BIOAUGMENTATION TECHNOLOGY FOR

WASTEWATER TREATMENT IN SOUTH AFRICA

by

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EXECUTIVE SUMMARY

MOTIVATION AND BACKGROUND

Bioaugmentation involves the use of specially selected and adapted microorganisms for the biodegradation of specific toxic, hazardous and often recalcitrant compounds. Its use could enhance the degradative potential of indigenous microbial populations to avoid predation, nutrient competition and subsequent biomass inactivation. Bioaugmentation may therefore make use of genetically manipulated organisms to enhance wastewater treatment efficiency.

Performance of wastewater treatment plants may be rapidly restored by bioaugmentation and the treatment performance under organic overload enhanced.

Laboratory studies have indicated the potential of bioaugmentation for the treatment of specific pollutants. Judicious implementation of the technology could therefore result in improving the overall treatment performance of biological systems by increasing its capacity, stability and shock tolerance to pollutants. It potentially offered an effective means of controlling the nature of biomass critical to wastewater treatment systems by boosting the concentration of bacteria and/or their enzymes which were necessary for the biodegradation of specific compounds.

Research in South Africa in the field of bioaugmentation was limited. Biosupplements have appeared on the market without having been tested under local conditions. This stressed the need for some form of control over the sale and use of such products. It was therefore necessary to establish guidelines on the evaluation of biosupplements to ensure that they met specifications and were safe to use.

Bioaugmentation could boost and replenish depleted populations of microorganisms, permitting rapid regrowth of the biomass. The use of biosupplements did not require alterations to existing treatment works. This represented a major benefit. The costs incurred were for the product to be added which was minimal compared to the construction of new or upgraded plants. An additional advantage was that biosupplements employ naturally adapted microorganisms which were environmentally friendly and did not pose a health hazard.

The technology may also find application in, e.g. the abattoir and tourist industry for the effective and inexpensive treatment of fats and oils and other associated contaminants. The performance of municipal treatment works could be boosted to treat toxic and hazardous wastes which inevitably were discharged, often in low concentrations but sufficient to disrupt their systems.

The growth rate of the microorganisms which preferentially biodegraded the more readily available substrates was generally greater than that of the augmented microorganisms since the former utilised and depleted essential nutrients more rapidly. Biosupplements therefore had to be added regularly to ensure that the required microorganisms for the biodegradation of the respective pollutant compounds were present in sufficient numbers. Maintaining the augmented population would prevent the accumulation of the more recalcitrant and toxic compounds at the expense of the more readily available substrates.

PROJECT AIMS

The original objectives of the project were:

- Establish the infrastructure and criteria required for the evaluation of biosupplements to ensure that they met specifications and were safe to use.

- Adapt and establish suitable procedures, including screening, isolation, culture and storage of microorganisms, for the successful implementation of bioaugmentation technology in the South Africa.

- Develop and/or improve biosupplements for the treatment of hazardous, toxic and recalcitrant pollutants in effluents and for the remediation of marine areas and contaminated soils.

ACHIEVEMENT OF OBJECTIVES

Results from preliminary experiments provided greater clarity on the direction to be set for the project. This resulted in redefining the project objectives by the Steering Committee as indicated below.

Delineation of project objectives by the Steering Committee

During the course of the investigation, the need for evaluating the biodegradation of phenol in effluent was identified. The Steering Committee therefore decided to focus on phenol as model compound for this investigation.

It was also decided that, since project K5/543 on "Bioremediation technology for the treatment of seepage water and soil in South Africa" was being funded by the Water Research Commission, the remediation of marine areas and contaminated soils was to be deleted from the current investigation.

Guidelines for bioaugmentation technology implementation in South Africa

No guidelines for the specification of products, there safe usage and expected performance existed in South Africa. In this report, the basis for the required guidelines has been outlined.

Guidelines for the legislative requirements for the implementation of bioaugmentation technology and complete specification of products in South Africa should be based on U.S.EPA regulations with simplifications as used in Germany (see sections 2.2 and 2.3).

The guidelines for the specification of augmentation products based on product description, safety and dosage scheduling (see section III) have been discussed. Case studies on product performance (see Appendix I) have been reported.

A register of bioaugmentation products should be compiled by the government departments of Water Affairs, Health and Environment together with specialists in the field of bioaugmentation. Registration should be simple to attract manufacturers and suppliers of products to have them listed without undue constraints. The onus should be on the supplier to guarantee product performance and provide necessary safety information and dosage requirements for the selected field of application. Listing, however, would not imply an endorsement of a product by the specialist technical review committee to be appointed by the authorities.

Methodology and procedures for bioaugmentation technology implementation in South Africa

The methodology and procedures required for the development and improvement of biosupplements has been demonstrated for phenol and polychlorinated biphenyls (see section IV). The capabilities have therefore been firmly established for the implementation of bioaugmentation technology in South Africa.

Full-scale practical application of bioaugmentation has been demonstrated (see Section V).

LEGISLATIVE REQUIREMENTS FOR BIOSUPPLEMENTS

The absence of effective legislation on the use of biosupplements in South Africa highlighted the need to rectify the situation. Products of quality should dominate on the local market. Careful control should result in the disappearance of ineffective products which could bring the technology into disrepute.

It should be the aim in South Africa to follow simple procedures, and legislation should encourage official listing. The regulatory policy should be guided by the following four principles:

- National government regulation should focus on the characteristics and risks of the biotechnology product and less on the process by which it is made.
- For biotechnology products that require review, such review should be designed to promote the review with the least burden and protect public health and welfare and the environment.
- Regulatory programmes should be designed to incorporate rapid advances in biotechnology and performance-based standards should receive high priority.
- Opportunity for the application of innovative and new products of biotechnology, all regulation of such products and regulation in the environmental and health arenas should use performance standards and not rigid controls or specific designs for compliance.

Since the USA has a well developed infrastructure for the control and application of augmentation products, the rules and regulations applicable should be used as guidelines for South Africa. However, simplicity of control and a minimum of formal documentation and administrative bureaucracy should be prescribed for the process.

Specialist technical committees would be needed to review the listing, specifications, health and environmental risks associated with the application of augmentation products at intervals. However, the onus should be on the manufacturer and/or the distributor of biosupplements to provide essential information and data on the formulation, biodegradative performance, list of microorganisms in the product, safety of use, method of application and any precautionary measures necessary in the use of such products. The manufacturers and distributors should be attracted to have their products listed in a national register. Their should not necessarily be an embargo on genetically manipulated organisms and their release should be handled by an organization on biotechnology such as SAGENE in South Africa.

SPECIFICATION REQUIREMENTS FOR BIOSUPPLEMENTS

It is difficult to lay down general guidelines on specifications, safety and usage applicable to all products because of their diversity. However, specifications should include a succinct product description, safety, performance expectations and dosage requirements for the specified pollutant.

Because of the importance of safety, the product safety characteristics should be submitted on a separate sheet to that for the general product specifications.

Generally speaking, each effluent is unique. To establish whether products performed as claimed, studies on the specific effluent should be performed. As examples, the biodegradation of phenol (gas works effluent), hydrocarbon oils (transport industry) and edible oils (lipids) has been reported. The products performed as expected but, in some cases, did not prove to be better than control activated sludge.

A number of products assessed did not perform according to their specifications. Laboratory evaluations should therefore be conducted to ensure their activity before full scale implementation is contemplated.

Should a treatment plant be disrupted by toxic compounds, it may be the most economical to introduce active biomass from another works which performs well, rather than purchasing biosupplements since sludge wastage is common in all treatment works. This does not imply, however, that bioaugmentation did not represent a viable option and the individual circumstances of the disruption must be carefully considered to arrive at the correct decision.

DEVELOPMENT OF BIOSUPPLEMENTS

The capabilities and methodology required for the development and improvement of bioaugmentation products was demonstrated, using phenol and polychlorinated biphenyl biodegradation as examples. The general procedure required for such development involved the procurement of a suitable source of inoculum of natural microorganisms from a contaminated site. From this inoculum, the selection and adaptation was carried out. For commercialization, extensive screening and testing would be required before patent applications could be considered. Suitable preservation and maintenance of stock cultures was essential to ensure that viable microorganism cultures capable of the specified biodegradative capabilities could be recovered for future use.

For the successful development of a biosupplement, a sound understanding of the biochemical catabolic pathways of the specific pollutant is essential as was shown for phenolics.

The formulation of a biosupplement generally requires extensive investigation into the best combination of microorganisms for best biodegradative performance. The microorganisms must be genetically stable to perpetuate their biodegradative capabilities. Consortia of microorganisms would generally be required.

PRACTICAL APPLICATION OF BIOSUPPLEMENTS

A case study on full-scale bioaugmentation for the biodegradation of hydrocarbon oils in an effluent is reported to illustrate the technological implementation in effluent treatment. An average of 30 m^3/d of effluent was produced by the particular industry. It was the aim to produce an effluent with a COD < 250 mg/ℓ and residual oils of < 30 mg/ℓ.

The estimated cost of implementation of bioaugmentation, at reigning prices in 1994, for the treatment of an effluent similar to that received for the evaluation amounted to R7,30/m^3 effluent which would be reduced to R3,70/m^3 once fully operational.

By comparison, the system in operation required an initial investment of R3.300 and monthly running costs for effective removal of oil averaged R195/month or R6,50/m^3.

A labourer was needed for at least 2 h/d compared to 1 h/d for the augmentation schedule once fully operational. Additional savings in running the bioaugmentation schedule could therefore be reflected.

Summary

Generally speaking, the use of bioaugmentation could potentially result in savings based on a higher flow-through rate, more efficient COD removal, suspended solids reduction, removal of odour, degradation of hazardous or toxic material, and overall improvement in operation, cost and effectiveness of the treatment system. The quick recovery and adaptation times of these organisms following a shock load would result in little or no downtime following an accidental spill or unfavourable cyclic conditions frequently encountered in normal plant operations.

GENERAL CONCLUSIONS

Biosupplements are imported into South Africa without much control. Varied success has been reported on the use of such products. Regulatory measures for the control and implementation of the technology should be based on USA and German legislation and control for the safe and competent application. Simple procedures must be implemented and a national register of products for specific treatment purposes compiled. The departments of Water Affairs, Health and Environment should be key participants in the appointment of specialist technical review committees essential for following up on the findings published in the report.

The investigation has resulted in establishing a firm basis for the successful and scientific implementation of bioaugmentation technology in South Africa.

TECHNOLOGY IMPLEMENTATION

Bioaugmentation was used in South Africa but with varied success. This stressed the need for performance assessments before embarking on full scale implementation. A number of commercial biosupplements did not perform as claimed and reliable manufacturers and suppliers of quality bioaugmentation products for the local market must be identified.

Proper safety measures must be adhered to. The safe usage would require inputs by expert scientists and health authorities.

The implementation of bioaugmentation technology must minimize risk to the end-user. Exploitation of the technology by incompetent suppliers must be safeguarded against, and protection of the end-user ensured, thereby ensuring the success of the technology in the country.

Microorganisms are rarely found in pure cultures in nature. Microbial communities in the natural environment represent a complex ecosystem. Application of biosupplements must therefore ensure that the added cultures are present in sufficient numbers at all times to be able to degrade the desired pollutants in the presence of the large numbers of natural microorganisms required for the biodegradation of the competing nutrients and more easily degradable pollutants.

FUTURE RESEARCH REQUIREMENTS

Research is required for the specific applications of biosupplements in the biodegradation of toxic and hazardous substances. Special applications in the field of bioremediation have been indicated. Industry should become more closely involved with this research.

Research efforts should concentrate on the interactions between microorganisms existing and developing in complex communities. Shifts in populations occurred which are currently not well understood. The influence of available nutrients, either in excess or if deficient, would affect the performance of the treatment works. Research into obtaining a better understanding of such complex micro-ecosystems would enhance our understanding of the biology of wastewater works, especially in cases where treatment was necessary at the source of the toxic or hazardous wastewater. The concentrations of such pollutants would generally be high at the source which posed a considerable challenge to achieve successful treatment on site to levels acceptable for discharge into a municipal sewer.

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LIST OF SYMBOLS AND ABBREVIATIONS

C	Centigrade
COD	Chemical Oxygen Demand
CWA	Clean Water Act
d	Days
DO	Dissolved Oxygen
g	Grams
GC-MS	Gas Chromatography - Mass Spectrometry
h	Hours
HPLC	High Pressure Liquid Chromatography
IUB	International Union of Biochemistry
ℓ	Litre
min	Minutes
mg	Milligrams
NCP	National Contingency Plan
OD	Optical Density
OPA	Oil Pollution Act
PCBs	Polychlorinated Biphenyls
R_h	Peak Retention Time in Minutes
SABS	South African Bureau of Standards
SE	Standard Error
TDS	Total Dissolved Solids
TKN	Total Kjeldahl Nitrogen
U.S.EPA	United States Environmental Protection Agency
v/v	Volume/Volume

I. INTRODUCTION

1.1 INTRODUCTION TO BIOAUGMENTATION

Nature has the ability to recycle and purify itself, but in recent years the demand placed on the environment by large amounts of xenobiotic, often recalcitrant and toxic hazardous pollutants exceeds its capacity to recover. Bioaugmentation technologies simply attempt to optimize the natural capacity of microorganisms to degrade and recycle such compounds by controlling the pH, oxygen supply or anaerobicity, and temperature, and supplying essential limiting nutrients to minimize the stress on such systems. Biodegradation techniques are versatile and can be utilized at various stages of treatment. Applications include removal of contaminants from raw materials prior to processing, treatment of wastes before discharge, treatment of effluent streams and decontamination of soils, sediments, surface water and groundwater.

There are three basic ways to accomplish the above.

- Supplemented microorganisms or their extracellular products may be released directly into the contaminated environment.
- Enhancement of the degradative potential of natural microbial populations to avoid predation, nutrient competition and subsequent biomass inactivation. This may be achieved primarily by supplementing the available supply of nutrients at the site with additional oxygen, nitrogen, phosphorus, essential vitamins or an organic compound essential for cometabolism.
- Microorganisms may be used in contained reactors to circumvent the problem of a complex and often unfavourable natural environment enabling the control for optimal biodegradation.

Bioaugmentation may therefore involve the use of genetically manipulated or specially selected and adapted microorganisms to enhance the biodegradation of specific toxic, hazardous and recalcitrant compounds which are difficult to remove from the environment by conventional treatment processes. In the wider sense, augmentation also infers the stimulation of microorganisms indigenous to the contaminated environment and wastewater to enhance and improve the treatment performance. The aim is to attack, as preferential substrates, such marginally available or difficult to degrade pollutants.

In the current investigation, bioaugmentation was applied specifically to the treatment of wastewaters. The breakdown of a variety of pollutants was investigated using commercially available supplemented microbes or specially selected and adapted microorganisms from acclimated biomass. This provided the basis for establishing the criteria essential for product specification and performance as well as the guidelines for their safe and controlled use.

1.2 MOTIVATION AND BACKGROUND

The main applications of bioaugmentation are:

- to remove specific toxic, hazardous and recalcitrant pollutants;
- to increase the specific rate of catabolic processes in a mixed culture of microorganisms;
- the rapid restoration of treatment plant performance after damage, inhibition, disruption or toxicity-induced failure;
- enhancement of treatment plant performance, e.g. under conditions of organic overload.

Laboratory studies have indicated the potential of bioaugmentation for the treatment of specific pollutants (Meyer & Oellermann, 1992; Oellermann & Scott, 1991). Judicious implementation of the technology can therefore result in the improvement and overall treatment performance of biological systems by increasing its capacity, stability and shock tolerance to pollutants. It potentially offers an effective means of controlling the nature of biomass critical to wastewater treatment systems by boosting the concentration of bacteria and/or their enzymes which are necessary for the biodegradation of specific compounds.

Research in South Africa in the field of bioaugmentation has been limited and laboratory infrastructure to assist industry with the scientific implementation of this technology was not available. Biosupplements have appeared on the market without having been tested under local conditions. It is imperative that some form of control must be exercised over the sale and use of such products. It is therefore necessary to establish guidelines on the evaluation of bioaugmentation products to ensure that they meet specifications and are safe to use. The analytical parameters which need to be assessed must be clearly determined. The needs which have been identified will be addressed and the research should contribute significantly to the development and implementation of bioaugmentation technology in South Africa.

The regeneration and restoration of performance in a disrupted wastewater treatment plant requires time. To speed up this process and to prevent spillage and discharge of inferior quality wastewater into the environment, biosupplements may prove beneficial. Supplementation would boost and replenish depleted populations of microorganisms, permitting rapid regrowth of the biomass. The use of biosupplements does not require alterations to existing treatment works. This represents a major benefit and the only costs incurred are for the product to be added which is minimal compared to the construction of new or upgraded plants. An additional advantage is that biosupplements employ naturally adapted microorganisms which are environmentally friendly and do not pose a health hazard.

The technology may also be applied in, e.g. the abattoir and tourist industry for the effective and inexpensive treatment of fats and oils and other associated contaminants. Municipal treatment works could benefit by boosting their capacity to treat toxic and hazardous wastes which inevitably are discharged, often in low concentrations but sufficient to disrupt their systems.

Contamination from oil spills by the Exxon Valdez during 1989 or during the Gulf war in the Middle East are eminent examples of hydrocarbon pollution. However, the danger of oil spills is also constantly present along the South African coastline. Such pollution is indicated by the remediation of the Petingo oil spill on the north-eastern seaboard of South Africa (Dehrmann, 1991). Remediation of hydrocarbon pollution is therefore of great importance and represents a specialist application of selected and adapted microorganisms for cleanup. Similarly, bioaugmentation can be applied to the remediation of landfill sites and the experience gained in this investigation could improve the management capabilities of waste sites in South Africa.

The growth rate of the microorganisms which preferentially biodegrade the more readily available substrates is higher than that of the augmented microorganisms since the former utilise and deplete essential nutrients more rapidly. Regular additions of biosupplements are therefore essential to ensure that the required microorganisms for the biodegradation of the respective pollutant compounds are present in sufficient numbers, relative to the concentration of the natural microorganisms responsible for the biodegradation of other more readily metabolizable organics present. Maintaining the bioaugmented population will prevent the accumulation of the more recalcitrant and toxic compounds at the expense of the more readily available substrates.

By virtue of its research in the biological treatment of effluent, the Division of Water Technology came in contact with the manufacturers and suppliers of biosupplementation products. A number of reliable products were obtained but an equal number of poorly performing supplements were encountered which lead to the exploitation of the market by incompetent suppliers. The need therefore arose and it was opportune to conduct the proposed investigation to ensure that bioaugmentation technology was soundly and scientifically established in South Africa.

1.3 PROJECT OBJECTIVES

The original objectives outlined in the project motivation were:

1.3.1 Establish the infrastructure and criteria required for the evaluation of biosupplements to ensure that they meet specifications and are safe to use.

1.3.2 Adapt and establish suitable procedures, including screening, isolation, culture and storage of microorganisms, for the successful implementation of bioaugmentation technology in South Africa.

1.3.3 Develop and/or improve biosupplements for the treatment of hazardous, toxic and recalcitrant pollutants in effluents and for the remediation of marine areas and contaminated soils.

1.4 ACHIEVEMENT OF OBJECTIVES

Only after completion of preliminary experiments, could greater clarity be obtained on the detailed direction to be followed in the study. The original objectives were thus reassessed and delineated by the Steering Committee. The subheadings in this section, therefore, do not parallel the sequence adopted in **section 1.3** above.

1.4.1 Delineation of project objectives by the Steering Committee

During the course of the investigation, the need for evaluating the biodegradation of phenol in effluent was identified. The Steering Committee therefore decided at its first meeting to select phenol as model compound on which the investigation should focus.

The overall objectives outlined in the initial project submission covered a diverse field of activities since it was impossible to predict the outcome of the envisaged research. Furthermore, project K5/543 on "Bioremediation technology for the treatment of seepage water and soil in South Africa" was accepted for funding by the Water Research Commission. This area was therefore not considered in the current investigation.

1.4.2 Guidelines for bioaugmentation technology implementation in South Africa

No guidelines for the specification of products, there safe usage and expected performance existed in South Africa. Such guidelines will have to be formally compiled based on this report.

Guidelines for the legislative requirements for the implementation of bioaugmentation technology and complete specification of products in South Africa should be based on U.S.EPA regulations with simplifications as used in Germany (see sections 2.2 and 2.3).

The guidelines for the specification of augmentation products based on product description, safety and dosage scheduling (see section III) and case studies on product performance (see Appendix I) have been reported.

A wide diversity of bioaugmentation products were available on the market. During the evaluation of commercially available products, it was established that, while some reliable products were available, a number did not effectively biodegrade the specified pollutant and marketing by incompetent suppliers posed a problem.

A register of products should be compiled by government authorities involving the departments of Water Affairs, Health and Environment. Registration should be simple to attract manufacturers and suppliers of bioaugmentation products to have them listed without undue constraints. The onus should be on the supplier to guarantee product performance and provide necessary safety information and dosage requirements for the selected field of application. Listing, however, would not imply an endorsement of a product by the local authorities. This should be done by a specialist technical review committee to be convened by the authorities.

1.4.3 Methodology and procedures for bioaugmentation technology implementation in South Africa

The methodology and procedures required for the development and improvement of biosupplements has been demonstrated for phenol and polychlorinated biphenyls (see section IV). The capabilities have therefore been firmly established for the implementation of bioaugmentation technology in South Africa.

Full-scale practical application of bioaugmentation has been demonstrated (see Section V).

1.4.4 Future research requirements

The investigation has successfully demonstrated the potential for bioaugmentation in South Africa. However, the expected performance of commercial products has not always been experienced. Feasibility assessments should therefore be conducted by competent institutions to ensure product performance before embarking on full-scale implementation.

Research should concentrate on specific applications of bioaugmentation products in the biodegradation of toxic, recalcitrant and hazardous compounds identified in South Africa. Fundamental research should focus on microbial interactions in consortia of microorganisms under a variety of conditions necessary for the catabolism of the selected pollutants.

II. LEGISLATIVE REQUIREMENTS FOR BIOSUPPLEMENTS

2.1 INTRODUCTION

Legislation on discharge standards has been enacted in the Water Act (1956), Act 54 of 1956, as amended in 1984 by the Water Amendment Act (1984), Act 96 of 1984. However, methods for the treatment of liquid and solid wastes has changed during the past decades. No legislation exists pertaining to the use of commercial biosupplements or specially selected and adapted microorganisms for the treatment of effluent. Genetically manipulated organisms are also being developed for the specific biodegradation of recalcitrant and often toxic and hazardous compounds. The specifications of commercially available biosupplements may be inadequate and general performance criteria may be difficult to define since each effluent is unique in one way or another. The uncontrolled use of such products may therefore pose health and environmental hazards.

The lack of guidelines on the use of biosupplements in South Africa requires the introduction of control measures on their specifications and usage. Regulatory measures introduced in the USA, Germany and Japan were looked into. These could assist in compiling suitable guidelines for South Africa with a view of developing adequate local legislation.

2.2 REGULATION AND CONTROL OF BIOSUPPLEMENTS - INTERNATIONAL SCENARIO

According to McCleary (1994), the United States Environmental Protection Agency (U.S.EPA) revised the National Contingency Plan (NCP) controlling the cleanup of spillages. It now requires that all bioremediation products be subject to efficacy testing. Toxicity testing has not been included since a less costly procedure is being investigated. The basic costs for the efficacy testing are estimated to be $10,000.

The EPA has defined bioremediation products as "microbial cultures, enzyme additives, or nutritive additives that are deliberately introduced into a discharge." The NCP currently lists 60 bioremediation products. It is anticipated to take one year to submit data in order to list new products. It is optional for suppliers of products to have them listed. The cost of the efficacy testing may act as deterrent to list products. However, efficacy testing contributed largely to eliminating products of poor quality from the list but did not guarantee performance nor indicate preference for one product over any other. The EPA also promotes field testing of listed products and collate a database on experimental data of various products.

Protocols for efficacy testing are updated at intervals and the latest update appeared in the Federal Register (1994). In this register, the EPA is promulgating rule changes or revisions for enactment in legislation such as the Oil Pollution Act (OPA) of 1990 or the Clean Water Act (CWA). The amended CWA may require presidential revision of the NCP to reflect the changes. The OPA specifies revisions to the NCP that enhance and expand on the current framework, standards and procedures. The previous revisions were promulgated in 1990. With each revision, supplementary information on statutory authority and background to the rulemaking, comments collected during the year after first publication and others are provided. It is therefore evident that a period of at least one year after first publication is required for the promulgation of the revisions.

In different sections of the register (Federal Register, 1994), the establishment of standards and procedures for treatment are provided and protocols on the efficacy testing published. Sections also provide information on the response plan "for the immediate and effective protection, rescue, and rehabilitation of, and the minimization of risk of damage to" the environment or natural habitats. The register therefore provides comprehensive guidelines on all aspects related to bioremediation and supplementation. Of considerable value is the section on comments and suggestions before finalization of the revision.

It is difficult to decide as to who should conduct the efficacy testing. It does appear that industry can conduct their own efficacy testing and generation of data on products. However, the EPA does ensure that the testing is conducted according to accepted norms and standards and procedures are used as promulgated in the Federal Register (1994). The EPA explicitly reserved the right to request additional documentation on test data and to conduct verification testing of the product effectiveness test results submitted by the manufacturers. It is also evident that toxicity testing is not a requisite and only products meeting or exceeding the effectiveness acceptability criteria must be tested according to accepted toxicity protocols.

Effectiveness testing should aim at simple procedures and minimize complex protocols to save on testing costs. Standard procedures should be clearly described (Federal Register, 1994). This is desirable but sometimes difficult to achieve for substances demanding highly sophisticated methods of analyses. Suitable standards or markers should be included in the assays and controls. This implies that a wide diversity of markers and appropriate controls have to be specified in an equally diverse range of products for the biodegradation and removal of specific substances to accepted norms.

The EPA clearly stipulates that retesting of products will be necessary with the promulgation of new legislative amendments, if applicable to the particular product listed (Federal Register, 1994). It demands the establishment of a schedule for this purpose and thus involves considerable administrative procedures and costs. It is also not clear in all cases, whether certain sections in the Federal Register is a guideline or a precise regulation. It is therefore necessary to ensure consistency in policy, instructions, guidance and requirements in documentation published by the EPA.

Data requirements may include (Federal Register, 1994):

- Name, brand, or trademark, if any, under which the product is sold.
- Name, address, and telephone number of the manufacturer, importer, or vendor.
- Name, address, and telephone number of primary distributors or sales outlets.
- Special handling and worker precautions for storage and field application. Tolerable and optimum storage temperature ranges are to be specified and could include temperatures under which product changes could be expected or alter its performance effectiveness.

- Shelf life.

- Recommendations on its application procedures, concentrations, and conditions for use, and any application restrictions.

- Effectiveness according to prescribed procedures and test methods. Manufacturers may be encouraged to provide additional data on product performance under conditions not specified for the "normal" test.

- Toxicity data is to be provided for those products listed that meet the effectiveness criteria.

- Reference standards could include pH, viscosity, presence of heavy metals and other ions such as cyanide, microorganism species and their percentage in the formulation, other biological additives and nutrients or special nutrient requirements, separate listing of special test methods for microorganisms such as *Salmonella*, faecal coliforms, *Shigella*, *Staphylococcus* coagulase positive, and ß-haemolytic *Streptococci*. For enzyme additives, enzyme name(s), International Union of Biochemistry (IUB), source of enzyme, units and specific activity, optimum pH and overall range, temperature, and salinity range for use of additive outside which its effectiveness is reduced by half its optimum capacity, enzyme shelf life and optimum storage conditions.

- Submission for listing may include confidential data and information. Confidential information, including technical data, must be submitted to the EPA on a separate list clearly marked. Such data, if agreed to its confidentiality, may not be made public.

- The EPA must be notified of any change in the formulation or composition of the product listed. This may require retesting for maintenance on the list.

- The listing of a product does not constitute approval of the product. Any advertisement, technical literature, or label referring to the placement on the list must either reproduce the EPA's written approval in its entirety or prominently include an approved disclaimer.

The required documentation on prescribed forms for application on the listing should include (Federal Register, 1994):

- Listed data for each analyte that has been analyzed, including all raw data.

- Tabulated and summarized statistics, including the mean, standard deviation, and sample size for each group at each day.

- The level of significance and the minimum significant difference value for each output.

- All computer outputs and the specific software package used to analyze the data should form part of the report.

In Germany, the application of bioaugmentation products apparently do not require precise legislative procedures (Pearce, personal communication). Efficacy testing is therefore not the norm and the onus is with the manufacturer or supplier of such products to provide accurate performance specifications, product descriptions, safety guidelines, and any other relevant information that may assist in its effective application. However, in the European Community, Germany in particular is pursuing the Biotechnology 2000 programme which endeavours to improve bioaugmentation products and will consider relevant legislation (Brauer, 1991). It was stated that Europe needs to scientifically integrate promising biotechnologies and coordinate options to apply modern improvements for the benefit of man and the environment by:

- Developing a regulatory framework for the best protection of man and the environment.
- Base safety regulations and standardization on scientific criteria by coordination in the European Community.
- Product sector regulations should be based on efficacy, safety and quality for man and the environment.
- Stimulate biotechnology research and development.
- Education and training of the public to improve understanding of the environmental benefits of biotechnology.
- By legislation and other means, establish biotechnology.
- Transfer technology into practical applications.

In Japan, the Waste Disposal and Public Cleansing Act was enacted in 1970, and amended in 1991. A programme on the updating of sanitary landfill technology and regulations has been reconsidered every 5 years to meet new requirements. This is heavily subsidized by the Japanese Government, amounting to 2,830 billion yen for the seventh 5-year period ending in 1995. Guidelines have been published on landfill regulations for municipalities. A year-long period is allowed for comments before promulgation of new guidelines. Each revision strives towards standardization of design and equipment employed at a landfill, and to develop design criteria for new and upgrading of existing facilities. The guideline revision is followed by intensive 2-year long discussions with the Ministry of Health and Welfare, municipalities, universities, consultants and the construction industry (Ikeguchi, 1994). It has unfortunately not been possible to establish what the situation is with respect to bioaugmentation products. However, it may be possible that similar programmes are followed for such technological applications because of the environmental awareness in Japan and the importance attached to a clean, safe and healthy environment.

2.3 REGULATORY GUIDELINES FOR THE CONTROLLED APPLICATION OF BIOSUPPLEMENTS IN SOUTH AFRICA

The absence of legislation on the application of bioaugmentation products in South Africa makes it imperative to rectify the situation. Effective products should dominate the local market to protect the technology which has considerable potential. Careful control should result in the disappearance of ineffective products which could bring the technology into disrepute. South Africa should glean the best of all available control, testing and legislation from the major developed countries.

It should be the aim in South Africa to follow simple procedures, and legislation should encourage official listing. The regulatory policy should be guided by the following four principles:

- National government regulation should focus on the characteristics and risks of the biotechnology product and less on the process by which it is made.

- For biotechnology products that require review, such review should be designed to promote the review with the least burden and protect public health and welfare and the environment.

- Regulatory programmes should be designed to incorporate rapid advances in biotechnology and performance-based standards should receive high priority.

- Opportunity for the application of innovative and new products of biotechnology, all regulation of such products and regulation in the environmental and health arenas should use performance standards and not rigid controls or specific designs for compliance.

Since the U.S. has a well developed infrastructure for the control and application of augmentation products, the rules and regulations applicable should be used as guidelines for South Africa. However, simplicity of control should be the aim.

Specialist technical committees would be needed to review the performance and possible health and environmental risks associated with the application of augmentation products. However, the onus should be on the manufacturer and/or the distributor of biosupplements to provide essential information and data on the formulation, biodegradative performance, list of microorganisms in the product, safety of use, method of application and any precautions necessary in the use of such products. This route should attract manufacturers and distributors to have their products placed on a national register. The minimum requirement should be some control in the importation of such products. Their should not necessarily be an embargo on genetically manipulated organisms. These should be considered for release by an organization on biotechnology such as SAGENE in South Africa.

The minimum specification criteria to be included in local regulation should be easy to measure and affordable such as, e.g. pH, microorganism species present in the formulation and their concentration(s), compounds degraded by the product and the residual concentration to be achieved. Safety specifications should form an essential part of the regulations and should be focused on in relative detail. Field testing under local conditions should be optional but would enhance the credibility of such products if supported by local case studies. In summary, regulation and control of bioaugmentation technology in South Africa should integrate the data requirements (see section 2.2, p. 6-7) into the proposed policy principles indicated above to achieve maximum effectivity with minimum constraints.

III. SPECIFICATION REQUIREMENTS FOR BIOSUPPLEMENTS

3.1 PRODUCT DESCRIPTION

It is essential to have an information sheet with a general description of the product. As a guideline the following could be listed in the description of a biosupplement (Table 3-1). It is not complete and will be determined by the nature of the product and the compounds to be degraded. It is therefore necessary to consider each product individually and no fixed rules can be laid down for the general description.

Table 3-1. Suggested characteristics to be included in commercial biosupplement specifications

Characteristic	Specification
Microbiological:	
Bacterial count	
Minimum aerobic	Number/g or mℓ
Minimum anaerobic	Number/g or mℓ
Type(s) of microorganism(s)	Name(s) of organism(s) in formulation
Biochemical:	
Enzyme activity	
Name(s) of enzyme(s) and compound(s) split	Activity in units
pH:	pH of preparation, range in which active and optimum
Physical:	
Appearance	Colour; Powder or Liquid
Odour	Brief Description
Screen mesh size	% size distribution
Moisture content	Mass %
Bulk density	g/cm^3
Usage:	Brief description of conditions for use, temperature at which to use
Storage:	Storage conditions and shelf life
Handling:	Precautionary measures for use of product
Packaging:	Nature of container and amount packaged
Performance Specification:	Brief description of formulation and its expected performance activity. Compounds degraded, tolerance to the compound and the residual level to be achieved.

Table 3-1 briefly indicates the specification of product performance. Manufacturers and suppliers of commercial biosupplements should provide succinct information and data on product performance. Since biosupplements are generally used for the biodegradation of specific compounds, the experimental conditions used for generating performance data should be clearly specified. The residual level of the compound achieved after treatment must be indicated and the time required to achieve such degradation shown in the data. Comparative data on the breakdown by biomass from wastewater treatment works could be beneficial but optional. Dosage requirements are important to enable effective implementation on full-scale and for costing of bioaugmentation. Supporting documentation on case studies, using a particular product for the treatment of a specified compound, would strengthen the credibility of product specifications.

Table 3-2. Suggested safety characteristics for the specification of biosupplements

Group Data	Characteristic
Physical data:	Boiling point Vapour pressure and density Solubility in water pH Appearance and colour
Fire and explosion hazard data:	Flash point Autoignition temperature Flammable liquids Extinguishing media
Reactivity data:	Stability Incompatibility (materials to avoid) Hazardous decomposition products Hazardous polymerization
Health hazard assessment:	General (screening test on cell cultures) Ingestion Eye contact Skin contact Skin absorption Inhalation Effects of overexposure First aid procedures for contact with skin, eyes and ingestion Spill or leak procedures Special protection information

3.2 PRODUCT SAFETY

According to the SABS, no safety guidelines and control on the use of biosupplements are available for South Africa. However, such guidelines for product safety exist in the private industry for the manufacture of biosupplements abroad since they have to comply with regulations of that country.

Certain guidelines were obtained from a US company which specializes in biosupplements. According to their safety sheets the following (Table 3-2) should be included to prove the safety of the biosupplement.

Some or all of these criteria may be applicable to a particular product and may have been included in the product description guidelines above. The data should not only be obtained on registration, but also after that, at reasonable intervals as biosupplements contain living organisms with a dynamic biochemistry. The genetic stability of the microorganisms may have to be taken into consideration. Suitable guidelines for South Africa could be based on those applicable to the USA provided they remain simple without excessive constraints to promote the registration of products in South Africa. The information sheets used for the USA may therefore suffice for South Africa without additional evaluation and collation of data locally.

There is an overlap in the information provided in Tables 3-1 and 3-2. This was intentional to provide separate information on product characteristics and safety to emphasise the importance safety.

3.3 PRODUCT DOSAGE

The dosing of augmentation products was dependent on the flow rates in a given system and not the amount of food coming into a treatment system. Table 3-3 indicates the minimum dosage per 1 000 m^3/d and it was important not to dose less than the minimum shown. This dosage schedule was for an activated sludge system and applied to a particular product with a specified cell concentration in the formulation. Aerated lagoons required slightly greater maintenance doses, but the given initial seeding schedule (day 1 - 18) applied. Biotowers, trickling filters and fluidised beds required a smaller dose, but the diversity in designs and methods of operation demanded a separate dosing schedule for each system. Although dosage levels vary widely with each application based on an evaluation of conditions, Table 3-3 can be used as a reference. More precise recommendations may be made after laboratory evaluation of field samples.

Table 3-3. Typical augmenting schedule proposed for an activated sludge system

Dosage (Day)	Biosupplement requirement for flows which are:	
	> 3 800 m^3/d	< 1 150 m^3/d
	kg/1 000 m^3	kg/1 000 m^3
1 + 2	3,2	1,35
3 + 4	1,9	1,1
5 - 11	1,25	0,7
12 - 18	0,7	0,4
Maintenance	0,3	0,15

Since the organisms in the biosupplement were in an inactive state, reactivation of the biosupplement products was necessary to ensure immediate action by the organisms and maximize the effectiveness of a bioaugmentation programme.

To assure proper biological activity, environmental prerequisites for aerobic microorganisms include maintenance of:

- DO greater than 1 mg/ℓ
- pH between 6 and 8
- sufficient nutrients.

Most suppliers of biosupplements recommend the use of dry cultures rather than liquid cultures. The dry cultures have about 10 times more activity per kg and are less bulky to handle than the liquid and are therefore recommended as seeding or start-up culture. The liquid cultures are more convenient to handle. Once the augmented bacteria have been established, a reduced dosage is required for their maintenance.

The addition of biosupplement into treatment systems could be made at various sites. Bioaugmentation usually occurs at the inflow pipelines, in a contact chamber where good mixing would occur, by spraying of bacterial suspensions over surfaces of static ponds or lagoons, to the recirculation stream, and on affected areas in the case of ground spills.

Supplementary information provided with the product could assist potential users of bioaugmentation in the correct implementation.

IV. DEVELOPMENT OF BIOSUPPLEMENTS

4.1 INTRODUCTION AND THEORETICAL BACKGROUND

The most important application was probably for the biodegradation of synthetic organics, often referred to as xenobiotics. Although foreign to the biosphere they do not of necessity pose an environmental threat. However, since many of them were chemically inert for their particular application, xenobiotics tended to be recalcitrant and resist biodegradation (Nörtermann & Hempel, 1991).

Xenobiotics are mostly introduced into the environment either by deliberate release, e.g. herbicides and pesticides, or involuntarily in wastewaters and by dumping or accidental spills. Special attention must therefore be given to industrial wastewater discharging recalcitrant, and often toxic and hazardous xenobiotic compounds on a continuous basis.

Microorganisms have been shown to possess a marked ability to evolve through mutation in a changing environment resulting in the breakdown of man-made compounds. This has raised hopes of finding ways to safely and effectively remove these substances from the environment. Laboratory studies, using specially selected and adapted or genetically manipulated microorganisms, supported such capabilities. However, the successful practical application remains difficult and often not achievable, especially in mixed and highly complex effluents (Thonart *et al.*, 1991). Treatment of such effluent requires well acclimated, mixed consortia of microorganisms (Wolfaardt *et al.*, 1994) and any disturbance in the chemical composition of the discharge will result in a change of the population and thus, in treatment performance. It is therefore essential to have some understanding of the synergistic interrelationships of microorganisms and the threshold concentrations below which enzymatic induction, growth or adaptation cannot take place.

4.2 SELECTION AND ADAPTATION OF MICROORGANISMS DEGRADING ORGANIC COMPOUNDS

4.2.1 Background

The conversion of organic carbon to carbon dioxide and methane was accomplished through enzymatic oxidation, with molecular oxygen involved as a final electron acceptor under aerobic conditions, or with, e.g. sulphate or nitrate as electron acceptor under anaerobic catabolism. Moreover, since catabolism of organic matter provides energy for the sustenance and growth of microorganisms, biological treatment of wastewaters in most cases produced innocuous end-products and biomass. However, if xenobiotic biodegradation is non-energy producing, an energetic co-substrate must be supplied for cometabolism.

For the selection of microorganisms, it is realistic to evaluate the potential to degrade the pollutants in the considered environment. This includes physico-chemical conditions such as temperature, pH, and the presence of possible inhibitory compounds as well as biological synergism or antagonism with natural microbial communities.

The first step in selection consists of screening the strains, most often isolated from natural contaminated sites. The aim of this screening, carried out on agar media in Petri dishes, is thus to assess the microbial performances in a wide range of situations related to the environment in which they will have to operate. For example, when some enzymatic capability is sought, it is common to use an insoluble or coloured substrate of this enzyme. The colonies that are able to utilize the substrate are surrounded by a cleared zone. The cleared area is generally proportional to the enzymatic activity.

As example, the enzymatic properties of a number of *Bacillus* strains are illustrated in Table 4-1 (Thonart *et al.*, 1991).

Table 4-1. Enzymatic properties of some *Bacillus* strains

Strain	Species	Enzymatic activity		
		Lipolytic	Proteolytic	Cellulolytic
4	*B. sphaericus*	+	++	++
10	*B. firmus*	++	+	++
23	*B. sphaericus*	++	++	+
28	*B. firmus*	++	+++	+
31	*B. brevis*	++	+++	+
36	*B. sphaericus*	+++	++	+
45	*B. sphaericus*	+++	+++	++
46	*B. sphaericus*	+	+++	++
50	*B. sphaericus*	++	+	++

+ = growth
++ = growth with cleared areas around colonies
+++ = growth with clearing on entire dish

It may be convenient to assess microbial activities based on the change in an indicator medium colour, as a consequence of a pH change. This may be applied to, e.g. urease activity which produces ammonia from urea. Staining procedures can be used to establish the disappearance of a substrate or the appearance of a catabolite. Nevertheless, the simplest screening method employs cell growth on media containing the pollutant under investigation as sole carbon source. This can also be used for cometabolism, since the selected strains must tolerate the cometabolite even though it may not be degraded. Equally important is the ability of the strains to compete with the natural microorganisms.

The preservation of cultures is important, firstly for maintaining stock cultures, and secondly for the packaging and distribution of the complete formulation. Several procedures can be used. Drying of cultures may be achieved by spray-drying, air drying of a thin layer of microorganisms or lyophilization of the cells. Protective agents are usually required to preserve the viability of the microorganisms (Thonart *et al.*, 1991).

The final step should be a laboratory as well as an *in situ* evaluation. The quality of the product should be controlled over as long a time period as possible to ensure reproducibility in quality of the supplement.

The general procedure for the development of a biosupplement is shown in Fig. 4-1.

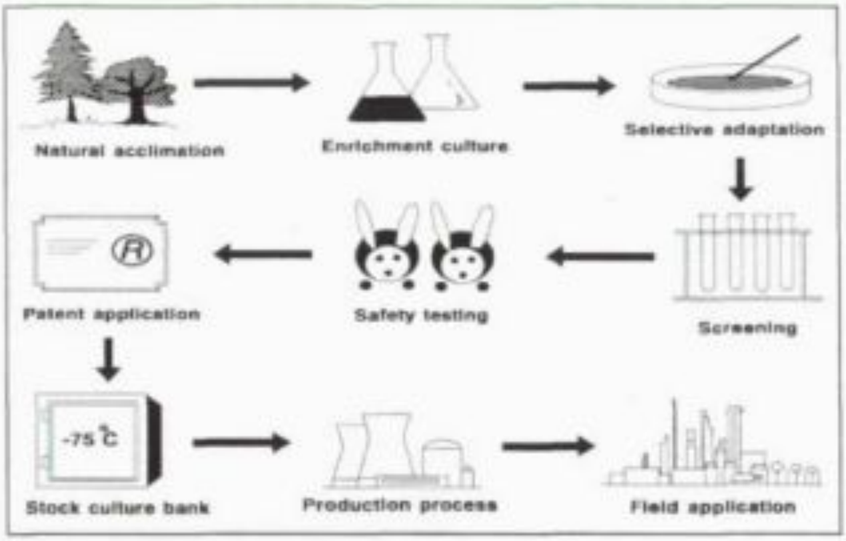

Fig. 4-1. Procedural outline for the development of a specially adapted biosupplement

4.2.2 Degradation of Phenol

Phenol was used as model compound to develop a biosupplement for its specific biodegradation. A series of experiments were conducted to gain experience and expertise in this field. The results of these experiments are presented in the following sections.

4.2.2.1 Phenol analysis

The aim was to adapt and develop a reproducible, accurate and sensitive high pressure liquid chromatography (HPLC) method for phenol determination.

A Waters HPLC, consisting of an automatic injector: WISP (Waters Intelligent Sample Processor), a resolve C-18 column, a detector and a recorder, was used for this investigation. The mobile phase was pumped through the system by a high pressure pump (model 590). The mobile phase was 40% acetonitrile:60% deionized water. The mobile phase was filtered and degassed before use, and kept under sterile conditions.

In order to establish the reproducibility of the procedure for phenol analysis, standards containing 1, 2, 5 and 10 mg/ℓ phenol were prepared in deionized water. The samples are filtered through 0,45 nm filters, before being injected into the system. The results are presented in Table 4-2 and the averages in Fig. 4-2. The analytical procedure is reproducible and the peak area is directly proportional to the concentration of phenol in the sample with a correlation coefficient of r = 0,99999.

Table 4-2. Reproducibility of analyses from duplicate injections of standard phenol samples after 0 h and 24 h storage

Sample	R_t* (min)	Area*	SE*
Std 1, 0 h	4,71 4,71	252754 241753	± 2,48%
Std 1, 24 h	4,80 4,80	260138 249772	
Std 2, 0 h	4,73 4,73	516527 501146	± 3,01%
Std 2, 24 h	4,82 4,82	516550 483099	
Std 5, 0 h	4,76 4,76	1248331 1244623	± 0,17%
Std 5, 24 h	4,82 4,82	1235719 1247692	
Std 10, 0 h	4,76 4,76	2522720 2514685	± 1,03%
Std 10, 24 h	4,82 4,82	2502601 2458886	

* R_t = Peak retention time in minutes
Area = Area is proportional to concentration
SE = Standard Error

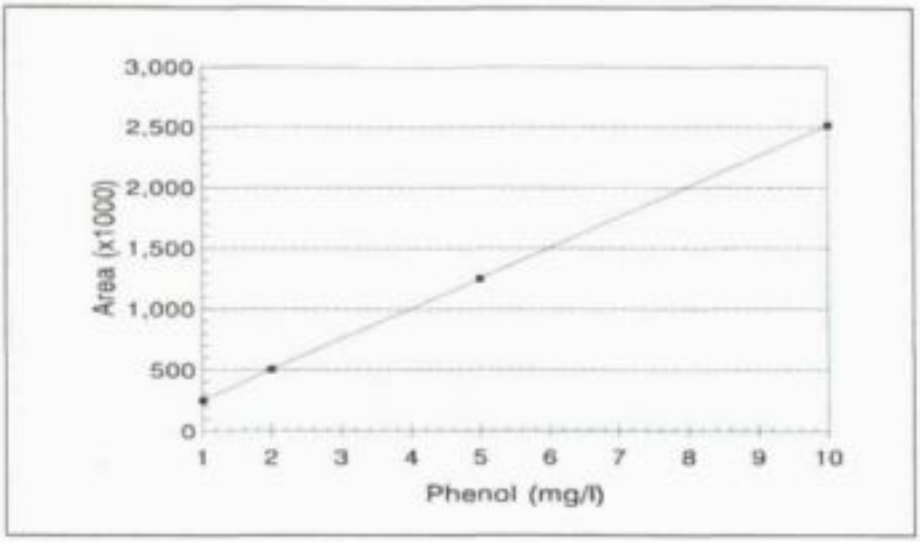

Fig. 4-2. Standard curve of phenol concentration

4.2.2.2 Isolation and enrichment of cultures

Activated sludge from the Daspoort Works of the Municipality of Pretoria was used as source of inoculum for the isolates.

Table 4-3. Colony morphology of phenol degrading bacterial isolates

Isolate	Gram reaction[1]	Diameter (mm)	Opacity	Elevation	Surface	Outline	Colour	Slime	Pigment[1]
1	-	3-5	Translucent	Convex	Glisten	Entire	Beige	+	-
2	-	>1	Translucent	Raised	Glisten	Entire	Yellow	-	-
3	+	2-3	Opaque	Flat	Glisten	Undulate	White	-	-
4	-	3-5	Opaque	Flat	Dull	Rhizoid	White	-	-
5	v	>1	Translucent	Convex	Glisten	Entire	Red	-	-
5.1	-	3-5	Opaque	Flat	Dull	Undulate	White	-	-
6	-	2	Transparent	Convex	Glisten	Entire	White	-	Y
6.1	v	3-5	Translucent	Raised	Dull	Rhizoid	White	-	-
7	-	1	Translucent	Convex	Glisten	Entire	Yellow	-	-
8	-	<5	Opaque	Flat	Dull	Rhizoid	White	-	-
8.1	+	3-5	Opaque	Flat	Dull	Rhizoid	White	-	-
9	v	3	Translucent	Raised	Glisten	Lobate	Beige	-	-
10	-	>1	Translucent	Flat	Smooth	Entire	Beige	-	-
11	-	2-3	Translucent	Convex	Glisten	Entire	Beige	-	G
12	-	3	Opaque	Flat	Rough	Undulate	Beige	-	-
13	-	2	Translucent	Raised	Smooth	Lobate	Beige	-	-
14	-	<5	Opaque	Flat	Dull	Undulate	Yellow	-	-
15	-	1-2	Transparent	Flat	Dull	Entire	Beige	-	-
16	+	<5	Translucent	Raised	Dull	Entire	White	-	-
17	+	3-5	Opaque	Flat	Dull	Rhizoid	Beige	-	-

1 **- = Gram negative; + = Gram positive; v = Gram variable**

2 **Y = yellow; G = green**

Microbial growth may be determined spectrophotometrically at 660 nm. However, total viable counts on a suitable agar medium after dilution in sterilised distilled water are preferred. The use of respirometry presents an alternative procedure to assess the microbial activity in the experiments. It is technologically highly advanced but demands considerable capital investment.

Primary isolations were made on minimal agar medium containing phenol as sole carbon source. Dominant bacteria, based mainly on colony morphology, were isolated and purified by streaking.

Twenty isolates were made and grown on nutrient agar plates and after 24 h incubation at 30°C, their morphological and cultural characteristics were determined. The pure isolates were also characterised according to their reaction to gram stain and light microscopic cell morphology.

The basic colony characteristics of the phenol degrading isolates are presented in Table 4-3.

The isolates were revived after freezing, to be described later (see **4.4.1 Storage and recovery of stock cultures**). Fourteen of the cultures survived. Although the cultures were considered pure before biofreezing, some appeared to be contaminated after thawing. The contaminants closely resembled the dominant species. It could not be established whether they represented strains of the dominant species. Before identification, therefore, serial dilutions were made and the dominant species reisolated. Six bacterial strains were identified as shown in Table 4-4.

Table 4-4. Identification of the phenol degrading bacterial isolates

Species identified
Bacillus cereus
Bacillus subtilis
Pseudomonas caryophylli
Pseudomonas putida
Sporosarcina urea
Staphylococcus cohnii subspecies II

4.2.2.3 Phenol tolerance

The twenty isolates were grown in minimal medium containing 1 g/ℓ phenol as carbon source for a period of 48 h. One mℓ of the isolate was added to minimal medium containing 100 mg, 200 mg, 400 mg, 500 mg and 1 000 mg/ℓ phenol, respectively. The isolates in the medium were placed in a shaking water bath at 30°C and samples were taken aseptically after 48 h. Growth was measured at 660 nm using the control medium as reference standard. The results are presented in Table 4-5. The twenty isolates grew at all of the selected phenol concentrations.

Table 4-5. Growth of isolates (OD 660 nm) at increasing phenol concentrations

Isolate	Phenol concentration (mg/ℓ)				
	100	200	400	500	1000
1	0,234	0,245	0,244	0,245	0,251
2	0,311	0,301	0,303	0,317	0,324
3	0,287	0,293	0,300	0,319	0,318
4	0,286	0,299	0,311	0,319	0,326
5	0,299	0,312	0,315	0,324	0,327
5.1	0,301	0,299	0,311	0,333	0,331
6	0,456	0,487	0,489	0,492	0,499
6.1	0,342	0,355	0,351	0,371	0,371
7	0,299	0,305	0,317	0,327	0,331
8	0,283	0,301	0,321	0,326	0,326
8.1	0,231	0,287	0,298	0,303	0,315
9	0,291	0,287	0,296	0,308	0,311
10	0,300	0,322	0,321	0,328	0,321
11	0,356	0,355	0,362	0,371	0,369
12	0,321	0,342	0,341	0,351	0,355
13	0,387	0,388	0,389	0,391	0,399
14	0,398	0,401	0,411	0,416	0,428
15	0,230	0,231	0,241	0,299	0,300
16	0,301	0,300	0,316	0,321	0,311
17	0,299	0,321	0,326	0,322	0,321

4.2.2.4 pH tolerance

To establish the influence of pH on the growth of the isolates, minimal medium at pH of 4, 7 and 9 respectively was prepared. The medium contained 500 mg/ℓ phenol as sole carbon source and was inoculated with the various isolates and growth estimated at 660 nm over a period of 9 d. The results are presented in Tables 4-6 to 4-8.

Table 4-6. Growth (OD 660 nm) of bacterial isolates over a period of 9 d in 500 mg/ℓ phenol at pH 9

Isolate	Days					
	1	2	3	5	7	9
1	0,451	0,495	0,511	0,418	0,400	0,390
2	0,365	0,397	0,411	0,315	0,310	0,290
3	0,299	0,365	0,381	0,349	0,241	0,201
4	0,311	0,365	0,385	0,351	0,314	0,284
5	0,456	0,511	0,513	0,461	0,412	0,402
5.1	0,451	0,498	0,511	0,476	0,455	0,402
6	0,301	0,361	0,399	0,372	0,251	0,202
6.1	0,311	0,386	0,388	0,348	0,302	0,248
7	0,211	0,294	0,321	0,286	0,215	0,191
8	0,481	0,513	0,525	0,444	0,405	0,399
8.1	0,442	0,491	0,504	0,466	0,401	0,385
9	0,444	0,487	0,501	0,414	0,400	0,364
10	0,300	0,321	0,382	0,311	0,298	0,218
11	0,301	0,311	0,386	0,300	0,286	0,254
12	0,299	0,354	0,411	0,371	0,315	0,265
13	0,318	0,344	0,399	0,362	0,321	0,241
14	0,351	0,368	0,400	0,376	0,371	0,288
15	0,201	0,222	0,281	0,218	0,197	0,157
16	0,222	0,251	0,361	0,248	0,197	0,161
17	0,231	0,251	0,298	0,211	0,194	0,156

Table 4-7. Growth (OD 660 nm) of bacterial isolates over a period of 9 d in 500 mg/ℓ phenol at pH 7

Isolate	Days					
	1	2	3	5	7	9
1	0,346	0,459	0,491	0,415	0,310	0,255
2	0,410	0,459	0,501	0,465	0,401	0,391
3	0,351	0,382	0,405	0,353	0,315	0,290
4	0,341	0,391	0,456	0,402	0,365	0,301
5	0,396	0,456	0,511	0,446	0,378	0,325
5.1	0,355	0,391	0,415	0,375	0,315	0,295
6	0,341	0,392	0,485	0,415	0,381	0,301
6.1	0,348	0,399	0,452	0,421	0,395	0,315
7	0,389	0,456	0,501	0,465	0,391	0,352
8	0,400	0,489	0,503	0,461	0,421	0,365
8.1	0,444	0,491	0,501	0,465	0,441	0,391
9	0,465	0,499	0,511	0,485	0,462	0,402
10	0,442	0,482	0,499	0,411	0,391	0,381
11	0,321	0,418	0,489	0,416	0,311	0,294
12	0,346	0,485	0,499	0,451	0,391	0,305
13	0,355	0,399	0,475	0,400	0,386	0,306
14	0,365	0,485	0,499	0,411	0,385	0,305
15	0,398	0,485	0,501	0,466	0,411	0,351
16	0,375	0,391	0,451	0,383	0,361	0,300
1	0,347	0,411	0,485	0,375	0,321	0,281

Table 4-8. Growth (OD 660 nm) of bacterial isolates over a period of 9 d in 500 mg/ℓ phenol at pH 4

Isolate	Days					
	1	2	3	5	7	9
1	0,221	0,256	0,356	0,246	0,215	0,190
2	0,195	0,199	0,251	0,200	0,190	0,110
3	0,186	0,199	0,251	0,196	0,185	0,141
4	0,191	0,211	0,248	0,201	0,194	0,171
5	0,185	0,198	0,251	0,218	0,165	0,138
5.1	0,258	0,311	0,398	0,385	0,308	0,214
6	0,165	0,211	0,281	0,198	0,151	0,110
6.1	0,185	0,222	0,256	0,200	0,186	0,163
7	0,286	0,295	0,344	0,265	0,201	0,195
8	0,199	0,241	0,281	0,205	0,185	0,155
8.1	0,356	0,425	0,481	0,423	0,323	0,301
9	0,389	0,451	0,475	0,401	0,341	0,301
10	0,311	0,385	0,421	0,400	0,315	0,291
11	0,181	0,212	0,247	0,199	0,169	0,152
12	0,185	0,222	0,252	0,201	0,153	0,131
13	0,189	0,215	0,251	0,231	0,199	0,115
14	0,196	0,235	0,285	0,205	0,151	0,135
15	0,251	0,281	0,296	0,211	0,185	0,115
16	0,298	0,351	0,361	0,204	0,186	0,143
17	0,211	0,285	0,314	0,264	0,211	0,165

All isolates grew at the three selected pH values. There was some inhibition of growth at pH 4 for some of the isolates, but isolates 5.1, 8.1, 9, 10, 15 and 16 grew well at this pH. Although the isolates can grow at a wide pH range, optimal growth is considered to be at a pH between 7 and 8.

4.2.2.5 Degradation of phenol by bacterial isolates

The twenty isolates were subjected to a preliminary phenol degradation study. The isolates were inoculated into liquid minimal medium containing 1000 mg/ℓ phenol and incubated at 30°C. Samples were taken after day 1, 2, 3, and 6 and analyzed for residual phenol. The results are presented in Table 4-9.

Most of the isolates showed the same trend. They adsorbed the phenol and then released it again, after which degradation took place. None of the isolates performed exceptionally well and five isolates (2, 6, 11, 13 and 14) were selected at random for further investigation.

To establish the time required for complete degradation of the phenol, the selected isolates were inoculated into minimal medium containing 1000 mg/ℓ phenol. Samples were taken over a period of 14 days and analyzed for phenol (Table 4-10). Every time a sample was removed from the flask, an equivalent volume of trace element and nutrient solution was added to the flasks.

Table 4-9. **Residual phenol (mg/ℓ) after degradation by bacterial isolates (initial phenol concentration was 1000 mg/ℓ)**

Isolate	Day			
	1	2	3	6
1	734	800	882	788
2	823	863	511	515
3	751	848	621	464
4	742	838	834	814
5	837	823	854	-
5.1	732	827	873	523
6	808	798	574	463
6.1	788	681	843	734
7	611	830	857	872
8	819	815	850	667
8.1	745	726	529	853
9	773	781	853	453
10	735	835	780	441
11	863	824	613	450
12	812	831	641	397
13	701	798	790	455
14	834	572	521	433
15	910	815	546	408
16	829	782	617	-
17	-	859	807	765

Table 4-10. **Residual phenol (mg/ℓ) after degradation with selected isolates**

Day	Isolate number				
	2	6	11	13	14
1	815	750	541	474	774
2	745	665	647	708	724
3	756	557	631	647	642
4	713	595	644	618	642
7	663	630	663	688	687
8	600	551	472	613	608
9	481	393	349	437	456
11	403	0	131	394	379
14	0	0	0	0	0

The phenol was degraded by all of the isolates within 14 days. Isolate 6 degraded the phenol more rapidly and a zero concentration was achieved by day 11. The isolates all had similar degradation capabilities. It was observed by light microscopy that some of the phenol was absorbed by the cells (isolates 2, 11, 13, 14), then released slowly again after which total degradation took place. Isolates 6 and 11 were the most promising with respect to their degradation rates and capabilities.

4.2.2.6 Influence of an additional carbon source on phenol biodegradation

The slow degradation observed by the isolates which were acclimated to phenol indicated a lack of a readily available energy supply. Supplementation of the minimal medium with 0,01%, 0,05%, 0,10% and 0,20% (mass/vol) glucose was then investigated. The residual phenol concentrations are presented in Figs. 4-3 to 4-6 respectively.

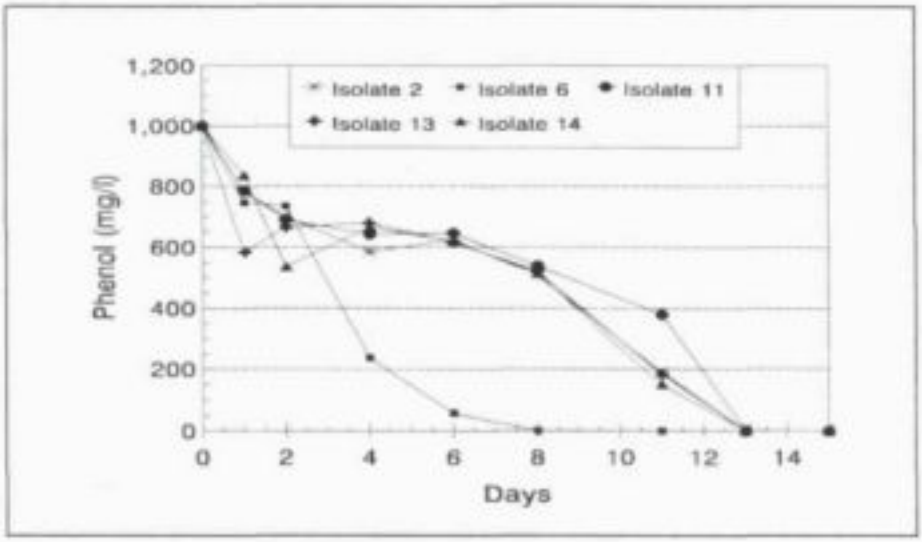

Fig. 4-3. Residual phenol in 0,01% glucose supplemented minimal medium

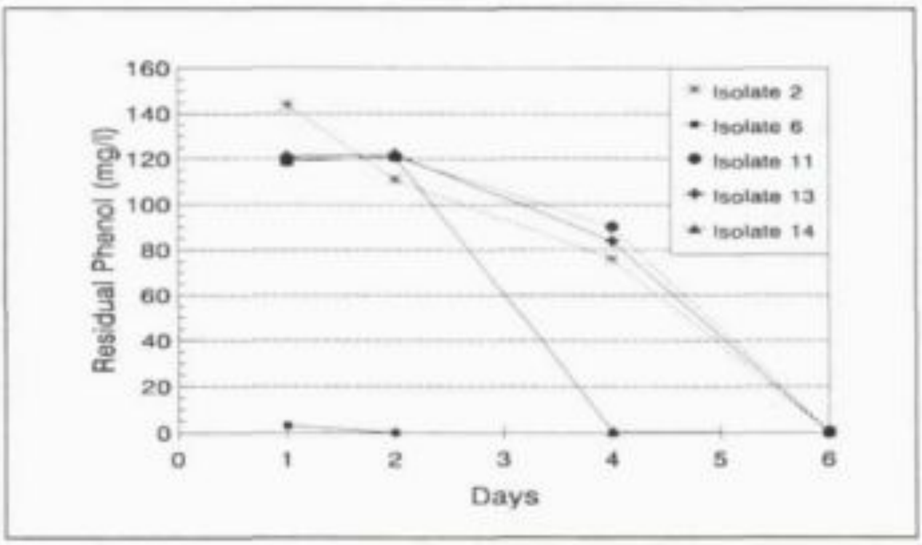

Fig. 4-4. Residual phenol in 0,05% glucose supplemented minimal medium

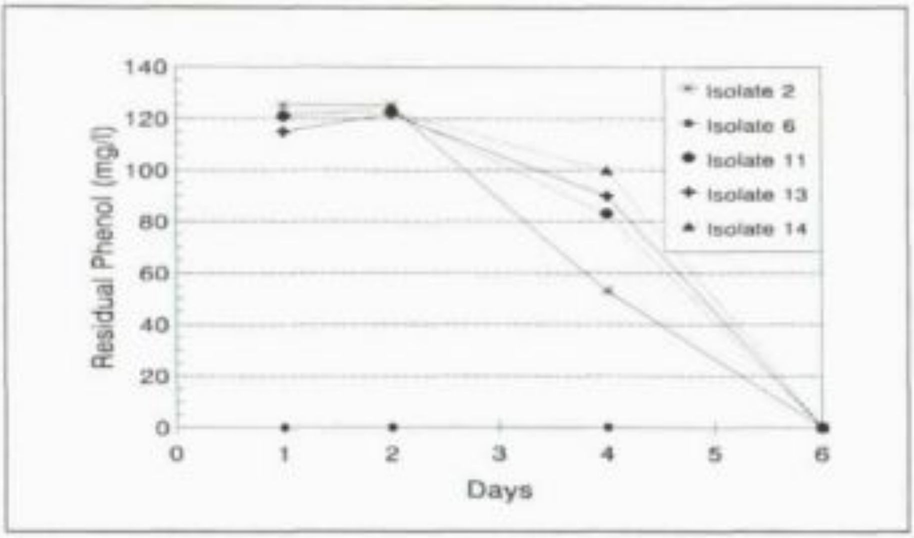

Fig. 4-5. Residual phenol in 0,10% glucose supplemented minimal medium

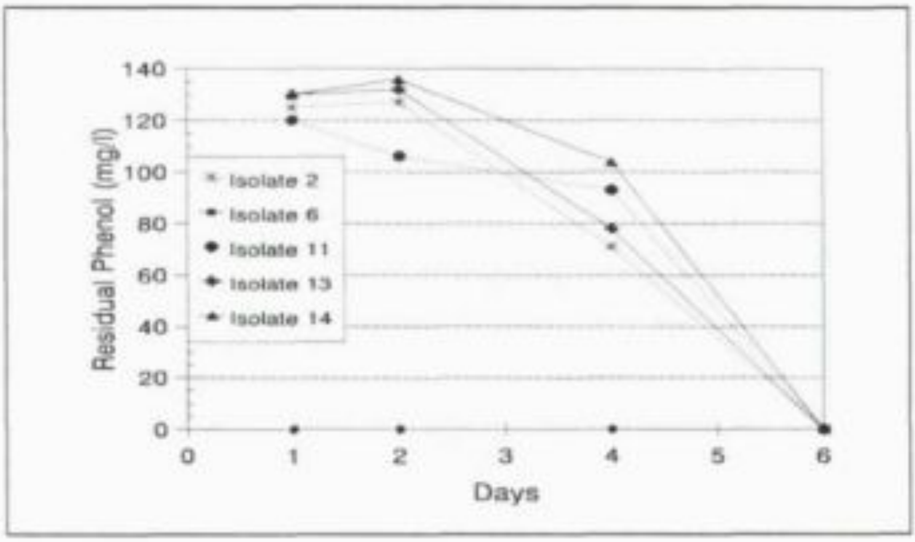

Fig. 4-6. Residual phenol in 0,20% glucose supplemented minimal medium

The addition of 0,05% to 0,10% glucose resulted in a marked improvement in the degradation of phenol. Within 1 day, more than 86% phenol was degraded and complete degradation was achieved by day 6. Isolate 6 performed best with virtually 99% removal achieved within 1 day and 100% within 2 days. This clearly demonstrates the need for a readily available carbon source to improve the metabolism of the isolates.

4.2.2.7 *Response to a shock load of phenol*

The effect of a shock load of phenol on the isolates was assessed. A load of 8 g/ℓ of phenol was added to the isolates which received no additional carbon source and to the isolates which received 0,1% glucose. The breakdown of phenol was improved slightly by glucose supplementation. Only the results of residual phenol and viable cell counts, in the absence of glucose, are presented in Fig. 4-7 and Table 4-11 respectively.

The cell concentration decreased approximately a 100 fold within 24 h from more than 10^8 cells/mℓ to 10^6 cells/mℓ. The viable counts gradually increased by an order of magnitude towards the end of the experiment.

Within 4 days, absorption and degradation of the phenol to between 2 000 mg/ℓ and 3 000 mg/ℓ occurred. The phenol was then released into solution and degraded further to between 1 200 and 1 600 mg/ℓ phenol after 23 days. This shows that degradation still took place in spite of the 100 fold cell loss after the initial shock load. To enhance and accelerate the degradation of the pollutant to acceptable levels, biomass supplementation will be required to ensure a quick recovery of the system.

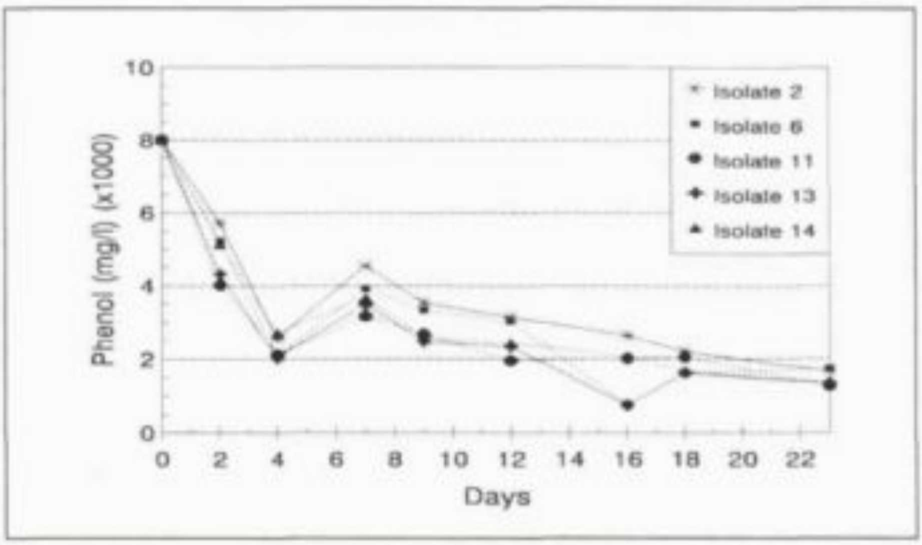

Fig. 4-7. Residual phenol after a shock load of selected isolates with 8 g/ℓ phenol

Table 4-11. Viable cell counts after phenol shock load of 8 g/ℓ and with no glucose addition

Day	Isolate				
	2	6	11	13	14
0	$2x10^8$	$5x10^8$	$4x10^8$	$4x10^8$	$2,6x10^8$
1	$8x10^6$	$3,4x10^6$	$3,3x10^6$	$3x10^6$	$5,2x10^6$
2	$5x10^6$	$5,4x10^6$	$3,1x10^6$	$5,6x10^6$	$4,5x10^6$
4	$3x10^6$	$6x10^6$	$2,5x10^6$	$1,1x10^7$	$3,1x10^6$
10	$2x10^6$	$6x10^6$	$1x10^6$	$8x10^5$	$3x10^5$
12	$1x10^5$	$1x10^6$	$1x10^6$	$2x10^6$	$4x10^5$
16	$3x10^5$	$1,1x10^6$	$4x10^5$	$5x10^5$	$6x10^6$
23	$7x10^6$	$2,9x10^7$	$1,2x10^7$	$6,5x10^6$	$7,2x10^6$

4.2.3 Degradation of Polychlorinated Biphenyls

4.2.3.1 Background

Polychlorinated biphenyls (PCBs) are a class of aromatic compounds which have found widespread applications because of their general stability and excellent dielectric properties (Hutzinger, 1974). The products of chlorination potentially include 209 congeners with one to ten chlorine atoms. Highly chlorinated PCBs have extremely low vapour pressures, are practically insoluble in water and are largely immobile in soil.

The persistence of PCBs in the environment has generated concern because of their tendency to bioaccumulate and because of their possible adverse health effects. Anaerobic and aerobic microorganisms have been isolated from contaminated sites and used in investigations on their biodegradation with varying degrees of success (Bedard & Haberl, 1990; Chaudhry & Chapalamadugu, 1991; Havel & Reineke, 1991; Quensen *et al.*, 1990; Safe, 1984; Unterman *et al.*, 1988). The reported studies are largely small-scale evaluations. However, the application of bacteria isolated by Unterman *et al.* (1988) for anaerobic remediation of the Hudson River sludge bed has been given the go-ahead (B. Ensley, Envirogen - personal communication) in the coming years and will be the first full-scale application of remediation of a PCB contaminated area.

Spillage is common at power stations resulting in the contamination of the surrounding soil. The recalcitrance of the compounds and the fact that some success for the use of a biosupplement in the removal of PCBs seems possible, has made the selection of the development of a biosupplement for PCBs a challenging task.

4.2.3.2 Isolation of microorganisms for the biodegradation of PCBs

Soil samples were taken after the occurrence of an Askarel (commercially available PCB transformer oil) spillage from a capacitor.

A sample of the soil was added to minimal medium. The broth was shaken with the soil and allowed to settle for ca. 30 min. The supernatant, containing the microorganisms, was then used to inoculate the medium containing 1,3 mℓ Aroclor 1248 per 500 mℓ. Agar plates containing Aroclor 1248 were also prepared and inoculated with the supernatant under aerobic conditions. Incubation was carried out at 37°C. A set of plates without yeast extract were similarly inoculated. After growth of the microorganisms had progressed, colonies were picked off and restreaked to obtain axenic cultures maintained for further use.

Isolated bacteria were identified using API strips. The identification is based on a number of biochemical and metabolic parameters and a code number is derived according to a prescribed protocol. The probability that an isolate is correctly identified is based on the degree of similarity between the unknown test organism and the behaviour of known bacteria under identical test conditions.

4.2.3.3 Assessment of microorganisms for PCB degradation capabilities

An experiment was set up using each of the aerobic bacteria individually and in five different combinations on PCB containing broth and their ability to degrade PCBs assessed after 7 days incubation. Growth under these conditions was found to be very slow.

Using high pressure liquid chromatography (HPLC) to monitor changes in PCBs, only slight changes in the ratios of different isomers were observed. Changes were most obvious in the combination of bacteria containing all three genera of bacteria previously identified.

This led to a second set of experiments in order to assess the biodegradation of PCBs by combinations of bacterial isolates. The design was basically the same as that of the first experiment, using Askarel or PCBs extracted from Askarel as carbon source.

To assess the breakdown of individual congeners, peak ratios relative to a preselected peak as reference (e.g. peak at retention time of 12.3 min) were calculated for the biodegraded sample and compared to the amount and ratio of an untreated PCB control. This procedure provides the most reliable method of calculating PCB isomer degradation since direct comparison of peak concentrations does not take into consideration any losses in one sample relative to that of the control sample which may have occurred during the experiment. Also, partial dechlorination of higher chlorinated isomers may result in the appearance of less chlorinated isomers creating the false impression that no degradation has occurred.

4.2.3.4 Methods of PCB analyses

Samples prepared in acetonitrile according to standard procedures were separated on a Waters Nucleosil ET125/8/4 5 C18 125x4mm HPLC column to obtain a quantitative and qualitative fingerprint of PCB isomers. Individual isomers could not be identified by HPLC but the resolution was sufficient to obtain an indication of the degree of biodegradation. Samples were then selected and prepared in dried petroleum ether for more detailed analysis by the Division of Materials Technology, CSIR, using gas chromatography - mass spectrometry (GC-MS) similar to the method outlined by Quensen *et al.* (1990). The chromatograms and mass data were compared with computer based standards.

4.2.3.5 *Results and discussion*

4.2.3.5.1 Resolution of PCBs by HPLC and GC

The spectral resolution of PCB isomers in Aroclor 1248 by HPLC is illustrated in Fig. 4-8 indicating the position of di- to hexa-chloro isomers. By comparison, the resolution of the same Aroclor congener mixture by GC is illustrated in Fig. 4-9 clearly indicating the improved resolving power by this procedure.

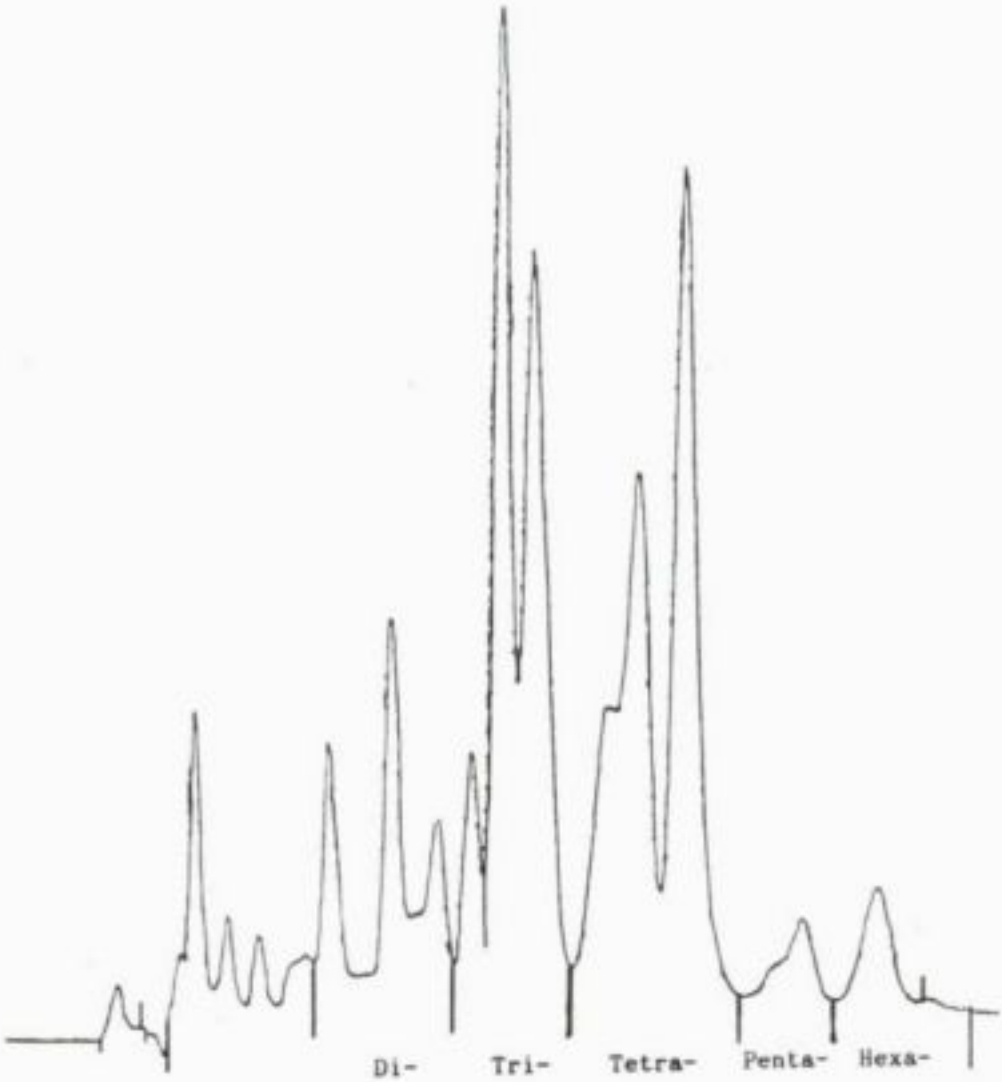

Fig. 4-8. Resolution of Aroclor 1248 by HPLC, indicating the positions of di-, tri-, tetra-, penta- and hexa-chloro isomers

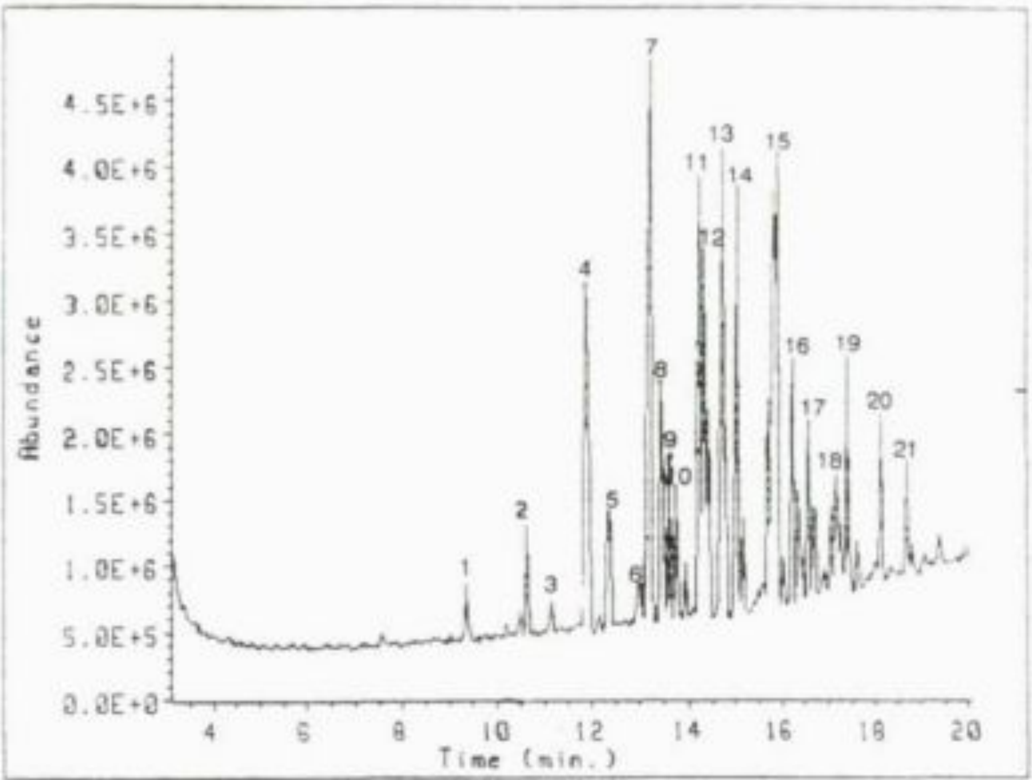

Fig. 4-9. **Resolution of Aroclor 1248 by gas chromatography**

4.2.3.5.2 Bacterial isolates

Bacteria were isolated using PCBs as sole carbon source. Growth on the selective medium therefore represents bacteria that have the ability to degrade PCBs and utilize them as source of energy.

As shown in Table 4-12, seven bacterial isolates from contaminated soil were identified by the API method.

Table 4-12. **Identification of bacteria isolated from PCB contaminated soil**

Species	Probability
Pseudomonas acidovorans	99,9 %
P. putida	99,9 %
P. maltophila	99,9 %
P. aeruginosa	99,9 %
P. vesicularis	99,9 %
Flavobacterium meningosepticum	99,9 %
Acinetobacter calco, var. lwoffi	40,6 %

The presumed *Acinetobacter* sp., identified at a probability of only 40,6%, may represent a new species of bacteria.

The successful isolation of bacteria using PCBs as sole carbon source proves that the biodegradation of PCBs is feasible and confirms reports in the literature (Bedard & Haberl, 1990; Havel & Reineke, 1991; Safe, 1984; Unterman *et al.*, 1988). From this study it is evident that *Pseudomonas* spp. tend to be the dominant aerobic microorganisms possessing the potential for the biodegradation of PCBs. Since they were obtained from contaminated soil, their application to bioremediation of soils is indicated.

4.2.3.5.3 Aerobic biodegradation of PCBs

The biodegradation of Askarel was assessed in the absence and presence of microorganisms. The percentage removal of PCB isomers from Askarel is presented in Fig. 4-10. A removal of between 30% and 95% was observed for the first four peaks representing the less chlorinated congeners after an incubation period of ten days.

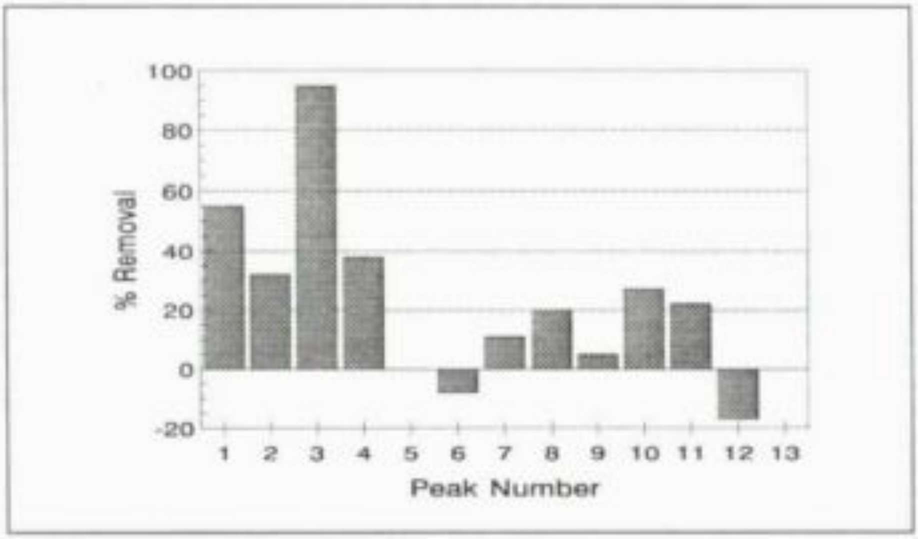

Fig. 4-10. The percentage removal of each isomer of the PCBs in Askarel after 10 days aerobic degradation

The biodegradation of Askarel-extracted PCBs by the bacterial isolates, as a percentage of removal, are presented in Fig. 4-11. It can be seen that 20-55% removal of each isomer was achieved for the first nine isomers representing a significant increase in the isomers which can be successfully degraded. It is also evident that more PCB isomers can be degraded once extracted from the Askarel. The reason for this difference could not be established.

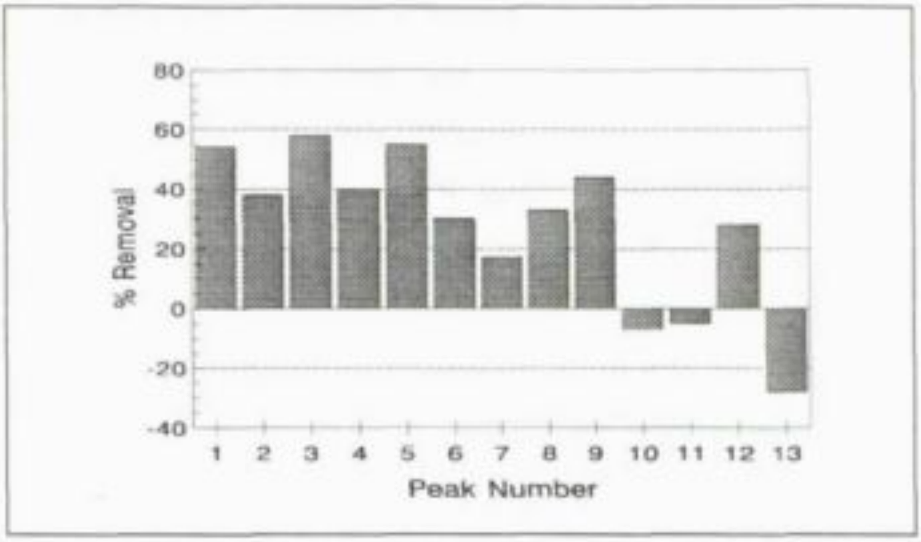

Fig. 4-11. The percentage removal of each isomer of the Askarel-extracted PCBs by aerobic bacterial degradation

4.2.3.6 Conclusions

Experimental evidence that aerobic bacteria can be successfully isolated from contaminated soil using PCBs as sole carbon source has been presented. Most bacterial isolates belong to *Pseudomonas* spp. Identification of anaerobic bacteria could not be established by conventional procedures.

In this investigation, removal of 30-90% of the lower chlorinated PCB isomers (up to tetra-chloro substitution) has been demonstrated using the bacteria isolated form the soil sample. More isomers were found to be degradable once extracted from the Askarel and exposed to the microorganisms

4.3 UNDERSTANDING CATABOLIC PATHWAYS

4.3.1 Catabolism of Phenol

The metabolic pathways for the biodegradation of phenol and o-, m- and p-cresol are illustrated in Fig. 4-12.

Phenol is hydroxylated by phenol hydroxylase to catechol which is broken down to 2-hydroxymuconic semi-aldehyde by catechol 2,3-dioxygenase. The aromatic ring is opened up in this step.

From this point onwards there are two possible degradation routes, viz. hydrolase splits off formic acid (HCOOH) to form 2-ketopent-4-enoate. Hydratase degrades the intermediate to 2-keto-hydroxyvalerate, and finally aldolase splits off acetaldehyde CH_3-CHO) to form pyruvic acid (CH_3-CO-COOH.)

Alternatively, isomerase degrades the semi-aldehyde to 4-oxalocrotonate (enol). A CO_2 molecule splits off by enzymatic degradation with decarboxylase to form 2-ketopent-4-enoate. The degradation then follows the pathway as previously described.

The breakdown of the cresols follows along similar lines.

An understanding of the catabolism of pollutants is therefore essential to enable the development of a treatment strategy. It is also necessary to establish whether a single microorganism species has the capability to completely degrade a substance without the accumulation of intermediates which may be toxic or not degradable to innocuous forms. In cases where the degradation of complex pollutants is aimed at, a group or consortium of microorganisms may be required to achieve effective breakdown (Vrdoljak *et al.*, 1991; Wolfaardt *et al.*, 1994)

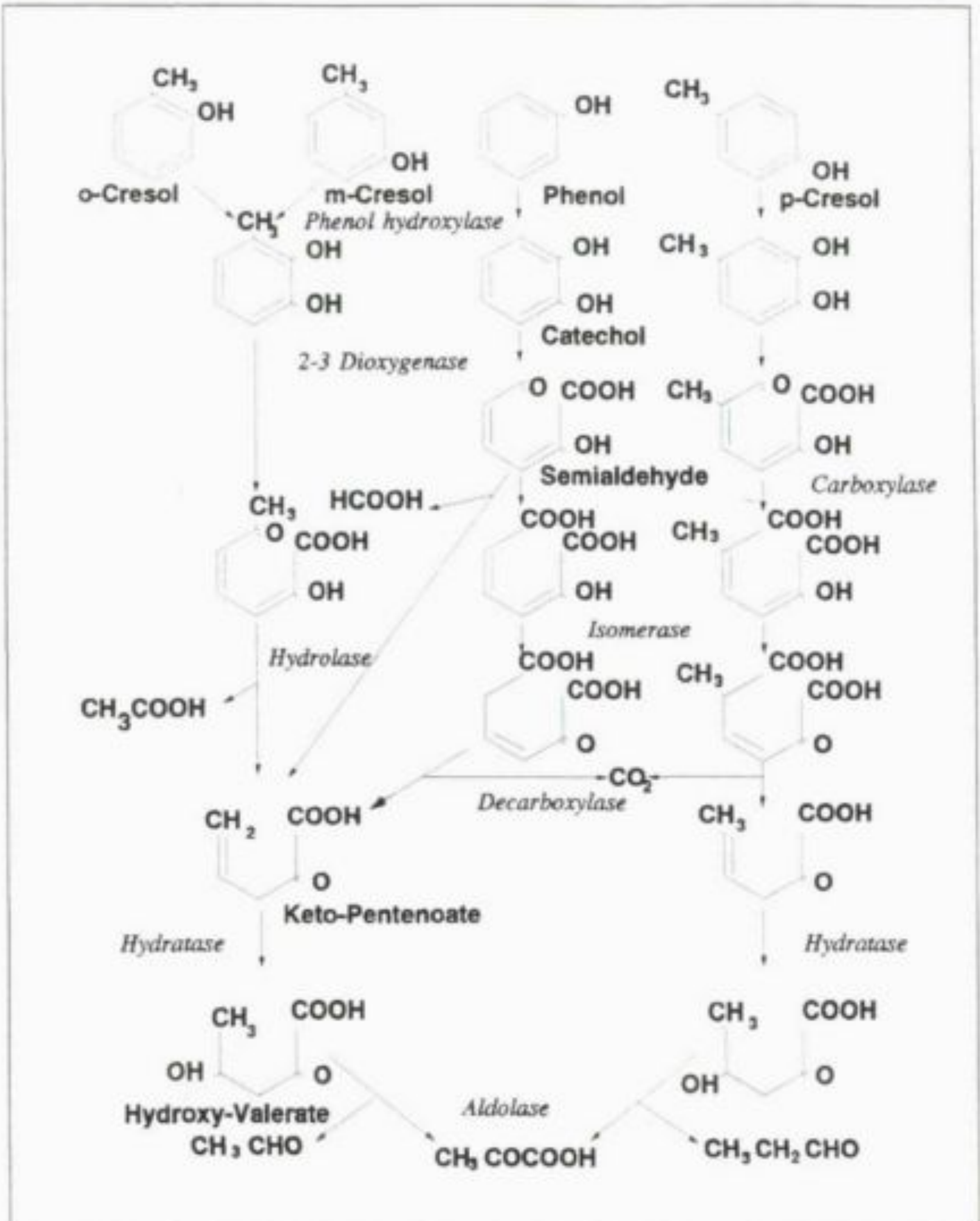

Fig. 4-12. Catabolic pathways of phenol and cresols by *Pseudomonas putida*

4.4 PRESERVATION OF STOCK CULTURES

4.4.1 Storage and recovery of stock cultures

Preservation of stock cultures of bacterial isolates is an important part of bioaugmentation. Suitable means of preservation must therefore be investigated. A common method is biofreezing at temperatures of -75°C. Protective media include glycerol, dimethyl-sulphoxide or protein-rich substances such as milk powder.

A series of experiments were conducted to establish the optimal conditions for storage. Glycerol (10% and 20% v/v) and dimethyl sulphoxide (10% v/v) were evaluated. Instead of freezing cell suspensions, sterile glass beads were added to the suspension and allowed to stand for approximately 10 min to allow adsorption of the cells to the glass surface. The supernatant was discarded and the beads frozen in sterile plastic vials.

After one week in the biofreezer, a recovery test was performed. Water and nutrient broth were used as media to suspend and recover the cells from the frozen beads. Beads were removed from the frozen bottles without thawing.

Three methods were evaluated for the recovery of cells from the frozen beads.

Method 1:

From each storage vial, two beads were aseptically removed using tweezers. One bead was transferred into a sterile test tube containing 1 mℓ sterilised distilled water and the other bead into 1 mℓ nutrient broth. Both test tubes were incubated for 24 h at 30°C. The same procedure was performed on all 20 phenol degrading isolates.

After incubation, serial dilutions were performed, the suspension plated on nutrient agar plates and incubated at 30°C. The total viability count was done after 24 h.

Method 2:

As for method 1, beads were transferred to a test tube containing either sterilised distilled water or nutrient broth. The tubes were shaken by a vortex mixture for 1 min in an attempt to remove the cells from the beads. Serial dilutions were made and the suspensions plated on nutrient agar plates and total viability counts performed after 24 h as before.

Method 3:

As before, after transferring the beads to the test tubes with the various media and shaking the tubes in the vortex mixer, the tubes were placed in a water bath at 30°C for 30 minutes. After 30 minutes, serial dilutions were performed and the isolates were plated onto nutrient agar plates and the total viable count monitored after 24 h.

It was shown that storage in 10% (v/v) sterile glycerol was the easiest and best method to use. Dimethyl sulphoxide appeared to be toxic to some isolates and poor survival was observed.

Recovery was best by using either method 1 or 3 (Table 4-13). Since method 3 is quicker, it is generally recommended. However, since different bacteria have different cell walls or membranes, it will always be necessary to establish the most appropriate medium and storage procedure for an isolate before deciding on a particular storage procedure.

Some cell loss occurred by biofreezing but not with all isolates. Residual cell adherence to the glass beads or floc formation could explain some of the reduction in counts experienced. It was also observed that the frozen cells on the beads require a growth medium and an initial period of reactivation to commence regrowth.

Table 4-13. Recovery of 2 isolates after biofreezing using three different methods of recovery

Method	Isolate	Cell concentration		
		Before freezing	Bead in Water	Bead in Nutrient broth
1	1	$1,5x10^8$	$2,5x10^4$	$4,5x10^7$
	3	$6x10^6$	$1,9x10^5$	$3,2x10^6$
2	1	$1,5x10^8$	no growth	no growth
	3	$6x10^6$	no growth	no growth
3	1	$1,5x10^8$	$2x10^4$	$1,1x10^7$
	3	$6x10^6$	$5,7x10^3$	$7,2x10^5$

Survival of the cultures was examined 1 year after the original freezing. Only 14 cultures had survived that period using method 3 for revitalization (see ***4.2.2.2 Isolation and enrichment of cultures***).

An experiment was conducted to establish the phenol biodegradation ability by the isolates. Although the bacteria grew on phenol as sole carbon source, very little or no degradation was observed, even in the presence of additional glucose as energy source which had previously been shown to be beneficial. It is difficult to explain this observation. However, Gaick *et al*, (1994) observed similar effects of freeze drying and storage on phenol degrading bacteria.

4.5 FORMULATION OF A BIOSUPPLEMENT

The formulation of a biosupplement for the biodegradation of a specific compound requires careful isolation and culture of microorganisms capable of specifically degrading the compound under investigation. Depending on the complexity and recalcitrance of the substance, more than one different microorganism may be necessary to achieve complete mineralization to innocuous end products. Consortia of microorganisms are generally required for efficient breakdown. The selection and adaptation process therefor requires considerable investigation and evaluation. If a number of different microorganism are required, combinations of these will have to be evaluated to establish that the best combination and relative concentrations of the microorganisms are present. Competitive inhibition by the microorganisms must be taken into consideration.

Once an effective and efficient combination has been obtained, it will require detailed testing under field conditions to ensure that the formulation performs as expected. Tolerance to other, possibly inhibitory and toxic, compounds also present in the effluent must be investigated. The safety aspects for the application of a biosupplement demands thorough control measures to be compiled. The microorganisms to be used must also be genetically stable to minimize loss of the biodegradative capabilities. This is particularly important if the genetic information for the degradative enzymes resides on plasmids which are extrachromosomal DNA elements frequently present in bacteria.

Maintenance of stock cultures is vital. The storage conditions must be selected to ensure that the degradative capabilities are maintained with maximum viability of the microorganisms during the storage over extended periods of time.

Every case will require detailed knowledge on the composition of the effluent to be treated. This may require the combination of more than one formulation of a biosupplement for the successful breakdown of pollutants which are difficult to degrade by indigenous microorganisms in a wastewater treatment works.

4.6 CONCLUSIONS

The methodology has been developed successfully for the formulation of biosupplements for the biodegradation of phenol and polychlorinated biphenyls. A problem was experienced with the extended preservation of phenol degrading bacteria. Loss of viability occurred to some extent but more important was the loss of their biodegradative capability.

The formulation of biosupplements requires lengthy developmental work followed by extensive testing to ensure expected product performance and reproducibility of the product to be marketed. Upscaling from laboratory- to full-scale culture of the bacteria for the formulation will require careful control.

V. PRACTICAL APPLICATION OF BIOSUPPLEMENTS

A case study is reported to illustrate the practical full-scale application of bioaugmentation in effluent treatment.

5.1 AIM

To establish the feasibility of bioaugmentation for hydrocarbon oil effluent breakdown to a chemical oxygen demand level of less than 250 mg/ℓ and oil concentration to less than 30 mg/ℓ as required by the applicable municipal regulations.

5.2 BACKGROUND

Approximately 30m^3/day of hydrocarbon oil-containing effluent was produced and discharged into a sump before disposal into the municipal sewer. The effluent contained light and heavy oils. The retention time in the sump was 24 h. The effluent analysis is given in Table 5-1.

Table 5-1. Analysis of hydrocarbon oil effluent before biotreatment

Determinant	mg/ℓ	Units
pH		6.63
COD	1 180	
Total Organic Carbon	97	
Total Kjeldahl nitrogen (TKN)	23,9	
Total Oils	117	
Suspended Solids	79,2	
NH_3-N	9,0	
ortho-PO_4	0,72	
SO_4	15,0	
Cu	0,18	
Fe	0,43	
Zn	0,39	
K	22,0	
Na	21,0	
Mg	1,39	
Ca	9,9	

5.3 INTRODUCTION

The removal of hydrocarbon oils and aromatics may be achieved by microbiological bioaugmentation. However, each effluent is unique demanding a biodegradability study to establish the most efficient augmentation product and dosage regime. Treatment of the effluent, therefore, could not be decided upon without the knowledge of its composition with regard to the pollutants and nutrients present. A biotreatability study was conducted to determine the best treatment option available.

The laboratory-scale evaluation was conducted with the plant layout in mind, so that the treatment to be recommended could be incorporated into the existing infrastructure with minimum change and expense. A mixture of commercially available biosupplements or bacteria obtained from an activated sludge plant were used as inoculum for the laboratory reactors.

The biosupplement was revived and dosed according to the manufacturer's recommendations.

After 24 h, a sample was taken from the reactors. The reactors were then emptied and replaced with fresh effluent simulating the emptying of a sump and refilling. The augmented reactor received a daily dose of 1 mℓ biosupplement and the control 2 mℓ of fresh activated sludge. To prevent the complete loss of bacteria during the daily replacement of the reactor contents, a synthetic material was used as support for bacterial adherence. Immobilization was beneficial, resulting in a reduction of the supplement dosage required to maintain the concentration of bacteria required for the oil degradation after the daily refill with effluent.

5.4 RESULTS

5.4.1 Reduction of COD

The COD was reduced from the influent concentration of 1180 mg/ℓ to 100 to 200 mg/ℓ after 48 hours and maintained at this level throughout the evaluation.

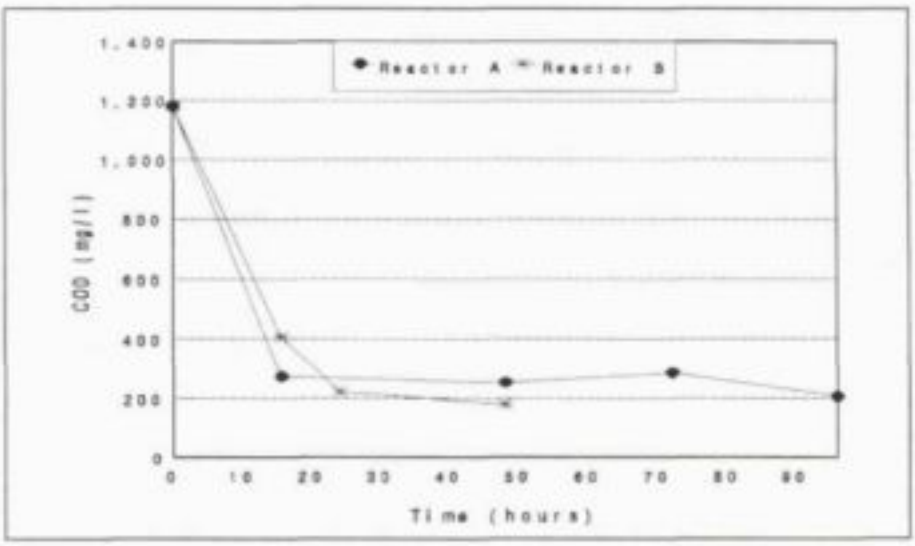

Fig. 5-1. Reduction of COD in reactors A and B initially supplemented with 1 and 10mℓ respectively

The recommended dosage of the particular augmentation product was 8 mℓ for our reactors. However, data in Fig. 5-1 show that the dosage of 1 mℓ was as effective as 10 mℓ and the reduced dosage was subsequently used. It also indicates that the product performs well with respect to COD removal.

5.4.2 Biodegradation of oil

An important aspect of the evaluation was the biodegradation of oil, although COD reduction was the major parameter for consideration. The analyses for oil in raw effluent and after treatment in the laboratory reactors are presented in Table 5-2.

Table 5-2. Analysis of oil in raw effluent before and after biotreatment

Sample	Oil concentration (mg/ℓ)
Raw effluent	190
Control	23
Reactor A	18

Both, the control and reactor A, achieved oil biodegradation to required concentrations within 24 h. While this might indicate that activated sludge could be used in the full scale application, the availability of the augmentation product compared to obtaining fresh activated sludge for daily dosage favoured the use of the commercia product. Its tolerance also to shock loads would be beneficial should the oil concentrations in the untreated effluent fluctuate considerably.

5.5 FULL-SCALE IMPLEMENTATION

The sump was fitted with a diffuse aerator and a medium suitable for immobilization of biosupplement bacteria. Addition of ammonium sulphate as nitrogen source was not required. Although the dosage of product would have to be established on site, it should be of the order of 1 mℓ/ℓ. A minimum hydraulic retention time of 24 h was advised. The point and frequency of dosage would be determined on site.

The estimated cost of implementation of bioaugmentation, at reigning prices in 1994, for the treatment of an effluent similar to that received for the evaluation would be:

Initial investment

Support material (60 m^2)	R 170
Frame for support medium	R 260
Surface aerator, 1,1 kW	R 3,500
Sub-total:	R 3,930

Bioaugmentation product

For startup:	Days 1-2	1kg/d	2,00 kg
	Days 3-4	500 g/d	1,00 kg
	Days 5-11	250 g/d	1,75 kg
	Days 12-18	150 g/d	1,05 kg
	Total product:		5,80 kg
	5,80 kg @ R98/kg		R 570
	Total initial investment:		R 4,500

Maintenance dosage cost

75 g/d for 30 d/month 2,25 kg/month

2,25 kg @ R98/kg R220,50/month

It was anticipated that, with time and the use of the synthetic support medium the dosage could be reduced to approximately 1,1 kg/month. The maintenance treatment cost would therefore amount to R7,30/m^3 effluent which would be reduced to R3,70/m^3 once fully operational.

Comparative cost of existing system

A mechanical moving filter type recovering the oil from the effluent in the sump was in operation. Because of poor maintenance, the system effectively removed approximately 50% of the oil content. Costs incurred for such a system would be for the filter, drive motor and oil sludge disposal. According to the industry, these costs represented an initial investment of R3,300 and monthly running costs for effective removal of oil of R195/month or R6,50/m^3.

Labour costs were not included in both calculations. However, for the oil sludge disposal and maintenance of the oil filter, a labourer was needed for at least 2 h/d compared to 1 h/d for the augmentation schedule once fully operational. Additional savings in running the bioaugmentation schedule could therefore be reflected.

5.6 CONCLUSIONS

The effluent analyses (Table 5-1) indicated the need for nutrient supplementation to obtain a ratio of 100:10:1 as COD:N:P generally accepted as the optimum for bacterial growth. However, the laboratory evaluation showed that added nitrogen and phosphate was not sufficiently beneficial to warrant its full-scale implementation.

The study has shown that the hydrocarbon oil is effectively biodegraded, and the effluent discharged had a COD of < 250 mg/ℓ and an oil concentration < 30 mg/ℓ at a residence time of 24 h. Final treatment costs would be less than the costs before implementation of bioaugmentation although the initial investment in the case study was higher. Daily routine required 1 h labour compared to 2 h for the mechanical oil removal.

In general, the use of bioaugmentation can potentially result in savings based on a higher flow-through rate, more efficient COD removal, suspended solids reduction, removal of odour, degradation of hazardous or toxic material, and overall improvement in operation, cost and effectiveness of the treatment system. The cultures in the bioaugmentation products are designed and selected to resist high concentrations of toxic, hazardous and recalcitrant pollutants in a waste treatment system. The quick recovery and adaptation times of these organisms following a shock load implies little or no downtime following an accidental spill or unfavourable cyclic conditions frequently encountered in normal plant operations. However, it is generally necessary to conduct laboratory evaluations to ensure that the supplement performs as expected. In the case of an emergency and when a treatment works has experienced washout or death of biomass for whatever reason, bioaugmentation can represent an economical and effective means for the quick recovery of a system with minimum downtime. This provides maximum environmental protection at minimum risk.

VI. GENERAL CONCLUSIONS

Biosupplements are imported into South Africa without much control. Their application is meeting with varied success and some products do not perform as expected. The use and implementation of the technology was therefore thoroughly investigated.

Regulatory measures in the USA, Germany and Japan were looked into. A national register is recommended and the procedures to be developed, should use the U.S.EPA regulations as basis but incorporating the simplicity of the German system. The system should attract manufacturers and suppliers of the local market to have their products listed without undue constraints. Listing does not, however, mean endorsement of the product and the onus should reside with the suppliers to prove the efficacy of their product. Adequate safety measures must be provided with the information sheets on products.

It is difficult to provide meaningful specifications on specific products and characteristics for inclusion have been submitted for consideration. The basis for South Africa should follow the USA requirements integrated into the regulatory policy principles proposed for South Africa.

Product performance varied with some successful products evaluated. Phenol, hydrocarbon oils and edible oils could be successfully biodegraded. However, the performance was often similar to that of an activated sludge from a municipal treatment works. The use of biosupplements therefore resides in their ability to tolerate shock loads of specific pollutants and restore the activity to normal after a plant disruption.

The methodology for the development of biosupplements has been outlined. It does entail lengthy investigations and evaluations to ensure the efficacy of the developed product and its safe application. Of particular importance is the preservation of stock cultures by appropriate methods. These methods will in most cases involve freezing at -75°C in the presence of a protective agent such as glycerol. The development of a biosupplement demands a thorough understanding of the catabolic pathways of product to innocuous forms. This will assist in selecting the best group of microorganisms since consortia of microorganisms are generally required for complete biodegradation of toxic, hazardous and often recalcitrant compounds.

The application of the technology requires a through knowledge of the composition of the effluent to be treated before contemplating bioaugmentation.

Future research should concentrate on microbial ecosystems and study the synergistic interactions which play an important role in the treatment of specific compounds in an industrial effluent. Treatment at source probably offers the best chance for the successful treatment before discharge into the sewer. Industry should become more closely involved in these investigations.

The technology has been placed on a sound basis for further implementation in South Africa. Specialist technical committees should be appointed for the compilation of regulatory requirements, product specifications and listing. The authorities responsible for following up on the regulatory and other requirements should be the departments of Water Affairs, Health and Environment.

VII. TECHNOLOGY IMPLEMENTATION

The application of bioaugmentation in wastewater treatment was originally the result of efforts to solve urgent operational problems, such as shock loads in treatment plants or to make a remedial response to spilling emergencies. In most cases addition of bacterial cultures either assisted the operation to return to normal or helped reduce the danger of the spilling pollutant. These successful applications have stimulated the application of bioaugmentation to municipal treatment works. They have also led researchers to conduct various studies aimed at a better understanding of bioaugmentation. However, a number of cases were reported with little or no advantage of bioaugmentation on the improvement of performance of treatment works (Yu & Hung, 1992).

Bioaugmentation is being used in South Africa with varied success. This stresses the need for proper feasibility assessments before embarking on full scale implementation. A number of commercial biosupplements do not perform as claimed and reliable manufacturers and suppliers of quality biosupplement products for the local market must be identified.

It is recommended that legislative requirements and guidelines for the successful implementation of the technology be compiled for South Africa. These should aim at simplicity of implementation with minimum administrative control. The onus should be on the manufacturer/supplier to ensure that the product offered performs as expected. A national register should be prepared with listings of products available for a specific application. Listing, however, does not imply endorsement of the product by the authorities responsible for the listing. This would require the formation of a specialist technical review committee to reconsider listings from time to time. The committee should also be responsible for the compilation of the guidelines based on the U.S.EPA and German regulations as reported on in this investigation. The responsibility for bioaugmentation technology implementation and control should reside with the departments of Water Affairs, Health and Environment.

Proper product specifications must be made available and safety measures adhered to. These requirements demand inputs by expert scientists and health authorities.

The implementation of bioaugmentation technology must minimize risk to the end-user. Exploitation of the technology by incompetent suppliers must be safeguarded against and the end-user protected, thereby ensuring the success of the technology in the country.

Microorganisms are rarely, if ever, found in pure cultures in nature. Microbial communities in the natural environment represent a complex ecosystem. Application of biosupplements must therefore ensure that the added cultures are present in sufficient numbers at all times to be able to degrade the desired pollutants in the presence of the large numbers of indigenous microorganisms required for the biodegradation of the competing nutrients and more easily degradable pollutants.

VIII. FUTURE RESEARCH REQUIREMENTS

The project has successfully demonstrated the potential of bioaugmentation in South Africa. However, the expected performance of biosupplements has not always been experienced. Implementation of the technology will therefore require proper feasibility assessments to be conducted by competent institutions in collaboration with the supplier and end-user.

Research is required for the specific applications of biosupplements in the biodegradation of toxic and hazardous substances such as polychlorinated biphenyls and other substances that may be identified. Special applications in the field of bioremediation are indicated. Industry should become more closely involved with this research.

Research efforts should concentrate on the interactions between microorganisms existing and developing in complex communities. Shifts in populations are bound to occur and which are currently poorly understood. The influence of available nutrients, either in excess or if deficient, will influence the performance of the treatment works. Research into obtaining a better understanding of such complex micro-ecosystems will enhance our understanding of the biology of wastewater works, especially in cases where treatment is necessary at the source of the toxic or hazardous wastewater. The concentrations of such pollutants is generally high at the source which poses a considerable challenge to achieve successful treatment on site to levels acceptable for discharge in a municipal sewer.

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APPENDIX

A.1 EVALUATION OF PRODUCT PERFORMANCE

It is important to evaluate the performance of biosupplements on industrial effluent, and not only the synthetic compound, since the effluent may contain toxic components which should at least be tolerated, if not co-degraded, by the biosupplement. Otherwise, such toxic components have to be removed before treatment.

Typical examples of using bioaugmentation in effluent treatment are reported in this section. The biodegradation of phenol, hydrocarbon oils and edible oils from industrial effluents were investigated. The composition of the three effluents used in this evaluation are presented in Table A-1.

Table A-1. Chemical composition of the three effluents used in the investigation

Parameter	Gas Works (Phenol)	Transport (Hydrocarbon)	Edible oils (Lipids)
pH	9,3	6,7	4,9
COD	7025	915	4879
Ammonia-N	1728	4,7	5,5
Nitrate-N	1,8	1,3	1,7
o-Phosphate	3,4	2,4	6,4
TKN	2100	12,2	13,1
Fats + Oils	-	150	1480
TDS	716	467	7515
Sodium	39	100	1771
Calcium	2	31	31
Magnesium	18	13	21
Sulphate	602	267	4113
Iron	3,05	3,3	4,6
Phenol	3725	1021	-

Note: All concentrations in mg/ℓ; pH in units

The effluents were diluted where necessary to obtain a maximum COD concentration of 2 500 mg/ℓ before being fed into the aerobic reactors. The reactors were aerated and the dissolved oxygen (DO) was maintained at levels >1 mg/ℓ. The pH was kept constant between 6,5 and 8,0, and nutrients (o-PO_4 and NH_3-N) were added to the reactors if the concentration levels were <1 mg/ℓ. In the case of the gas works effluent, the pH was adjusted to 10,5 and the effluent was aerated overnight to strip off the excess ammonia which would be toxic to the reactor biomass. The final pH of the reactor was 7,5.

Activated sludge from the Daspoort Works of the Municipality of Pretoria was used as control and compared with the performance of a commercial biosupplement formulation. Alternatively, activated sludge from the Pretoria Rooiwal Works was used.

A.1.1 Phenol degradation in a gas works effluent

The results of phenol removal using Daspoort activated sludge as control are presented in Fig. A-1. Phenol was removed well by the control and almost equalled the performance by the commercial biosupplement. Daspoort has a history of good phenol removal and the results were therefore not surprising. The commercial biosupplement tolerated phenol at concentrations of at least 1,6 g/ℓ. The initial phenol concentration was reduced from 1600 mg/ℓ to 3,3 mg/ℓ in the control and 2,4 mg/ℓ by the biosupplement.

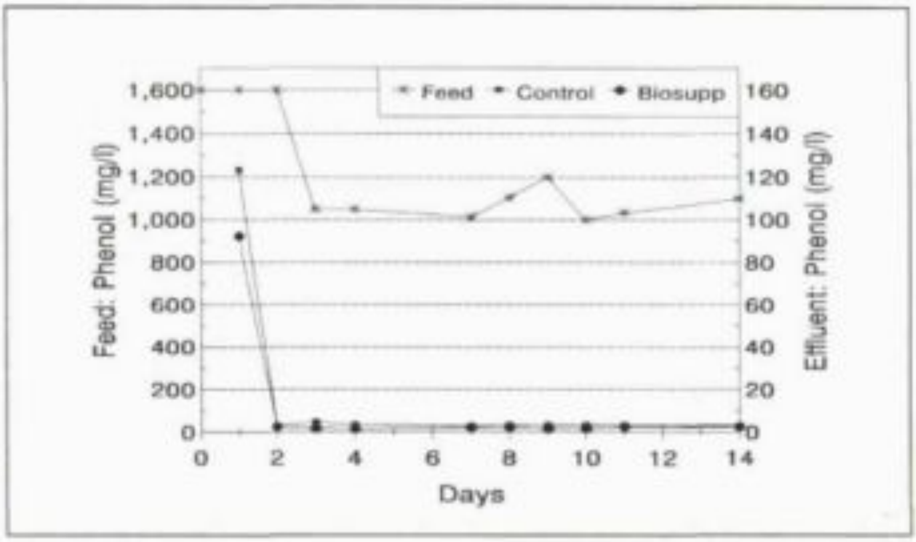

Fig. A-1. Phenol removal from gas works effluent using Daspoort activated sludge as control

To investigate the importance of the source of activated sludge as control, activated sludge from the Rooiwal Works was used in the repeat experiment. The results are shown in Fig. A-2.

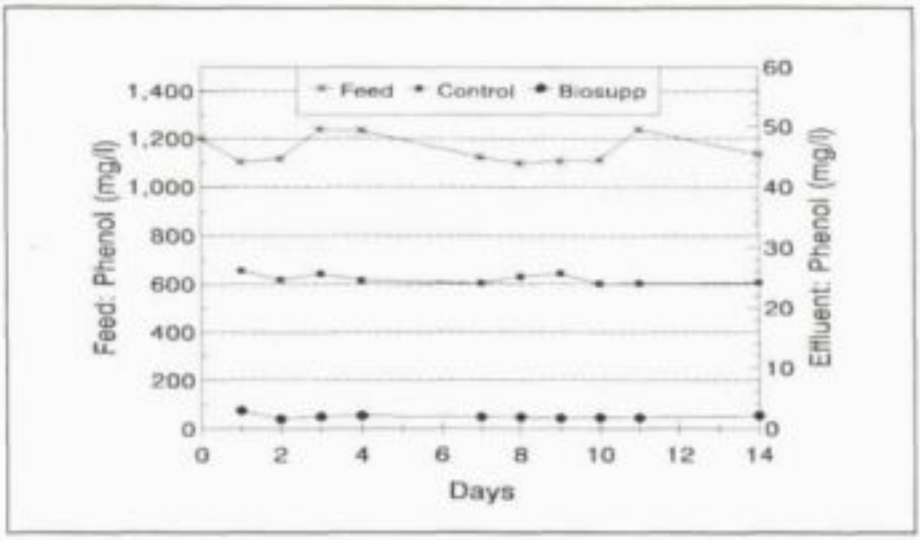

Fig. A-2. **Phenol removal from gas works effluent using Rooiwal activated sludge as control**

The Rooiwal activated sludge performed well but was less efficient than the Daspoort sludge. This clearly illustrates the importance of specifying the source of control sludge used should such a bioreactor be included for comparative purposes.

A.1.2 Hydrocarbon oil degradation in an industrial effluent

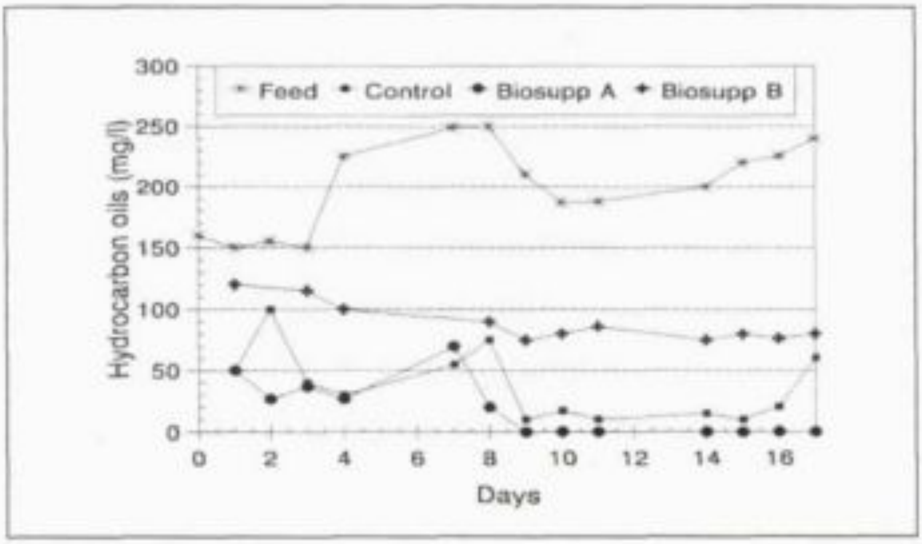

Fig. A-3. **Removal of hydrocarbon oils from effluent**

The results of the hydrocarbon oil removal from an oil-contaminated effluent are illustrated in Fig. A-3. Biosupplements were dosed according to the specifications of the manufacturer. They were claimed to remove hydrocarbon oils to < 20 mg/ℓ. Approximately 79% of hydrocarbon oils were removed by the control compared to 90%

by the commercial biosupplement A. Biosupplement B did not perform as expected. It is therefore important to conduct laboratory trials before full-scale implementation is contemplated to ensure that the products perform as expected.

A.1.3 Edible oil degradation in an industrial effluent

The results of edible oil removal from an effluent are illustrated in Fig. A-4. The control reactor with Daspoort activated sludge reduced the oil levels to between 100 and 200 mg/ℓ. The commercial biosupplement performed better with a removal to between 0,1 and 25 mg/ℓ.

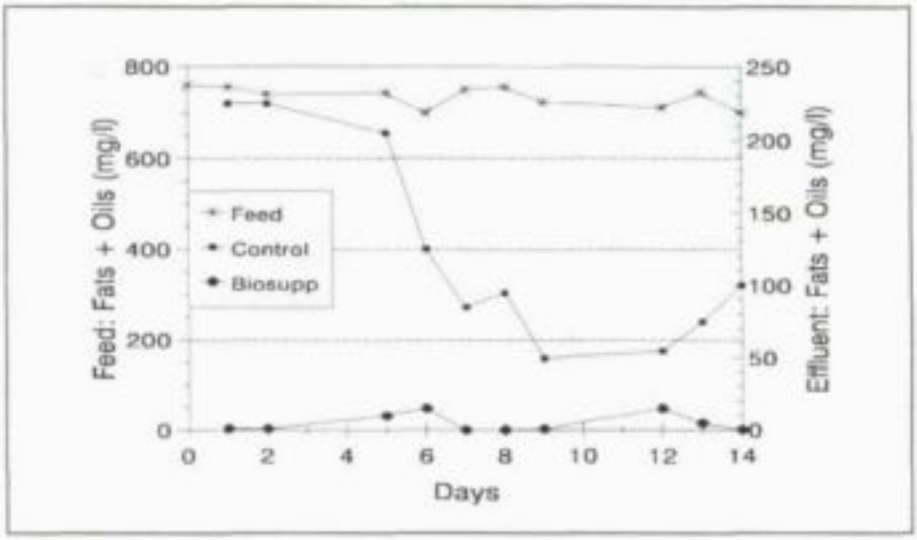

Fig. A-4. **Removal of edible oils form an effluent**

A.1.4 Miscellaneous product evaluations

A product which was claimed to degrade lignins in pulp and paper effluent was evaluated. It is known that lignins are difficult to degrade and the product did not perform well. Long exposure times were required and no benefit could be derived from bioaugmentation.

A number of products claimed to reduce suspended solids and COD in municipal effluent were tested. In no case could the suspended solids be reduced and the COD reduction was marginal compared to a control activated sludge. The settling properties of the suspended solids were improved resulting in their displacement to the bottom of the clarifier. This again highlights the importance of conducting laboratory investigations before full-scale implementation at a treatment works.

Should a treatment plant be disrupted by toxic compounds, it may be the most economical to introduce active biomass from another works which performs well, rather than purchasing biosupplements since sludge wastage is common in all treatment works. This does not imply, however, that bioaugmentation is not a viable option and the indiv:dual circumstances of the disruption must be carefully considered to arrive at the correct decision.