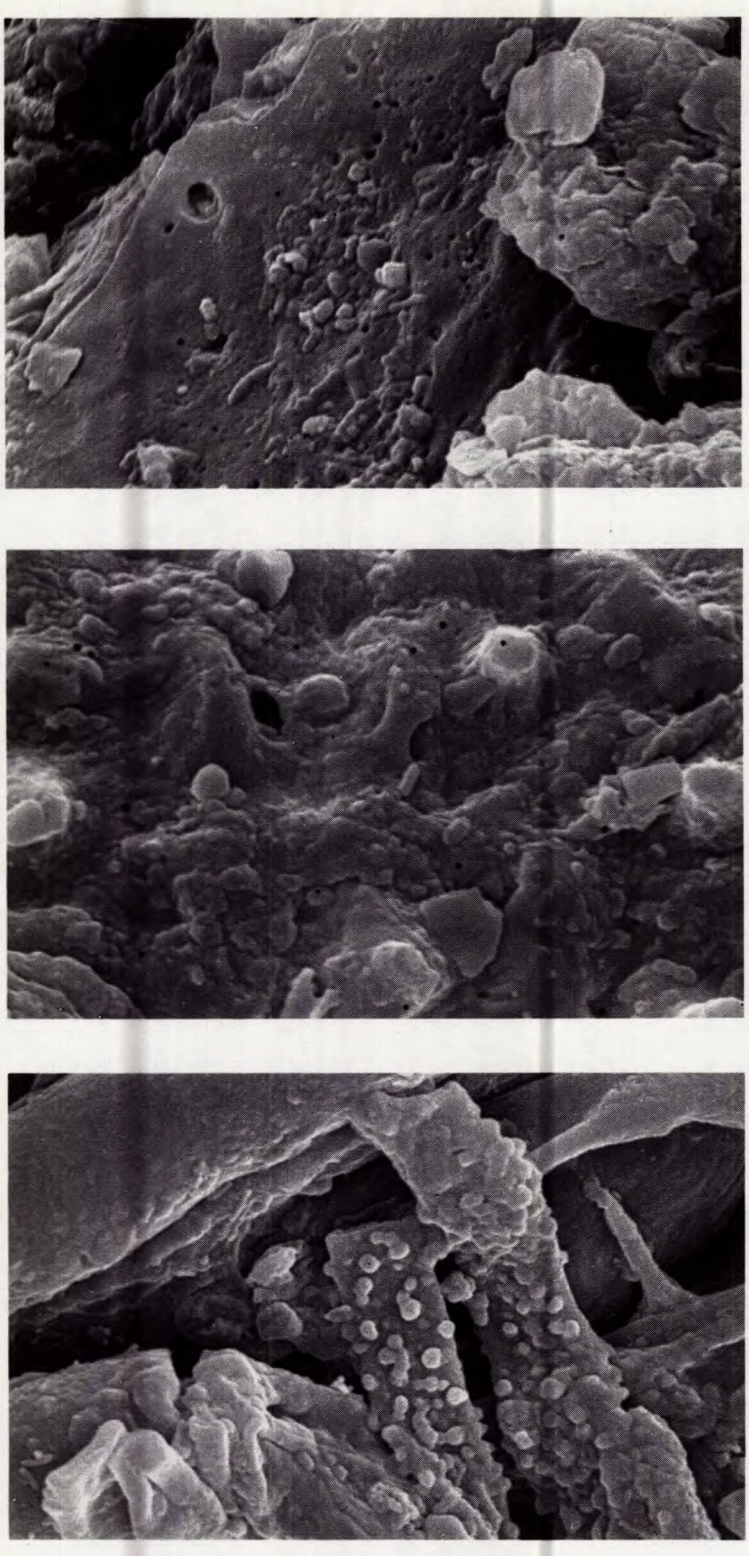


Figure 4: Electron micrographs from biofilter[1]

[1] Lower micrograph is from the influent end of the biofilter, the middle micrograph is from the mid-point of the biofilter and the upper micrograph is from the effluent end of the biofilter.

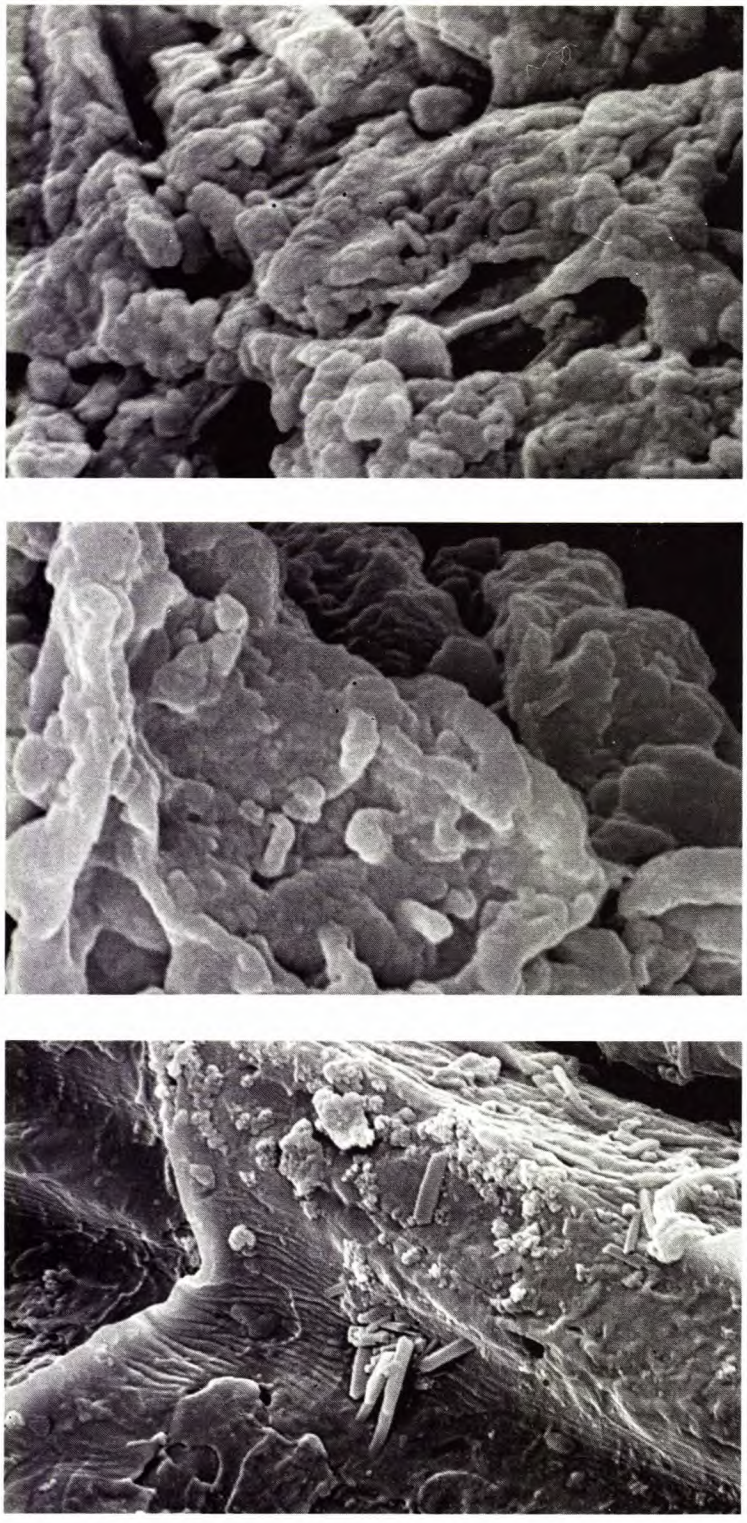


Figure 5: Electron micrographs from biofilter 2[1]

[1] Lower micrograph is from the influent end of the biofilter, the middle micrograph is from the mid-point of the biofilter and the upper micrograph is from the effluent end of the biofilter.

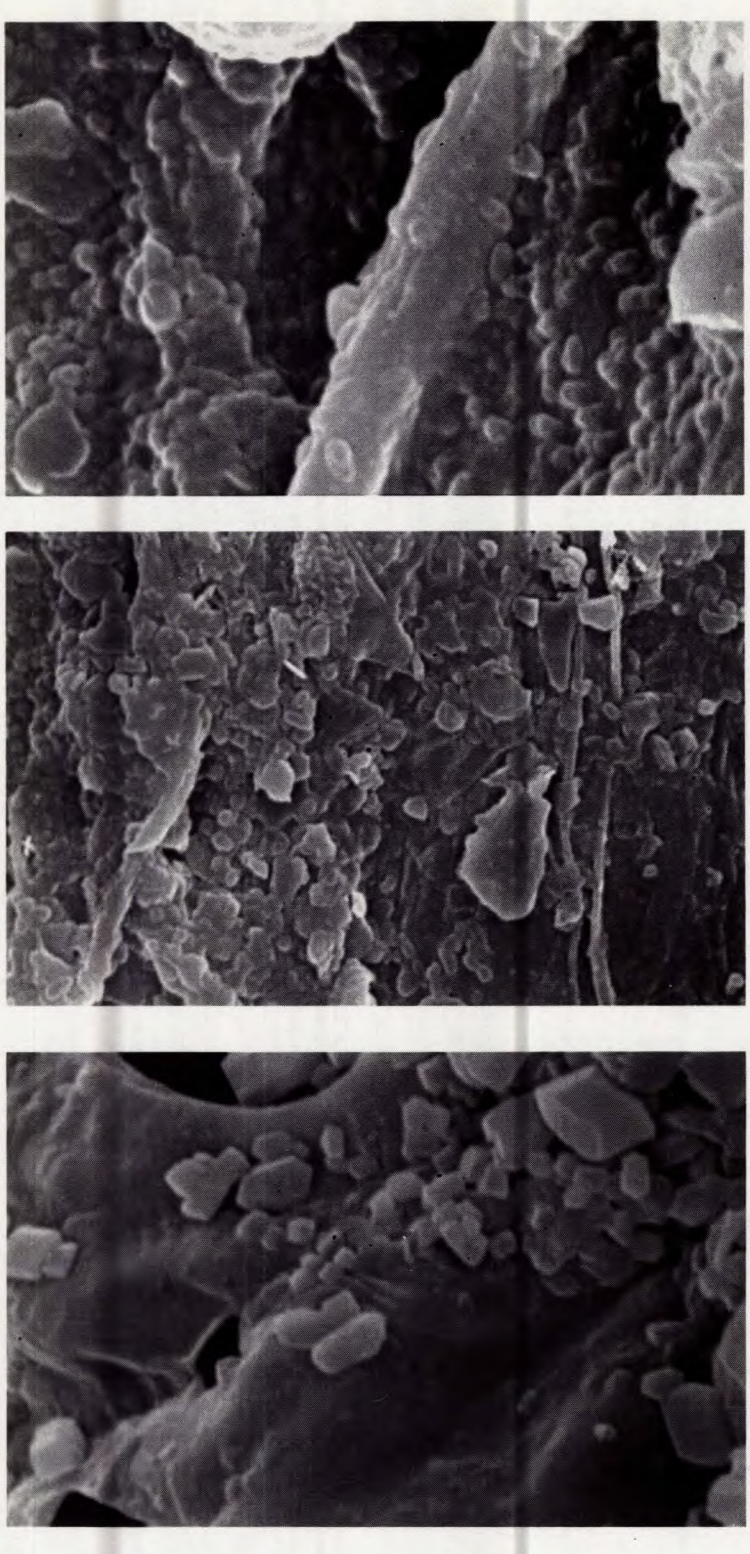
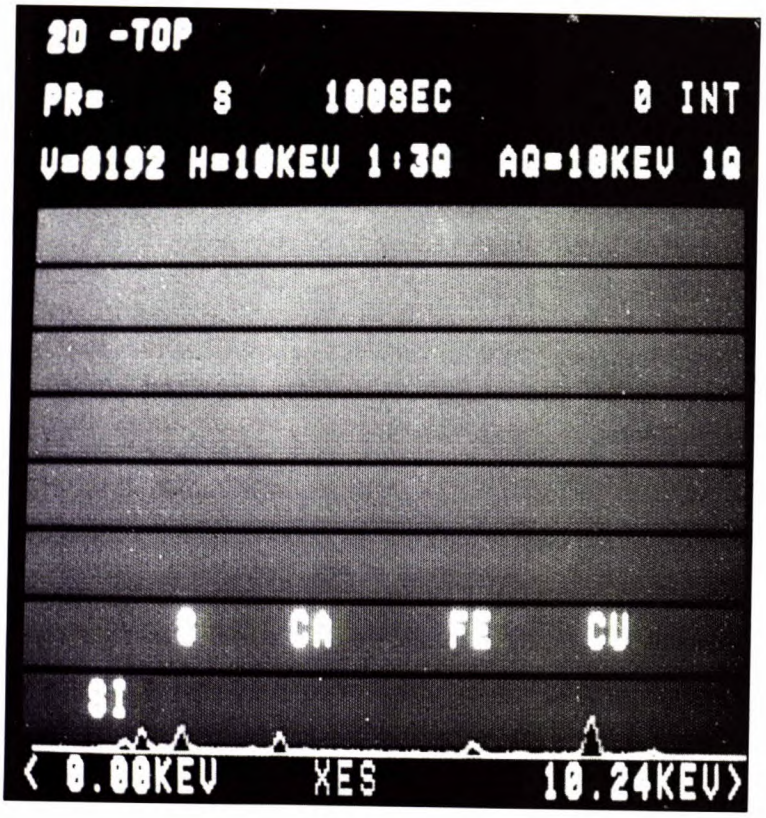


Figure 6: Electron micrographs from biofilter 3[1]

[1] Lower micrograph is from the influent end of the biofilter, the middle micrograph is from the mid-point of the biofilter and the upper micrograph is from the effluent end of the biofilter.



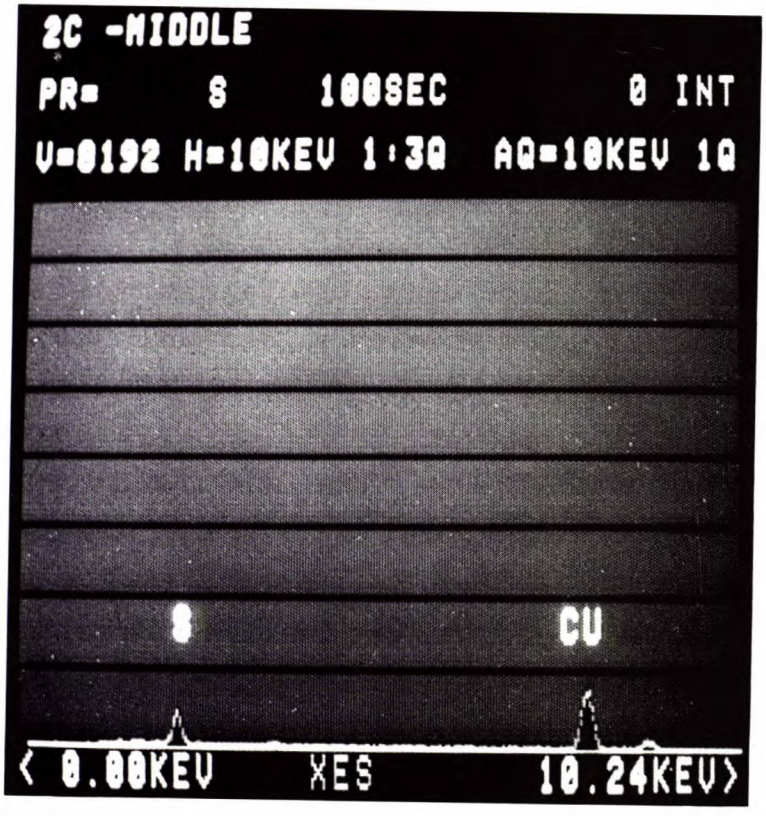


Figure 7a: EDX spectra from biofilter core

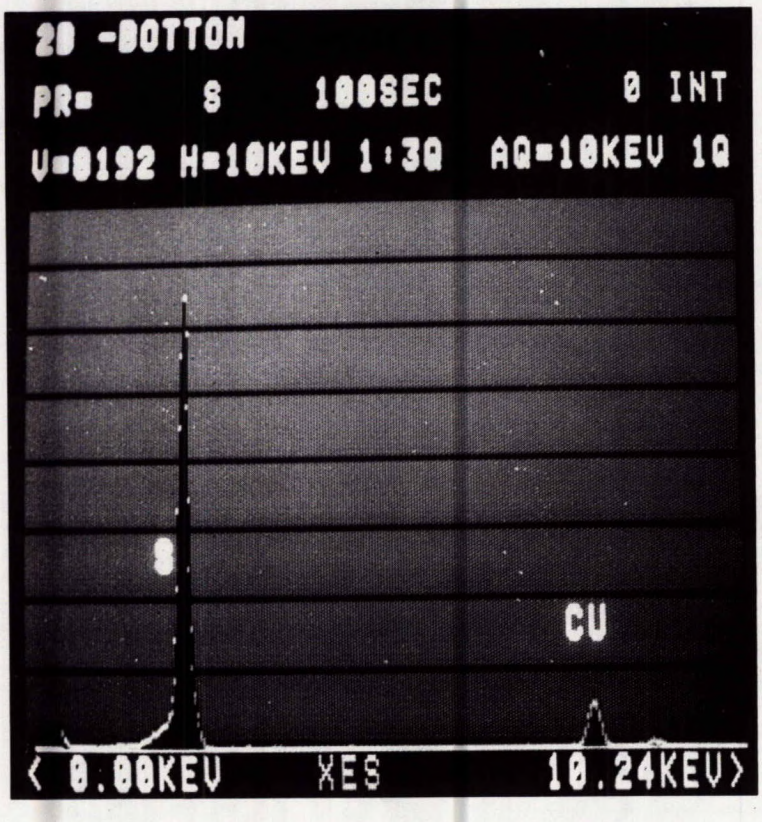


Figure 7b: EDX spectra from biofilter core

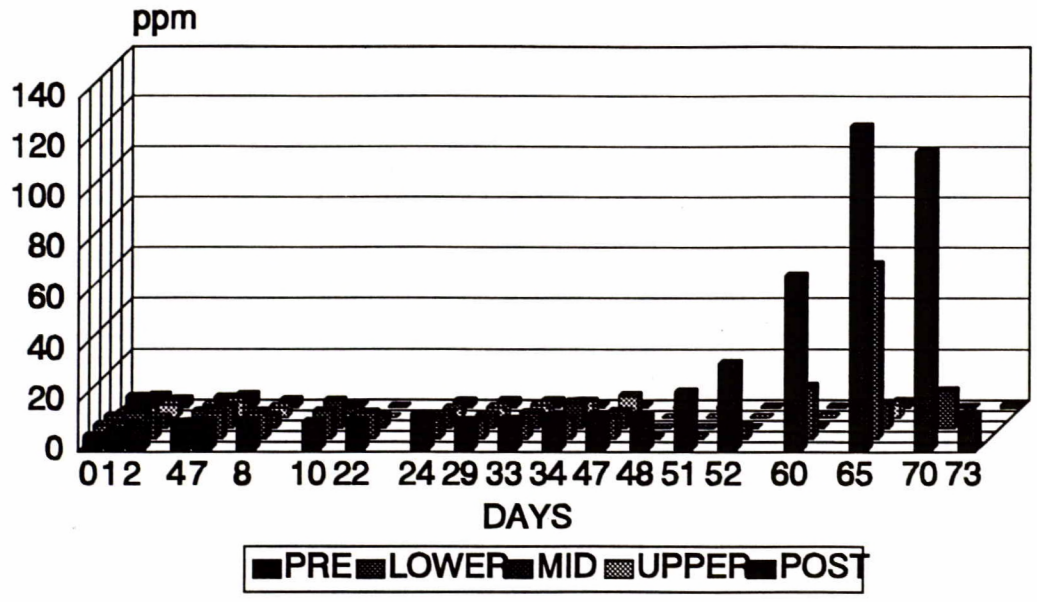
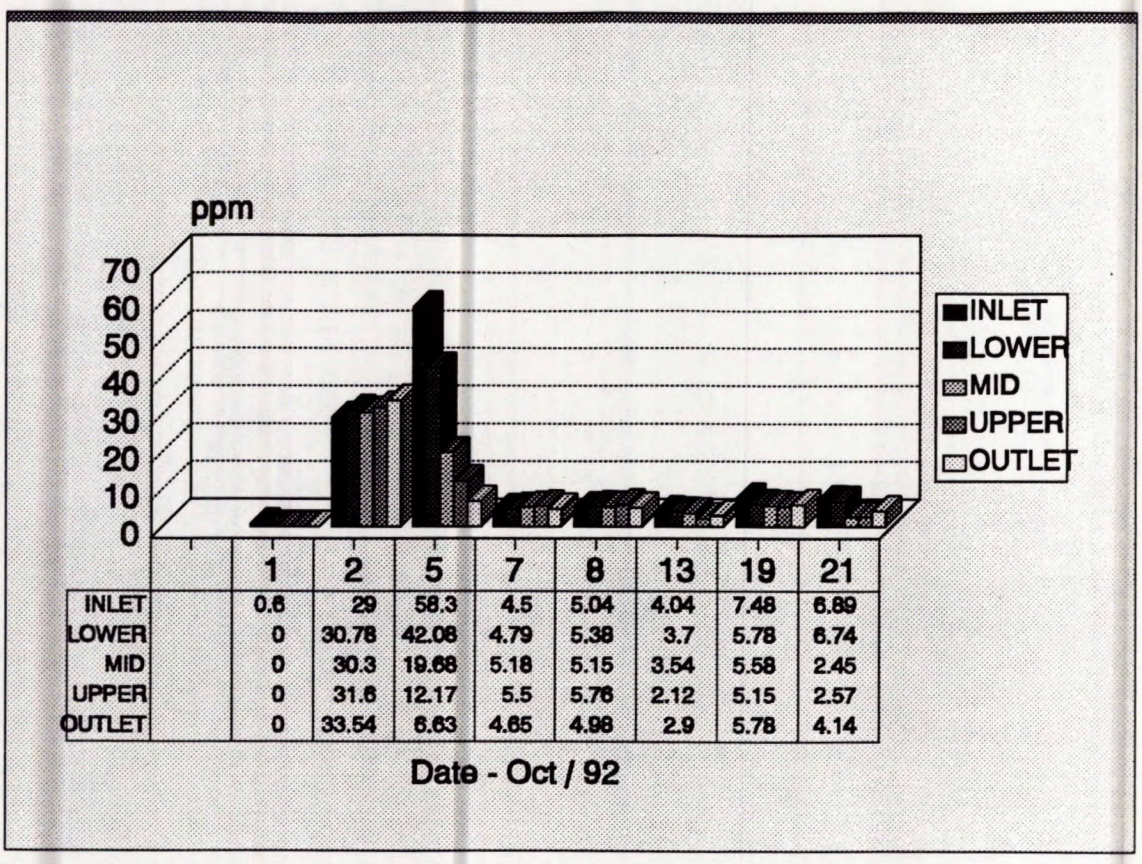


Figure 8: Hydrogen sulphide profile throughout biofilter 2



	1	2	5	7	8	13	19	21
INLET	0.6	29	58.3	4.5	5.04	4.04	7.48	6.89
LOWER	0	30.78	42.08	4.79	5.38	3.7	5.78	6.74
MID	0	30.3	19.68	5.18	5.15	3.54	5.58	2.45
UPPER	0	31.6	12.17	5.5	5.76	2.12	5.15	2.57
OUTLET	0	33.54	6.63	4.65	4.98	2.9	5.78	4.14

Figure 9: Hexane profile throughout a biofilter

Table 1 : Bacterial counts in various biofilters

Treatment	Description	R2A CFU[a]	S6 CFU[a]
H_2S	biofilter #1 H_2O effluent	86 [d]	2 [bd]
	biofilter #1B lower	1.9×10^8	$<1.1 \times 10^4$
	biofilter #1C mid	1.5×10^8	2.8×10^4
	biofilter #1D upper	3.6×10^9	$<1.5 \times 10^4$ [bc]
H_2S	biofilter #2 H_2O effluent	410[d]	15 [bd]
	biofilter #2B lower	1.6×10^6	[c]
	biofilter #2C mid	1.1×10^6 [b]	[c]
	biofilter #2D upper	2.0×10^8	[c]
H_2S Hexane Thiophene	biofilter #3 H_2O effluent	5.0×10^5 [bd]	[c]
	biofilter #3B lower	8.3×10^6	4.7×10^4
	biofilter #3C mid	1.9×10^7	[c]
	biofilter #3D upper	9.3×10^8	3.1×10^8

[a] Colony forming units.
[b] Based on numbers below counting limits.
[c] CFU's were observed in numbers that did not reflect the dilution of homogenous sample preparation.
[d] CFU per ml of sample.

Table 2 : Hydrogen sulphide profile for biofilter 1

Date	Time (24 hour)	H_2S (ppm)				
		Inlet	Bottom	Middle	Top	Outlet
De 7[1]		ND[10]	ND	ND	ND	ND
De 9[2]	1415	11.61	10.72	9.59	10.91	10.83
De 10	1350	7.03	7.69	3.35	8.45	8.66
De 10	1540	12.78	12.44	11.14	12.29	12.46
De 11	1115	11.02	11.37	9.94	11.34	11.91
De 15	1045	12.53	12.36	11.36	12.16	12.10
De 15[3]	1330	13.33	11.55	10.28	11.59	11.83
De 15	1540	12.25	7.54	9.94	9.92	8.61
De 16	1045	11.69	7.02	10.86	11.64	9.56
De 16	1105	10.07	6.79	10.59	11.53	7.55
De 17	1105	11.16	8.00	6.74	6.95	6.58
De 17	1455	5.33	8.85	6.90	7.44	7.91
De 21	1115	10.12	6.56	6.46	4.80	4.57
De 22	1045	10.08	7.11	7.25	6.64	5.61
Fe 3	1440	9.29	9.43	7.34	6.82	4.51
Fe 4	1640	12.22	12.38	13.36	11.33	3.86
Fe 5[4]	0940	12.97	9.45	9.77	7.96	6.01
Fe 8	1350	11.89	10.99	8.57	8.71	7.04
Fe 10	1555	13.67	11.74	11.19	8.54	3.54
Fe 12	1220	12.00	11.10	9.93	8.54	4.42
Fe 17	1525	13.48	3.19	ND	0.023	0.008
Fe 18[5]	1030	12.74	5.17	0.060	0.022	0.016
Fe 18	1047	31.38	21.40	1.25	ND	ND
Fe 18	1425	33.60	13.87	ND	ND	0.51
Fe 22	1335	37.22	30.66	14.88	1.99	0.139
Fe 24	1100	26.91	25.82	26.26	ND	0.041
Fe 26	1345	39.12	41.88	28.07	25.41	11.00
Fe 27[6]		ND	ND	ND	ND	ND
Fe 28[7]		ND	ND	ND	ND	ND
Mr 1[8]	1055	11.99	ND	ND	ND	4.19
Mr 1	1115	17.90	ND	ND	ND	0.61
Mr 1[9]	1325	19.67	ND	ND	ND	7.44

[1] Biofilter packed with 2.1 L (196 g) of coarse peat. Air turned on.
[2] H_2S turned on at 1100h December 9.
[3] Water turned on at 1350h December 15.
[4] Inoculate with seed bacteria 1400h February 5.
[5] H_2S flow increased 1045h February 18.
[6] H_2S flow turned off 2000h February 27 due to safety sensor system shutdown.
[7] H_2S flow turned back on 1330h February 28.
[8] H_2S flow increased slowly March 1.
[9] H_2S flow turned off 1345h March 1 due to safety sensor system shutdown.
[10] ND - not determined.

Table 3 : Hydrogen sulphide profile for biofilter 2

Date	Time (24 hour)	H_2S (ppm)				
		Inlet	Bottom	Middle	Top	Outlet
No 16[1]	1600	4.96	5.33	4.72	1.69	4.15
No 17	1600	6.68	5.93	5.75	2.12	4.57
No 18	1050	8.83	6.24	4.18	5.23	3.11
No 18	1535	9.72	7.13	6.45	5.38	6.00
No 20	1105	10.60	6.57	6.70	5.06	3.74
No 23	1055	11.19	10.36	8.76	6.37	4.85
No 23[2]	1350	10.19	10.68	5.40	5.64	6.68
No 23	1550	11.24	10.77	4.98	4.12	2.74
No 24	1555	11.23	11.38	10.14	1.62	1.06
No 25	1120	9.85	10.65	8.16	0.38	4.80
No 25	1525	10.72	10.90	10.94	1.57	0.71
No 26	1605	11.02	11.14	11.12	2.16	2.72
No 27	1135	10.81	10.33	4.93	NIL[11]	NIL
De 9	1100	8.28	6.16	3.89	0.80	0.90
De 9	1520	9.11	9.39	6.23	3.54	2.34
De 10	1410	13.83	7.91	5.20	3.68	2.33
De 11	1140	13.35	7.68	5.86	4.37	2.76
De 15	1350	11.35	7.03	5.20	4.08	2.58
De 16	1315	12.07	7.82	6.18	5.24	3.86
De 17	1050	11.65	6.67	6.23	5.18	2.55
De 17	1540	11.29	7.80	5.60	5.57	3.46
De 21	1335	12.93	7.95	10.34	6.48	0.67
De 21	1555	10.30	7.70	7.43	8.99	1.06
De 22	1540	13.50	8.05	6.74	8.70	0.50
Fe 3	1455	14.91	1.73	0.086	0.136	NIL
Fe 4	1620	13.77	0.77	NIL	0.025	NIL
Fe 4[3]	1715	19.14	1.37	0.126	0.075	0.013
Fe 5	0915	22.76	0.53	0.11	0.060	0.005
Fe 5[4]	1030	39.32	4.23	0.78	0.42	0.027
Fe 5	1047	37.93	2.20	0.63	0.27	0.023
Fe 5	1107	34.22	5.05	0.056	ND[12]	0.035
Fe 5	1128	33.25	3.21	0.49	0.179	0.033
Fe 8	1200	34.03	5.07	0.16	0.03	0.06
Fe 8[5]	1315	65.93	25.99	0.76	0.28	0.04
Fe 8	1330	55.02	9.48	5.17	0.34	0.08
Fe 8	1413	67.20	17.63	1.06	0.53	0.09
Fe 8	1613	67.77	25.56	2.64	1.34	0.26
Fe 9	1415	69.46	19.62	1.12	0.41	0.09
Fe 10	1525	68.24	20.76	0.87	0.29	0.035

Date	Time (24 hour)	H_2S (ppm)				
		Inlet	Bottom	Middle	Top	Outlet
Fe 12	1155	61.81	14.56	0.69	0.109	0.046
Fe 17	1325	67.91	12.91	0.566	0.088	0.031
Fe 17[6]	1345	90.54	63.86	3.676	2.655	0.717
Fe 17	1415	127.68	68.43	9.856	5.264	2.179
Fe 17	1643	114.99	60.85	18.30	14.08	8.067
Fe 18	1400	80.35	51.29	12.18	7.06	5.35
Fe 22	1400	102.08	114.76	27.63	9.80	0.13
Fe 24	1600	117.55	LA	14.97	1.24	0.088
Fe 26	1410	101.62	56.72	18.91	0.916	0.050
Fe 27[7]		ND	ND	ND	ND	ND
Fe 28[8]		ND	ND	ND	ND	ND
Mr 1[9]	1055	14.10	ND	ND	ND	0.765
Mr 1	1115	25.94	ND	ND	ND	3.06
Mr 1	1325	52.85	ND	ND	ND	6.05
Mr 4[10]		ND	ND	ND	ND	ND

1 H_2S turned on at 1100h November 16.
2 Inoculated with seed bacterial culture at 1330h November 23.
3 Increase H_2S flow 1640h February 4.
4 Increase H_2S flow 1028h February 5.
5 Increase H_2S flow 1313h February 8.
6 Increase H_2S flow 1343h February 17.
7 H_2S flow turned off 2000h February 27 due to safety sensor system shutdown.
8 H_2S flow turned on 1330h February 28.
9 Gas flows increased slowly March 1 after safety system shutdown. H_2S flow turned off 1345h March 1 due to safety sensor system shutdown.
10 Biofilter dismantled.
11 NIL - not detected, below lower detection limit.
12 ND - not determined.

Table 4 : Sulphate and pH profile for a biofilter treating hydrogen sulphide

Date	Day Number	Volume (mL)	pH	SO_4 (mg/L)	SO_4 (mg/day)	Cumulative SO_4 (mg)	Pressure (inches of water)
Nov 16[1]	0	ND[11]	ND	ND	ND	ND	0.6
Nov 18	2	1480	4.75	151.0	75.5	151.0	0.7
Nov 20	4	1474	4.74	239.0	119.5	390.0	0.6
Nov 23[2]	7	ND	ND	ND	2.1	396.2	0.3
Nov 25	9	45	3.95	10.4	2.1	400.4	0.2
Dec 2	16	498	4.57	1532.4	218.9	1932.8	0.7
Dec 4	18	870	5.76	473.3	236.6	2406.1	0.7
Dec 7	21	475	4.03	468.4	156.1	2874.5	0.7
Dec 9	23	700	3.75	616.0	308.0	3490.5	0.7
Dec 10	24	260	3.83	149.2	149.2	3639.7	0.7
Dec 11	25	2.75	3.80	157.0	157.0	3796.7	0.7
Dec 14	28	730	3.78	458.4	152.8	4255.1	0.8
Dec 16	30	840	3.53	914.8	457.4	5169.9	2.8
Dec 26	40	410	3.66	1560.5	156.0	6730.4	1.6
Jan 11	56	2230	2.02	5773.5	360.8	12503.9	1.2
Jan 14	59	240	1.85	879.7	293.2	13383.6	0.5
Jan 22	67	1330	1.79	5161.7	645.2	18545.3	1.2
Feb 1	78	1360	1.78	4925.9	447.8	23471.2	0.5
Feb 3	80	385	1.80	1083.8	541.9	24555.0	0.4
Feb 4[3]	81	ND	ND	ND	772.2	25327.2	0.4
Feb 5[4]	82	355	1.78	1544.3	772.2	26099.3	0.4
Feb 8[5]	85	395	1.78	1714.5	571.6	27813.8	0.4
Feb 10	87	440	1.64	5051.1	2525.6	32864.9	0.4
Feb 12	89	260	1.67	2022.5	1011.2	34887.4	0.4
Feb 17[6]	94	405	1.52	11202.5	2240.5	46089.9	0.4
Feb 19	96	515	1.61	5693.8	2846.9	51783.7	0.8
Feb 22	99	570	1.55	11286.8	3762.3	63070.5	0.8
Feb 24	101	380	1.52	9588.7	4794.4	72659.2	1.2
Feb 27[7]	104	ND	ND	ND	2493.8	80141.1	0.1
Feb 28[8]	105	ND	ND	ND	2493.8	82634.9	0.1
Mar 1[9]	106	ND	ND	ND	2493.8	85128.7	0.2
Mar 3[10]	108	230	1.46	17457.1	2493.8	90116.3	0.2

1 H_2S turned on at 1100h November 16.
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8 H_2S flow turned on 1330h February 28.
9 Gas flows increased slowly March 1 after safety sensor system shutdown. H_2S flow turned off 1345h March 1 due to safety sensor system shutdown.
10 Column dismantled.
11 ND - not determined.

Table 5 : Sulphur mass balance

Molecule	mMoles In	mMoles Out	% In	% Out	%
H_2S	1,006.8	35.3	100	3.5	96.5 Unaccounted
SO_4^{2-}	0	934.8	0	92.8	-
Total	-	-	-	-	3.5 Unaccounted
Conversion: Overall	-	-	-	-	92.8
Conversion: Peak	-	-	-	-	99.9998

Table 6: Counts from EDX sulphur emission throughout a biofilter treating hydrogen sulphide

	Counts[2]	
Location	A[3]	B[4]
Upper	2,990	3,380
Centre	12,287	83,496
Lower	112,416	147,944

1 For preparation method see section 1.3.5.
2 Relative abundance from H_2S specific emission wavelength.
3 Sample air dried and examined.
4 Sample prepared in ethanol, air dried and examined.

Table 7 : n-Hexane profile throughout a biofilter[1]

Day	Hexane (ppm)				
	Inlet	Bottom	Middle	Top	Outlet
1	0.60	NIL[2]	NIL	NIL	NIL
2	29	30.78	30.30	31.60	33.54
5	58.3	42.08	19.68	12.17	6.63
7	4.50	4.79	5.18	5.50	4.65
8	5.04	5.38	5.15	5.76	4.98
13	4.04	3.70	3.54	2.12	2.90
19	7.48	5.78	5.58	5.15	5.78
21	6.89	6.74	2.45	2.57	4.14

[1] Biofilter was packed with 2.1 L (196 g) of coarse peat the air turned on and inoculated with soil enrichment culture.

TRAITEMENT DES REJETS CHIMIQUES TOXIQUES PAR BIOFILTRATION SUR SUPPORT ORGANIQUE: ÉTUDES DE LABORATOIRE

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RÉSUMÉ

Des travaux préliminaires de recherche visant à sélectionner des souches de microorganismes capables de dégrader les HAP et à établir la performance du procédé BIOSOR® (biofiltration sur support organique) pour le traitement des effluents liquides d'origine industrielle, ont été réalisés en laboratoire.

Les études microbiologiques nous ont permis d'isoler 49 souches et d'observer leur capacité à dégrader le naphtalène (2 cycles), le phénanthrène (3 cycles), le 9-méthyl anthracène (3 cycles) et le pyrène (4 cycles). Les produits détectés dans les rejets industriels bruts sont, en majorité, des composés aromatiques mono et polycycliques dont la famille des quinolines et des pyridines. Les résultats des essais hydrodynamiques montrent une efficacité épuratoire variant de 80 % à 100 %. Les comptes bactériens réalisés sur le garnissage organique révèlent une bonne résistance des microorganismes face à l'apport de polluants toxiques.

TREATMENT OF TOXIC SUBSTANCES FOUND IN EFFLUENTS BY ORGANIC BIOFILTERS: BENCH SCALE STUDIES

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ABSTRACT

Preliminary research for the purpose of selecting microorganism strains able to degrade polycyclic aromatic hydrocarbons (PAHs) and determining the performance of the BIOSOR® process (organic biofiltration) in the treatment of liquid industrial effluents was conducted on laboratory columns. In our tests, we studied the effect of different effluents on the behaviour and effectiveness of the biofilter system.

The microbiological studies enabled us to isolate 49 strains and observe their ability to degrade naphthalene (2 cycles), phenanthrene (3 cycles) and pyrene (4 cycles). Tests are under way to determine the potential of the bacteria and fungi isolated for degrading PAHs with 5 and 6 cycles.

The products detected in industrial wastes are mostly monocyclic and polycyclic aromatic compounds of the family of pyridines, phenols, quinolines, benzoquinolines and phthalates. The results of the hydrodynamic tests show a cleaning efficiency varying between 80% and 100%. Further studies will enable us to establish the biodegradability of the compounds retained in the biofilter through chemical analyses and respirometry tests of the lining. These tests will also allow us to identify the competent micro-organisms performing the biodegradation.

INTRODUCTION

La décharge d'effluents industriels pose un problème environnemental important tant du point de vue de la nature toxique de la charge polluante que de la quantité des rejets produits. L'industrie québécoise rejette annuellement dans le fleuve Saint-Laurent et ses affluents, 265 000 tonnes de déchets liquides chimiques dangereux (1). Bien que la nature des contaminants d'origine industrielle soit très variable, les contaminations par des substances organiques toxiques représentent une partie importante des déchets identifiés. Nous faisons donc face à des problèmes de contamination par des substances telles que huiles et graisses, hydrocarbures aromatiques mono et polycycliques (HAM, HAP), composés phénoliques et produits halogénés volatils.

Les normes environnementales relatives aux rejets dans le milieu naturel devenant de plus en plus exigeantes, il est nécessaire d'effectuer un traitement des effluents. Quoique plusieurs systèmes de traitement permettent de réaliser une décharge contrôlée des rejets, pour des raisons économiques et techniques, la plupart des procédés traditionnels ont certaines limitations qui motivent la recherche d'autres techniques pour le traitement des rejets industriels.

Parmi les méthodes applicables à la destruction, la décontamination ou la détoxification des composés organiques toxiques présents dans l'eau, le traitement biologique se révèle être une voie intéressante. De manière plus spécifique, la biofiltration sur support organique utilisant une microflore fixée et naturellement sélectionnée représente une technique efficace et peu coûteuse. En effet, sa fiabilité, sa stabilité, sa performance et ses faibles coûts d'investissement et d'opération constituent les plus importants avantages du procédé (2).

Le système de biofiltration sur lit organique consiste en un procédé de filtration lente à biofilm fixe (figure 1). Comme agent dépolluant, le support organique peut intervenir à deux niveaux, soit comme une résine naturelle capable de fixer plusieurs types de polluants ou comme support pour différents microorganismes aptes à dégrader les substances retenues. Ces polluants sont dégradés en CO_2 et H_2O grâce à l'activité microbienne (3). Les constituants des supports organiques, particulièrement la lignine et les acides organiques, possèdent de nombreux groupes fonctionnels polaires : alcools, phénols, aldéhydes, cétones, acides, éthers. Ce caractère polaire lui confère une bonne capacité d'adsorption pour des molécules organiques et pour des métaux de transition (4). Les propriétés d'adsorption pourraient aussi être reliées à la présence d'une structure poreuse, propice à l'adsorption physique (5).

Différentes études ont été faites sur l'utilisation des biofiltres sur lit organique dans le domaine de la dépollution de l'eau (6). Les travaux de Dufort et Ruel (7), Poots *et al.* (8), McKay et Allen, (9), Poots et McKay (10), Thun *et al.* (11), Cloutier *et al.* (12), Lecours (13), Toller et Flaim (14), ont clairement établi l'applicabilité de la biofiltration

sur lit organique pour le traitement (l'enlèvement) de divers polluants tels les hydrocarbures, les pesticides, les teintures organiques acides ou basiques, etc. Par ailleurs, les travaux d'Ottengraf et Van Den Oever (15), Van Der Hock et Oosthoek (16), Don (17), Werner *et al.* (18), Saberiyan *et al.* (19), démontrent l'efficacité des biofiltres lors du traitement des effluents gazeux contaminés par des composés organiques toxiques tels le benzène, le toluène, le xylène (BTX), les composés organiques volatils (COV), les composés carbonés aliphatiques ou cycliques, les composés organo-sulfurés, organo-chlorés et azotés, les aldéhydes, les acides organiques, etc.

Compte tenu des performances du système et du marché potentiel de cette technologie dans le domaine de l'assainissement industriel, il y a un avantage certain à développer le processus de biofiltration afin de l'adapter au traitement des rejets chimiques toxiques. C'est pourquoi l'objectif de ce projet est d'évaluer le potentiel du procédé BIOSOR® pour réaliser un traitement économique et performant des décharges liquides de l'industrie chimique.

Ce travail présente les résultats des travaux préliminaires de développement consacrés à mettre en évidence la capacité des souches microbiennes naturellement sélectionnées à dégrader les HAP et à établir le comportement général du procédé BIOSOR® lors du traitement des eaux usées de l'industrie chimique.

MATÉRIEL ET MÉTHODES

Méthode Analytique

La méthode utilisée pour l'extraction et l'analyse des HAP présents dans les effluents liquides est basée sur la méthode 610 de l'agence américaine pour la protection de l'environnement (20). Celle-ci consiste en une extraction liquide-liquide. Le solvant d'extraction est le chlorure de méthylène. Les échantillons sont par la suite analysés par chromatographie en phase gazeuse couplée à la spectroscopie de masse (chromatographe HP-5890-II, détecteur MSD HP-5971A). Le détecteur de masse spécifique est utilisé en mode SIM (Selected Ions Monitoring) avec les trois principaux ions provenant de la fragmentation de chacun des produits analysés.

Compte tenu de la diversité des substances chimiques aromatiques dans les effluents industriels bruts, nous avons vérifié le comportement général et l'efficacité des biofiltres à l'aide d'un suivi des composés organiques totaux solubles dans le chlorure de méthylène et des produits majeurs regroupés par famille. Les familles sélectionnées sont les quinolines et les pyridines. Dans chacune des familles, la plupart des produits identifiés étaient, soit des dérivés méthylés des unités de base ou soit des isomères de même masse. L'efficacité d'épuration était calculée à partir du rapport des intégrations des pics de chacun des produits, mesurées sur des échantillons recueillis à l'entrée et à la

sortie des biofiltres. Les données étaient par la suite regroupées en famille.

Études Microbiologiques

Afin de mettre en évidence le potentiel de dégradation des HAP de souches isolées d'effluents industriels liquides et de sols contaminés, des essais de croissance en milieu liquide et solide ont été réalisés. Afin de mettre en évidence la capacité des bactéries isolées des différents effluents, les essais ont été réalisés sur un milieu solide vaporisé d'une préparation de HAP dans l'éther et, par la suite, l'inoculum a été déposé sous forme de gouttes. Un résultat positif de dégradation est observé lorsqu'il y a formation d'une zone claire autour des colonies. La procédure employée s'inspire de la méthode élaborée par Kiyohara *et al.* (21). De leur côté, les moisissures ont été mises en culture dans un milieu liquide contenant une concentration connue d'un HAP donné. Des échantillons ont été prélevés à différents intervalles de temps et, grâce à des analyses chimiques, la concentration en HAP restante en solution était déterminée. Ainsi, en comparant les résultats des fioles essais et de leurs fioles témoins respectives (non inoculées), il a été possible d'évaluer les pertes de HAP occasionnées par l'activité des microorganismes (biodégradation). Les essais de dégradation ont été réalisés sur les produits suivants; le naphtalène, le phénanthrène, le 9-méthyl anthracène et le pyrène.

Par ailleurs, des comptes bactériens ont été effectués sur les biofiltres pour établir la résistance des microorganismes face à l'apport de polluants toxiques. Pour ce faire, un échantillon de 2 g de garnissage humide est prélevé au sein des 10 premiers cm du lit filtrant de chaque biofiltre, ceci après homogénéisation de cette partie du garnissage. Cet échantillon a été broyé et homogénéisé par un passage de 2 min au broyeur Sorwall (1200 tr/min). Après plusieurs dilutions appropriées (eau peptonée 0,1 % avec ajout de Tween 80 à 0,1 %), 1 mL est ensemencé sur milieu nutritif organique (Plate Count Agar - Difco). Le comptage s'effectue après 2 j d'incubation à 35°C.

Tests de Biofiltration

Afin d'étudier et de comparer le comportement épuratoire de plusieurs types de garnissages organiques (écorces, copeaux de bois, compost, tourbe, tourbe + inoculum (sol contaminé), charbon activé (témoin), des essais ont été réalisés sur des pilotes de type colonne (tube SDR 35) de 7 L (diamètre de 9,5 cm et une hauteur de garnissage de 1 m). Trois points de prélèvement ont été placés sur toute la hauteur de chacun des biofiltres, ceci afin de vérifier différents paramètres tels l'activité biologique et le taux d'humidité. Le débit d'alimentation a été, pour ces différents biofiltres, de 1 L/j en moyenne.

RÉSULTATS ET DISCUSSION

Essais Qualitatifs avec les Bactéries

Le tableau 1 résume l'ensemble des résultats obtenus pour ces essais. Parmi les 17 souches isolées et testées, 5 ont été identifiées comme ayant la capacité de dégrader certains HAP : isolat 2 : *Brahamella* sp.; isolat 4 : *Streptomyces*; isolat 17 : *Pasteurella* sp.; isolat 47: *Achromobacter xylosoxidans* et isolat 49 : *Pseudomonas stutzeri.*

Pasteurella sp. s'est avéré le plus polyvalent des isolats, en démontrant un potentiel à dégrader les HAP de 2, 3 et 4 cycles. Cependant, aucune des souches isolées n'a été en mesure, à première vue, de dégrader le 9 méthyl-anthracène, un HAP de 3 cycles avec un groupement méthyle en position 9.

Essais Quantitatifs avec les Moisissures

Le tableau 2 résume l'ensemble des résultats obtenus lors de ces essais. Les isolats 40, 41, 42, et 43 ont tous la capacité de dégrader un ou plusieurs HAP. Une souche (43) a même démontré la capacité de dégrader, après 6 jours, tous les HAP testés. Ces résultats prouvent que les microorganismes présents dans le sol contaminé et donc susceptibles de se retrouver dans le biofiltre composé d'un mélange tourbe-sol, possèdent un potentiel de dégradation des HAP fort intéressant. Cependant, on ne peut conclure qu'il y a dégradation complète (minéralisation) des HAP, puisque les essais réalisés n'ont pas été orientés dans le but de vérifier cette possibilité.

Finalement, en comparant entre eux les résultats obtenus avec les bactéries et les moisissures, on note que les moisissures ont enregistré des performances très intéressantes par rapport aux bactéries concernant la dégradation du 9 méthyl-anthracène et du pyrène.

Étude du Comportement et des Performances de Différents Garnissages de Biofiltration

Caractéristiques de l'Effluent Industriel

Les caractéristiques physico-chimiques moyennes de l'effluent industriel utilisé sont rapportées au tableau 3. Il est à noter que les composés organiques totaux solubles dans le chlorure de méthylène regroupent plusieurs substances chimiques aromatiques dont les HAM, les HAP, les composés phénoliques, les quinolines, les pyridines, etc. De plus, d'autres produits tels les composés fluorés et sulfurés ont été également répertoriés dans l'effluent.

Performance Épuratoire et Stabilité du Système

Les figures 2, 3 et 4 présentent l'évolution des capacités de piégeage des différents garnissages pour les composés organiques totaux solubles dans le chlorure de méthylène, les quinolines et les pyridines respectivement. Dans les trois cas, l'efficacité épuratoire du charbon actif, considéré comme témoin, est supérieure à 99 %. Les autres types de garnissage tels le mélange tourbe-sol, la tourbe ou le compost donnent des taux d'efficacité supérieurs à 80 % et ceci, après 40 jours de fonctionnement. Seuls les copeaux montrent une efficacité nulle au démarrage des biofiltres, efficacité qui augmente progressivement au cours du temps pour atteindre 80 % au 40^{e} jour. Dans ce cas, un temps d'adaptation d'une trentaine de jours semble nécessaire pour obtenir une bonne épuration du système.

Au niveau des performances des garnissages, la tourbe plus inoculum de sol donne de bons résultats. Les copeaux et les écorces quant à eux pourraient, au sein d'un garnissage de type BIOSOR ®, constituer des agents structurants tout en participant activement à l'épuration de l'effluent toxique. Il est à noter que le compost même s'il présente de bons résultats épuratoires pose, de par sa texture très compacte, des problèmes hydrodynamiques (saturation en eau, compaction et colmatage).

Suivi Microbiologique

En parallèle avec le suivi chimique de l'affluent et des effluents des biofiltres, nous avons effectué des comptes bactériens totaux afin de vérifier l'évolution des populations au cours du temps (figure 5).

Ces résultats montrent une croissance importante de la biomasse surtout en ce qui concerne le biofiltre de Tourbe et Sol. La biomasse ne semble pas être, d'un point de vue général, affectée par la nature des eaux usées traitées. De plus, une simple observation des colonies se développant sur les géloses en Pétri montrent initialement une représentation importante de *Penicillium* sp. dans tous les cas. Cette tendance se maintient dans les biofiltres jusqu'au 13^{e} jour et dans le biofiltre écorces jusqu'à la 3^{e} semaine. Entre le 13^{e} et le 41^{e} jour de fonctionnement, on observe un changement dans les populations, certaines disparaissant au profit d'autres. Après le 41^{e} jour, les populations semblent se stabiliser entre 10^{9} et 10^{10} UFC/g.

CONCLUSION

La biofiltration à l'aide d'une microflore fixée et naturellement sélectionnée représente une technique efficace et peu coûteuse de remplacement pour les traitements physiques, chimiques et thermiques actuellement utilisés et qui entraînent des coûts élevés d'investissement et d'opération.

Les moisissures ont enregistré des performances très intéressantes par rapport aux bactéries concernant la dégradation du 9 méthyl-anthracène et du pyrène. Cependant, on ne peut conclure, pour le moment, qu'il y a dégradation complète (minéralisation) des HAP.

Bien que les biofiltres à base de tourbe, copeaux de bois et d'écorces permettent d'atteindre une efficacité épuratoire variant de 80 % à 100 %, des études additionnelles demeurent nécessaires pour établir la biodégradabilité des composés retenus par ceux-ci.

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RÉFÉRENCES BIBLIOGRAPHIQUES

1. Centre Saint-Laurent. 1990. Les toxiques dans le Saint-Laurent. Feuillets d'information sur l'état du Saint-Laurent. Environnement Canada.

2. **Buelna, G., G. Bélanger**. 1990. Biofiltration à base de tourbe pour le traitement des eaux usées des petites municipalités. Sciences et techniques de l'eau, **23**: 259-264.

3. **Bélanger, A., D. Potvin, R. Cloutier, M. Caron et G. Thériault**. 1987. La tourbe: Une ressource d'avenir. Étude réalisée pour le Centre québécois de valorisation de la biomasse, 125 p.

4. **Coupal, B., J.M. Lalancette**. 1976. The treatment of wastewaters with peat moss. Water Research, Vol. **10**, pp. 1071-1076.

5. **Tinh, V.Q., R. Leblanc, J.M Janssens et M. Ruel.** 1971. Peat moss - A natural adsorbing agent for the treatment of polluted water. Can. Min. Metall. Bull. Vol. **64**, pp. 99-104.

6. **McLellan, J.K., C.A. Rock.** 1986. The application of peat an environmental pollution control: a review. International Peat Journal. Vol **1**, pp. 1-14.

7. **Dufort, J., M. Ruel.** 1972. Peat moss as an adsorbing agent for the removal of coloring matter. Proc. 4th Int. Peat Congr., Otanieni, Finland.

8. **Poots, V.J.P., G. McKay et J.J Healy.** 1976. The removal of acid dye from effluent using natural adsorbents. I - Peat. Wat. Res., **10** (12): 1061-1066.

9. **McKay, G., S. J. Allen.** 1980. Surface mass transfer processes using peat as **a** absorbent for dyestuffs. Can. J. Chem. Eng., **58** : 521-526.

10. **Poots, V.J.P., G. McKay.** 1980. Flow characteristics and parmeters relating to the use of peat and wood as cheap adsorbent materials for waste water purification. Sci. Proc. R. Dublin Soc., Ser. Al, **6**(15): 409-440.

11. **Thun, R., L. Fagernas et J. Brandt.** 1983. Use of thermally treated peat for water purification. Int. Symp. Peat Util. (Proc.): 365-379.

12. **Cloutier, J.N., A. Leduy et R.S. Ramalho**. 1985. Peat Absorption of herbicide 2.4 - D - from wastewaters. Can. J. Chem. Eng., **63**: 250 257.

13. **Lecours, M.** 1988. Mémoire de maîtrise des sciences appliquées, Université Laval, Sainte-Foy, Janvier 1988, 160 p.

14. **Toller, G., G.M. Flaim.** 1988. A filtering unit for the removal of pesticide residues from aqueous solutions. Wat. Res., **22**(5): 657-661.

15. **Ottengraf, S.P.P., H.C. Van Den Oever.** 1983. Kinetics of organic compound removal from waste gases with a biological filter. Biotechnology and Bioengineering, vol. XXV, pp. 3089-3102.

16. **Van Der Hoek, K.W., J. Oosthoek.** 1984. Composting : Odour emisssion and odour control by biofiltration. Proceedings of seminar organized by Commission of the European Communities, pp. 271-281.

17. **Don, J.A. 1986.** The rapid development of biofiltration for the purification of diversified waste stream. Div. Technol. Soc. 561, pp. 63-73.

18. **Werner, W., H.G. Liebe et B. Striefler.** 1986. Emission control with biofilters: Application, design and operational experience. 7th World Clean Air Congress, Sidney, Australia.

19. **Saberiyan, A., M. Wilson, E. Roe, J. Andrilenas, C. Esler, H. Kise et P. Reith.** 1993. Removal of VOCs via air-biofiltration: a technique for treating secondary air emissions from vapor extraction and air-stripping systems. *In situ* and On Site Bioreclamation. The Second International Symposium. San Diego, California. April 5-8 .

20. Environmemental Protection Agency. 1982. Test method 610. Polynuclear Aromatic Hydrocarbons. Cincinnati, Ohio.

21. **Kiyohara, H., K. Nagao et K. Yana.** 1982. Applied and Environmemental Microbiology, pp. 454-457.

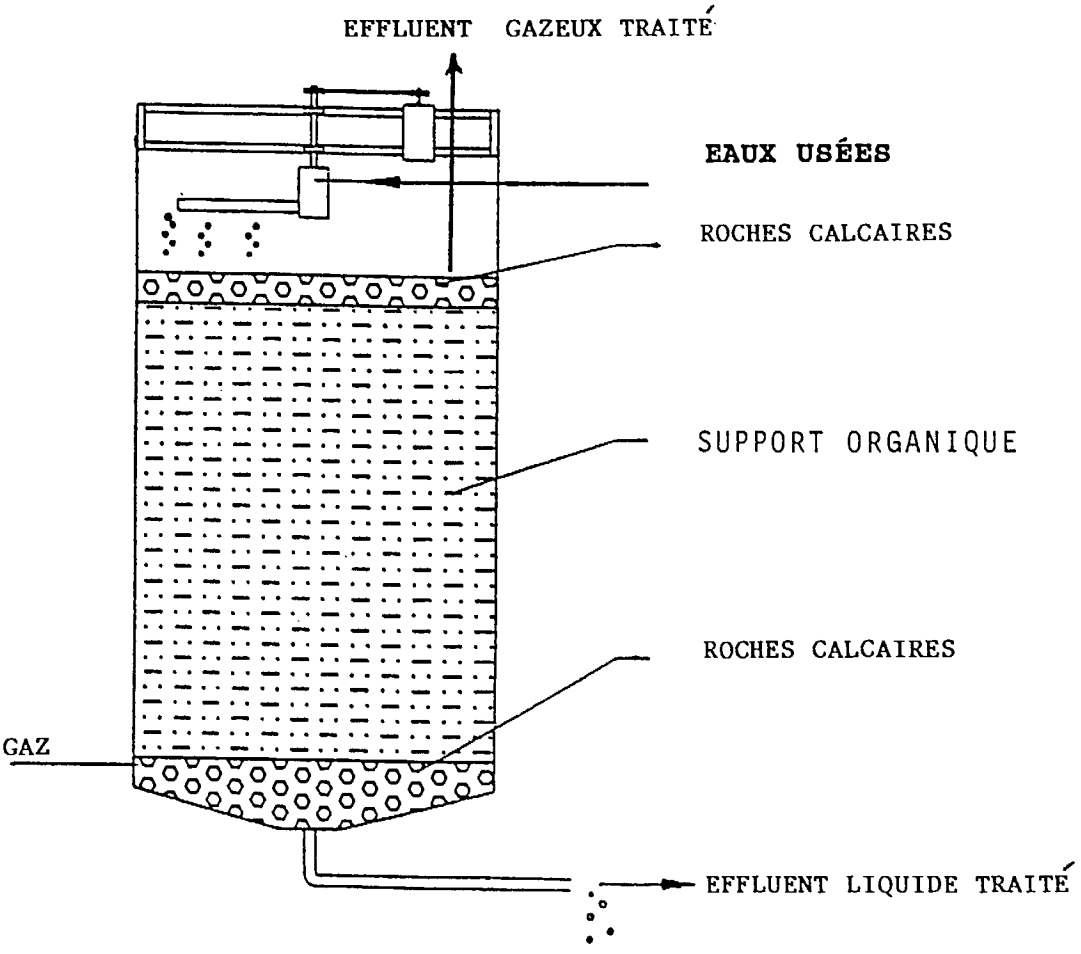

Figure 1. PRINCIPE DE FONCTIONNEMENT DE LA BIOFILTRATION SUR LIT ORGANIQUE

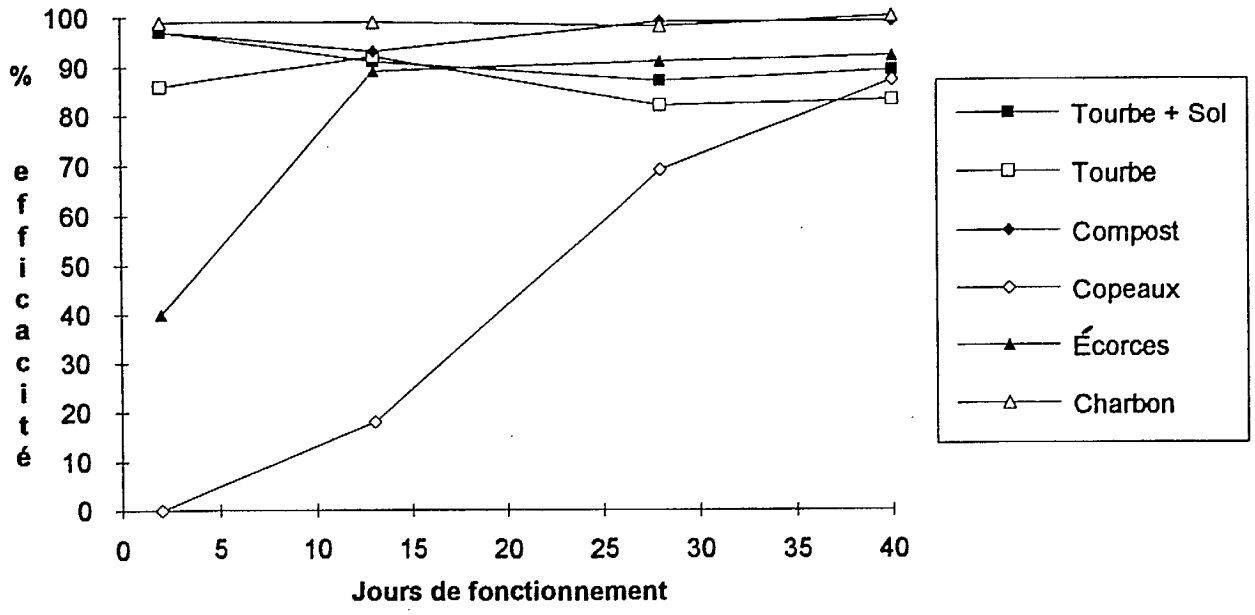

Figure 2 . ÉVOLUTION DE L'EFFICACITÉ DES DIFFÉRENTS BIOFILTRES POUR LA PRISE EN CHARGE DES COMPOSÉS ORGANIQUES TOTAUX SOLUBLES DANS LE CHLORURE DE MÉTHYLÈNE

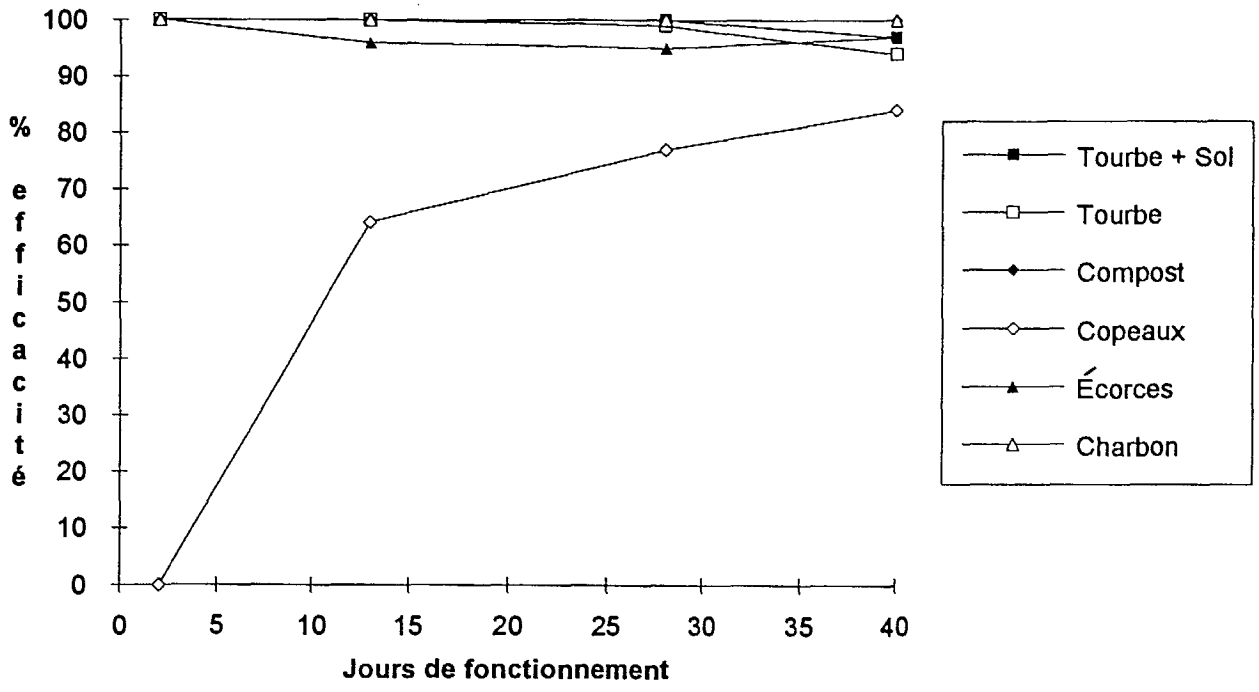

Figure 3 . ÉVOLUTION DE L'EFFICACITÉ DES DIFFÉRENTS BIOFILTRES POUR LA PRISE EN CHARGE DES QUINOLINES

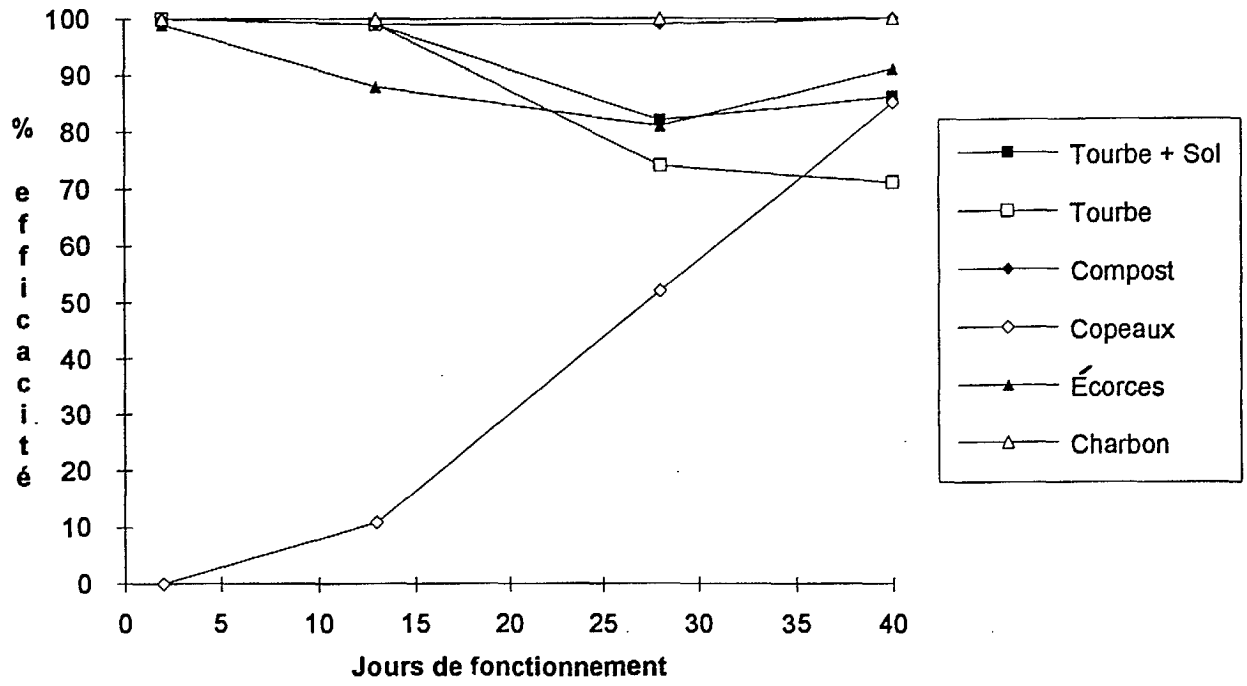

Figure 4 . ÉVOLUTION DE L'EFFICACITÉ DES DIFFÉRENTS BIOFILTRES POUR LA PRISE EN CHARGE DES PYRIDINES

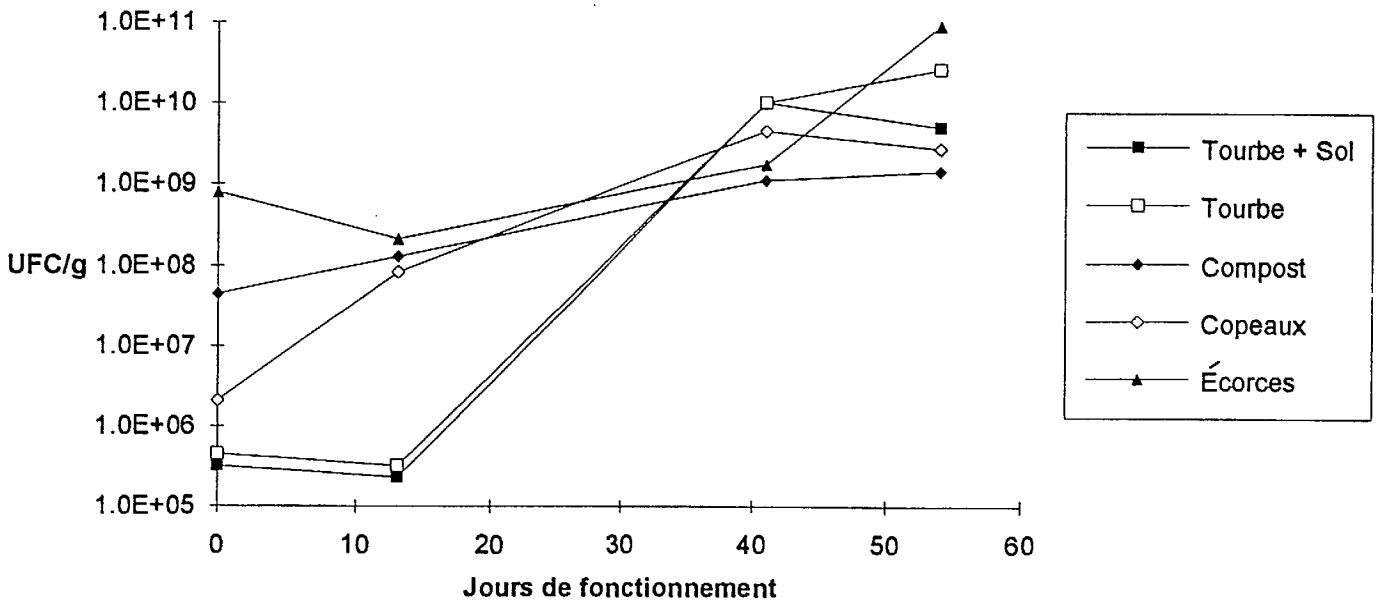

Figure 5 . ÉVOLUTION DES COMPTES BACTÉRIENS TOTAUX AU COURS DU TEMPS POUR LES DIFFÉRENTS BIOFILTRES

TABLEAU 1 . RÉSULTATS CUMULATIFS DES ESSAIS DE DÉGRADATION QUALITATIFS RÉALISÉS AVEC LES BACTÉRIES

N° de l'isolat	Capacité de dégradation			
	Naphtalène	Phénanthrène	9 Méthyl Anthracène	Pyrène
2	+	+	-	-
4	+	-	-	-
17	+	+	-	+
47	-	+	-	-
49	+	-	-	-

TABLEAU 2 . **RÉSULTATS CUMULATIFS DES ESSAIS DE DÉGRADATION QUANTITATIFS RÉALISÉS AVEC LES MOISISSURES (après 6 jours)**

N° de l'isolat	Capacité de dégradation observée			
	Naphtalène	**Phénanthrène**	**9 Méthyl Anthracène**	**Pyrène**
Concentration initale (ppm)	281-415	102-118	0,1 - 1,6	0,046 - 0,165
40	++	+/-	+	+
41	-	+/-	+	+
42	+	++	-	-
43	+	+	++	+

Légende : **- = 0 %; +/- = -10 %; + = 10-50 %; ++ = 50 % et plus**

TABLEAU 3 : CARACTÉRISTIQUES PHYSICO-CHIMIQUES DE L'EFFLUENT INDUSTRIEL

Paramètres	Moyenne
pH	6,2
DCO (mg O_2/L)	785
DBO_5 (mg O_2/L)	86
Oxygène dissout (mg/L)	5
N_{tot} (g/L)	1,1
P_{tot} (mg/L)	0,53
MES (mg/L)	7
COTSCM* (ppm)	37

* : Composés organiques totaux solubles dans le chlorure de méthylène

DEVELOPMENT AND APPLICATION OF TECHNIQUES FOR MONITORING THE BIOREMEDIATION OF PETROLEUM HYDROCARBON-CONTAMINATED SOILS

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ABSTRACT

The feasibility of using biological techniques to remediate soils contaminated with organic compounds generally requires laboratory evaluation before proceeding to a larger scale operation. Accurate assessment of the biological potential in soil requires techniques that determine the types of microorganisms present and their activities. A serie of tests has been assembled to specifically examine bioremediation potential in soil, and to monitor performance during the treatment operation. Tests include physical and chemical characterization of the soil, microbiological assessment and catabolic gene probing, mineralization of labelled test substrates, respirometric activity and toxicological evaluation.

Chemical characterization of the soil provides information on the types of organics, their concentrations and whether there are any interfering materials present. Microbiological assessment involves the culturing of bacterial populations in the soil on selective or nonselective growth media and the examination of the colonies to determine which have the genetic potential to degrade the soil contaminants. Catabolic gene probes, derived from the bacterial degradation pathways for aliphatic and aromatic compounds, are used to survey viable bacteria from petroleum hydrocarbon contaminated soils. Contaminated soils consistently demonstrate the presence of bacteria possessing the genetic capacity to degrade simple straight chain alkanes (*alkB* probe-positive), and aromatic compounds (*xylE* probe-positive). Mineralization and respirometric studies are indicators of the biological activity in the soil, and can be directed at microbial activity towards specific substrates.

Gene probe monitoring of a petroleum hydrocarbon contaminated soil during biopile treatment demonstrated that hydrocarbon-degrading bacterial numbers and activity were temperature dependent. The results showed that the activity of the indigenous bacteria as measured by hexadecane mineralization also correlated with the disappearance of the oil and grease.

The application of this protocol has provided a useful means to screen contaminated soils for bacteria with desirable catabolic properties and to monitor pollutant-degrading bacteria during biotreatment.

MISE AU POINT ET MISE EN PRATIQUE DE TECHNIQUES DE SURVEILLANCE DE LA BIODÉCONTAMINATION DES SOLS CONTAMINÉS PAR DES HYDROCARBURES DÉRIVÉS DU PÉTROLE

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RÉSUMÉ

Pour pouvoir utiliser des techniques biologiques de remise en état des sols contaminés par des composés organiques, on doit généralement procéder à une évaluation en laboratoire avant de passer à toute opération de plus grande envergure. En vue d'évaluer avec exactitude le potentiel biologique du sol, il faut faire appel à des techniques permettant de déterminer les types de microorganismes présents et l'activité de ceux-ci. On a regroupé une série de tests pour étudier spécifiquement le potentiel de biodécontamination, et aussi pour surveiller la performance du traitement. Les essais portent sur la caractérisation physique et chimique du sol, son évaluation microbiologique et le sondage génique catabolique, la minéralisation des substrats expérimentaux marqués, l'activité respirométrique et l'évaluation toxicologique.

La caractérisation chimique du sol nous fournit de l'information sur les types de produits organiques, sur leur concentration et sur la présence éventuelle de produits causant des interférences. L'évaluation microbiologique consiste à cultiver les populations bactériennes dans le sol sur des milieux de culture sélectifs ou non sélectifs, et à examiner les colonies pour déterminer lesquelles sont génétiquement capables de dégrader les contaminants du sol. Les sondes pour gènes cataboliques, qui viennent des voies de dégradation bactérienne des composés aliphatiques et aromatiques, sont employées pour étudier les bactéries viables provenant de sols contaminés par des hydrocarbures dérivés du pétrole. Les sols contaminés montraient toujours la présence de bactéries génétiquement capables de dégrader de simples alcanes à chaîne linéaire (positives pour les sondes *alkB*) et des composés aromatiques (positives pour les sondes *xylE*). Les études de la minéralisation et les études respirométriques sont des indicateurs de l'activité biologique du sol et peuvent porter sur l'activité microbienne vis-à-vis de substrats spécifiques.

La surveillance par sonde génique du traitement d'un sol contaminé par des hydrocarbures a démontré que l'abondance et l'activité des bactéries capables de dégrader les hydrocarbures dépendaient de la température. Les résultats ont indiqué que l'activité des bactéries indigènes, telle que mesurée par la minéralisation des hexadécanes, était également corrélée avec la disparition des huiles et graisses. L'application de ce protocole a été un moyen pratique de dépistage des sols contaminés contenant des bactéries qui possèdent des propriétés cataboliques désirables, et de surveillance des bactéries capables de dégrader les polluants pendant le biotraitement.

INTRODUCTION

The development of biological processes for the remediation of contaminated sites is a relatively recent phenomenon. This approach has become widespread in Europe and the United States and more recently in Canada. Bioremediation of soils and sediments contaminated with organic pollutants can provide a more cost effective alternative to conventional physical and chemical procedures. The biodegradation of a large number of organic pollutants has been demonstrated in the laboratory, and has provided fundamental information on the biochemistry, genetics and ecology of microorganisms responsible for these events. Demonstration of *in situ* biodegradation, however, is an altogether different challenge (1). A complex interaction of biological, chemical and physical factors can profoundly influence biodegradation in soil and sediments, and technical limitations make it difficult to verify the role of microorganisms in the disappearance of the contaminant. Three major classes of factors that influence biodegradation are the type of matrix (soil type and particle size distribution, concentration of organic matter, the presence of essential nutrient elements, oxygen diffusibility), the contaminant (type and concentration, mixtures of different substrates and non-substrates, substrate bioavailability) and the microorganism (diversity, genetic potential to degrade the contaminant, competition, predation) (Fig. 1).

Several molecular biology techniques have recently been applied to the study of microbial ecology and environmental microbiology, in some cases with particular reference to contaminated soils. The techniques include the use of gene probes and DNA hybridization techniques (2, 3, 4), the polymerase chain reaction (PCR) (5) and the application of reporter genes (6, 7). The use of specific gene probes, or specific oligonucleotide primers and PCR, to detect and monitor bacteria in environmental samples can dramatically reduce the detection time and increase detection sensitivity. The application of these techniques promises to answer many questions about natural microbial communities. In addition, the possibility of releasing genetically engineered microorganisms into the environment to aid in the biodegradation of harmful pollutants, has necessitated the development of rigorous methods to monitor their ultimate fate (8). The identification of specific pollutant degrading bacteria from a contaminated soil can provide a means to enhance the biodegradation rate with indigenous, well adapted bacteria, possibly overcoming limitations inherent in the soil (Fig. 2).

This work describes the development and application of a protocol for assessing the feasibility of biotreatment, including the use of several catabolic gene probes to specifically detect, isolate and monitor organic pollutant-degrading bacteria in contaminated and noncontaminated soils. The technique is also applicable to monitoring the performance of the biotreatment system during the course of its operation.

MATERIALS AND METHODS

Biotreatability Protocol

The biotreatability protocol developed to assess the applicability, and to monitor the performance, of a biological process for the remediation of a contaminated site is depicted in Figure 3. The different stages of the assessment are described below:

Level 1: Microbiological performance.

1. Analysis of the physical characteristics of the soil provides information on the soil's ability to adsorb contaminants and on the difficulty which bacteria will encounter in reaching the pollutants.
2. Chemical analysis of the soil. This will indicate whether the presence of certain pollutants (*e.g.*, heavy metals, chlorinated solvents) can create inhibitory conditions. If chemical analysis indicates the presence of known "biodegradable" pollutants, a biological process will have a greater chance of success. In addition, analysis of the pH, the moisture content, the major ions and the C/N ratio offers a better indication as to whether the soil environment is favourable for microbial growth.
3. Determination of the presence of hydrocarbon-degrading bacteria using fragments of nucleic acid (DNA probes) derived from bacterial pathways known to be involved in the dissimilation of hydrocarbons. These bacteria, which specialize in the degradation of both aliphatic and aromatic hydrocarbons, are often found in small quantities in contaminated soils (9, 10). While these microorganisms can be isolated and characterized by means of standard microbiological enrichment techniques, the use of DNA probes offers rapid, quantitative data on the presence of these microorganisms (11).
4. Mineralization studies of targeted pollutants. In this test, carbon-14-labelled pollutants are added to contaminated soil and the production of labelled CO_2 is measured. Depending on the nature of the contaminants present in the soil, compounds such as hexadecane, dotriacontane, naphthalene, toluene, etc. can be used.
5. Assessment of the respiratory activity of soil microorganisms. This test involves measuring the oxygen consumption by the microorganisms in a soil sample from the remediation process. Without adding nutrients or an additional carbon source, the actual respiration rate in the contaminated soil can be determined. This test is an excellent indicator of the soil's overall biological activity.

Level 2: Toxicological assessment.

Level 2 of this protocol consists of verifying whether the biotreatment has been successful in eliminating the toxicity associated with the pollutants. Series of biotests are performed on the soil and its leachates to determine whether the toxicity has been reduced and/or transferred. If necessary, the groundwater is sampled to detect any transfer of toxicity. The biotests currently employed are Microtox, SOS Chromotest, and growth

inhibition in the phytoplanktonic algae *Selenastrum capricornutum.*

Soil Sampling

Soil samples were collected using a hand auger equipped with inner copper liners. Samples were collected from the treatment pile as indicated in Figure 4, placed on ice and transported to the laboratory where they were processed immediately.

Chemical Analyses

The concentration of petroleum hydrocarbon (TPH) was monitored using the mineral oil and grease method derived from the American Public Health Association (APHA) method 5520 (1989), including methods 552OF (mineral oil separation) and 5520C (infrared measurement). The pH, percentage humidity, concentration of NH_4^{3+}, NO_3^-, PO_4^{3+} , and the C/N ratio were determined in all soil samples using standard methods previously described (12).

Mineralization of Test Substrates

The activity of hydrocarbon-degrading microorganisms was determined using ^{14}C-labelled hexadecane and dotriacontane. Aliquots of soil (20 g) were added to serum bottles and a total of 100 mg/kg hexadecane or 100 µg/kg dotriacontane was added. A CO_2 trap was included and the bottles were sealed. The rate of mineralization was determined by following the rate of production of $^{14}CO_2$.

Respirometry

The respiration rate in soil samples was monitored using an electrolytic respirometer (Bioscience Inc., model ER-100, Bethlehem, Pennsylvania, U.S.A.). Unamended soil (200 g) was placed in 1 L reactors and incubated at room temperature for 10 days with continuous measurement of oxygen consumption based on the rate of re-supply of oxygen to the reactor.

Soil Treatment System

The soil was treated in closed reactors, each with a capacity of approximately 1500 m^3. The soil was first cleared of stones and enriched with nutrients, sawdust and gypsum in concentrations not exceeding 10% of the total volume. The reactor aeration system did not go into operation until May. Samples were taken at two levels (0.6 and 1.2 m) at different points within the reactor (Fig. 4). Thermistors provided continual measurements of the temperatures at both sampling levels and a gas detector measured the percentage of CO_2 leaving the aeration system. Details of the treatment system and analyses performed during monitoring have been published previously (12).

Bacterial Viable Counts

To determine the number of viable bacterial cells per gram of soil (CFU/g), an aliquot of soil was aseptically added to preweighed sterile test tubes containing glass beads (3 mm dia.). The tubes were re-weighed and sterile minimal salts medium (MSM) (13) was added to three times the weight of the soil. The mixture was vortexed for several minutes before preparing a 1:10 dilution series in MSM. An aliquot (0.1 mL) from the dilution series was spread plated in triplicate onto MSM containing yeast extract, tryptone and starch (250 mg each), and the plates were incubated at room temperature for at least one week before counting colonies.

Gene Probe Preparation

Gene probes for the present study were prepared by the polymerase chain reaction (PCR) using primers, generally 30 nucleotides (nt) in length, derived from the published sequences of specific catabolic genes. The *xylE* gene probe is an 834 nt fragment from within the coding sequence of the *xylE* gene from *Pseudomonas putida* mt-2 (ATCC 33015) carrying the Tol plasmid (14). The *alkB* gene probe is an 870 nt fragment from within the coding sequence of the *alkB* gene of *P. oleovorans* ATCC 29347 (15). The probe fragments were prepared as previously described (4), visualized by agarose gel electrophoresis, extracted and purified from the gel using the Geneclean kit (Bio 101 Inc.). These two genes, the enzymes they encode and the reactions they catalyze are depicted in Figure 5.

The probes were labelled with [^{32}P]dATP using the "Multiprime DNA labelling system," (Amersham).

The principle of gene probing is presented in Figure 6. The target DNA which is composed of two polymeric strands is held together by hydrogen bonding between complementary bases (A with T and G with C). The strands are separated and fixed to a surface which is then exposed to a labeled gene probe. The probe will stick to the target DNA where it finds its complementary base sequence. The procedure by which gene probes are applied to the microbial characterization of the degradation potential of contaminated soils is presented in Figure 7. Bacterial colonies can be transferred onto membranes, the cells lysed and the DNA denatured and fixed onto the membranes to provide a mirror image of the positions of the colonies on the original petri plate. Alternatively, the DNA can be extracted directly from the whole soil sample, purified, and then analyzed for the presence of target sequences. The method used in this study involved the initial growth of bacterial colonies on solid media and the gene probing of the colonies following their transfer onto membranes.

DNA (Southern) Hybridization

Bacterial colonies were lifted onto nylon membranes, the cells were lysed, the

DNA denatured and fixed to the membranes as previously described (4).

Southern hybridization of the membranes with labelled probe was performed using the Zeta-probe protocol (Bio-Rad, Richmond CA). Probed membranes were sealed in plastic blotting bags and exposed to Kodak X-AR2 films at -80 °C.

RESULTS AND DISCUSSION

Characterization of Contaminated Soil

The soil was characterized as a high clay-content soil (33% clay, 31 % silt and 36% sand). It was contaminated by petroleum hydrocarbons (approximately 6700 mg/kg in May 1991) consisting essentially of aliphatic chains with 12 to 30 carbons. No volatile compounds or polycyclic aromatic hydrocarbons (PAHS) were present. The pH (approximately 7.6), moisture content (approximately 20%), C/N ratio (approximately 25) and concentrations of ammonium, nitrate and phosphate ions indicated that this soil was chemically capable of supporting microbial activity. The addition of nutrients was deemed to be necessary to maintain a proper C/N ratio.

Removal of Oil and Grease

In a process treating soils contaminated by petroleum hydrocarbons, mineral oil and grease (MOG) measurements are the major criterion for determining when remediation is complete. Mineral oil and grease concentrations from May 1991 to August 1992 showed that the most rapid disappearance was during the first six months (Fig. 8). The value for May 1991 represents the soil in its initial state prior to any treatment. In September 1991 placement of the soil in the reactors was started. The reactors began operation in October, at which time the concentration of oil and grease was 2090 mg/kg. Monitoring of the treatment process using the described protocol began in December 1991. The most active period for hydrocarbon biodegradation occurred in the first six months, prior to any manipulation of the soil, except for its transport and placement on site.

Analyses of mineral oil and grease never showed concentrations below criterion B (Fig. 8). The results remained relatively constant at around 1400 mg/kg throughout the study. The coefficient of variation for the entire study (117 samples) was only 22%. This coefficient of variation is very low, given the fact that the variability due to sampling and soil preparation is approximately 15%. In August 1992, results indicated that only two of eight sampling holes had concentrations of mineral oil and grease below 1000 mg/kg. GC/MS analysis of specific hydrocarbons (pristane and hexadecane) from the same extracts, confirmed their biodegradation beginning in May 1992, when the aeration system was initiated.

Over the one-year monitoring period, climatic conditions had a pronounced effect on the biodegradation of the hydrocarbons in the reactors. The most important factor was temperature. Figure 9 shows the relationship between temperature, respiration and the mineralization of hexadecane and dotriacontane in the centre reactor. Although the outdoor temperature averaged well below 0 °C from December through March, the average temperature in the active biopile never went as low as 0 °C. Mineralization and respiratory activity closely paralleled the biopile temperature profile (Fig. 9). As the outside and biopile temperatures dropped from December to March, the mineralization of hexadecane and dotriacontane decreased proportionately, and as the temperatures rose in the spring and summer, so did mineralization activity. With the exception of one outlier, which occurred in May, the respiratory activity in the soil showed a very similar profile to the mineralization activity. The exceptionally high respiratory activity observed in May could have been associated with the start-up of the aeration system.

Monitoring of Hydrocarbon-Degrading Bacteria

The total viable bacterial population in the treatment biopile was found to be very high initially, possibly through the contribution of foreign bacteria with the agents used to supplement and bulk up the soil (Fig. 10). The total population declined very rapidly and seemed to become relatively stable for the rest of the treatment period. In contrast, the bacteria that tested positive for both the gene probes were initially present at a relatively low population density in comparison to the total bacterial population. The probe-positive bacteria did increase in number initially but then, likely due to the decreasing temperature, their numbers decreased and did not start to increase again until the temperature had increased substantially in the spring, and the aeration system was in operation. The *alkB* and *xylE* probe-positive bacterial population levels correlated well with the mineralization and respiratory activity in the soil (Figs. 9 and 10). These results indicate that monitoring the population levels of a specific catabolic genotype may provide information on their relative activities. Although the presence of an appropriate genotype may be demonstrated by the gene probe, this is not necessarily an indication of metabolic activity of that component of the bacterial population. Monitoring techniques that are able to specifically detect metabolic activity are preferable, but at present, a number of technical problems must be overcome before this can be realized.

The existence of significant biological activity during the winter indicates that soil can be treated in reactors at any time of the year in Canada. It might be preferable to start reactor treatment during the autumn to avoid a composting effect, which is sometimes encountered when large concentrations of hydrocarbons are present. Several biotreatment operations have reported temperatures exceeding 50 °C and even 60 °C during the summer. At temperatures this high the soil is pasteurized and a large proportion of the total bacterial population, including hydrocarbon-degrading bacteria, could be rendered inactive or eliminated. Starting a bioreactor in the late fall, when outside temperatures approach the freezing point at night, could therefore avoid these composting effects.

CONCLUSIONS

A detailed protocol was developed to assess the feasability of biological treatment of petroleum hydrocarbon contaminated soils, and to monitor the treatment system during its operation. The protocol included the following assessments; the physical and chemcal characterization of the contaminated soil, the biological activity using catabolicgene probes derived from bacterial pathways for the dissimilation of petroleum hydrocaron components, mineralization studies using carbon-14-labeled representative test substrates, respiratory activity in the soil, and the toxicity of the soil before and during the treatment process. The application of this protocol to a biopile treatment process demonstrated that biodegradation of the contaminants had occurred. The use of catabolic gene probes demonstrated the presence of hydrocarbon-degrading bacteria and showed that their numbers correlated well with degradation activity, which was dependent on ambient temperature conditions.

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REFERENCES

1. **Madsen, E.L.** 1991. Determining *in situ* Biodegradation: Facts and Challenges. Environ. Sci. Technol. **25**(4):1663-1673.

2. **Olsen, B.H.** 1991. Tracking and Using Genes in the Environment. Environ. Sci. Technol. **25**(4):604-61 1.

3. **Sayler, G.S., A.C. Layton.** 1990. Environmental Application of Nucleic Acid Hybridization. Ann. Rev. Microbiol. **44**:625-648.

4. **Greer, C.W., L. Masson, Y. Comeau, R. Brousseau, and R. Samson.** 1993. Application of Molecular Biology Techniques for Isolating and Monitoring Pollutant Degrading Bacteria. Water Poll. Res. J. Canada **28** (in press)

5 . **Bej, A.K., M.H. Mahbubani.** 1992. Applications of the Polymerase Chain Reaction in Environmental Microbiology. PCR Meth. Applicat. **1**: 151-159.

6. **Drahos, D.J., B.C. Hemming, and S. McPherson.** 1986. Tracking Recombinant Organisms in the Environment: Beta-galactosidase as a Selectable, Non-antibiotic Marker for Fluorescent Pseudomonads. Bio/Technol. **4**:439-44.

7 . **Masson, L., Y. Comeau, R. Brousseau, R. Samson, and C.W. Greer.** 1993. Construction and Application of Chromosomally Integrated *lac-lux* Gene Markers to Monitor the Fate of a 2,4-Dichlorophenoxyacetic Acid-Degrading Bacterium in Contaminated Soils. Microb. Releases **1**:209-216.

8 . **Colwell, R.R., C. Somerville, I. Knight, and W. Straube.** 1988. Detection and Monitoring of Genetically-engineered Micro-organisms. In: The Release of Genetically-engineered Micro-organisms, M. Sussman, C.H. Collins, F.A. Skinner and D.E. Stewart-Tull, (Eds). London: Academic Press, pp. 47-60.

9. **Atlas, R.M., R. Bartha.** 1972. Degradation and Mineralization of Petroleum by Two Bacteria Isolated from Coastal Waters. Biotechnol. Bioeng. **14**:201-308.

10. **Atlas, R.M.** 1987. Biodegradation of Hydrocarbons in the Environment. Environmental Biotechnology, New York, Plenum Press. pp. 211-222.

11. **Richardson, K.J., M.H. Stewart, and R.L. Wolfe.** 1991. Applications of Gene Probe Technology to the Water Industry. J. Am. Water Works Ass. **9**:71-81.

12. **Samson, R., C.W. Greer, and J. Hawari.** 1992. Demonstration of a New Biotreatability Protocol to Monitor a Bioprocess for the Treatment of Contaminated Soils. National Research Council Canada, Biotechnology Research Institute, collaborative project report, Dec. 9.

13. **Greer, C.W., J. Hawari, and R. Samson.** 1990. Influence of Environmental Factors on 2,4-Dichlorophenoxyacetic Acid Degradation by *Pseudomonas cepacia* Isolated from Peat. Arch. Microbiol. **154**:317-322.

14. **Nakai, C., H. Kagamiyama, M. Nozaki, T. Nakazawa, S. Inouye, Y. Ebina and A. Nakazawa.** 1983. Complete Nucleotide Sequence of the Metapyrocatechase Gene on the TOL Plasmid of *Pseudomonas putida* mt-2. J. of Biol. Chem. **258**(5):2923-2928.

15. **Kok, M., R. Oldenhuis, M.P.G. van der Linden, P. Raatjes, J. Kingma, P.H. van Lelyveld, and B. Witholt.** 1989. The *Pseudomonas oleovorans* Alkane Hydroxylase Gene: Sequence and Expression. J. Biol. Chem. **264**(10):5435-5441.

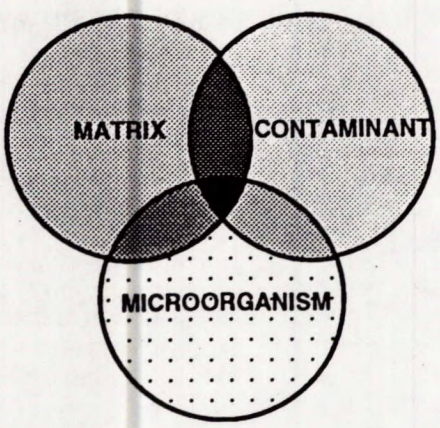

Figure 1. Schematic of Essential Interactions Between the Matrix, Contaminant and Microorganism in Order for Biodegradation to Occur

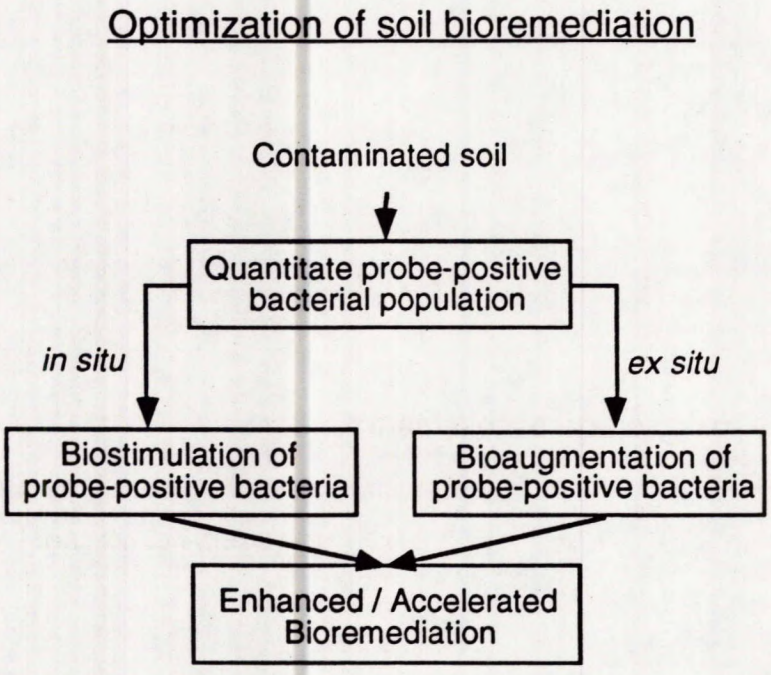

Figure 2. Biodegradation of contaminants in soil can be enhanced by identifying the indigenous bacteria responsible for the degradation and enhancing or accelerating their activity by biostimulation (supplementation with nutrients) or by bioaugmentation (increasing the amount of active biomass in the soil)

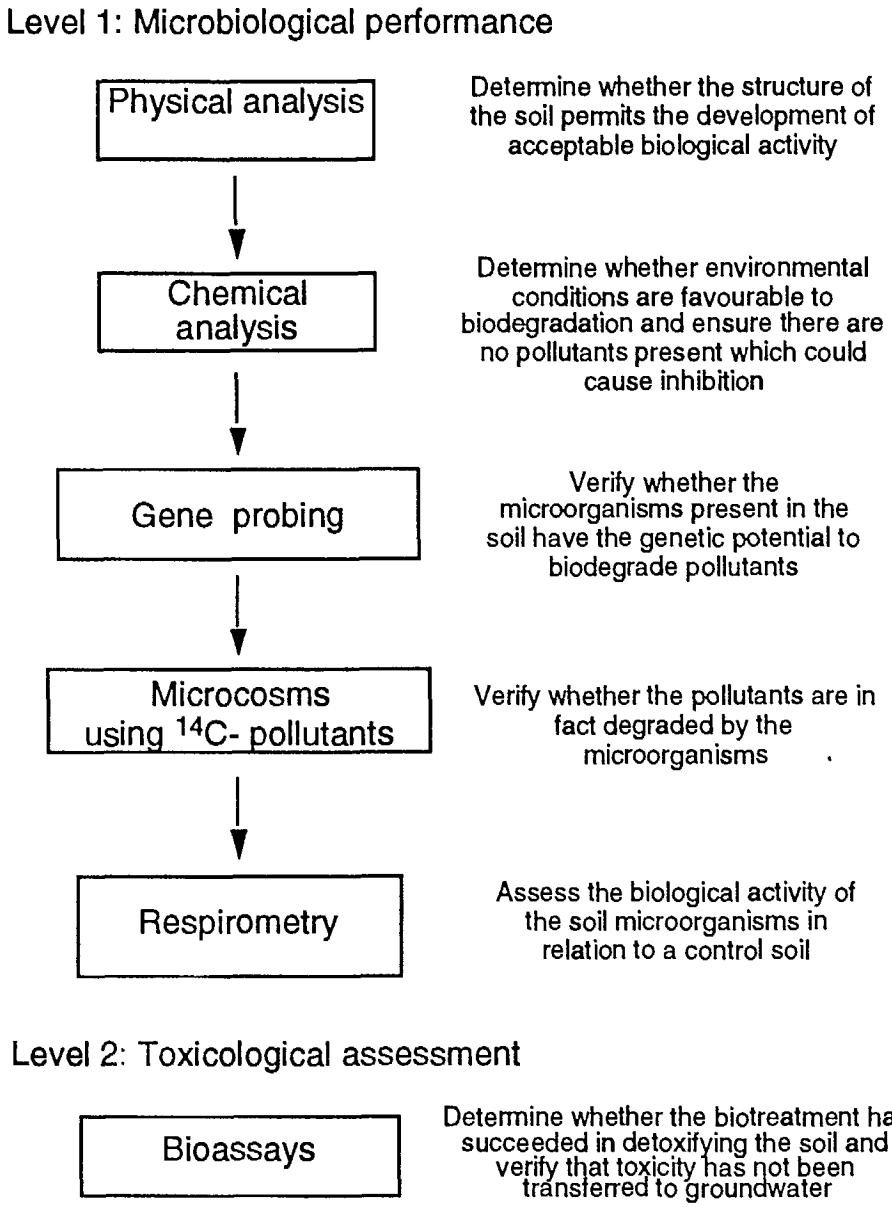

Figure 3. Outline of the biotreatability protocol

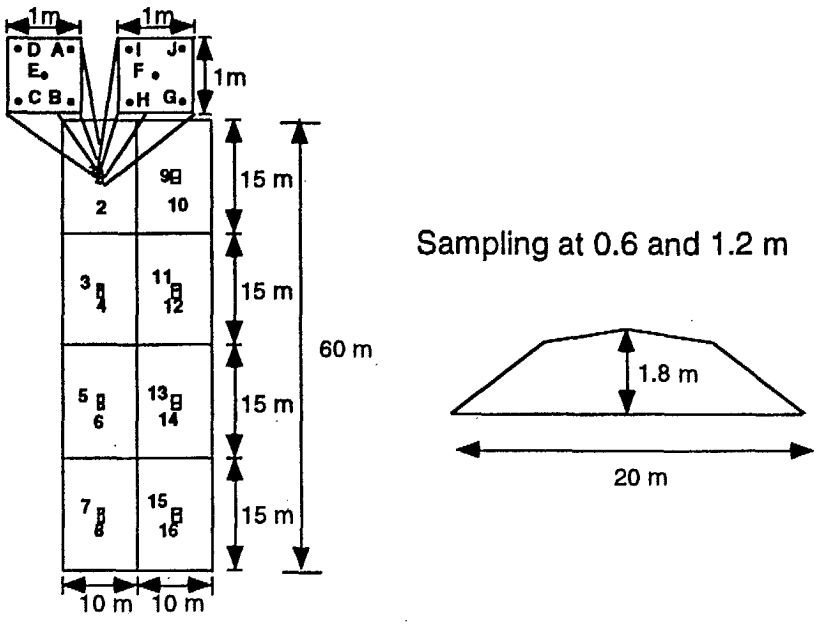

Figure 4. Sampling of the Soil Treatment Pile. Sixteen soil samples were collected each month for 10 months from 2 depths at 8 different sampling locations in the pile, as indicated

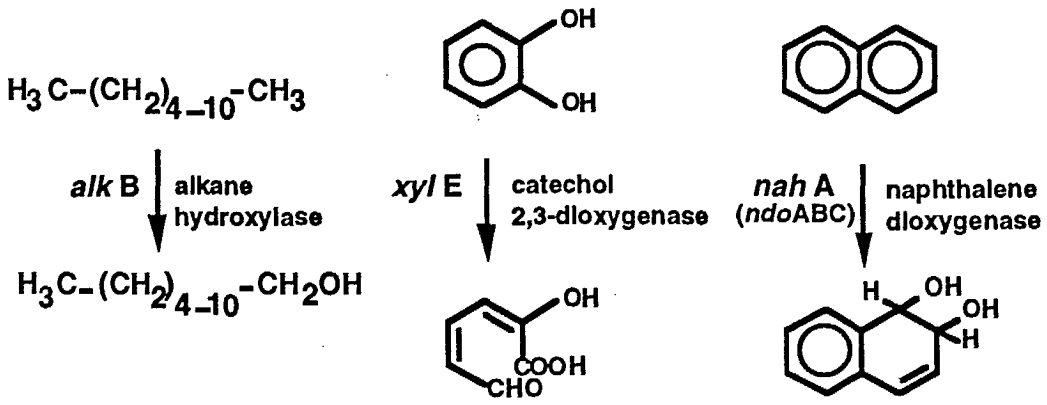

Figure 5. Key Genes, the Enzymes They Encode, and the Metabolites Produced During the Biodegradation of Hydrocarbons. The gene probes were derived from within the coding sequence of each of the genes depicted

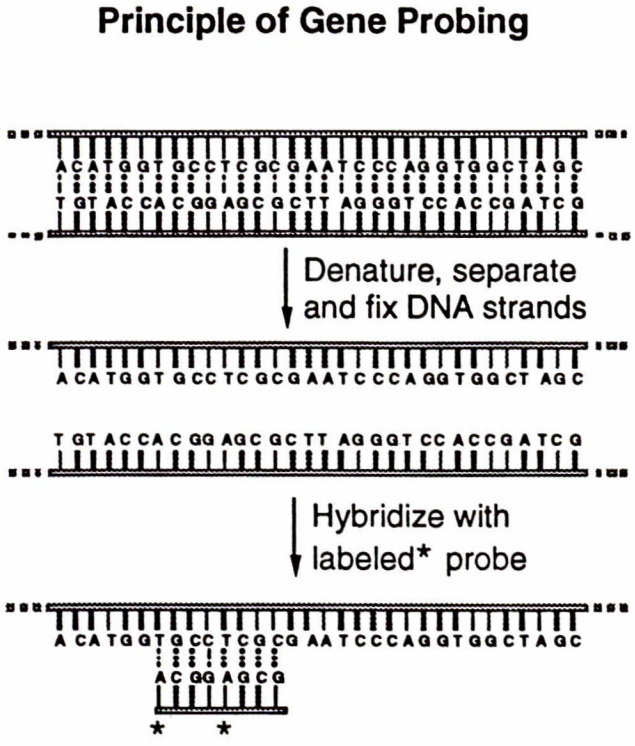

Figure 6. The Principle of Gene Probing. Target DNA is composed of complementary strands held together by base pairing. The strands are separated (denatured) and fixed onto a solid support. A labeled gene probe is then contacted with the support and will bind to a region of the target DNA that is complementary to its own base sequence

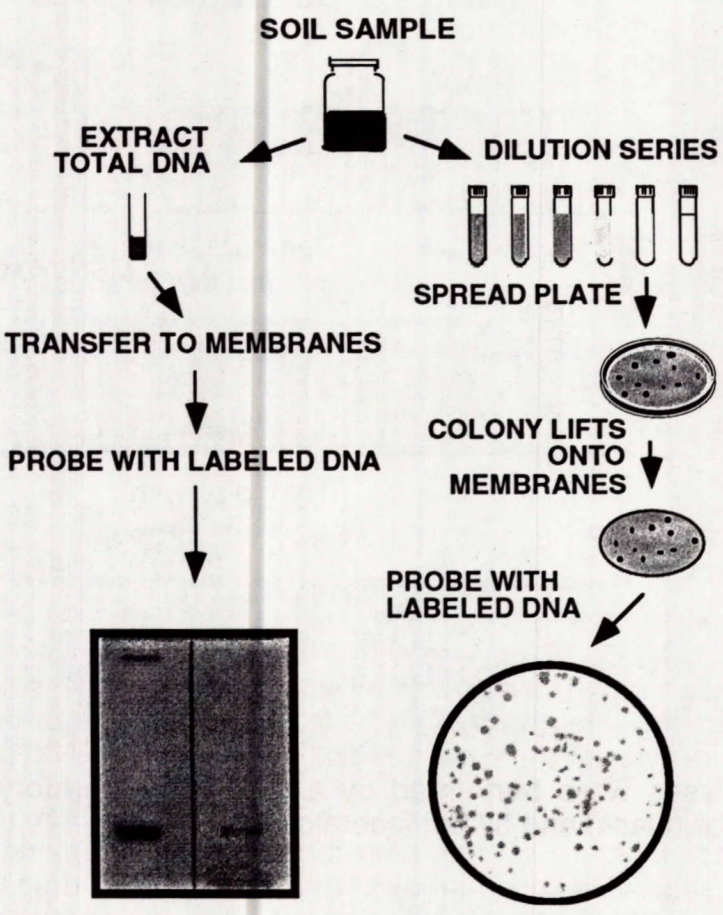

Figure 7. Monitoring Contaminated Soil. The DNA can be extracted directly from the contaminated soil or indirectly by first growing up colonies on solid media. In either case, target DNA is denatured and fixed onto membranes and contacted with a labeled gene probe. In the first case, total DNA from the sample is probed, and in the second case, individual bacterial colonies are probed

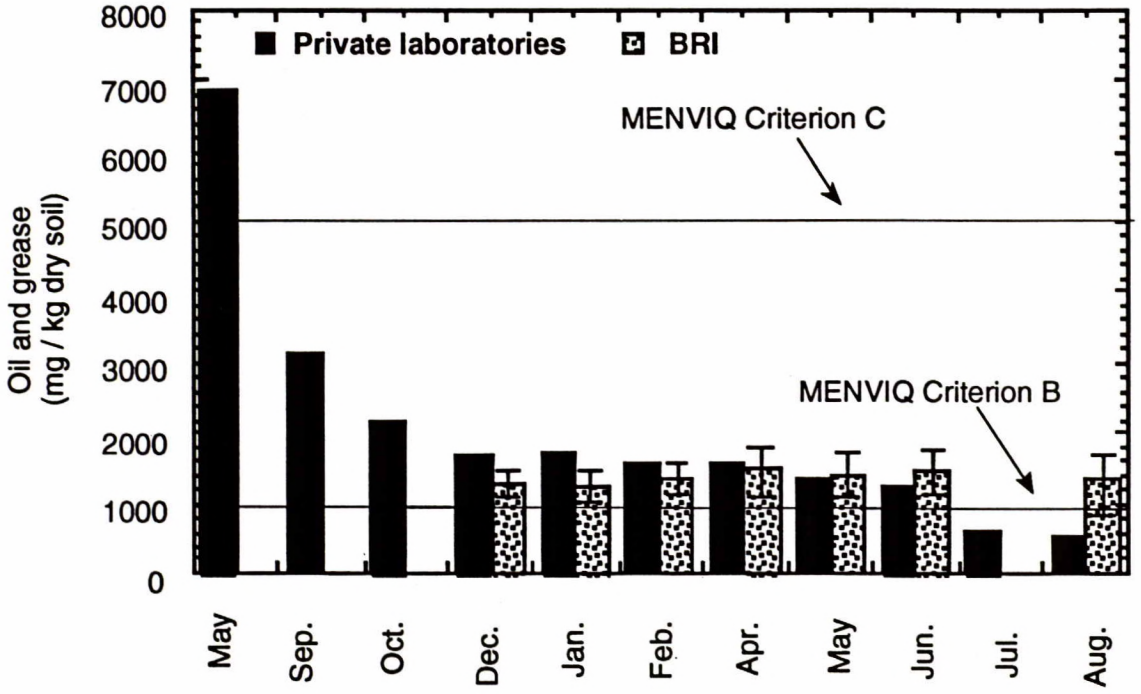

Figure 8. Concentration of mineral oil and grease in the soil during biopile treatment. Regulatory criteria for residual levels of oil and grease of the Minister of the Environment of Quebec (MENVIQ) are indicated. Analyses were performed by a qualified laboratory in the private sector (black) and by our laboratories (stippled)

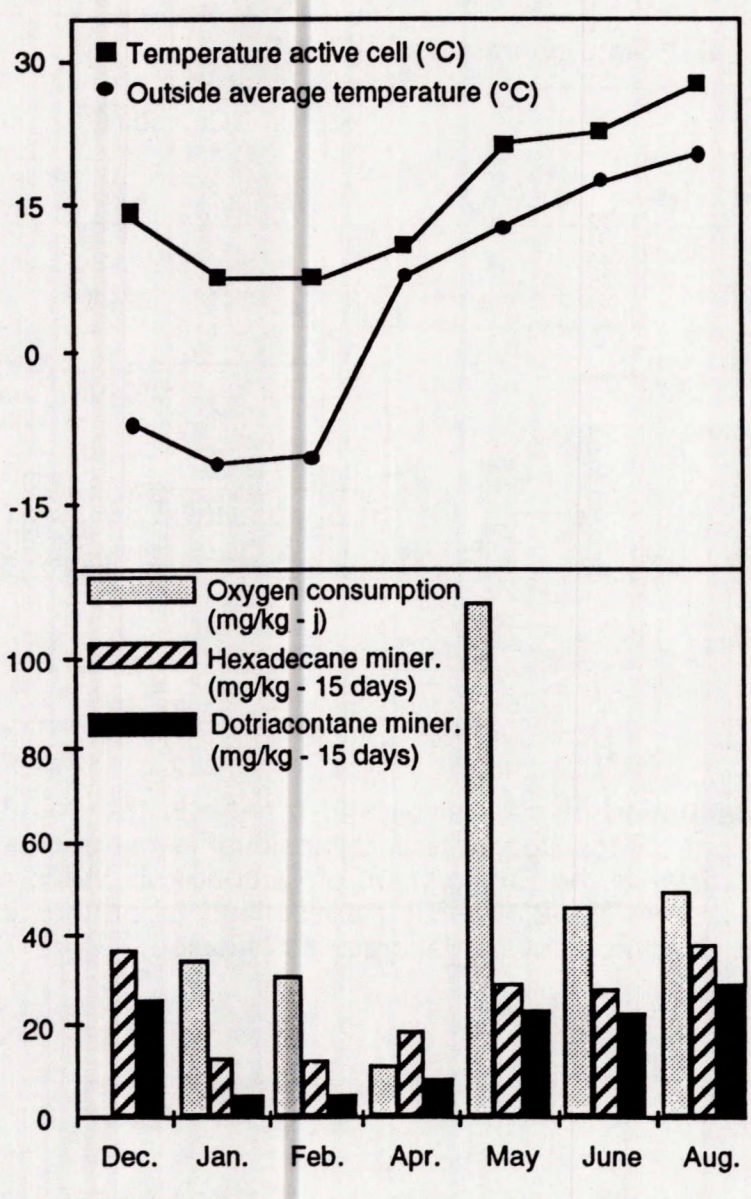

Figure 9. Microbial Activity in the Soil Biopile During Treatment. The respiration rate (as oxygen consumption) and the mineralization rates for hexadecane and dotriacontane during treatment are shown. The temperature of the active biopile cell and the outside air temperature during operation are also indicated

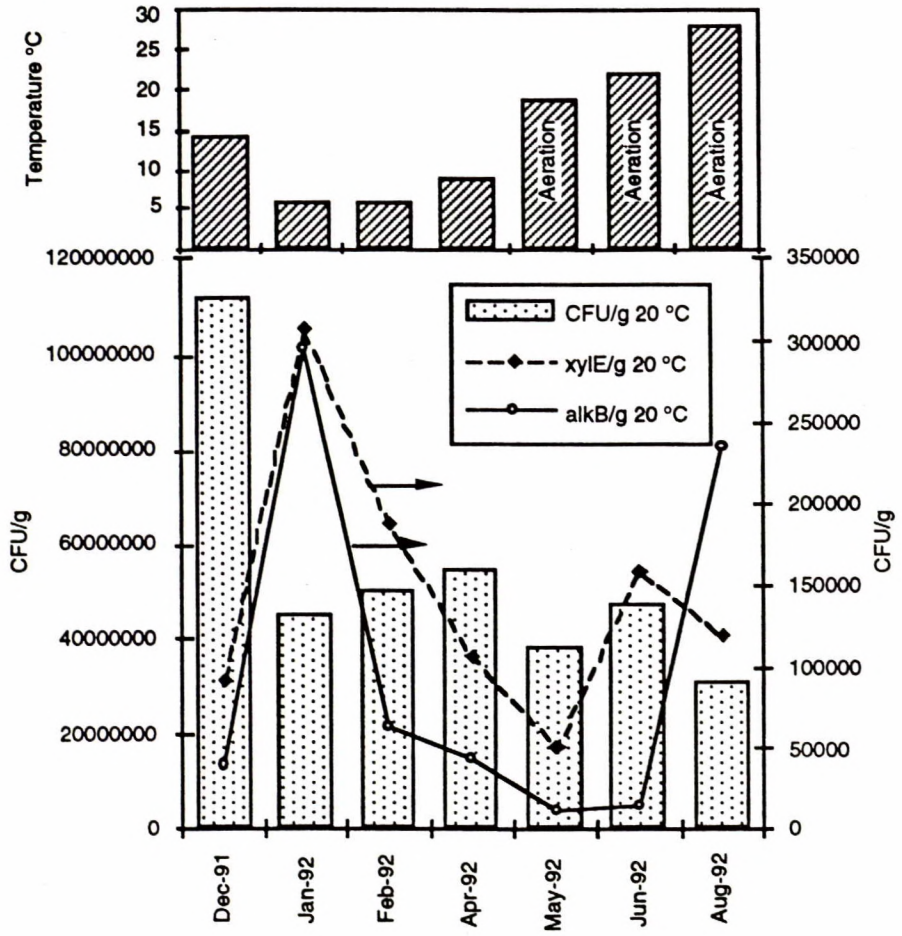

Figure 10. Bacterial Populations in hte Biopile During Operation. Total viable bacteria, as colony forming units per gram of soil (CFU/g) were monitored in the contaminated soil, as well as the population levels of bacteria that were positive for the *alkB* and *xylE* gene probes. The soil temperature in the treated pile is indicated, as well as the start of the aeration system

THE ENVIRONMENTAL AND REGULATORY DIMENSIONS OF BIOTECHNOLOGY UNDER THE CANADIAN ENVIRONMENTAL PROTECTION ACT: AN OVERVIEW

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ABSTRACT

The new substances provisions of CEPA require that any substance not on the Domestic Substance List (DSL) be "notified" and assessed for "toxicity" as defined by CEPA. Biotechnology products, including microorganisms, are considered as substances. Environment Canada and Health Canada are currently developing, through a multistakeholder consultative mechanism, the notification requirements for microorganisms that would fall under CEPA. The inclusion of naturally occuring microorganisms (NOM's) and the absence of these organisms from the DSL indicate that many applications under mining and bioremediation would fall under new substances provisions. In the development of the notification categories, Environment Canada and Health Canada have recognized that many of the current applications of microorganisms in this sector will pose little incremental risk when used under appropriate conditions. Other applications may pose increased risk and by definition, all genetically modified organisms would need to be assessed at their first introduction. The consequences, potential costs and benefits will be discussed.

LES DIMENSIONS ENVIRONNEMENTALES RÉGLEMENTAIRES DE LA BIOTECHNOLOGIE SOUS LA LOI CANADIENNE DE LA PROTECTION DE L'ENVIRONNEMENT: UN APERÇU

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RÉSUMÉ

D'après les mesures pour les nouvelles substances sous le régime de la Loi canadienne sur la protection de l'environnement (LCPE), toute substance n'apparaissant pas sur la liste des substances domestiques (LSD) doit être rapportée et sa toxicité doit être évaluée, tel que défini dans la LCPE. Les produits dérivés de la biotechnologie, incluant les microorganismes, sont considérés comme des substances. Environnement Canada et Santé Canada, par un mécanisme consultatif, élaborent en ce moment les exigences de notification pour les microorganismes dans le cadre de la LCPE. L'inclusion de microorganismes de l'environnement et l'absence de ces organismes de la LSD suggère que plusieurs applications dans le domaine minier et de la biorestauration seraient régies par les mesures pour les nouvelles substances. En établissant les catégories pour la notification, Environnement Canada et Santé Canada ont reconnu qu'actuellement l'utilisation de microorganismes dans ces secteurs représente généralement peu de risque lorsqu'ils sont utilisés dans les conditions appropriées. D'autres applications peuvent augmenter les risques et par définition, tous les organismes modifiés génétiquement devraient être étudiés au moment de leur première utilisation. Les conséquences, coûts et bénéfices vont être ici discutés.

INTRODUCTION

The Canadian Environmental Protection Act (CEPA) was proclaimed in 1988 and many sections came into effect immediately. Other sections have come into force as the necessary regulations have been developed by the two Departments with regulatory responsibility and authority under the Act, namely Environment Canada and Health Canada. CEPA is the one of the legal instruments whereby the Government will put in effect the Green Plan for ensuring the protection of the environment and human health.

CEPA is one of a continuing series of activities relating to the regulation of biotechnology products initiated under the Green Plan, which draw together the fabric of the government commitments under the Green Plan to protect human health and the environment. Associated activities include the formation of the Interdepartmental Committee on Biotechnology (ICB), a senior level policy committee to overview government activities in biotechnology, and two sub-committees, the Biotechnology Coordination Committee (BCG) and the Sub-Group on Safety and Regulation (SGSR) with responsibilities for non-regulatory activities and regulatory activities respectively. All regulatory initiatives in biotechnology, when first proposed and developed, are reviewed by these committees to ensure consistency in government approach, including CEPA biotechnology aspects.

Green Plan

The Green Plan calls for the development of a federal regulatory framework for biotechnology products by 1993. In addition, the Green Plan calls for the development and publication of national standards and codes of practice for environmental protection and protecting human health following accidental or deliberate release, and the development of specific regulations requiring notification of new biotechnology products prior to release or introduction, to ensure the continuing protection of the environment and human health.

The principles of the federal regulatory framework have been developed and were made public in January 1993.

Principles of a Federal Regulatory Framework for Biotechnology:

- Build on existing legislation and instruments.
- Uphold health and environmental safety standards.
- Harmonize with national priorities and standards.
- Use risk based assessment methodologies.
- Assess products not processes.
- Develop a favourable climate for investment, development and innovation by adoption of sustainable products and processes.

A critical principle in the framework is that no new institutions or legislation respecting the regulation of biotechnology products will be developed. Both Departments and Acts that are currently involved in regulation will be the basis for future regulation.

A national standard for environmental protection is a definition of those aspects of the environment that must be considered and evaluated in assessing the possible effects of an environmental release or activity that may impact on the environment. It includes both a description of the specific aspect of the environment that must be considered, *e.g.* effects on wildlife populations, and where reasonable, some measure of what is acceptable. The standard is null on the product type. Health Canada is responsible for developing national standards for the protection of human health. Environment Canada is developing standards for the release of four generic classes of substances.

National Standards (Under Development by Environment Canada):

-Microorganisms and Biochemical Products.
-Plants.
-Animals Including Fish and Birds.
-Chemicals.

The biotechnology national standard that is most developed is specific to microorganisms, and biochemical products from microorganisms. The development of a national standard for plants is progressing in consultation with Agriculture Canada and other affected Departments.

The development of regulations for the notification and assessment of biotechnology products under CEPA is the third component of the Green Plan commitment on biotechnology. Biotechnology products fall under the provisions for new substances in CEPA. Against the backdrop of the national standard, Departments administering other Acts, or Environment Canada in administering CEPA, will develop information requirements specific to particular product types. The information provided will be used in performing an assessment of environmental safety and public health. There is therefore a hierarchy of information requirements that can be seen as:

Hierarchy of Information Requirements for Assessment:

-National Standard.
-CEPA.
-Biomining/Bioremediation.
-Specific applications.

CEPA New Substances: Biotechnology

Microorganisms can be considered under two headings, naturally occurring (NOM), and genetically modified organisms (GMO). There is no evidence that the large scale production of naturally occurring microorganisms and their release into the environment, particularly into ecosystems from which they were not isolated, is environmentally safe. There is evidence that increased exposure to naturally occurring microorganisms can result in adverse health effects. As a result, a decision was made to include both NOM's and GMO's under the CEPA New Substance Notification Regulations. Both are biotechnology products, there are simply some additional information requirements for GMO's that relate specifically to the modified micro-organism.

Sections 25-32 of CEPA refer to substances "new to Canada", and require notification and an assessment of whether or not the substances are or could be "toxic" as defined under Section 11 of CEPA. There are three pieces to this puzzle, **substance, new, and toxic.** These terms are clearly defined in CEPA sufficient to leave no doubt as to what was intended. In addition, the Interdepartmental Committee on Biotechnology has also developed a government wide definition of biotechnology.

Biotechnology:

> "The application of science and engineering in the direct or indirect use of living organisms or parts or products of living organisms in their natural or modified forms " (CEPA, Section 3(1)).

Substance:

> "Any distinguishable kind of organic or inorganic matter, whether animate or inanimate" (CEPA, Section 3(1)).

New:

> "A substance not on the Domestic Substance List".

Toxic:

> " A substance is toxic if it is entering or may enter the environment in a quantity or concentration or under conditions;
>
> a) having or that may have an immediate or long-term harmful effect on the environment,
>
> b) constituting or that may constitute a danger to the environment on which human life depends; or,
>
> c) constituting or that may constitute a danger in Canada to human life or health" (CEPA, Section 3(1)).

Following notification and assessment the Act calls for one of several actions:

1. Recommend addition to the DSL, unconditional manufacture/importation and use.
2. Recommend permit to manufacture or importation with conditions.
3. Recommend prohibition of manufacture/importation based on assessment as toxic.

Application of CEPA New Substance Notification Regulations

CEPA calls for notification and assessment prior to the importation /manufacture and release in a commercial activity of any new microorganism. Notification means that any new substance must be notified prior to manufacture or importation for use in Canada. CEPA defines "new" as not appearing on the DSL. The DSL is a list of substances derived from the 1985 TSCA inventory and from nominations from other sources, that are known to be in use in Canada between January 1st 1984 and December 31st 1986. There are no NOM's or GMO's on the TSCA inventory, therefore industry must provide the nominations. Notice of intent to develop the DSL was published in the Canada Gazette in May 1993, and some nominations have been received. A provisional list will be published this year. Other nominations are still expected.

One component of notification is the identification of the organism. This is a critical issue for several reasons. The level of identification is a determinant in the unique identification of the "substance" for listing on the DSL. In the absence of unique identification the DSL includes information on specific use and location. Also, identification is a means of providing information on potential toxicity by indicating possible toxin production, pathogenicity and the level of information available on the organism.

CEPA requires that the notifier provide Environment Canada and Health Canada with sufficient information to be able to make a determination of potential toxicity. Information on use and location is important because the specific application will in many cases, determine which components of the "national standard" are appropriate for consideration, and to what extent they are applicable in determining toxicity. The questions posed relate to:

-Entry.
-Exposure.
-Effect.

For a substance to be toxic it must enter the environment in sufficient quantity that exposure to target or non-target organisms or systems brings about an adverse effect. The assessment methodology is a risk based process, it is driven by the requirement to identify and, if possible, quantify the risk to the environment and human health.

The information requested is categorized in four sections:

Identification.
Exposure.
Fate and Effect
- i: Human.
- ii: Environment.
  - a) Organisms.
  - b) Biosphere.

Identification

Identification should be based on acceptable authorities such as ATCC or other recognized institutions, in addition, physiological characteristics should be utilized to more precisely identify the organism. It is to the proponents benefit to identify as clearly as possible the microorganism since the lower the level of characterization the more information is needed in other areas to permit an assessment of potential toxicity.

For example, some Pseudomonades are pathogenic to both humans and animals. A notification for an organism identified as *Pseudomonas* sp. would result in a need to generate information on potential toxicity to answer concerns in later sections. If the identification was *Pseudomonas viridans* this would of itself provide much of the information that would be necessary for human or environmental assessments.

Entry

Exposure is assumed since these products are intended for commercial use, and not for use in a Level 4 containment facility. It is presumed that there will be release into the environment and concomitant exposure of both human and biota. There are however mitigating circumstances relative to entry that are taken into account in determining the type and degree of information required to make an assessment. These substances are "new" to Canada, and as such there is theoretically no information available on entry to the Canadian biosphere.

Information relative to entry comes in many forms, the most immediate of which is the intended use. This not only describes what the substance is intended to do, but also to a degree defines the expected level of entry or release. There would be significant differences between substances intended for widespread dispersion in the environment such as biofertilizers, and substances intended for use in contained facilities such as settling lagoons or even more so in some types of bioreactors.

Entry is a component of the exposure assessment, and therefore part of the assessment equation, for risk:

Risk = Hazard Exposure (Entry and presence).

Exposure

Exposure poses the question: "Based on how much has been released, what organisms or systems may be exposed and for how long?" Consider the case for direct acute exposure. If there is limited release (entry into the environment), there will be little or no exposure and therefore, if the appropriate level of identification indicates little hazard, the risk is small. Conversely, if the organism has a high inherent hazard such as a human pathogen or known plant pathogen, or if there is no information on the organism, then a small level of entry may pose a risk to exposed non-target organisms. The information requirements in the standard relate to unconditional release into the environment, for specific uses in specific locations the information requirements would be tailored to the likely target and non-target organisms or systems.

The next level of information concerns the effects of substances that are known to, or suspected to, persist and multiply in the environment. In this case, and one that must be considered for any live organism, there is a potential for chronic effects that may be quite different to those for acute direct effects.

If the information provided indicates that the organism may persist and multiply in the environment, and if it is indicated that it may exert an adverse effect on the environment, information will be sought on the "fate and effects" of the organism specific to the location of intended use. This would obviously require field tests of some form and accumulation of data on survival, persistence and dispersal.

Information requirements on non-target exposure will depend on the location and intended use of the microorganism. It also depends on the level of concern about the organism. The effects could be on single species or process, an ecosystem, or a population.

Effect

It is not possible to separate exposure from effect in making an assessment, nor indeed in discussing the information needs for exposure. In the instance of a known organism for which available information on human, animal and plant pathogenicity, and on survival and dispersal indicate no hazard or effect, the information needed for an assessment would be minimal since the information would indicate no effect, and therefore little risk. Where there is evidence of an effect on a non-target system, plant, animal or process, such as mineral recycling, where there could be population displacement and a change in the status of an ecosystem, information would be sought

to enable Environment Canada to make an estimate of risk. A topic of concern in determining potential effects especially relevant to bioremediation, biomining and bioleaching is the effect of the microorganism on metabolite production and the consequences of the use of microorganisms. This is a secondary effect that must be considered in making an assessment.

Genetically modified organisms

There are no schedules specifically designed for GMO's. The basic premise is that GMO's are treated as another strain of microorganism. By definition, at this time, they are new substances. The information relative to genetically modified organisms is included in the requirements for strain-history. In the strain history information should be provided on the modification, the source of the transferred material, to provide assurance that it comes from non-pathogenic sources, the mechanism of action should be provided if known since this will limit the need for data to be generated on non-target processes. The last and potentially most critical information is on the level of gene flow and potential for transfer to other organisms. This is determined by estimating the stability of the transferred genetic material.

Biomining and Bioremediation

CEPA new substances notification regulations are residual, they apply to those new substances that are not notified and assessed under other Acts, *e.g.* pesticides. Major applications to which these regulations will apply are for the biomining and bioremediation industries. Environment Canada and Health Canada have worked closely with a Multistakeholder Consultation in developing the regulations and have taken into account many of their proposals relating to reducing the information requirements for specific applications. These proposals, in general, apply to the degree of entry/exposure and the potential risk resulting from the use of indigenous organisms. As indicated above, entry is a prime determinant in any assessment of risk. This has been taken into account in determining the amount of information needed to make the assessment. The current draft of the notification regulations contains information schedules based on the expected degree of entry into the environment, and therefore the possible exposure.

Uncontained (Schedule XIV).
Contained (Schedule XV).
Enclosed (Schedule XVI).
Same site/Same type (Schedule XVII).
Containtment (Schedule XX).
Use in continental US (Schedule XVII).

The information schedule for uncontained use is the basic requirement for unconditional release in any quantity into the environment. It contains the most detailed information requirements in the schedules and is the basis for addition of the

microorganism to the DSL.

Containment may result from physical or biological factors. A containment facility is defined by the MRC guidelines, and results in a degree of entry with minimal exposure, and therefore minimal risk. Biological containment could result from lack of nutrient, minimal dispersal or reduced survival due to abiotic factors such as temperature, humidity, pH or oxygen tension. In considering containment, other than claiming an MRC guideline containment facility, the various means of dispersal must be considered, wind, water and the organism.

The application of large inocula of organisms that have been isolated from the location of use and grown in culture is not usually expected to result in significant adverse effects, and this limits the information requirements. Possible effects are due to scale-up of local populations. Environment Canada is currently working on the definition of same site, through consultation with microbial ecologists, to determine the scope of application of this schedule. It is possible that this schedule could apply both to individual locations in a region, or to specific applications throughout the country as in similar ecosystems for similar results.

There are no organisms listed on the TSCA inventory of 1985, the basis of the DSL for any substances other than those for which evidence is provided indicating use in Canada in the period 1984-1986. However, there are microorganisms in commercial use in the continental United States that could be beneficial in Canada. To ensure that Canada is not economically disadvantaged there is a schedule with reduced information requirements specifically for those microorganisms.

Consequences

Following a notification Environment Canada will have information on expected releases on a commercial scale of microorganisms into the environment. Following publication of the regulations in Canada Gazette II each new substance must be notified within a given time. Environment Canada and Health Canada will review and evaluate the information in the appropriate schedule and provide a response within the appropriate time frame. It is unlikely that many notifications for microorganisms currently in use will result in significant changes to current procedures, notifications for brand new, or genetically modified microorganisms will pose some problems since there may be limited information available on which to base an assessment. This would result in a need for data to be generated.

Actions Following Assessment Under CEPA New Substances

Recommendations

1. Addition to the DSL - Unconditional importation/manufacture and use.
2. Conditional - Permission to import/ manufacture and use not time limited.
3. Prohibition - Development of a regulation within 2 years.

Note: Assessed as toxic does not mean automatic prohibition.

Costs and Benefits

As with any regulation, there will be a cost associated with their implementation. What will that cost be? Prior to any regulation being put in place there is a requirement for a Regulatory Impact Analysis. This analysis is developed for the Department and Cabinet and specifically addresses the cost of compliance with the proposed regulations. The information for this analysis comes basically from two sources; the proposed regulations come from the government, the cost information will in most part be provided by industry. Industry will be asked which companies are willing to provide information on a confidential basis. The analysis will be provided in part by Industry Science Canada. If the conclusion is that the regulations are too costly or provide for economic disadvantage for Canada they will be returned for revision.

The first benefit is that the likelihood of significant environmental damage or adverse human health effects is diminished by prior notification of use. This translates into significant potential economic gain in two ways. The first is that the country does not have to pay for environmental remediation or health care. Costs for a major clean-up can be very high. The industry is protected from association with environmental damage. The second benefit is in international trade, both the products from industry and the technology being applied are potential trade items. In addition, throughout the world there is a developing requirement for environmental risk assessments for both products and technologies. The Canadian regulations have been developed in conjunction with the US, EC and OECD approaches. The other piece of international legislation that may become binding is the UN Convention on Biodiversity. This draft legislation contains quite specific recommendations on approaches to safety in use of biotechnology. Canadian products that pass through the new substance notification regulations will be acceptable throughout the world.

In comparison with our trading partners, specifically the US and the EC. CEPA notification requirements compare with TSCA. TSCA requires notification but does not necessarily mean that the substance will enter commerce, only 33% of the substances on the TSCA inventory enter commerce. The CEPA regulations are geared to substances entering commerce, it is one stop shopping. When a microorganism is used in the United States, depending on the use and location, it may require application under one or several

State or Federal statutes with requirements in excess of CEPA. The CEPA regulations parallel those of the EC for the same reasons.

SUGGESTED READINGS

Department of the Environment. 1993. Biotechnology Component of the Domestic Substances List - Provisional List, Canada Gazette, Part I, November 20.

Medical Research Council *et al*. 1990. Laboratory Biosafety Guidelines. Minister of Supply and Services Canada, MR 21-1/1990E.

Government of Canada. 1990. Canada's Greenplan for a Healthy Environment. Minister of Supply and Services Canada, En 21-94/1990E.

Government of Canada News Release. 1993. Federal Government Agrees on New Regulatory Framework for Biotechnology, January 11. (Contact R. Parent, Media Relations, (613) 995-8963).

MICROBIALLY MEDIATED METAL REMOVAL FROM ACID MINE DRAINAGE

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POSTER ABSTRACT

In 1989, a test cell system was constructed at the foot of an Inco tailings dam at Makela, Ontario to treat an acid mine drainage (AMD) seepage for Fe (200-320 mg/L), Ni (25-40 mg/L), S (800-1000 mg/L), and an acidity of 380-750 mg/L equiv. $CaCO_3$. The system was designed to expose the AMD first to oxidizing conditions to promote the oxidation, hydrolysis and precipitation of Fe (as $Fe(OH)_3$). After oxidization and acidification, the AMD is exposed to reducing conditions to promote microbially mediated alkalinity generation. This results in an increase in pH and the consequent precipitation of metals as sulphides and hydroxides. The system comprises four cells, two of which are for oxidation and two for the removal of acidity and metals in reducing conditions. The ARUM (Acid Reduction Using Microbiology) or reducing cells have a constructed sediment of organic materials and a floating cattail cover. Floating cattail covers help reduce wind-induced water mixing and promote reducing conditions; they also provide organic material in the form of detritus and root exudates to provide electron donors for the alkalinity generating processes. After two and a half years of experimentation and tuning the system to achieve flow control, water treatment was achieved with a flow rate of 1 L/min and a total residence time of approximately 300 days. Ni concentration declined by 77-88 %, Fe by 88-98 %, S by 39-51 % and acidity by 72-79 %. The system continued to operate effectively in 1993.

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ÉLIMINATION MICROBIOLOGIQUE DES MÉTAUX CONTENUS DANS LE DRAINAGE MINIER ACIDE

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RÉSUMÉ D'AFFICHE

En 1989, un système de cellules d'essai a été construit au pied d'un tas de résidus de l'Inco à Makela (Ontario) pour traiter les eaux d'infiltration de drainage minier acide contenant du Fe (200-320 mg/L), du Ni (25-40 mg/L) et du S (800-1000 mg/L) et dont l'acidité s'élève à 380-750 mg/L de $CaCO_3$ équivalent. Le système a été conçu pour soumettre les eaux de drainage acides à des conditions d'oxydation afin de promouvoir l'oxydation, l'hydrolyse et la précipitation du Fe (sous forme $Fe(OH)_3$). Après l'oxydation et l'acidification, les eaux acides sont soumises à des conditions de réduction pour favoriser l'alcalinisation microbiologique, ce qui a pour effet d'accroître le pH et de précipiter les métaux en sulfures et hydroxydes. Le système est composé de quatre cellules, dont deux servent à l'oxydation et deux à la neutralisation des eaux et l'élimination des métaux dans des conditions réductrices. Les cellules de neutralisation microbiologique ARUM (Acid Reduction Using Microbiology) contiennent un sédiment composé de matière organique et une couverture flottante de joncs. Cette couverture aide à réduire le mélange de l'eau par le vent et favorise la réduction; elle fournit également de la matière organique sous forme de détritus et d'exsudats de racines, produisant ainsi des donneurs d'électrons nécessaires à l'alcalinisation. Après deux ans et demi d'essais et de mise au point du système pour régler le débit, l'eau a été traitée à un débit de 1 L/min et un temps de rétention d'environ 300 jours. La concentration de Ni a chuté de 77-88 %, celle de Fe de 88-90 %, celle de S de 39-51 % et l'acidité de 72-79 %. Le système a continué de fonctionner efficacement en 1993.

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