

UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA VEGETAL



*Performance evaluation of microbial inocula as
biological reference material for
biodegradability and ecotoxicity tests*

Susana Maria Teixeira Paixão Alves

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On the Cover - Scanning or transmission electronic microscopy photos of some bacteria from soil and sewage: *Nitrosomonas* spp. (left, Copyright ©Dennis Kunkel Microscopy, Inc.), *Bacillus subtilis* (center and bar, Copyright ©Kansai International Public Relations Promotion Office), *Pseudomonas* sp. (right, Copyright ©James A. Sullivan - www.cellsalive.com).

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***Avaliação de inóculos microbianos como material
biológico de referência para testes
de biodegradabilidade e ecotoxicidade***

Susana Maria Teixeira Paixão Alves

Tese orientada pelo Prof. Doutor Rogério Paulo de Andrade Tenreiro, da FCUL,
e pela Doutora Ana Maria Carneiro Anselmo, Investigadora Principal do INETI

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Na presente dissertação incluem-se resultados que foram alvo de publicação com outros autores. Para efeitos do disposto no nº 2 do Art. 8º do Decreto-Lei 388/70, a autora da dissertação declara que interveio na concepção e execução do trabalho experimental, na interpretação dos resultados e na redacção dos manuscritos publicados ou enviados para publicação.

Lisboa, 28 de Fevereiro, 2005

Susana Paixão Alves

(Susana Maria Teixeira Paixão Alves)

To my parents, my husband, and my son with love.

Whenever a new discovery is reported to the scientific world, they say first, "It is probably not true". Thereafter, when the truth of the new proposition has been demonstrated beyond question, they say, "Yes, it may be true, but it is not important". Finally, when sufficient time has elapsed to fully evidence its importance, they say, "Yes, surely it is important, but it is no longer new".

Michel de Montaigne

"The farther one pursues knowledge, the less one knows."

Lao Tzu

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Abbreviations

AS – Activated sludge	MC – Model community (CM – comunidades modelo)
BOD – Biochemical oxygen demand	MITI – Japanese Ministry of International Trade and Industry
BOD₅ – 5-Day biochemical oxygen demand, (CBO₅ – Carência bioquímica de oxigénio - 5 dias)	NOAEL – No-observed-adverse-effects-level
BRM – Biological reference material	NOEC – No-observed-effect-concentration
CEN – Comité Européen de Normalisation	NP – Norma Portuguesa (Portuguese norm)
EN – European Norm	O₂ – Oxygen
CFU – Colony forming units	OECD – Organization for Economic Cooperation and Development
ChV – Chronic value	PC – Principal components
CI or IC – Confidence interval	PCA – Principal component analysis
CLPP – Community-level physiological profile	PEC – Predicted environmental concentration
CO₂ – Carbon dioxide	PNEC – Predicted no effect concentration
COD – Chemical oxygen demand	r – Correlation coefficient (Pearson)
DI – Designed inoculum	r² – Determination coefficient
DL – Deliverable	RB – Ready biodegradability
DOC – Dissolved organic carbon	RM – Reference material
EC – Effective concentration	SCAS – Semi-continuous activated sludge
EC₅₀ – Median effective concentration	SD – Standard deviation
ECO – BIOLOG Ecoplate	ST – Stages
EEC – European Economic Community (⇒ EC = European Community)	ThCO₂ – Theoretical amount of carbon dioxide
ELV – Emission limit values	ThOD – Theoretical oxygen demand
ESR – Existing substances regulation	TI/RE – Toxicity identification / reduction evaluations
EU – European Union	TPI – Treatment plant inoculum
GN – Gram-negative	TOC – Total organic carbon
GP – Gram-positive	TU – Toxic unit
IB – Inherent biodegradability	UPGMA – unweighted pair group method of arithmetic averages
IC₅₀ – Median inhibitory concentration	USEPA – United States Environmental Protection Agency
ISO – International Organization for Standardization	VC or CV – Variation coefficient
LC₅₀ – Median lethal concentration	WET – Whole effluent toxicity
LOEC – Lowest-observed-effect-concentration	WTP(s) – Wastewater treatment plant(s)

Resumo

A avaliação dos riscos ambientais de substâncias, efluentes ou qualquer outro poluente passa obrigatoriamente pela realização de um conjunto de ensaios normalizados, que permitem obter informações relativas aos potenciais efeitos no ambiente, nomeadamente no que diz respeito à sua biodegradabilidade e ecotoxicidade, entre outros.

De facto, a determinação da biodegradabilidade é essencial para avaliar o destino final de poluentes orgânicos e prever em parte o seu impacte ambiental, visto que as consequências da sua introdução no ambiente dependem essencialmente da rapidez com que são eliminados pelos organismos vivos.

A toxicidade é uma propriedade inerente a todas as substâncias ou misturas de produtos químicos, a qual descreve o seu potencial para produzir efeitos adversos nos organismos vivos. A avaliação da biodegradabilidade e da toxicidade de poluentes orgânicos libertados no ambiente é realizada utilizando bioensaios, i.e. testes que utilizam organismos vivos. Devido ao seu importante papel ecológico, as bactérias são muito utilizadas para a realização de bioensaios, quer como organismo de ensaio para determinar o efeito tóxico de um poluente ou como inóculo para testes de avaliação da biodegradabilidade.

Existem diversos tipos de testes de biodegradabilidade e de toxicidade normalizados, que utilizam bactérias para avaliar o destino dos poluentes e o seu impacte ambiental e muitos destes testes utilizam as lamas activadas, um inóculo microbiano heterogéneo de uma estação de tratamento biológico de águas residuais, tratando essencialmente resíduos domésticos, como cultura microbiana padrão.

Esta utilização, que remonta ao princípio do século passado, relaciona-se directamente com a necessidade de avaliar o oxigénio necessário para oxidar a matéria orgânica carbonácea presente no efluente doméstico a tratar, por via biológica.

Em estudos de biodegradabilidade/tratabilidade, os métodos normalizados mais utilizados são:

- Teste da carência bioquímica de oxigênio (CBO), método das diluições e método manométrico. Implica a determinação da quantidade de oxigênio dissolvido necessário para oxidar um poluente orgânico utilizando para o efeito microrganismos.
- Teste para a inibição do consumo de oxigênio pela lama activada. Esta norma internacional especifica um método para determinar o efeito inibitório de um dado poluente a testar no consumo de oxigênio pelos microrganismos da lama activada. O efeito inibitório pode incluir o efeito na respiração e na nitrificação. Este método dá informação acerca dos efeitos inibitórios ou estimulantes após uma curta exposição (3 horas) do poluente a testar aos microrganismos da lama activada.
- Teste de Zahn e Wellens. Teste para avaliar a biodegradação final potencial de substâncias orgânicas não voláteis solúveis em água, quando expostas a concentrações relativamente elevadas de microrganismos num ensaio estático, de longa duração, tendo em consideração a capacidade adaptativa das estirpes microbianas presentes.

Estes testes têm sido também utilizados para avaliar a eficiência de tratamentos, em termos da biodegradabilidade e toxicidade, de efluentes contendo compostos químicos recalcitrantes ou inibidores para os microrganismos normalmente encontrados nos processos de tratamento biológico tradicionais.

No entanto, as lamas activadas são compostas por uma comunidade mista de microrganismos competitivos, que crescem devido às substâncias orgânicas presentes na água residual. Portanto, as comunidades microbianas presentes nas lamas activadas dependem das condições ambientais, da composição da água residual influente e do modo de funcionamento da estação de tratamento. Variações na comunidade microbiana tornam impossível a realização de ensaios controlados utilizando lamas activadas, causando problemas na reprodutibilidade dos resultados dos testes biológicos e portanto na comparação de diferentes resultados.

Além disso, a utilização de um inóculo microbiano, proveniente de uma estação de tratamento de lamas activadas, levanta problemas de diversa ordem: dificuldades na sua

obtenção, manutenção da viabilidade metabólica, impossibilidade prática de controlo das características microbiológicas, e eventual presença de organismos patogénicos.

Estes factos têm vindo a dificultar a normalização de análises e ensaios biológicos que envolvem a utilização de inóculos de lamas activadas, indicando uma necessidade óbvia para a existência de um material biológico de referência padronizado, o qual deverá ser biologicamente definido, estável, pronto a usar e apresentar as mesmas capacidades metabólicas do que as existentes nas lamas activadas.

Neste âmbito, o objectivo principal deste trabalho foi desenvolver e propor pelo menos um material biológico de referência padronizado, para ser utilizado como inóculo alternativo em bioensaios normalizados baseados no uso de lamas activadas, nomeadamente os que estão relacionados com a determinação da biodegradabilidade para substâncias ou efluentes ou com a avaliação da toxicidade. Concomitantemente, estudou-se o efeito de diferentes procedimentos utilizados nas etapas de preparação do inóculo, descritas nas referidas normas, de forma a avaliar o seu efeito no resultado final do teste realizado. Isto permite definir um procedimento com regras específicas e normalizado para a preparação do inóculo, uma vez que esse procedimento pode afectar a composição qualitativa/quantitativa da estrutura da comunidade microbiana do inóculo, contribuindo também como fonte adicional de erro.

Deste modo, cada material biológico de referência foi preparado de forma a cumprir os seguintes critérios previamente definidos:

- apresentar um perfil metabólico semelhante ao das lamas activadas;
- ser um consórcio microbiano composto por um número reduzido de estirpes bacterianas (3-6), de modo a permitir a sua normalização, controlo e produção;
- ser formado exclusivamente por bactérias heterotróficas, as quais são consideradas as principais responsáveis pela degradação da matéria orgânica e actividade metabólica em geral;
- ser uma mistura de culturas bacterianas homogénea e estável;
- não conter microrganismos patogénicos, nem bactérias nitrificantes;

- não conter microrganismos adaptados a determinados poluentes; e
- apresentar adequabilidade como inóculo alternativo nos bioensaios baseados no uso de lamas.

De facto, a utilização de inóculos microbianos adequados e padronizados, como culturas representativas, pode actuar como um complemento e importante ferramenta suplementar aos testes que utilizam as lamas activadas, contribuindo para uma maior precisão e reprodutibilidade dos resultados desses bioensaios.

Neste contexto, numa primeira fase deste trabalho, diversas amostras de lamas activadas foram recolhidas e preparadas de acordo com dois procedimentos diferentes, geralmente descritos nos testes normalizados para a preparação do inóculo. Estes inóculos de lamas activadas foram caracterizados microbiologicamente para padrões de referência, estabelecendo-se os seus perfis metabólicos com base na utilização dos diversos substratos contidos nas microplacas BIOLOG (GN, GP, ECO). Os resultados deste estudo mostraram que o procedimento utilizado na preparação do inóculo influencia o seu comportamento final, traduzido no respectivo perfil metabólico exibido (ex. diminuição do número de substratos utilizados pela comunidade com a lavagem do inóculo de lamas).

Do mesmo modo, foram traçados perfis metabólicos individuais para bactérias heterotróficas não patogénicas, quer estirpes predominantes ou comuns isoladas das lamas activadas, quer estirpes equivalentes seleccionadas de colecções de culturas. Estes dados metabólicos (respostas para 31 ECO, 95 GN e/ou 95 GP substratos) foram comparados com os perfis das lamas activadas, utilizando métodos estatísticos adequados, para determinar as estirpes relevantes que mais contribuíam para os perfis metabólicos das lamas. Com base nestes resultados, diversos consórcios microbianos padronizados, i.e. comunidades modelo (CM) compostas por 3 a 6 estirpes bacterianas, foram preparados e analisados como inóculos representativos em termos da sua semelhança metabólica com os perfis das lamas activadas.

Simultaneamente, outros três consórcios padronizados foram analisados como culturas representativas das lamas relativamente aos seus perfis metabólicos: um inóculo de solo

padronizado (“designed inoculum” - DI) - consórcio composto por 6 bactérias não patogênicas seleccionadas de estirpes isoladas de solos, uma vez que as suspensões de solo são também descritas como um inóculo opcional às lamas activadas em alguns testes biológicos (ex. teste do CBO), e dois inóculos comerciais - o BI-CHEM[®] e o BIOLEN M112, misturas de culturas bacterianas especializadas e adequadas como materiais biológicos.

De modo a identificar os potenciais efeitos da utilização de diferentes passos no procedimento da preparação do inóculo nos perfis metabólicos finais destes três consórcios, vários passos de preparação, normalmente descritos nos testes normalizados, foram aplicados aos referidos consórcios. Os resultados demonstraram que diferentes meios de cultura conduzem a diferentes comunidades modelo (CM), apesar de terem origem no mesmo consórcio inicial, traduzido pelo diferente perfil metabólico exibido por cada uma das CM resultantes. Do mesmo modo, a densidade celular inicial do inóculo microbiano também influencia o seu perfil metabólico.

Neste trabalho, a exibição de um comportamento metabólico semelhante aos das lamas activadas era um pré-requisito para seleccionar culturas mistas representativas que pudessem ser um inóculo alternativo ao das lamas, apesar de também ter sido testada uma cultura pura, de *Pseudomonas* sp., como representante das bactérias heterotróficas ubíquas no ambiente aquático e terrestre e contendo um elevado potencial metabólico. Com base na análise dos dados metabólicos obtidos para as diversas CM e para as lamas activadas, utilizando técnicas estatísticas multivariadas e de correlação, as comunidades CM5 (DI), CM9 (BIOLEN) e a CM12 (BI-CHEM[®]) foram as que apresentaram a maior semelhança com os perfis das lamas activadas.

Consequentemente, das várias CM caracterizadas metabolicamente apenas três foram avaliadas como inóculos alternativos às lamas activadas em testes biológicos seleccionados. Assim, o potencial e a sensibilidade do BI-CHEM[®], do BIOLEN e do DI, como materiais de referência, foram avaliados em testes de biodegradabilidade (teste do CBO₅, teste Zahn-Wellens) e de toxicidade (teste da inibição do consumo de oxigénio), sendo os resultados comparados e correlacionados com os obtidos utilizando inóculos de lamas activadas. Os

bioensaios foram realizados com os produtos químicos de referência, para validar o procedimento e a viabilidade do inóculo microbiano utilizado. Um significativo número de réplicas (≥ 10) foi também realizado (teste do CBO_5 e teste de toxicidade) para permitir comparar a repetibilidade e a variabilidade observada nos resultados com os potenciais materiais de referência e com as lamas activadas. A biodegradabilidade foi avaliada para a solução padrão de glucose-ácido glutâmico (teste do CBO_5 , método das diluições ou método manométrico) e para o dietilenoglicol, DEG (teste Zahn-Wellens), e a inibição das taxas respiratórias dos microrganismos foram estimadas para o 3,5-diclorofenol (3,5-DCP).

Os resultados obtidos com estes inóculos para o CBO_5 da solução padrão variaram entre 176,8-216,9 mg/l, pelo método das diluições, e entre 209,2-228,6 mg/l, pelo método manométrico, situando-se dentro da gama descrita para as lamas activadas, $198 \pm 30,5$ mg/l.

No teste Zahn-Wellens, os perfis das curvas de biodegradação para o BI-CHEM[®] e o BIOLÉN foram semelhantes entre eles e dependeram da concentração do DEG testada, cumprindo o critério de validade ($>70\%$ biodegradação aos 14 dias) apenas no ensaio com a menor concentração de DEG. Para o DI, as curvas de biodegradação do DEG obtidas mantiveram um perfil semelhante para as duas concentrações testadas (400 ou 600 mg/l) com declives acentuados, apresentando uma degradação completa do DEG em 12-14 dias, em menos tempo do que os inóculos comerciais. Estes resultados foram os mais semelhantes aos obtidos com as lamas activadas, as quais apresentaram uma degradação completa do DEG em 10-11 dias; no entanto, a duração da fase de biodegradação foi idêntica à observada para o DI diferindo essencialmente na fase de adaptação. Os resultados para a degradação do DEG, pelo DI, estão de acordo com o critério de validade para o inóculo de referência do teste Zahn-Wellens. O potencial biodegradativo do DEG e de outros dois compostos de referência (benzoato de sódio e acetato de sódio) foi também avaliado utilizando placas BIOLOG MT. Contrariamente ao DEG, que só foi utilizado pelas lamas activadas, os outros dois compostos foram biodegradados por todos os inóculos durante os três dias de incubação.

Os valores de toxicidade aguda (IC_{50}) obtidos variaram entre 9,39-22,66 mg/l, tendo sido o BI-CHEM[®] o mais sensível e com o comportamento mais semelhante ao das lamas activadas testadas; no entanto, os resultados obtidos com os restantes inóculos microbianos incluem-se todos na gama estipulada na norma do teste para o 3,5-DCP (5-30 mg/l).

Em conclusão, o DI salienta-se como material de referência nos testes de biodegradabilidade, enquanto o BI-CHEM[®] parece ser a alternativa mais adequada no teste de toxicidade, devido à sua sensibilidade ser mais semelhante à observada para as lamas activadas. Além disso, em termos de potencial metabólico global, o DI e o BI-CHEM[®] foram os inóculos mais representativos dos perfis metabólicos das amostras de lamas activadas, entre os vários consórcios microbianos testados.

Adicionalmente, para além de outros inóculos também estudados, o DI e o BI-CHEM[®] foram validados como material de referência para o teste do CBO_5 (método manométrico e método das diluições), testando-se diversas amostras reais (efluentes) num estudo comparativo com um inóculo de uma estação de tratamento (TPI). As elevadas correlações ($r: 0,96 - 1,00$, $p < 0,05$, $n \geq 7$) observadas entre os resultados obtidos com o DI ou o BI-CHEM[®] e o TPI, independentemente do método utilizado, mostraram que o teste do CBO_5 com qualquer um destes consórcios microbianos como material de referência é válido. O método manométrico utilizando um destes materiais de referência pode ser uma boa metodologia a adoptar para a determinação do CBO_5 de poluentes, em estudos de biodegradabilidade/tratabilidade, devido à sua fácil manipulação.

Passado mais de um século sobre a utilização das lamas activadas como cultura microbiana padrão, o conhecimento científico e a experiência adquirida na realização dos testes normalizados envolvendo o uso desta cultura mista microbiana permitem repensar a sua função, nomeadamente em termos de protecção ambiental. Proteger obriga ao estabelecimento de limites, os quais devem garantir a inocuidade vista sobre um ponto de vista integrado, ou seja, do equilíbrio populacional das espécies presentes num determinado ecossistema. Nesta perspectiva, consórcios microbianos definidos, de fácil manutenção e

manipulação, deverão ser utilizados para a normalização de ensaios biológicos, conduzindo a uma maior qualidade no controlo e monitorização da poluição ambiental.

Palavras-Chave: materiais biológicos de referência, comunidades modelo, perfis metabólicos, BIOLOG, lamas activadas, biodegradabilidade, toxicidade.

Abstract

Several biological methods employ activated sludge (AS) as inoculum to assess toxicity or biodegradability of pollutants, since pollutants that ultimately enter the environment are often discharged through wastewater treatment plants. The use of AS to evaluate the environmental impact of chemicals and wastewaters suffers from several drawbacks related with the heterogeneity, absence of standardization and health risk associated with this mixed sewage population.

To search for reliable testing inocula alternatives, the potential of several well-defined microbial inocula as reference material, for ready and inherent biodegradability and toxicity screening tests (BOD₅ test, Zahn-Wellens test and respiration inhibition test, respectively), was evaluated and the results were compared with AS' results. In overall, the most relevant results in terms of environmental application were obtained with bacterial mixtures, namely with three standardized microbial consortia: two commercial inocula (BI-CHEM[®] and BIOLEN M112) and a designed inoculum (DI, a standardized soil inoculum). A community-level physiological profile (CLPP) analysis was also performed for each inoculum tested, being its metabolic fingerprint compared with AS' CLPPs.

From biodegradability results obtained, DI stands out a good choice as reference material for biodegradability tests, while from toxicity results the BI-CHEM[®] appears as the suitable alternative to AS, since its sensitivity is similar to that of AS. Moreover, in terms of the overall metabolic potential, DI and BI-CHEM[®] were the most representative inocula of AS' CLPPs among the consortia tested.

In addition, beyond other inocula also studied, DI and BI-CHEM[®] were validated as seed for BOD₅ test, by testing several real samples in a comparative study with a treatment plant inoculum.

The use of well-predefined microbial consortia, with simple maintenance and manipulation, can be helpful for the normalisation of biological assays leading to an improvement of the quality of chemical and effluent testing and monitoring.

Keywords: biological reference materials, model communities, community-level-physiological profile, BIOLOG, activated sludge, biodegradability and toxicity tests.

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Chapter 1

POLLUTION AND ENVIRONMENTAL SCIENCES

With the increased worldwide industrialization and with the concomitant higher demand for chemicals, both the technologically advanced and developing nations faced increasing ecological and toxicological problems from the release of toxic contaminants to the environment.

The environment consists of various types of ecosystems; these include: air, water (freshwater or marine), soil/sediments, and various forms of biota. The effects of any pollutant on such a diverse system are further influenced by several factors, such as chemical, physical, biological, geological, climatic, and socio-economic and their interrelationships.

The dramatic increase in public awareness and concern about the state of the global and local environments that has occurred in recent decades has been accompanied and partly prompted by an ever-growing body of evidence on the extent to which pollution has caused severe environmental degradation. The introduction of harmful substances into the environment has been shown to have many adverse effects on human health, agricultural productivity and natural ecosystems (Alloway and Ayres, 1997).

Environmental contamination can be defined as a human action able to modify properties of environmental conditions or the availability and quality of resources over a given space range and time interval. Environmental contamination does not necessarily imply measurable damage to living organisms. On the other hand, environmental pollution occurs when contamination produces measurable damage to single organisms, populations, or biological communities (Bacci, 1994).

The first approaches to the study of environmental contamination were essentially directed toward preserving human health from the possible backlash of harmful substances released in the environment.

The main objective of toxicology is to assess the toxic effects chemicals may cause, both under the analytic plan as well on the physiologic and biochemical point of view. This science is at the same time descriptive and explicative, searching to precise the action mechanisms of the toxics. The whole research, from analytic point of view, of harmful substances present in a variety of media or inside of the living organisms is related with this discipline, as well as the evaluation of the toxicity of the pollutants on the living organisms (Ramade, 1979). Traditionally, based in a pharmacological background, toxicology was mainly concerned with the health effects of chemicals on humans, studying the effects in individual organisms, using a limited number of species of mammals, usually rodents (Francis, 1994; Lynch and Wiseman, 1998).

Later, the idea that the human health cannot be protected unless in conjunction with wildlife protection, led to a definition of a new branch in environmental sciences, ecotoxicology. The term was introduced by René Truhaut, in 1969, who defined ecotoxicology as a branch of the toxicology dealing with direct and indirect effects of natural substances and xenobiotics on living organisms (Bacci, 1994; Hoffman *et al.*, 1995). Ecotoxicology is the study of toxic or adverse effects of chemicals on biota, particularly on communities and populations, and their interactions with processes controlling the functioning of defined ecosystems. So, ecotoxicology, or environmental toxicology, can be defined as the study of the fate and effect of toxic agents on ecosystems (Bascietto *et al.*, 1990; Cairns and Mount, 1990, Hoffman *et al.*, 1990). This implies that the environmental chemistry is also involved, attempting to describe, quantify and understand the environmental distribution and

transformations of chemicals released in the environment. Ecology is obviously implied in the evaluation of the effects on the ecosystems, which substantially differ from the effects on individual organisms or populations: for example, the death of an individual or the disappearance of a population are dramatic for whoever dies or disappears, but may have no significant influence on the functions of an ecosystem. On the other hand, subtle sublethal effects on certain species may produce significant ecological modifications (Bacci, 1994).

Therefore, ecotoxicology is an interdisciplinary field of science that has been developed over the last 60 years, and its main supporting disciplines are toxicology, environmental chemistry and ecology. The goal of ecotoxicology consists of producing criteria for the prevention or minimization of contamination. Ecotoxicology can also apply predictive instruments able to produce criteria even for hypothetical chemicals or potential contaminants, before their release into the natural system. Ecotoxicology developed along two lines, first as an applied field within the regulatory framework towards the environmental protection, and secondly as a more scientific field dedicated to the mechanisms underlying the fate of the pollutants in the environment and the effects on organisms (Lynch and Wiseman, 1998).

CHEMICAL IMPACT ASSESSMENT: REGULATORY NEEDS

The total number of existing chemicals is enormous and the global use of synthetic organic chemicals is increasing annually in number and diversity. Regulatory agencies around the world face an enormous challenge in the environmental management of chemicals. This is due to the vast array of chemicals that require evaluation of their potential environmental behaviour, fate processes, and their adverse effects. The regulatory framework is continuing to evolve with the increase

in the knowledge about the environmental effects of chemicals. Environmental science must continue in its search for the most appropriate assessment tools. The aim should be to distinguish between actual means of meeting legislated regulatory objectives and the scientific knowledge to achieve the ultimate goals set forth in the legislation. Thus, scientific data and understanding of the chemicals and their effects are needed not only to support the existing regulations but, more importantly, also to guide the continuous process of developing better regulations and guidelines.

Specific data requirement varies with the type of chemical, applicable regulation, and the component of the environment to be protected. Regulations on toxic substances control and environmental protection deal with an array of chemicals that may or may not prove to be toxic, may have levels at which they do not cause adverse effects, and may and may not enter ecological systems. A recommended base set for the premanufacture testing of new chemicals, by USEPA (United States Environmental Protection Agency) and OECD (Organization for Economic Cooperation and Development), basically contains three types of data required in assessing environmental effects of a pollutant: (1) physical/chemical data (water solubility, partition coefficient, dissociation constant, hydrolysis (as a function of pH), etc); (2) ecotoxicity data (acute and chronic toxicity); and (3) degradation/accumulation data (ready and inherent biodegradability; bioaccumulation) (Ramamoorthy and Baddaloo, 1991).

In Europe there is much debate at present concerning the assessment of risks and their management from chemicals produced in the industrial domain. This is being motivated by a perception of failure to deliver by current legislation, for example on existing substances. According the Existing Substances Regulation (ESR) or the EEC Council Regulation 793/93, the evaluation of the existing chemicals is carried out by four steps, namely data collection, priority setting, risk assessment, and, if

necessary, risk reduction (Hansen, 1999). Equally, the development of thinking associated with the Water Framework Directive (2000/60/EC) is raising issues about defining quality in the ecological domain. Moving from observations on changes picked up by monitoring and surveillance of the ecological domain, retrospectively, to their causes in the industrial domain challenges science. The development of relevant, but reliable robust test systems will therefore be of crucial importance in completing this causal chain and developing appropriate and cost-effective management programmes (Calow, 1999). Also, more recently, legislation has taken into account the role of soil as fundamental basis of life and habitat for human beings, animals, plants and soil organisms. The definition of testing requirements for the assessment of the hazard potential of substances in relation to the development of soil related protection targets has strengthened the need of the development of new specific test methods or the adaptation of existing test methods, originally developed for testing chemical substances, to the requirements of soil quality testing (Riepert and Felgentreu, 2003).

BIOLOGICAL MONITORING AND ECOTOXICOLOGY

Biological monitoring can be defined as the systematic use of biological responses to evaluate changes in the environment with the intent to use this information in a quality control program. Biological monitoring thus not only has a signalling and predictive function but also a controlling function (Persoone and Pauw, 1991).

In the environmental context, biological monitoring refers to the gathering of information on biological organisms to assess the environmental impact or the biological health of the environment. In a strict sense, the term applies to a continuing collection of data used to establish whether explicitly stated quality control conditions are being met (Smith, 2002).

In biological monitoring, attention should be given to ecosystems because they reflect interactions between both biotic and abiotic factors. An ecosystem is a complex, autonomous, functioning unit. It has been defined as “a functional entity with internal homeostasis, identifiable boundaries, and recognizable relationships between subcomponents”. As a consequence, the analysis of ecosystems should be holistic. In addition to evaluate individuals and populations within a system, relevant structural and functional parameters must be considered. This holistic approach is particularly important in the analysis of disturbed ecosystems. In biological monitoring, changes in the physical, chemical, and biological structure of the receiving system, as well as changes in functioning of these elements, must be considered (Mathews *et al.*, 1982).

The essence of the entire field of biological monitoring is that no one can protect the health, condition, or quality of a natural system without obtaining information directly about the condition of that system and the organisms that inhabit it. Furthermore, the organisms must not only be able to survive but be able to function normally as well. As a consequence, one needs an array of information based on diverse and dissimilar methodologies in order to have a reasonable expectation of adequately protecting the ecosystem receiving the potential pollutant. If this information is not gathered on a systematic basis, it would not fulfill the requirements of a quality control system. The field of biological monitoring was developed in order to control and effectively maintain environmental quality at socially and biologically desirable levels (Cairns and Schalie, 1980).

Biological monitoring has attracted increasing attention in the field of water pollution for two primary reasons: (1) the response of organisms to various toxicants is often mediated by the chemical-physical qualities of the receiving system water and these may change rather frequently, thus changing the toxicity of the pollutant;

and (2) there is a possibility of interactions with other waste discharges which will markedly alter the nature of the toxic response. Thus, both environmental interactions and interactions with other potential toxicants may substantially alter the response of an organism from that obtained with a pure chemical in a laboratory situation (Cairns and Schalie, 1980).

In the environment, pollutants occur in multiple combinations rather than individually. Assessing their combined effects on organisms and evaluating the potential risks for man and the environment are the task of biomonitoring.

Liquid industrial wastes are one of the major sources of pollution in freshwater and estuarine systems. Thousands of industries throughout the world discharge effluents that contain mixtures of toxicants such as organic compounds and heavy metals, plant nutrients such as phosphate and nitrate, and a variety of other organic substances such as sodium chloride, ammonia, sulphates and sulphites. Properties of wastes such as pH and temperature may affect organisms in receiving waters (Walsh *et al*, 1982).

The degradation of the aquatic resources due to effluent discharges containing pollutant substances, some of which persistent, toxic and/or bioaccumulable, has coming to put in risk their natural function of life support and their utilization for the most variety of human activities. It was necessary to establish limits for the quality of the effluents discharged by industries or by urban agglomerates, to protect the aquatic environment from an unsustainable degradation. The regulation of the discharges came to imply the treatment of the effluents.

Currently the chemical specific approach plays a major role in water quality policy. However, when considering complex mixtures as effluents, the possibilities of a chemical specific assessment are limited since (GFEA, 1999):

- There are many substances that cannot be identified.

- Not all substances are analysable/detectable. The number of substances can be so large, that a chemical specific approach is unattainable.
- There is a lack of data on effect-parameters for many substances. Data on the environmental characteristics are not available or incomplete.
- Micro-pollutants and degradation products are undefined, and therefore not accounted for.
- Combined effects of substances, present in the discharges, are not being considered. A mixture can have very different environmental characteristics in comparison to the separate substances.

Some of the disadvantages of the specific chemical approach can be avoided by using chemical group parameters (e.g. suspended solids, biochemical oxygen demand, chemical oxygen demand, total organic carbon) which give a better impression of the constituents of an effluent as all substances are considered regardless of their chemical specification. These parameters provide valuable information about the efficiency of wastewater treatments and can basically characterise the organic loading of industrial effluents.

Most of the existent regulatory framework based the actions of quality control of the discharges in the establishment of emission limit values (ELV), for specific chemicals or for quality group parameters. In Portugal, the legislation in force is according to DL 236/98, which specifically refers that the preservation of the aquatic environment from the pollution provoked by certain substances, toxic and bioaccumulable, denominated hazardous substances, it is made through their elimination or reduction. The fixation of emission limit values for the toxic substances is based on ecotoxicological studies for each substance, individually, similar to what are actually requested at many countries in the ambit of the specific legislation for the notification and control of commercialised chemicals (Barros *et al.*, 1993).

The use of toxicity tests for monitoring and regulating the effects of toxic pollutants on the aquatic life is an important aspect of the regulatory process. The evaluation of the toxicity in one or more components of any ecosystem is made by ecotoxicology. This science aims predicting effects of potentially toxic agents on the natural systems and in nontarget species, with the goal of protecting entire ecosystems and not merely isolated components (Connel, 1987; Harris *et al.*, 1990; Warren-Hicks and Parkhurst, 1992; Hoffman *et al.*, 1995).

In Portugal, there is no legislation available on bioassays on effluents monitoring (Brito *et al.*, 1999). However, when the conditions of effluents discharge for the aquatic environment are considered, and the goal is the protection of the integrity of aquatic ecosystems, a methodology based only in ELV for pollutants specifically identified in those effluents has gaps, once it doesn't consider the effect of all the toxic compounds present in the effluents (only the chemicals specified in list of the hazardous pollutants are considered), the total effect of the mixture neither the receiving medium.

Toxicity testing has become an integral part of the evaluation of the effects of waste discharges on the environment, because the physical and chemical tests alone are not sufficient to assess potential effects on biota. The effects of chemical interactions and the influence of complex matrices on toxicity cannot be determined from chemical tests alone (APHA, 1998; Brito *et al.*, 2002; Wharfe, 2004). The toxicity tests have a number of advantages over chemical-specific tests. For example, toxicity tests aggregate the toxicity of all constituents into a single measurement, and therefore all kinds of hazardous substances including their degradation products are considered. In addition, toxicity test endpoints integrate the bioavailability and interactions of multiple toxicants, as additive, synergistic or antagonistic effects, into a single measurement (Warren-Hicks *et al.*, 1992; GFEA, 1999).

So, biomonitoring of whole effluent has been used as an indicator of the total effluent effects, which may not be easily identified with specific chemical analysis (Dorn *et al.*, 1991; Arbuckle and Alleman, 1992; USEPA, 2000a,b). The use of ecotoxicity tests permits a direct evaluation of the toxicity of an effluent (environmental samples) which has not been chemically characterized, integrating the impact of all components to which the biota are exposed. Therefore, the evaluation of the whole effluent toxicity (WET), in addition to the chemical-specific approaches, has becoming an essential and obligatory parameter to control the complex discharges. In this context, the characterization of the effluents to be discharged in the aquatic systems should be performed not only in terms of their physical-chemical characteristics but also in terms of their toxicity.

When an effluent fails to meet biomonitoring permit limits, toxicity identification/reduction evaluations (TI/RE) may be required to separate and identify sources of toxicity. The TI/RE process uses a series of physical/chemical fractionation procedures coupled with toxicity tests to isolate and characterize the toxic component(s) in the wastewater for the development of options for reducing or eliminating the toxicity (Lankford and Eckenfelder, 1990; USEPA, 1991, 1993a,b; Hall, 1998; Ausley *et al.*, 1998; Hutchings *et al.*, 2004). From these evaluations, permittees can alter manufacturing and discharge processes to reduce or eliminate the toxicity of their effluent.

RISK ANALYSIS

Ecological risk assessments evaluate the likelihood that adverse ecological effects will occur as a result of exposure to stressors related to human activities, such as the release of chemicals, and natural catastrophes. The term *stressor* is used to describe any chemical, physical, or biological entity that can induce adverse effects on

ecological components, that is, individuals, populations, communities, or ecosystems. Adverse ecological effects include a wide range of disturbances ranging from mortality in an individual organism to a loss in ecosystem function (Hoffman *et al.*, 1995; De Cohen *et al.*, 2000).

Risk analysis is a process in which prediction and evaluation are combined to estimate the probability or frequency of harm (risk) for a given hazard (an event which has the potential to be harmful). Manifestation of a risk requires an event, a pathway for transport, and a receptor that could be harmed at the exposure point (Petts and Eduljee, 1994). In essence, risk analysis provides a structured approach for ascertaining the nature and extent of the relationship between cause and effect.

Generally, risk analysis is viewed to consist of three main stages (Lynch and Wiseman, 1998):

- (i) Hazard identification,
- (ii) Risk assessment (or risk estimation),
- (iii) Risk evaluation.

Hazard identification refers to exposure assessment and determines potential hazards and the conditions that result in adverse consequences to individuals or populations that contact with the pollutant. The pollutant (substances or mixtures) physical-chemical characteristics, the emission conditions and the behaviour and interaction with receptor media are determinants to evaluate the potential of the existent exposure (Barros *et al.*, 1993; Lynch and Wiseman, 1998).

Risk assessment refers to the effects of the pollutant on individuals, populations and ecosystems. The toxicity characteristics are determinant to evaluate the potential to occur an adverse effect. Laboratorial toxicity tests are used to characterize ecological effects. The results of toxicity tests are used to quantify the relationship

between the amount of pollutant and the magnitude of the response, and to evaluate the cause-effect relationship.

Risk evaluation refers to risk characterization. The profiles of exposure and ecological effects serve as input to risk characterization whenever risks are estimated and described. The risks are estimated by integrating the exposure (predicted environmental concentration) and effects data to yield an expression of the likelihood of adverse effects occurring as a result of exposure to a certain stressor (Norton *et al.*, 1992). This final stage utilizes comparisons and judgments to determine the magnitude and significance of environmental risks, and establish whether the estimated level of risk is tolerable (Petts and Eduljee, 1994; Lynch and Wiseman, 1998).

An important objective of laboratory testing of the effects of chemical compounds or complex mixtures (effluents) is to assess the impact on natural ecosystems and to calculate risk factors. A simple ecotoxicological risk analysis can be performed by the extrapolation of acute or chronic toxicity data for various test species by regression techniques (Vindimian *et al.*, 1999). Alternatively, laboratory-to-field extrapolation procedures may use distribution models (usually the log-logistic) for sets of acute and/or chronic toxicity test data (Kooijman, 1987; Wagner and Løkke, 1991; OECD, 1998), to give concentrations at which no effects are expected (NOECs - No Observed Effect Concentrations) and comparing these levels with the estimated exposure ones. Risk analysis models have gained worldwide consideration by regulatory bodies as essential tools for setting and achieving environmental goals.

Using basic data on the volume of a substance produced or processed and the estimated releases to the environment, and properties of the chemical (e.g. volatility, water solubility, (bio)degradation, and partitioning behaviour between water and air), the environmental distribution of a chemical can be modelled. This results in a

series of Predicted Environmental Concentrations (PEC) at each industrial site, and also over a defined region, for each environmental compartment (air, water, soil). When available, representative measured data are used to refine or replace the modelled data.

For the environmental effects assessment a Predicted No Effect Concentration (PNEC), using the acute or chronic data and an assessment factor, should be calculated for species representative for the environmental compartment under investigation (Girling *et al.*, 2000). For the environmental protection goals, risk characterisation is expressed as the PEC/PNEC. If the predicted no effect concentration is exceeded, i.e. $PEC/PNEC > 1$, there is considered to be a risk. For the human populations, risk characterisation is expressed as No Observed Adverse Effects Level (NOAEL) / Exposure, with a margin of safety.

ECOTOXICOLOGICAL BIOASSAYS

The pollutants have certain intrinsic properties, which determine the likely effect that they will have after emission or discharge into the environment. These properties include: short- and long-term toxicity, persistence, dispersion properties, chemical reactions that the compound undergoes (transformation and/or biodegradation), bioaccumulation, and biomagnification. The research within the field of ecotoxicology includes studies of the distribution of pollutants and toxic compounds in the environment, their transport, bioaccumulation, degradation, and biological and ecological effects.

Bioassays are crucial for the detection of pollution in the environment and the assessment of toxicity of wastewaters and chemical substances and their fate.

Bioassays are a common type of biological monitoring that may be required as a method of measuring water quality to determine compliance with discharge limits.

Two types of ecotoxicological bioassays can be distinguished:

- Assays of biodegradation, bioaccumulation and biomagnification in which is followed the behaviour of the chemical compound tested along the time.
- Assays of inhibition, stimulation or behavioural alteration in which are studied the reactions of living organisms in defined conditions (e.g. toxicity tests).

A bioassay can be defined as a procedure that uses living material to estimate the effects of pollutants. The idea behind the bioassays is that the test organism will react in a predictable way to various types of environmental contaminants.

Biological indicators can be used to assess either the actual or the potential impact of xenobiotic substances or chemical compounds mixtures on the natural ecosystem and can be applied in two different ways: (i) a posteriori assessment – to monitor the actual effects on nature, or (ii) a priori assessment – to predict the impact of a pollutant prior to its release (Janssen and Persoone, 1992).

Living organisms constitute a vast diversity of taxonomy, life history, physiology, morphology, behaviour, and geographical distribution. For ecotoxicology, these biological differences mean that different species respond differently to a compound at a given concentration (i.e. different species have different sensitivities) (Posthuma *et al.*, 2002).

The main goals of the use of bioassays have been defined as: to rank hazards (screening toxicity tests), to set discharge limits, to predict the environmental consequences of discharges to environmental resources (waterbodies, soil), to control effluents, to protect important species and to protect ecosystem structure and

function (biomonitoring), to perform biomarker research and to develop biosensors. Depending on their application they meet specific and different requirements.

TOXICITY EVALUATION

Toxicity is an inherent property of all substances or chemical compounds mixtures, which describes its potential to produce an adverse effect on a living organism. Toxicity is a function of the pollutant concentration (dose) and the duration of the exposure, and can be influenced by factors exterior to the individual but related to its habitat, and also by factors resulted from its vital functions and life cycle.

Every chemical has various effects on human beings, the environment and its components. The major factors that determine the toxicity of a chemical to biological species are dependent upon the quantity of the substance. In short, the dose makes the poison. Another important variable that determines the magnitude of the toxic effects of the chemical is the duration of exposure. For the same toxic substance, the living organisms can present different physiological disturbances according to the quantity absorbed and the duration of the exposure to the toxic.

To evaluate the toxicity is necessary, in first place, to define “toxic effect” i.e. choose an endpoint to be measured, and then decide how to measure the chosen effect. In the toxic effects are included lethal effects (death, survival) and sublethal effects, as changes on motility, growth, development and reproduction, or biochemical, physiological, histological or behavioural alterations.

Effect and response, in ecotoxicology, have two different meanings:

- Effect indicates the damage, or the biological function compromised by the action of the toxic (e.g. survival, motility, growth rate).
- Response is the proportion of exposed organisms showing a determinate effect due to the toxic action (% incidence).

Effects and responses depend on several factors, other than those related to the tested biological species and treated group characteristics. These may be related to the test species or to some abiotic factors; in the first case, the factors that modify toxicity include health conditions, eventual acclimatization, genetic variability and age. Abiotic factors relate to temperature, oxygen availability, pH, hardness and salinity of water, and concentration of particulate matter. Assuming that the test species were correctly selected and the experiment conducted in optimal conditions, for a given effect (e.g. lethality) the responses (expressed in percentage of incidence) are quantitatively related to both the exposure level (dose or concentration) and the exposure time (Bacci, 1994).

Ecotoxicity testing

The evaluation of the toxicity is performed with tests using living organisms, which are based on the existence of a correlation concentration (dose) – response (effect). Despite the response could be variable in nature and extension according to the type of organisms, due to biological diversity, the concept of correlation concentration - response is usually applicable and it implies that for each substance or mixture tested there will be a threshold concentration below which there is no observed effect.

Ideally, toxicity bioassays are used to predict the levels of chemicals that produce observable effects on biological systems, e.g. populations, communities and ecosystems, and to identify the biological resources at risk. The bioassays evaluate the degree of sensitivity (or resistance) to a toxicant for several species, belonging to different phylogenetic groups, and usually are carried out to estimate the “safe” or “no adverse effect” concentration of a pollutant in the environment, which is defined as the concentration that will permit normal propagation and development of the organisms in the receiving system (Janssen and Persoone, 1992; GFEA, 1999). This

can be done assuming that the test systems and organisms are surrogates for all organisms occurring in natural ecosystems.

Toxicity tests should reflect the level of exposure to the chemical that is expected to be present in the environment, the possible effects and consequences of this chemical, and the concentration that is likely to produce adverse effects.

The toxicity tests can be divided in different groups, according to the type of test organism used. Aquatic toxicity tests are designed to describe the effects of toxic agents (natural or xenobiotic pollutants) on aquatic organisms. Several species from different taxonomic groups, and from freshwater, salt and brackish water, are used in these tests for the aquatic environment (water quality): fish (*Brachydanio rerio*, *Pimephales promelas*), crustaceans (*Daphnia magna*, *Ceriodahnia dubia*, *Artemia salina*), rotifers (*Brachionus calyciflorus*, *Brachionus plicatilis*), cnidarians (*Hydra attenuata*), insects, annelids, bivalves (*Crassostrea gigas*), aquatic plants (*Lemna minor*), algae (*Scenedesmos subspicatus*, *Selenastrum capricornutum*) and bacteria (activated sludge, *Pseudomonas putida*, *Vibrio fischeri*) (GFEA, 1999; APHA, 1998, Pica-Granados *et al.*, 2000). Wildlife toxicity tests are designed to describe the effects of toxic agents on terrestrial organisms: birds, mammals (rodents), amphibians and reptiles. Soil toxicity tests are designed to describe effects of environmental contaminants to soil organisms, as earthworms, soil arthropods and soil microorganisms (Keddy *et al.*, 1995; Hoffman *et al.*, 1995; Lynch and Wiseman, 1998).

Bioassays with selected species representative for the biological communities of the environments under consideration are now applied more or less regularly to determine toxic and genotoxic effects. Taking into account the species-specific and chemical-specific character of toxicity to biota, the necessity of an approach based on a battery of tests with species of different trophic level is currently also generally

accepted and implemented. Greater environmental realism is also a reason for using a test battery with one species from each of the four different trophic levels: bacteria, algae, herbivores and carnivores (trophic level approach) in order to find out the most sensitive trophic level. The test organism chosen within a taxonomic group may have a drastic influence on the test result. The selection of the test species depends on the objective of the study (Buikema *et al.*, 1982; Weber, 1993; APHA, 1998). The taxonomic groups most commonly used for the toxicity evaluation of pollutants (chemical substances, chemical mixtures and effluents) have been bacteria, microcrustaceans, algae and fish. Toxicity tests use organisms with various ages and life stages, because younger biota are often more sensitive to the pollutants. Sensitivity of a species to pollutants is also dependent on the duration of the exposure and the evaluated endpoints.

There are two opposite philosophies in the choice of the test organisms: one tendency is to standardize test organisms as far as possible (low genetic variation) to reduce variability and increase reproducibility and comparability; the other tendency is to use geographically and biologically representative organisms and /or wild populations to have a better basis for interpretation of results based on environmentally realistic conditions (GFEA, 1999).

Most of the toxicity testing to date has been performed with single-species laboratory test systems, highly standardized and reproducible – prerequisites for regulatory purposes. This inevitably means low environmental realism, using a few well-understood test species. More complex multispecies tests (microcosm, mesocosm), which have a higher degree of realism, have been suggested as a means to increase the predictive capacity of ecotoxicity assessments. More complex bioassays are more expensive, presenting problems of reproducibility and standardization, but in contrast with the simplified standardized single species tests,

may use the same endpoints to evaluate the health of natural systems (Cairns and Niederlehner, 1995). However, single species testing is the best for screening and range finding to determine priorities (compliance with water quality criteria, effluent requirements and discharge permits), to compare relative toxicity of chemicals or wastes (effluents) to selected test organism, determining their concentration-response curves, or to assess the relative sensitivity of different organisms to different conditions (temperature, pH).

Toxicity tests are classified according to (a) duration: short-term, intermediate, and/or long-term; (b) method of adding test solutions (exposure system): static, renewal, or flow-through, and (c) purpose: permit requirements (e.g. water quality criteria), effluent quality monitoring, single compound testing, relative toxicity, relative sensitivity, etc. The type of the test selected depends of the aim of the work (APHA, 1998; Paixão, 2001).

The sustainability of the environment is dependent on the preservation of the biological chain and consequently on the effective prognosis and prevention of impacts of pollutants on it. Currently, more than 100,000 chemical compounds are used worldwide, and the release of these compounds into the environment as well as the constant increase of municipal and industrial effluents discharges can cause a serious environmental problem for aquatic and terrestrial ecosystems. Faced with this enormous and urgent task to screen these products for potential hazard and biomonitoring effluent discharges, the ecotoxicologists will need to rely on information obtained from some of the well-established, standard ecotoxicity tests.

Toxicity tests are one of the essential tools for evaluating the effects of natural and anthropogenic contaminants in environment, in supplement to chemical specific analysis. During the last decades the scientific and regulatory community has gradually realized that biological methodologies have to be taken into consideration

for an ecologically meaningful assessment of the toxicological hazards of contaminants. Effect evaluations obtained with biological techniques indeed integrate the impact of all contaminants to which living biota are exposed.

There are several publications describing ecotoxicity test methods, where are specified the principle of each test method, the test organism, the test conditions and its applicability, as well as the test validation conditions (Grothe *et al.*, 1996, Hall and Golding, 1998; APHA, 1998; ASTM, 2002; USEPA, 2000a,b). Several of the toxicity test methods using aquatic organisms are standardized and have been proposed by national/international standardization groups (ISO, EN, NP), and/or by regulatory bodies (USEPA, OECD).

Acute toxicity tests are short-term tests designed to measure effects of toxic agents on the test organisms during a short portion of their life span. Acute toxicity concerns effects occurring after a short exposure time: 24 to 96 hours, although shorter exposures (e.g. 15, 30 min) may be applied in particular tests, e.g. bacterial tests. The typical endpoint is lethality, however sublethal effects (inhibition of an important biological function, e.g. immobilization) are also estimated. Acute toxicity tests generally involve a short exposure of a test organism to 4-6 different increasing concentrations (dose) of a pollutant (% effluent, or mg/l for chemical substances), a control and a vehicle control (i.e. solvent control), if a vehicle is used. Two or more replicas are used for each tested concentration and control. A total of at least 20 test organisms should be exposed per toxicant concentration.

Generally, data generated in acute toxicity tests are quantal, that is, responses are measured with yes/no - type observations (e.g. death or not?). Of particular concern, in acute toxicity tests, is the 50% incidence and the concentration provoking this response. This concentration is the median lethal concentration (LC_{50}) when the mortality is the test endpoint, or the median effective concentration (EC_{50}) when a

sublethal effect is the endpoint. In comparing $L(E)C_{50}$ values, the pollutant with higher $L(E)C_{50}$ value is less toxic because a greater concentration is required to produce 50% incidence (mortality/sublethal effect). For a direct lecture of the ecotoxicity results was defined the term toxic units (TU), as $TU=100/L(E)C_{50}$. Tonkes *et al.* (1999) present a ecotoxicity scale for environmental samples.

In chronic toxicity tests the organism is exposed for a significant part of the life cycle. The endpoints generally used in chronic toxicity are sublethal effects such as reduced growth, reproduction capacity, altered development or biochemical effects, within a test duration of 16 hours to 7 days in short-term chronic tests, and of 7 to 60 days (or more) in long-term chronic tests (GFEA, 1999). Data generated in chronic tests are quantal (long-term survival), continuous (growth), or count (number of young produced). In chronic studies, the traditional goal is to calculate thresholds, or those levels of exposure to toxicants that are not able to induce any detectable adverse effect. The chronic value, ChV, is a point estimate determined as the geometric mean of the LOEC (lowest-observed-effect concentration) and NOEC (no-observed-effect concentration). LOEC is the lowest concentration tested having a statistically significant difference from control response, and the NOEC is the highest concentration with a response not statistically different from control response. NOEC can be used for analysis of any biological endpoint in ecotoxicity testing. Chronic toxicity testing permits the assessment of the possible adverse effects of a pollutant during long-term exposure at sublethal concentrations.

The most well-developed and widely used bioassays for routine control, screening of newly developed chemicals and monitoring applications (e.g. effluent monitoring) are acute toxicity tests using aquatic organisms. One major constraint of the standardized conventional test methods (e.g. Daphnia Test), which presently limits the application of bioassays to a restricted number of highly specialized laboratories,

is the intrinsic need and the high costs for continuous culturing/maintenance of stocks of live test organisms.

During the last two decades, intensive research in ecotoxicological bioassays have been performed to develop alternative microbiotests which are independent of recruitment, maintenance and/or culturing of live stocks of test organisms, which allows routine testing at low cost with basic laboratory equipment and materials. A microbiotest can be defined as the exposure (< 24 hours) of a unicellular or small multicellular organism to a liquid sample in order to measure a specific effect (Blaise, 1991). These small scale tests, miniaturized into kits (Toxkits) were developed in the Laboratory of Biological Research in Aquatic Pollution at University of Ghent in Belgium, by Prof. Guido Persoone and collaborators. A variety of microbiotests, using invertebrates, microalgae and bacteria, are now available (Persoone, 1992; Snell and Janssen, 1998; Janssen, 1998; Janssen *et al.*, 2000, Persoone *et al.* 2000), several of which have been substantially validated, which should be considered as valuable alternatives to the restricted number of conventional bioassays (van der Wielen *et al.*, 1993; Persoone *et al.*, 1995; Persoone, 1998; Paixão *et al.*, 1999; Latif and Zach, 2000).

Information generated from several ecotoxicity tests can be used in the pollution management for the purposes of: (a) prediction of environmental effects of a pollutant (chemical substance, waste, effluent), (b) comparison of pollutants, organisms or test conditions, and establishment of the concentration-response curves for pollutants; (c) regulation of discharges of the pollutants, and (d) monitoring.

Concentration (dose) – response relationship

The relation concentration-response, or more classically, a dose-response relationship is the most fundamental concept in toxicology and ecotoxicology. This concept assumes that there is a causal relationship between the dose of a toxicant (or

concentration for toxicants in solution) and a measured response. Using this concept, ecotoxicity tests are designed to describe a concentration-response relationship, aiming to estimate as precisely as possible the pollutant concentration that produces an observable and quantifiable response on test organisms, under controlled laboratory conditions. The response may be any measurable biochemical or biological parameter that is correlated with exposure to the toxicant (Ramamoorthy and Baddaloo, 1991; USEPA, 2000a). The concentration-response relationship extends from acute to chronic response and demonstrates that for every pollutant there is a threshold concentration below which no adverse effect is observed. When the measured effect (response) is plotted graphically with the concentration, is traced the concentration-response curve.

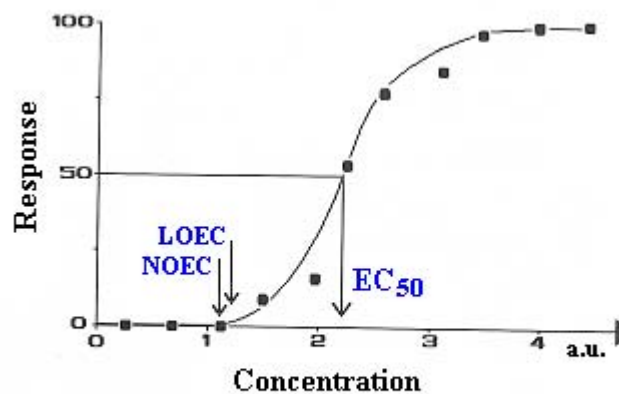


Fig. 1. Classical concentration-response curve, with schematic representation of EC_{50} , NOEC and LOEC concepts. (a.u. = arbitrary units).
(Adapted from Bacci, 1994)

In Fig. 1 is presented the classic concentration-response curve, obtained when testing groups of organisms with different concentrations (doses) of the same pollutant and observing the effect over a fixed period of time. The curve could better be called response-concentration, as the response (% incidence) depends on the concentration (independent variable) and not vice versa. The classic concentration-

response curve is depicted as a sigmoidal shaped curve, however, the particular shape of the concentration-response curve may differ for each coupled toxicant and response pair (Ernst, 1998; USEPA, 2000a). In addition, the shape of the curve depends on the organism used in toxicity test and is specific to the pollutant.

The sigmoidal concentration-response curve present a more or less wide linear middle portion, and is not generally symmetrical, especially when concentrations are in arithmetic progression, due to a slower increase in responses with increasing exposure level when approaching the maximum (100% incidence). The reason for the sigmoid trend, as in cumulative frequency distribution functions, relies on the fact that the observed responses at each concentration level are actually cumulative.

In general, more severe responses (acute effects) occur at higher concentrations of the toxicant, and less severe responses (chronic effects) occur at lower concentrations. The slope of the linear portion of the curve is also an important parameter, once indicates the scattering of % incidence data, due to the extent of the individual variability in reacting to the toxic agent. The steeper the slope of the middle portion of the curve, more intense is the response over a narrow range of concentrations. A homogeneous population produces a tight curve with a very steep slope, with the most of the population going from 0% to 100% response over a very small concentration range, while a heterogeneous population produces a less steep slope in concentration-response curve, which shows much more scatter around the mean (Bacci, 1994; Francis, 1994).

The concentration-response curve is the basis for the determination of point estimates (EC50, EC10, LOEC, NOEC, etc) in pollutant toxicity testing. Numerous procedures are available for analyzing quantal toxicity data, including manual techniques for graphical interpolation or computer software packages (e.g. TOXCAL[®], Tidepool Scientific Software, McKinleyville, CA). Regression methods,

both parametric (e.g. log-probit transform) and nonparametric (Fry, 1992; Weber, 1993; Kerr and Meador, 1996; APHA, 1998), may be used to generate effective concentrations (ECs), evaluate conventional concentration-response test designs, and provide confidence intervals for point estimates (L(E)C₅₀) interpolated from the appropriate models, as well as goodness-of-fit estimates of the model outputs. Hypothesis testing methods and ANOVA data analysis have been used for chronic toxicity tests results, to estimate LOEC, NOEC and ChV (Guimarães and Cabral, 1997; Sparks *et al.*, 1999; Isnard *et al.*, 2001).

BIODEGRADATION EVALUATION

The 1980s have been characterized by concern about the presence of toxic and other xenobiotic organic compounds in the environment. Biodegradation is widely recognized as one of the key factors determining the fate and distribution of biogenic and xenobiotic compounds in the environment. Biodegradation refers to the breakdown of organic chemicals due to biological attack. In natural ecosystems, microorganisms play a major role in mineralising various organic pollutants. Microbial degradation of wastes and pollutants is essential for maintaining environmental quality. Biodegradation results in a decrease in the mass or load of chemicals and may prevent accumulation in the receiving environment.

Biodegradability of chemicals is one of the most important aspects of their environmental behaviour because a biodegradable substance is expected to cause less ecological problems in the long term than a persistent one. The users of chemicals need biodegradation data as well to decide, for example, if a substance can be treated in a biological wastewater treatment plant. For the generation of these biodegradation data, especially in the laboratories of the chemical industry, and their

acceptance by customers and authorities, internationally harmonized and standardized test methods are required (Pagga, 1997).

Industries and government regulators increasingly seek to know the fate of chemicals in the environment and thus prevent potential negative impacts on human or ecosystem health (Wackett and Ellis, 1999). Regulatory requirements for biodegradability have been introduced to support classification and labelling of dangerous substances, and other environmental management practices such as discharge permits. Several regulatory agencies are introducing persistency (and bioaccumulation) categories to allow regulatory action based on intrinsic properties only. Knowledge of the processes for removing chemicals by biodegradation, and particularly the rate of the biodegradation, is therefore of great importance in environmental safety assessments of all kinds (Hales *et al.*, 1997).

Biodegradation in the environment

After the release of a chemical, natural or anthropogenic, into the environment, its distribution is governed by a number of physical, chemical and biological processes, which are known as environmental fate processes. Transport of the chemical and its subsequent partitioning between media, such as air, water, soil/sediment, and biota distribute the chemical in the environment. Biotic and abiotic processes can cause the chemical to transform (minor or major alteration of its original form) and even undergo degradation to its fundamental building units of water, carbon dioxide, ammonia, etc. The greater the resistance of a chemical to the transformation and/or degradation processes, the longer is the persistence of the chemical in its initial form in the environment. Fig. 2 illustrates the fate processes of chemicals (xenobiotics) in the environment.

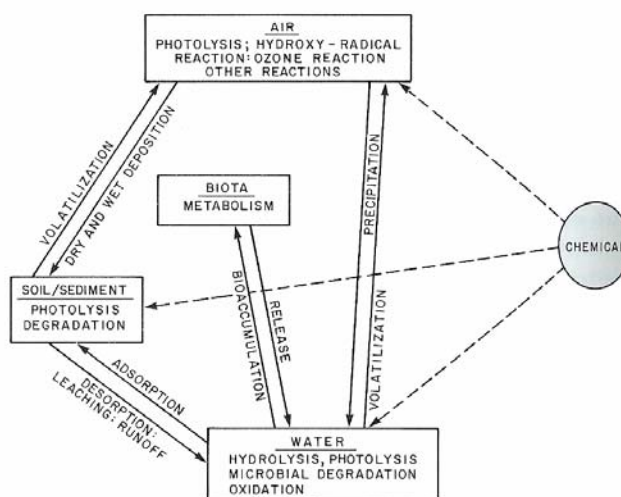


Fig. 2. Schematic illustration of transport and degradation processes of a chemical. (Reprinted from Ramamoorthy and Baddaloo, 1991)

Degradation processes are constantly taking place on a large scale in the natural environment, especially in the waterbodies (freshwater and marine ecosystems). Water is the most important medium of transport and distribution for many substances and an absolute prerequisite for all biological processes. Other important environmental compartments with high degradation activity are soil, especially humus-rich tilled soil, and residues of plants in woods and fields. In these complex ecological systems, as well as in technical systems such as wastewater treatment plants, the natural cycle of substances (N, C, P, S, O) occurs, in which is extremely important the role of the decomposers. They break down the available biomass of the producers (plants) and the consumers (animals), as well as their excretion products, and also anthropogenic wastes, into carbon dioxide, water and other low molecular weight degradation products (catabolites), such as inorganic substances (Pagga, 1997).

Aerobic biodegradation is considered to be one of the most important fate processes for natural and industrial organic chemicals cycling in the aquatic environment,

sediments and soil. Biotic species such as oxygen-consuming microorganisms, particularly bacteria and fungi, use their metabolic activities to break down complex organic chemicals to simple end-products.

Collectively, microorganisms possess the greatest enzymatic diversity found on the earth and metabolise millions of organic compounds to capture chemical energy for growth. This metabolism, called catabolism or biodegradation, is the principal driving force in the half of the degradative earth's carbon cycle (Wackett and Ellis, 1999). Microorganisms are increasingly used in technical systems to biodegrade hazardous xenobiotic compounds for cleanup of environmental contaminants (e.g. bioremediation application), and also to treat solid residues and wastewaters (e.g. activated sludge treatment plants, aerobic composters). In these systems, the same biodegradation processes occur as in natural environments, however under optimised conditions (Pagga, 1997).

Biodegradability testing

It is widely recognized that any hazard or risk assessment of environment pollution from the release of organic chemicals must include an evaluation of their biodegradability (or persistency) in the receiving systems, soil, water and/or wastewater treatment plants (Nyholm, 1996). The knowledge of their biodegradability is important, as it is one of the key factors determining the fate of organic chemicals in both natural and engineered environments; a high persistence level is undesirable.

Biodegradability can be defined as the intrinsic capacity of a chemical to be degraded by the action of microorganisms. The terms primary (partial) or ultimate (complete) are often used to quantify the extent of biodegradability. Primary biodegradation is used to denote an alteration of the chemical structure and loss of specific properties, whilst ultimate biodegradation indicates the total mineralization of the chemical to inorganic products (Vazquez-Rodriguez *et al.*, 1999). The

biodegradation occurs by means of different mechanisms, which include successive oxidation-reduction reactions and hydrolysis, as already described. The knowledge of microbial growth and substrate utilization kinetics is important to the prediction of the fate of organic compounds in the environment. The models used to describe the kinetic parameters for prediction of biodegradation rates are derived from Monod equation, which is currently applied to express the growth of microbial populations (Blok and Struys, 1996; Grady *et al.*, 1996; McAvoy *et al.*, 1998; Blok, 2001).

Biodegradable organic pollutants can be grouped into two categories according to the amount of energy captured by microorganisms during their biotransformation: primary substrates and secondary substrates. The oxidation of primary substrates provides energy and carbon source for cell growth and maintenance, i.e. the cells grow by consuming primary substrates if nutrients are available. On the other hand, the degradation of secondary substrates supplies no or negligible energy for cell synthesis and maintenance, consequently, cell growth is impossible or negligible when these substrates are the only organic compounds, even when nutrients are available (Sáez and Rittmann, 1991). The biodegradation of secondary substrates can occur by cometabolism, i. e. the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound, with no mineralization and not associated with microbial growth (Painter, 1995). These microbial processes may lead to environmental detoxication, the formation of new toxicants, or the biosynthesis of persistent products. Detoxication is a common outcome of mineralization, except when one of the products itself is of environmental concern, as in the case of nitrate in certain waters. Cometabolism of a toxicant may also result in detoxication, and certain synthetic compounds that appear to be cometabolized in nature are converted to products that are not of ecological concern.

Methods for measuring biodegradability can be categorized into two main types: direct measurement of parent compound concentration, by using substance-specific analytical methods such as gas chromatography (GC), high pressure liquid chromatography (HPLC), capillary electrophoresis; and indirect measurement of parent compound bioconversion, such as cumulative oxygen uptake (respirometric tests as biochemical oxygen demand (BOD) determination tests), carbon dioxide evolution, increase in cell concentration, changes in pH, removal of dissolved organic carbon (DOC), chemical oxygen demand (COD) or total organic carbon (TOC), etc. (Pitter and Chudoba, 1990; Painter, 1995; Gotvajn and Zagorc-Končan, 1999a,b; Jiang *et al.*, 2002; Painter *et al.*, 2003).

Substance analytical methods are required for the primary biodegradation and especially in the case of low concentrations of the test material, permitting to quantify the disappearance of the parent compound. Measurements of BOD and CO₂ release in closed batch systems clearly indicate biodegradation processes, however they don't take the transformation of the test material into biomass into account. Biodegradation test results are obtained by comparing the measured value with a theoretical value (ThOD – theoretical oxygen demand and ThCO₂ – theoretical amount of carbon dioxide produced). Tests on the basis of these parameters are also used for insoluble and poorly water-soluble chemicals, whereas detection of DOC (COD) removal is limited to sufficiently water-soluble compounds and may also include abiotic elimination processes such as adsorption on biomass, sedimentation or stripping (Koziollek *et al.*, 1996; Pagga, 1997). Indirect measurement of biodegradation is easy and can be automated. However, quantification of biodegradation kinetics using any indirect measurement may be misleading. The BOD (oxygen uptake) measurements can demonstrate initial biotransformation but cannot prove that the contaminant was mineralized to carbon dioxide; furthermore the occurrence of nitrification reactions can also confuse oxygen uptake

measurements. However, direct measure of CO₂ evolution allows an overall carbon balance and can be used to demonstrate mineralization of a contaminant to carbon dioxide (Govind *et al.*, 1996). The CO₂/DOC – combination test (Strotmann *et al.*, 1995) allows two important parameters to be determined in parallel and to present unequivocally evidence about the biodegradation of the compounds involved. The ratio BOD/COD or BOD/TOC can also be viewed as an indicator of biodegradability or treatability. The disadvantage of using summary parameters is that relatively high concentrations of test compounds are required, and for prediction of the kinetic factors and parameters the test concentration should be as close as possible to real environmental conditions.

The BIOLOG microplate technique developed by Biolog Inc. also provides a rapid, convenient approach to assay carbon source utilization, in which tetrazolium violet is used as a redox dye to indicate utilization of the carbon sources (Bochner, 1989). Those commercially available microplates are 96-well plates and allow the simultaneously testing of several organic compounds. BIOLOG MT microplates contain only the redox dye and nutrients preloaded and dried into the 96-wells, permitting that selected carbon sources can be loaded into those wells for testing their biodegradability. If a carbon source is utilized by the inoculated microorganism suspension, the tetrazolium dye is reduced and turns purple. A microplate reader has the capability of quantifying both the extent and the rate of color formation, and therefore can provide sufficient data to test for biodegradation (Yu and Yu, 2000).

Laboratory studies of pollutant biodegradation are best conducted by using mixed culture systems taken from the field, for example, bacteria from soil, wastewater treatment plants and surface water. The goal of such studies is to use degradation rates measured in the laboratory to predict degradation rates in the environment. The use of pure culture systems, despite highly reproducible, suffers from a lack of

environmental realism and are consequently unsuitable as predictors of environmental fate of chemicals. Several factors influence the rate and extent of biodegradation, such as temperature, salinity, pH, redox potential, nitrogen and phosphorus levels, adequate availability of a suitable electron acceptor, chemical concentration, molecular structure, physico-chemical properties, availability of alternate carbon sources, basal salts medium, water hardness, source and concentration of the inoculum, number of degrading microorganisms, and microbial acclimation due to prior exposure of the microbial population to the chemical (Spain and van Veld, 1983; Blok and Booy, 1984; Watson, 1993; Struijs *et al.*, 1995; Thouand *et al.*, 1995; Percherancier *et al.*, 1996; Zaidi *et al.*, 1996; Ingerslev *et al.*, 1998, 2000). Another important factor that also affects biodegradation is the toxicity of the organic chemical itself. Very often chemicals may be susceptible to biodegradation at low concentrations, yet may be toxic to the degrading population at higher concentrations, thus inhibiting their own biodegradation (Dean-Ross and Rahimi, 1995). The conditions and properties of the test systems used, as well as the test duration, also influence biodegradation.

Considerable effort has been expended in developing biodegradation tests, standardizing methods, and evaluating and correlating results. Standardized test methods of the International Organization for Standardization (ISO), the Organization for Economic Co-operation and development (OECD Guidelines) and directives of the European Union (EU), known and accepted by industry and authorities, are used to characterize the environmental behaviour of chemicals (pure substances), namely biodegradability. In general, the available standardized biodegradability tests can be categorized into three groups: 1. ready biodegradability (RB), 2. inherent biodegradability (IB), and 3. simulation tests. A brief critical evaluation of past and improved methods has been presented by Nyholm (1991), and a detailed overview of the methods and test principles is given by Painter (1995).

Other recent reviews on biodegradability testing were also published contributing for a better comprehensive overview in this issue (Kaiser, 1998; Pagga, 1997, Hales *et al.*, 1997).

OECD has proposed a three-tiered biodegradability test system for the protection of the environment and human health, which was adopted by EU. According to EU legislation, its extent depends on type of the chemical, produced or marketed quantity, persistency, toxicity, bioaccumulation, etc. The first tier (level 0) involves simple, inexpensive and stringent tests for ready biodegradability (Nyholm, 1991; Struijs and van der Berg, 1995), which provide limited opportunity for acclimation and biodegradation to occur. So, they are believed to possess less degradation potential than most real environment. These screening tests are carried out with a restricted microbial biomass, ranging from less than 10^2 to about 10^6 cfu/ml (Painter, 1995). Ready biodegradability is an indication of the ease with which a chemical is oxidatively degraded by microbial action under conditions typically found in a polluted river or in sewage treatment plants. This process normally results in the formation of CO_2 , bacterial biomass and inorganic products. A positive test result indicates ready biodegradability in most environmental conditions (at least under aerobic conditions), whereas a negative result does not exclude environmental biodegradability, but indicates that further investigation is necessary. A second tier (level 1) is followed including powerful tests for inherent (potential) biodegradability performed with a high biomass, about 10^7 cfu/ml (Struijs and van der Berg, 1995), which provide a prolonged exposure of the substance to the microorganisms and thus favourable conditions for biodegradation. The final tier (level 2) involves simulation tests, which should be quantitative predictors of the biodegradation behaviour under specified environmental realistic conditions such as soils, surface waters, activated sludge plants, etc, and biodegradation rates should be estimated from such simulation studies (Ingerslev *et al.*, 1998; Gotvajn and Zagorc-

Končan, 1999b). The test methods of the first two tiers are based upon measurements of simple chemical summary parameters (e.g. DOC) or on respirometry, feasible because the test substance is added as the only or dominating carbon source, while simulation tests usually involve specific chemical analysis or the use of radiolabelled substances due to the low concentrations of the test substance. The three-level tests differ in the procedure, in the parameters used to evaluate the biodegradability, the concentration of test substance, the amount and origin of inoculum used, and the mode of operation (Gotvajn and Zagorc-Končan, 1996; Vazquez-Rodriguez *et al.*, 1999). Pagga (1997) presented an overview of standardized methods on biodegradability in water and compost (OECD guidelines and corresponding ISO standards).

No-similar agreed scheme has yet been developed for biodegradability assessments of industrial effluents, probably partly due to the technical and conceptual problems that arise because effluents are usually complex mixtures of partly unknown composition. Traditional biodegradability assessments are rather simplistic and based on BOD/COD ratios. As only the most readily degradable effluent material is included in a 5-day BOD, longer-term BOD tests (e.g. 14 - 28 days of duration) are sometimes used. In order to assess the potential for biodegradation (treatability) in a municipal or in an industrial wastewater treatment plant (WTP), various laboratory- or pilot-scale treatability tests (mostly non-standardized) are commonly used, but these are normally carried out also by measuring only DOC or COD. All these methods suffer from the fact that the degradability of minor wastewater constituents, including possible environmentally harmful contaminants, cannot be examined by respirometry or by measuring chemical summary parameters. Alternatively to this whole effluent approach, based on summary parameters, assessments can be made based on biodegradability data for individual effluent constituents, which can be both well defined single chemical substances or classes of substances with similar

properties, i.e. the individual compound approach. However, very frequently the specific compound approach may not provide sufficient information, due to the several limitations of the chemical analysis techniques (Nyholm, 1996), as already referred.

Current standard biodegradation tests were designed as pass/fail tests to determine the general biodegradation potential of specific chemicals. The biodegradation result is generally stated as a degradation level in percentage, which is determined from a degradation curve. For each chemical tested a full biodegradation curve should be obtained and considered; in particular, the duration of the lag and biodegradation phases (days), the slope of the biodegradation portion of the curve (% biodegradation per day), and the duration of the plateau phase as well as its average percentage of biodegradation should be quantified (Fig. 3). This presupposes that a sufficient number of data points (e.g. COD, DOC) have been made for describing the whole curve.

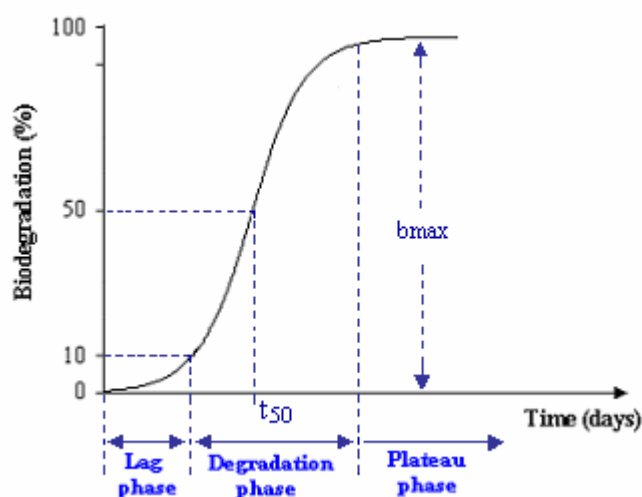


Fig. 3. Biodegradation curve (idealised).
(Adapted from Painter, 1997)

Typically, biodegradability tests show an initial phase of very slow degradation, the lag phase. This period can last from a few hours to several weeks or longer, and is explained by the time required for acclimation or adaptation by the inoculum to metabolize the test substance efficiently. A value of 10% is universally used to describe the end of the lag time, after which the regular degradation period begins, in which the bacteria use the substances as food and grow (Fig. 3).

In the OECD regime, a substance is considered readily biodegradable if it reaches a certain level of degradation (“pass level”) within a 10 days period (10 days window), from the beginning of mineralization (lag period) to the start of the final plateau, during a RB test with relatively low degrading power. The “pass level” ranges between 60 and 80% of biodegradation, depending on the assay parameter used (e.g. DOC removal: 70%; ThOD: 60%; ThCO₂: 60%; disappearance of parent compound: 80%) (Kaiser, 1998; Gotvajn and Zagorc-Končan, 1998, 1999a). For RB tests, the total time to reach the plateau phase must not exceed 28 days. For IB and other tests, the specified times to reach the plateau phase are greater than 28 days, and this additional time could be due to a longer lag time and/or biodegradation phase. Experience shows that a readily biodegradable substance will generally be completely degraded in the environment after a relatively short time, or by 90% in a wastewater treatment. So, chemicals passing the RB tests do not have to be further tested.

A substance may not be readily biodegradable, yet be inherently biodegradable, if complete degradation is demonstrated in an IB test of high degrading power. Such a substance is not persistent and will be degraded in the medium or long term in the environment (Pagga, 1997). However, inherently biodegradable compounds are assumed not to be degraded in wastewater treatment, unless a simulation test shows otherwise. Any substance that fails to degrade in an IB test is assumed to be non-

biodegradable in the environment (Painter, 1997). If more accurate data regarding the degradation behaviour in natural or technical environment are required, simulation tests must be performed.

Biodegradability and risk assessment

A large variety of biodegradability test methods have been used for various purposes, but only recently has interest grown in the use of biodegradability test data for environmental risk assessment (EEC, 1996; Blok, 2000). For this purpose quantitative predictions are required for the behaviour of the substances in various environmental conditions such as those present in sewage treatment plants, rivers, soil, or marine systems. Therefore, assessing the risk of chemicals for the environment requires an estimation of degradation rates in order to evaluate a realistic exposure concentration for organisms, i.e. to estimate the PEC (predicted environmental concentration). Extrapolation studies from results of ready and inherent biodegradation tests to real environment with mathematical models for the estimation of biodegradation rates, which are essential for deriving exposure concentrations of relevant compartments, have been reported by several authors (Struijs *et al.*, 1991; Struijs and van den Berg, 1995; Ahtiainen *et al.*, 2003).

Recently, Blok (2000) have proposed a new classification system for organic compounds ranking them in increasing order of persistence into eight biodegradability categories, based on a concept of probability of biodegradation which combines parameters as the maximum specific growth rate, a specific fraction of the total viable biomass and the percentage of mineralization reached.

BIOASSAYS WITH MICROORGANISMS

About three decades ago, interest began to focus on the potential impact on health and environment by releases of persistent synthetic (xenobiotic) organic substances,

through the discharges of municipal and/ or industrial wastes (effluents, solid residues). The evaluation of toxicity and biodegradability or persistency of such pollutants is essential for their hazardous impact and risk assessment, either on a biological treatment plant or on the environment of their ultimate disposal (e.g. water, soil, etc), and is performed by using bioassays.

Due to their important ecological role, bacteria are extensively used for the performance of those bioassays, either as a test organism to determine the effect of a pollutant (inhibition, toxicity), or as inoculum in the screening biodegradability tests to determine if the pollutant is removed (biodegradability/ treatability) (Blessing and Süßmuth, 1993). Bacteria can be found ubiquitously in nature, and they are the main biological agents responsible for the removal of both naturally occurring organic substances and waste xenobiotic chemicals from the environment. They are also involved in the recycling of mineral nutrients and their activities are essential to self-purification processes in the aquatic environments. Therefore, toxic effects on bacteria could impair these processes and have serious implications for the environment (Bitton and Dutka, 1986).

Moreover, the knowledge of the toxic effects of wastewaters and substances on sewage bacteria is important to protect wastewater treatment plants from toxic shock loading and to choose non-inhibiting concentrations when biodegradability is tested in aquatic systems (Strotmann and Pagga, 1996; Kelly *et al.*, 1999).

Several types of biodegradation and toxicity assessment tests, using bacteria (pure or mixed cultures), are nowadays available for evaluating the fate of a pollutant and its environmental impact, in both natural and technical systems (WTP). During the past few years much effort has been made to standardize the analytical procedures of these tests (e.g. OECD, ISO, CEN), which aim:

- The control of wastes, which guarantee the safety of industrial products/ processes,

- The environmental protection,
- The protection/control of the wastewater treatment plants (WTPs).

Many of these test methods (Table 1) employ activated sludge (AS), a heterogeneous microbial inoculum from a biological WTP, to assess toxicity or biodegradability of pollutants, since pollutants that ultimately enter the environment are often discharged through wastewater treatment plants. The activated sludge process is a widely used biological method of wastewater treatment in which the carbonaceous organic matter of wastewater provides an energy source for the production of new cells for a variable and mixed community of microorganisms in an aerobic aquatic environment. These microorganisms convert carbon into cell mass and oxidized end products that include carbon dioxide and water (heterotrophic active biomass). In addition, a limited number of microorganisms may exist in activated sludge that obtains energy by oxidizing inorganic compounds, e.g. ammonia nitrogen to nitrate nitrogen, in the process known as nitrification (autotrophic active biomass). This consortium of microorganisms, the biological component of the treatment process, is known collectively as activated sludge (Rader, 1999).

Bacteria, fungi, protozoa, rotifers and higher forms of invertebrates constitute the typical microbiology of AS. The microorganisms that are of greatest numerical importance in activated sludge are bacteria ($\approx 95\%$), which occur as microscopic individuals from one micron in size to visible aggregates or colonies of individuals or flocs. The flocs are composed of microbial biomass held together by bacterial slimes produced by *Zooglea ramigera* and similar microorganisms (Atlas, 1997). The nonbiological component of activated sludge flocs are organic and inorganic particles of the wastewater and extracellular microbial polymers that are generally made up of polysaccharides, glycoprotein fibers, organic acids, proteins and lipids (Jenkins *et al.*, 1993; Örmeci and Vesilind, 2000; Abreu, 2004). So, AS flocs are

aggregates of inert suspended solids, containing different groups of microorganisms embedded in a polymeric network.

Table 1. Standardized test methods for biodegradability and toxicity (OECD Guidelines and ISO Standards), which use activated sludge as microbial inoculum (Adapted from Pagga, 1997; Ahtiainen, 2002).

OECD GUIDELINES (OECD, 1993) – ISO STANDARDS

BIODEGRADABILITY TESTS

Tests on ready biodegradability

- 301 A: DOC Die-Away Test (equivalent to ISO 7827)
- 301 B: CO₂- Evolution Test (Modified Sturm test) (equivalent to ISO 9439)
- 301 C: MITI (I) Test
- 301 D: Close Bottle Test (equivalent to ISO 10707)
- 301 E: Modified OECD Screening Test (equivalent to ISO 7827)
- 301 F: Manometric Respirometry Test (equivalent to ISO 9408)

Tests on inherent biodegradability

- 302 A: Modified SCAS Test (equivalent to ISO 9887)
- 302 B: Zahn-Wellens/EMPA Test (equivalent to ISO 9888)
- 302 C: Modified MITI Test (II)
- ISO 5815 (1989). Water quality - Determination of biochemical oxygen demand after 5 days (BOD₅): Dilution and seeding method.

Simulation tests

- 303: Couple Units Test (equivalent to ISO 11733 – AS simulation test)

TOXICITY TESTS

- ISO 8192 (1986). Water quality - Test inhibition of oxygen consumption by activated sludge (equivalent to OECD 209)
- ISO 9509 (1989). Method for assessing the inhibition of nitrification of activated sludge microorganisms by chemicals and wastewaters.
- ISO 15522 (1999). Water quality – Determination of the inhibitory effect of water constituents on the growth of activated sludge microorganisms.

In AS, some bacteria are strict aerobes whereas others are anaerobes, but the preponderance of species are facultative bacteria, able to live in either the presence or absence of dissolved oxygen. While both heterotrophic and autotrophic bacteria

reside in activated sludge, the former predominate. The heterogeneous nature of the organic substrates in sewage allows mostly the development of diverse heterotrophic bacterial populations, primary floc-forming bacteria and some filamentous bacteria. Table 2 shows some examples of the most important genera, from different bacterial metabolic groups, present in activated sludge consortia.

Table 2. Examples of important genera of different metabolic groups of bacteria residing in activated sludge (Adapted from ¹Jenkins *et al.*, 1993; ^{2,3}Wanner, 1994a,b; ⁴Lopes, 1996; ⁵Vazoller, 1996; ⁶Atlas, 1997; ⁷Seviour and Blackall, 1999; ⁸Gerardi, 2002; ⁹Abreu, 2004; ¹⁰Sidat *et al.*, 1999; ^{11,12}Maszenan *et al.*, 1998, 2000; ¹³Wanner, 2001).

METABOLIC GROUP		EXAMPLES OF GENERA / SPECIES
Chemoheterotrophs	Aerobic bacteria	<ul style="list-style-type: none"> • <u>Floc-forming bacteria</u>: <i>Escherichia</i>, <i>Enterobacter</i>, <i>Bacillus</i>, <i>Pseudomonas</i>, <i>Micrococcus</i>, <i>Alcaligenes</i>, <i>Moraxella</i>, <i>Flavobacterium</i>, <i>Arthrobacter</i>, <i>Citrobacter</i>, <i>Zoogloea</i>, <i>Achromobacter</i>, <i>Flavobacterium</i>, <i>Comamonas</i> [1, 2, 4-9] • <u>Several filamentous bacteria</u>: <i>Sphaerotillus natans</i>, <i>Beggiatoa</i>, <i>Thiothrix</i> I, II, <i>Leucothrix</i> sp, <i>Microthrix parvicella</i>, <i>Nocardia</i>, <i>Gordonia</i>, <i>Nostocoida limicola</i>, <i>Haliscomenobacter hydrossis</i>, <i>Flexibacter</i>, <i>Rhodococcus</i>, <i>Mybacterium</i>, <i>Corynebacterium</i>, etc [3, 5, 7, 8, 9]
	Fermentative bacteria	<ul style="list-style-type: none"> • <i>Aeromonas</i>, <i>Pasteurella</i>, <i>Alcaligenes</i> [2, 4]
	Denitrifying bacteria (anaerobic/aerobic)	<ul style="list-style-type: none"> • <i>Pseudomonas</i>, <i>Bacillus</i>, <i>Alcaligenes</i>, <i>Achromobacter</i>, <i>Flavobacterium</i>, <i>Arthrobacter</i>, <i>Moraxella</i>, <i>Paracoccus denitrificans</i>, <i>Glucobacter</i> sp., <i>Zoogloea ramigera</i> [4, 7]
	Poli-P (aerobic bacteria P-accumulating)	<ul style="list-style-type: none"> • <i>Acinetobacter</i>, <i>Pseudomonas</i>, <i>Moraxella</i> [5, 7], <i>Aeromonas</i>, <i>Staphylococcus</i>, <i>Streptococcus</i>, <i>Micrococcus</i>, etc [10] • G-bacteria [11], <i>Tetrasphaera</i> [12], <i>Amaricoccus kapliciensis</i> [13]
	SO ₄ -reducing bacteria (anaerobic)	<ul style="list-style-type: none"> • <i>Desulfovibrio</i>, <i>Desulfobacter</i> [4]
Chemoautotrophs	Nitrifying bacteria (aerobic)	<ul style="list-style-type: none"> • <i>Nitrosomonas</i>, <i>Nitrobacter</i>, <i>Nitrospira</i> [2, 4, 5, 7]
	Sulfur-oxidizing bacteria (aerobic)	<ul style="list-style-type: none"> • <i>Beggiatoa</i>, <i>Thiothrix</i>, <i>Thiobacillus</i> [2, 4, 7]

Activated sludge is a complex mixture of various constituents that shows a wide variation in its physical, chemical and biological properties. The biodiversity in the AS microbial community depends on environmental conditions, process design and mode of the treatment plant operation, and the characteristics of influent wastewater. Variations in the microbial community make it almost impossible to carry out controlled bioassays using AS, causing problems with the reproducibility of the biological tests results and therefore in the intercomparison of different test results. This fact puts difficulties and constitutes a barrier to the normalisation of biological analysis and assays, which involve the use of an activated sludge inoculum.

In addition, the use of AS as inoculum for biological assays leads to several problems, such as: inoculum sampling, maintenance of the viability of the inoculum, difficulty in controlling the quantitative and qualitative characteristics of the inoculum, and possible presence of pathogenic microorganisms (bacteria, viruses and intestinal parasites - Seviour and Blackall, 1999).

There is a clear need for a biological surrogate for AS to assess toxicity or biodegradability of pollutants in AS-based biological tests, which should be a standard biological reference material (BRM). Such reference material (RM), or reference inoculum, should be biologically well-defined, stable and ready-to-use, and successfully present the same metabolic properties as activated sludge.

There currently exist some commercial products (from USA) formulated for use in biodegradability and toxicity tests, e.g.:

- PolySeed[®] - a blend of broad spectrum bacteria designed specifically as seed inoculum for the biochemical oxygen demand (BOD₅) test (APHA, 1998). PolySeed[®] is an USEPA approved BOD₅ inoculum that has been used to seed both municipal and industrial wastes. InterLab Supply.
- Polytox[™], Nitrotox[™] – USEPA recommended inocula constituted each one by a different specialized blend of microbial cultures for measuring the toxicity of wastewater. InterLab Supply.

- Bi-Chem[®] BOD Seed – a specialized blend of microbial cultures tested to provide BOD results equivalent to municipal seed inoculum (Novozymes Biologicals, Inc).

In the last few years several disposable-type microbial sensors (with a great lifetime >1 month), have been developed to simplify the traditional standardized BOD₅ test, a crucial environmental index for monitoring organic pollutants in wastewater, permitting a faster measure of BOD (in 1-30 minutes). These biosensors resolved two problems associated to this method: reduced the time required for the assay, which prevented its use for control process, and overcame the problems inherent to the use of AS as seed.

The biosensors, consisting of an oxygen electrode with a microbial membrane attached to its tips, usually use pure strains, e.g. *Bacillus subtilis* (Qian and Tan, 1998); *Trichosporon cutaneum* (Yang *et al.*, 1996; Marty *et al.*, 1997); *E. coli* (Pasco *et al.*, 2000); *Pseudomonas fluorescens* (Yoshida *et al.*, 2001) and microorganisms isolated from special WTPs (An *et al.*, 1998; Trosok *et al.*, 2001), which despite seem to be more suitable than mixed inocula suffer of lower environmental realism, because a mixed bacterial population probably shows a response different from that of pure cultures. Nevertheless, also some mixtures of microorganisms were proposed as BOD biosensors, including AS obtained from WTPs (Strand and Carlson, 1984; Kong *et al.*, 1996; Lukasse *et al.*, 1997; König, 1999). Still, the microbial sensors consisting of AS containing mixed populations presented several difficulties to give reproducible results.

None of these biological products, the commercial inocula or the microbial sensors, are proposed, certified or validated as reference materials to be used in the standardized biological assays, at European level.

SCOPE OF THE THESIS

The aim of this work was to develop and propose a valid biological reference material, to be used as alternative seed in relevant standardized AS-based bioassays, namely those related to biodegradability assessment for substances and effluents or toxicity evaluation (ISO/TR 15462, 1997; ISO 8192, 1896; ISO 5815,1989; see Table 1).

The development of a standard biological reference material, showing the behaviour of AS, i.e. a surrogate culture, can be used as calibrant for quality control, namely of wastes and their biological treatments. Furthermore, this surrogate culture should help to improve biological assays standardization and to provide traceability and reproducible data when performing AS-based tests.

So, this biological reference material was designed according to the previous defined criteria:

- exhibit a similar metabolic profile and adequate resistance as real AS;
- be a mixed microbial consortium composed by a reduced number of microorganisms (3-6 strains), in order to allow feasible standardization, control and production;
- be formed exclusively by heterotrophic bacteria, which are considered the main responsables of organic matter degradation and overall metabolic activity;
- be a homogeneous and stable blend of bacterial cultures;
- not contain any pathogenic microorganism and nitrifier;
- not contain acclimatised microorganisms to particular pollutants;
- be of easy preservation, preparation and manipulation; and
- present feasibility as seed in AS-based bioassays.

This standard biological material may act as a complement and important tool for existing activated sludge tests, which use non-standardized AS from a WTP.

Therefore in this study, with the main goal of propose a biological reference material that satisfy all the above criteria, several standard microbial inocula: commercial inocula (Bi-Chem[®] BOD Seed and Biolen M112), a pure culture of *Pseudomonas* sp. and various model communities prepared with selected well-known strains isolated from AS or soil (e.g. soil inoculum, design inoculum, etc), were metabolic characterized and evaluated as alternative seed in AS-based tests, namely: the test for inhibition of oxygen consumption (ISO 8192, 1986 or OECD 209), the BOD₅ test (ISO 5815, 1989; APHA, 1998; WTW, 1997), and the Zahn-Wellens test (ISO 9888, 1999) in comparison with AS. Moreover, from these standard microbial inocula, those most similar to AS, either in terms of their metabolic fingerprinting or in their responses in the bioassays, were chosen as potential biological reference materials. These representative inocula (AS surrogates) were validated as reference materials to be used as seed in the BOD₅ test, by testing several real effluents.

In this context, and as displayed in Fig. 4, several stages were performed aiming to attain different objectives:

- Stage 1 (**ST1**): Microbiological characterization of AS communities, collected from different urban WTPs with different effluent compositions, using classical, microscopic and biochemical methods (API identification system and BIOLOG system). In this stage was performed the isolation and identification (at genus or species level) of the predominant and common microbial species (aerobic heterotrophic bacteria) to be used in ST2, for metabolic studies. This task contributed also for the selection of the equivalent strains, with the adequate characteristics (non-pathogenic, etc), from culture collections (CCMI - Cultures Collection of Industrial Microorganisms, from Laboratory of Industrial Microbiology in INETI, Portugal), which could compose the biological reference material (representative inoculum).
- Stage 2 (**ST2**): Metabolic characterization of AS microbial communities by establishment of the metabolic fingerprints, i.e. the Community Level Physiological Profile (CLPP). Because several standards do not use directly the raw samples of AS, the metabolic characterization was also performed in samples

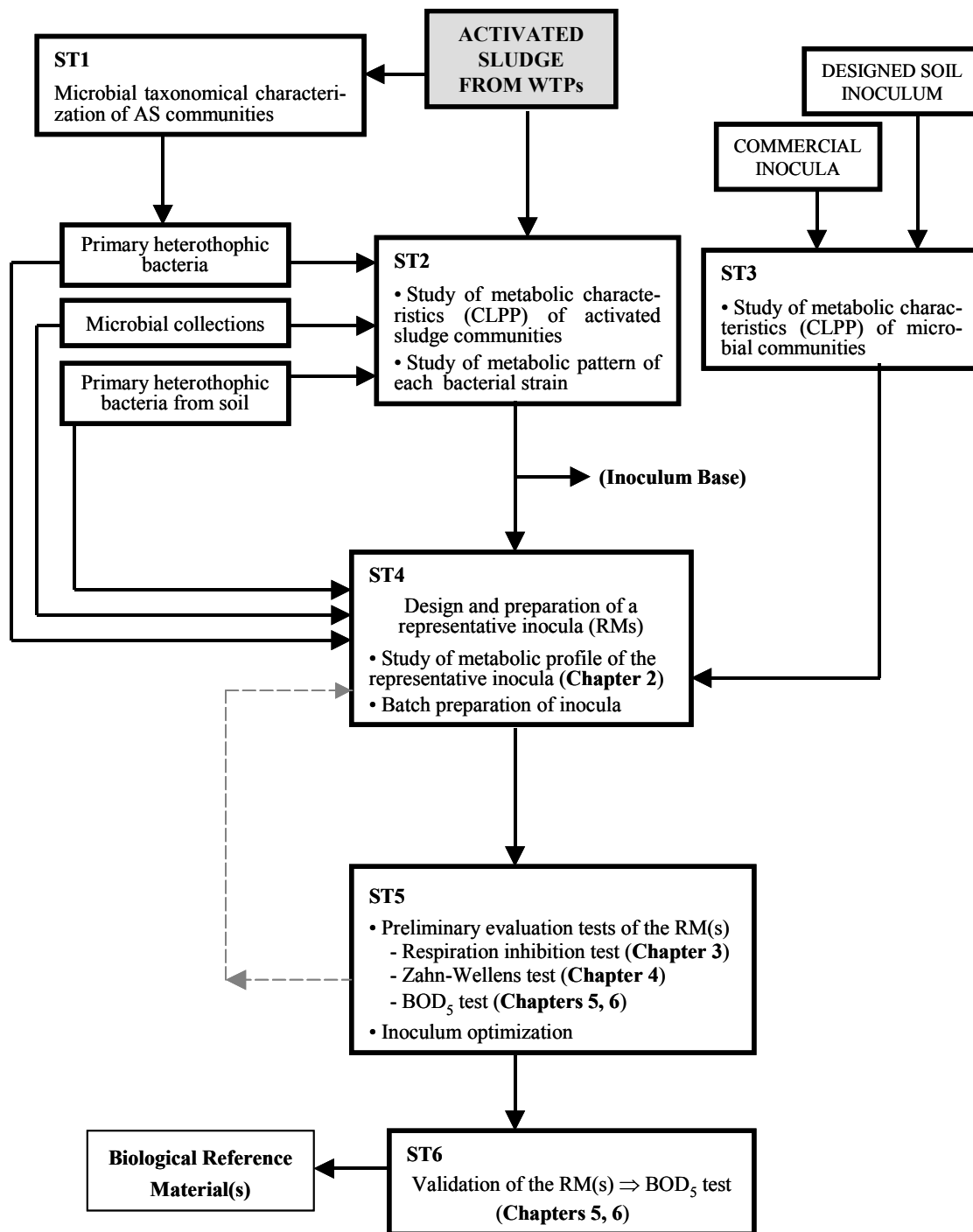


Fig. 4. Schematic overview of the workplan of the study developed in this thesis.

prepared as described in those standards. The objectives of this stage were: 1) study of the metabolic behaviour of different AS microbial communities to establish a representative metabolic fingerprint; and 2) study of the metabolic pattern of individual predominant strains (those isolated in the previous ST from AS and the relevant equivalent microbial species from CCMI) to evaluate its own contribution for each community metabolic behaviour, and to decide the basic composition of a representative inoculum. BIOLOG MicroPlates™ (GN, GP and EcoPlates) were used for the metabolic profile analysis. From this stage, a reference inoculum base was produced.

- Stage 3 (**ST3**): Study of the metabolic profiles of some existing commercial inocula selected, namely: Bi-Chem® BOD Seed (Novozymes Biologicals, Inc) and Biolen M112 (Gamlen Industries S.A., St. Marcel-Vernon, France) and a designed soil inoculum (consortium of six non-pathogenic bacteria isolated from soil, despite of being ubiquitous in all environments), using the BIOLOG MicroPlates™, as in prior stage. The objective of this stage was to obtain CLPPs of the commercial inocula and the soil inoculum.
- Stage 4 (**ST4**): Design and preparation of representative microbial inocula (AS surrogate cultures), according the defined criteria (see above). This stage initiated with the study of metabolic profile of the reference inoculum base, which was carried out as in ST2. The reference inoculum base is composed by a set of defined strains from culture collections and well-known non-pathogenic bacterial isolates (from AS) and must reflect the biodiversity of the AS. Some variations of this inoculum base (different model communities prepared) were studied and the impact of the addition of other strains in the global metabolic fingerprint was investigated. The main goal was the design of representative inocula and the establishment of a batch preparation of the potential biological reference materials.
- Stage 5 (**ST5**): Preliminary tests for the inocula evaluation to assess their potential as reference materials (RMs). The objective of this stage was the evaluation of the performance of the representative inocula, in terms of their utilization as seed in selected AS-based bioassays, with their potential optimization. A comparative study with AS and the proposed representative inocula, was carried out using the standards: ISO 5815 (1989) and ISO 8192 (1986). These preliminary tests were performed with the respective reference solutions/ compounds: glucose-glutamic acid solution for BOD₅ tests (dilution and manometric methods) and 3,5-dichlorophenol for the respiration inhibition test. A statistical significant number of tests (about 10 replicates) was carried out to establish and compare repeatability and variability in the experimental results with the potential RMs and the AS. The representative inocula most similar to AS (surrogate cultures) were also tested as seed in an IB test, the Zahn-Wellens test (ISO 9888, 1999) for the reference

compound: diethylene glycol. In this stage, the behaviour of the potential RMs must be according to the several specifications described in the standards.

- Stage 6 (**ST6**): First validation of the potential reference materials as seed in a BOD₅ test (ISO 5815, 1989; WTW, 1997), by performing this test for a significant number of real effluent samples and comparing the results with those obtained with AS (RMs *versus* AS). From this stage, one or more standard biological reference materials, to be used as calibrant for BOD₅ test and prone to be evaluated and validated for other biodegradability and toxicity standards, were expected to be obtained.

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Chapter 2

MICROBIAL COMMUNITIES PRESENTING A METABOLIC PROFILE SIMILAR TO ACTIVATED SLUDGE

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ABSTRACT - To search for reliable testing inocula alternatives to activated sludge (AS) cultures, several model microbial consortia were compared with AS populations for their metabolic behaviour. The community-level characterization of these mixed microbial populations was performed using the BIOLOG EcoPlates™, GN and GP MicroPlates™. The metabolic profiles obtained for model communities and AS samples were analysed by Principal Component Analysis (PCA) and hierarchic clustering methods, in order to evaluate the ability of BIOLOG plates to distinguish among the different microbial communities and to compare them with AS communities. The impact of different inocula preparation methodologies on the community structure was also studied. The metabolic profiles obtained with both EcoPlates and GN microplates led to the identification of three similar clusters in the studied microbial communities, demonstrating that EcoPlates, despite contains 31 substrates against the 95 GN substrates, are able to screen communities with a metabolic profile similar to AS. New well-defined, standardized and safe inocula presenting the same metabolic community profile as AS were selected and can be tested as surrogate cultures in activated sludge based-bioassays.

Keywords: standardized biological reference material, model communities, community level analysis, BIOLOG, activated sludge.

INTRODUCTION

Biological assays are crucial for detection of pollution in the environment and the assessment of toxicity of wastewaters and chemical substances. During the past few years much effort has been developed to standardize analytical protocols (e.g. OECD, ISO, CEN) that guarantee the safety of industrial products and processes as well as the protection/control of wastewater treatment plants (Cordis, 2000). Many of those bioassays utilize activated sludge (AS) as the biological reference material for the study of biodegradability (Pagga, 1997; Strotmann *et al.*, 1995) and toxicity (Pagga and Strotmann, 1999; Strotmann *et al.*, 1994; Strotmann and Pagga, 1996).

However, besides the distinct abiotic characteristics of such habitats, the microbial population of AS depends on the waste composition and the operation mode of the treatment plant. Variations in microbial population affect the reproducibility of bioassays and therefore complicate the comparison of different tests. So, a strong need exists for the development of a standard and certified biological material, with the metabolic behaviour of AS, that can be used as calibrant for quality control, improving standardization of biological assays and providing traceability (Cordis, 2000).

Previous studies have shown that microbial communities produce habitat-specific and reproducible patterns of carbon source oxidation (Gamo and Shoji, 1999; Glimm *et al.*, 1997; Harch *et al.*, 1997; Kaiser *et al.*, 1998; Smalla *et al.*, 1998) and the method used to discern temporal and spatial differences among microbial communities was the substrate profiling by BIOLOG microtiter plates (BIOLOG Inc).

Since the proposal of the community-level BIOLOG assay for the characterisation of the heterotrophic microbial communities in environmental samples (Garland and Mills, 1991), this method has become the most used because of its rapidity and

simplicity. The BIOLOG plates contain multiple sole carbon sources and a control without carbon source. Each well also contains a minimal growth medium and the redox dye tetrazolium violet that turns purple in the presence of electron transfer, indicating the substrate utilization by the inoculated microbes.

In almost all previous environmental studies employing BIOLOG plates, the Gram-negative (GN) microplates were the most used. However, there were also a few studies using Gram-positive (GP) plates. Choi and Dobbs (1999) have demonstrated that GN microplates and EcoPlates are equally efficacious in distinguishing among heterotrophic bacterial communities from a variety of environments. BIOLOG data are also well-suited for multivariate statistical analyses such as principal component analysis and cluster analysis, tools which can distinguish among microbial communities from various environments (van Heerden *et al.*, 2002; Choi and Dobbs, 1999; Glimm *et al.*, 1997; Garland, 1997; Victorio *et al.*, 1996).

The main objective of this study was to compare the metabolic fingerprints of several well-defined microbial consortia with those of activated sludge inocula, the reference communities, using the EcoPlates, GN and GP MicroPlates™ from BIOLOG Inc. (Hayward, CA), in order to select those that present a similar community level physiological profile (CLPP) to AS, which then can be tested as alternative inocula for toxicity and biodegradability assessment. Different inoculum preparation methodologies (inoculum washing, ≠ growth medium, ≠ optical density) were also applied to the microbial consortia to identify differences in metabolic profiles.

MATERIALS AND METHODS

Microbial inocula

In this study several microbial inocula were used: activated sludge inocula as reference communities and distinct bacterial model communities, as potential standardized biological reference materials. Each one of the model communities was designed to follow the predefined criteria: 1) exhibit a similar metabolic profile and adequate resistance as real AS; 2) consist in a mixed microbial consortium composed by a reduced number of strains, in order to allow feasible its standardization, control and production; 3) consist exclusively in heterotrophic bacteria, which are considered the main responsible of organic matter degradation and overall metabolic activity; 4) be a homogeneous and stable blend of bacterial cultures; 5) not contain any pathogenic microorganisms and nitrifiers; 6) not contain acclimated microorganisms to particular pollutants; and 7) be of easy preservation, preparation and manipulation. So, the model communities consisted of (a) well-defined bacterial mixtures containing 4-8 bacterial strains, in equal proportions, belonging to non-pathogenic species from genera commonly found in sewage and/or soil, namely: *Acinetobacter*, *Enterobacter*, *Xanthomonas*, *Pseudomonas*, *Bacillus* and *Staphylococcus*, and (b) commercial inocula (biological seed standardized by its manufacturer, containing non-pathogenic bacteria): the BIOLIN M112 (Gamlen Industries S.A., St. Marcel-Vernon, France) and BI-CHEM[®] BOD SEED (Novozymes Biologicals Inc., Salem, VA).

Activated sludge inocula

Activated sludge (AS) samples were collected from the aeration tank of two municipal wastewater treatment plants, the Beirolas'WTP (WTP1), which predominantly treats domestic sewage and the S. João da Talha'WTP (WTP2) which

treats urban wastewaters with $\approx 60\%$ industrial sewage. The concentration of activated sludge samples was 2-4 g/l (suspended solids) and the inocula were used within 36 h of collection. Each sample was allowed to settle 10 min to obtain a turbid supernatant of the sludge with an $OD_{600} \approx 0.30$ (Guckert *et al.*, 1996; Kaiser *et al.*, 1998) (AS1 - WTP1 and AS3 - WTP2, respectively). AS washed samples were also prepared, by centrifuging (6000 rpm, 10 min) and washing twice the samples with sterile phosphate buffer (50 mM, pH 7) or NaCl 0.85%, and resuspended in phosphate buffer to an $OD_{600} \approx 0.30$ (AS2 - WTP1 and AS4 - WTP2, respectively).

Preparation of model communities (MC)

Fifteen bacterial consortia (MC5 - MC7 with predominantly GP bacteria, and MC14 - MC25 with predominantly GN bacteria) were prepared with known pure cultures previously characterized in terms of their individual metabolic profiles, which have been selected from CCMI (Cultures Collection of Industrial Microorganisms, from Laboratory of Industrial Microbiology in INETI, Portugal), and from bacterial isolates of environmental samples (soil, activated sludge). The pure cultures were previously grown on tryptic soy agar (TSA) and transferred onto tryptic soy broth (TSB) and incubated at 28°C for about 20 hours (exponential phase), with agitation (150 rpm). To prepare these model communities, the selected pure cultures were added in equal parts (5-10 ml) and then each mixture was centrifuged at 6000 rpm for 10 min and washed twice with sterile 50mM phosphate buffer (pH 7) or NaCl 0.85%. The pellet was resuspended in 50 mM phosphate buffer (pH 7.0) to an optical density (OD) at 600 nm of 0.30-0.35 ($\approx 10^8$ cfu/ml). Model communities 5-7 were the same bacterial consortium but grown on two different culture media: synthetic medium (ISO, 1986) and TSB, MC5 and MC6 - MC7, respectively. Final suspensions in phosphate buffer were adjusted to an $OD_{600} \approx 0.3$ (MC7) - 0.5 (MC5,

MC6), corresponding to $\approx 10^8$ cfu/ml. Two commercial inocula (microbial consortia of GN and GP bacteria), the BIOLEN and BI-CHEM[®], were used to prepare six bacterial mixtures (MC 8-10 and MC 11-13, respectively). For each commercial inoculum, a suspension was prepared in isotonic solution (ISO, 1986), aerated for 10 min to rehydrate the inoculum and adjusted to an $OD_{600} \approx 0.30$ (MC8 and MC11). These suspensions (30-50 ml) were inoculated on synthetic medium and on TSB and incubated at 28°C for about 18-20 hours, with agitation (150 rpm). Each grown culture was centrifuged (6000 rpm, 10 min) and washed twice with sterile NaCl 0.85% or phosphate buffer. The pellet was resuspended in the phosphate buffer to an $OD_{600} \approx 0.30 - 0.35$ for MC9 and MC12 (synthetic medium) and to an $OD_{600} \approx 0.5 - 0.6$ for MC10 and MC13 (TSB).

BIOLOG assays

BIOLOG EcoPlates[™], GN and GP MicroPlates[™] (BIOLOG Inc.) were used to evaluate community-level metabolic responses. The EcoPlate contains three replicate wells of 31 carbon substrates (Choi and Dobbs, 1999; Insam, 1997) and a control well (A1), with no added carbon substrate. Any colour development in A1-well presumably indicates utilization of carbon sources inherent to the inoculated microbial suspension. GN and GP microplates consist of 96-well microtiter plates with 95 different carbon sources (Zak *et al.*, 1994) and a control well, so these plates were used in triplicate for each microbial community analysed.

For the BIOLOG assays with the several microbial communities, each suspension of bacterial cells prepared as described above was used to inoculate BIOLOG EcoPlates, GN and GP microplates. Aliquots of 150 μ L were added per well using a multi-channel pipettor (BIOLOG - 8 channels, BIOLOG Inc., Hayward, USA), and the BIOLOG plates were evaluated for OD changes at 590 nm using a microtiter

plate reader (model MRX_{REVELATION}, Dynex Technologies Inc., Chantilly, USA). Following an initial reading (T_0), the plates were incubated at 28°C for 72 hours. The plates were read periodically at 17.5, 20, 22.5, 41, 45.5, 48.5, 66 and 72 h, during the incubation period (Guckert *et al.*, 1996). For each inoculum, the results were expressed as the mean OD₅₉₀ (OD_{T_i}) for the blanks and the ECO, GN and GP substrates. For each reading time (T_i), a relative value (RV) corresponding to the optical density ratio (OD_{T_i}/OD_{T₀}) was then calculated.

BIOLOG data analysis

In the analysis of BIOLOG data two different approaches (Paixão *et al.*, 2003a) were used. For a qualitative assay of each substrate, matrices of blanked relative values were obtained by subtracting the RV of the blank well (RV_{blank}) to the RV of the substrate (RV_{substrate}). These matrices were converted in boolean ones, considering positive (+) the substrates for which $(RV_{\text{substrate}} - RV_{\text{blank}}) > 0.40 \times RV_{\text{substrate}}$ and negative (-) the substrates for which $(RV_{\text{substrate}} - RV_{\text{blank}}) \leq 0.40 \times RV_{\text{substrate}}$. The correction factor of 40% was applied to BIOLOG data (RV) to eliminate false negatives, as described in the BIOLOG system for strain identification.

A quantitative analysis was also performed by plotting the optical density ratio (OD_{T_i}/OD_{T₀}) *versus* time for the 32 wells of EcoPlates, the 96 wells of GN plates and the 96 wells of GP plates. Each substrate curve was blanked (blank A1 curve subtracted) and then the curve-integration approach was used (Guckert *et al.*, 1996; Hackett and Griffiths, 1997). Metabolic profiles of BIOLOG results were primarily expressed as the net area under the curve for each of the 31+95+95 response wells over 3 days of incubation.

The relationships between the BIOLOG profiles of the several microbial communities were determined by principal component analysis (PCA), using the

ANDAD software (CVRM-IST 1989-2000, Portugal), and by cluster analysis and Pearson linear correlations between sample data, using the program NTSYSpc version 2.02h for Windows (Exeter Software, New York). PCA, applied to quantitative data, is an ordination method which projects the original set of data points into new axes or principal components (PC) so that intrinsic patterns of clustering become apparent. Each PC extracts a percentage of the variance in the original data, with the greatest variance extracted by the first axis. PCA also allows identify the major discriminating variables associated with a given PC (Victorio *et al.*, 1996). In cluster analysis, applied also to quantitative BIOLOG data (ECO-GN, ECO-GN-GP), the microbial communities were clustered using the unweighted pair group method of arithmetic averages (UPGMA) and the linkage distance between profiles, based on Pearson' correlation coefficient (1 - Pearson r).

RESULTS AND DISCUSSION

Phenotypic fingerprints of the microbial communities

The phenotypic fingerprints obtained for the different microbial communities after 72 h of incubation are presented in Table 1. The AS samples (AS1-AS4) were considered positive for almost all substrates (95-99%) of the three plate types (ECO-GN-GP). The model communities 12, 15, 17-25, were considered positive for 96-100% of 221 substrates tested; the model communities 5, 9, 14, 16 and 25 were positive for 86-93% of ECO-GN-GP substrates, respectively. The model communities 6, 7 and 11 were positive for about 87-98% of 126 substrates tested (ECO-GN), the model community 10 was considered positive only for 21% out of 126 substrates and the model communities 8 and 13 were considered negative for all ECO-GN-GP substrates, according to the criteria used. So, the model communities that presented the most similar fingerprinting pattern to AS were the consortia 12, 15, 17-25.

Table 1. Phenotypic fingerprints obtained with BIOLOG plates (ECO, GN and GP) for the 25 microbial communities tested.

Microbial communities ^a	Substrates with negative (-) response (Well No.)			Total No. of substrates with positive (+) response
	ECO	GN	GP	
AS 1	---	64	30, 87, 95, 96, 33	215
AS 2	---	4, 72, 92	30, 68, 87, 93, 95, 49	212
AS 3	---	---	30, 87	219
AS 4	---	4, 6, 23, 37, 64, 72	7, 30, 55, 62, 87, 93	209
MC 5	19	64	12, 16-18, 41, 48, 55, 64, 67, 68, 84, 87, 89	206
MC 6	19, 32	2, 48, 56, 64, 69, 87, 95	ND ^b	117
MC 7	16, 19	9	ND	123
MC 8	2-32	2-96	ND	0
MC 9	11, 14, 19, 21-24, 27, 29	13, 14, 19, 21-23, 28, 30, 37, 39, 40, 56, 60, 61, 64, 75, 76, 79, 80, 85, 87, 92	7, 12, 41, 48, 49, 51, 53, 54, 68, 69, 78, 83, 87, 89, 93, 94	174
MC 10	2-32	2, 4, 6, 7, 13, 14, 16, 17, 21-24, 26, 28-30, 32, 34, 37-44, 46, 48-50, 52-54, 56, 58-77, 79-88, 91-93, 96	ND	27
MC 11	11, 16, 19, 21, 23	2, 42, 64, 89-96	ND	110
MC 12	19	14, 64	93	217
MC 13	2-32	2-96	ND	0
MC 14	11, 14, 19, 21	2, 4, 13, 25, 60, 61, 64, 66	6, 29, 30, 48, 64, 68, 89	202
MC 15	11, 19, 22	2, 13, 23, 64	6, 48, 89	211
MC 16	19	2, 4, 13, 23, 25, 64, 66, 92	6, 7, 29, 48, 49, 56, 60, 64, 87, 89, 91, 93	200
MC 17	22	4, 13, 72	9, 49, 64, 89	213
MC 18	---	60	---	220
MC 19	---	92	64, 89	218
MC 20	---	---	9, 49, 56, 81, 89	216
MC 21	---	92	9, 89	218
MC 22	---	---	---	221
MC 23	---	13, 64, 72	93	217
MC 24	14	9, 60	---	218
MC 25	5, 14	2, 9, 39, 60	17, 56, 64, 93	211

^a AS: Activated Sludge; MC: model community. ^b ND: not determined.

Principal component analysis of BIOLOG data

Based on the quantitative metabolic patterns data, expressed as response curve net areas for 72 h, for ECO, GN and GP substrates, a principal component analysis (PCA) was carried out to determine the relationships between the different inocula tested in comparison with AS.

PCA of metabolic profiles allows for comparison of microbial samples on the basis of differences in the patterns of carbon source utilization. The comparisons by PCA determine how the samples are different, but do not test among samples for specific differences. The separation of samples in PC space can be related to differences in carbon source utilization by examining all the possible correlations of the original variables to the PCs. The most important carbon sources in differentiating among microbial communities typically give high positive or negative correlations, which are reflected in the ordination plots. Poor correlation of a carbon source does not necessarily mean that it was poorly utilized by the inocula, but rather that its utilization was not different among microbial samples and therefore is not useful in differentiating inocula (Victorio *et al.*, 1996).

The statistical analysis for the microbial communities functional differences was based on PCs of each one of the three microplate types: ECO (Fig. 1), GN (Fig. 2) and GP (Fig. 3) to compare the ability of each BIOLOG plate to distinguish among the microbial communities. PCA was done based on the correlation matrix. Throughout, two principal components (PC1, PC2) were used in this data analysis, since about 75% of the total variance in the data is explained.

In the PCA for ECO data, the PC1 explained 65% of the total variation observed while the PC2 explained 10%. Analysis of the correlation of C source utilization on PC1 and PC2 (see Table 2) indicated that PC1 was associated to the utilization capacity of almost all ECO-substrates, with the higher correlations ($r \geq 0.85$)

Table 2. Correlation of C source utilization variables on first two principal components (PC) in analysis of EcoPlate data. These values correspond to the graphical analysis presented in Fig. 1.

Well No.	Substrate category ^a	ECO substrate	PC1	PC2
2	CA	pyruvic acid methyl ester	0.92	^b
3	POL	tween 40	0.89	
4	POL	tween 80	0.76	
5	POL	cyclodextrin	0.58	-0.55
6	POL	glycogen	0.64	-0.61
7	CH	D-cellobiose	0.95	
8	CH	α -D-lactose	0.83	
9	CH	β -methyl-D-glucoside	0.91	
10	CH	D-xylose	0.90	
11	CH	i-erythritol	0.80	
12	CH	D-mannitol	0.97	
13	CH	N-acetyl-d-glucosamine	0.96	
14	CA	D-glucosaminic acid		-0.76
15	PHO	glucose-1-phosphate	0.87	
16	PHO	D,L- α -glycerol phosphate	0.86	
17	CA	D-galactonic acid γ -lactone	0.89	
18	CA	D-galacturonic acid	0.94	
19	PHE	2-hydroxybenzoic acid	0.78	
20	PHE	4-hydroxybenzoic acid	0.82	
21	CA	γ -hydroxybutyric acid	0.75	
22	CA	itaconic acid		-0.74
23	CA	α -ketobutyric acid	0.75	
24	CA	D-malic acid	0.71	
25	AA	L-arginine	0.85	
26	AA	L-asparagine	0.90	
27	AA	L-phenylalanine	0.77	
28	AA	L-serine	0.91	
29	AA	L-threonine	0.72	
30	AA	glycyl-L-glutamic acid	0.82	
31	AM	phenylethylamine	0.74	
32	AM	putrescine	0.76	

^a CA: carboxylic acid; POL: polymer; CH: carbohydrate; PHE: phenolic compound; AA: amino acid; AM: amine; PHO: phosphorylated chemical. ^b When no value is given, the correlation is not relevant (<0.45 and >-0.45).

observed for the carbohydrates group in general, for the amino acids: L-arginine, L-asparagine and L-serine (r : 0.85-0.91), for the carboxylic acids: pyruvic acid methyl ester ($r=0.92$), D-Galactonic acid γ -lactone ($r=0.89$) and D-galacturonic acid ($r=0.94$), and for the two phosphorylated chemicals (r : 0.86-0.87), while PC2 was associated mainly to the utilization of two carbohydrates, D-glucosaminic acid and itaconic acid. Positive correlation, as verified for substrates associated for the first principal component can indicate a greater response for those carbon sources in communities with higher coordinate scores for the axis, while a negative correlation, as observed for the substrates associated to PC2, would indicate a greater utilization of those substrates in communities with lower scores for the axis (Garland, 1996).

The bidimensional-plot (PC1xPC2) presented in Fig. 1 shows the relationships between 25 microbial communities according to ECO-carbon source pattern utilization, which could be grouped in three major clusters (see figure). The model communities 18, 19, 21-24, from the predominant cluster, were the most responsive to ECO substrates associated to PC1, in opposite to the model communities 8, 9, 10 and 13, which were the least responsive to those ECO substrates. AS communities (1-4) were the most responsive relatively to the utilization of the two carbohydrates mainly associated to PC2 that present a negative correlation: D-glucosaminic acid ($r=-0.76$) and itaconic acid ($r=-0.74$), as well as the model communities 11 and 12. In contrast, MC25 showed the lower utilization of these substrates, as well as MC14 and MC15 (see Fig 1).

According to this PCA analysis (Fig. 1), the metabolic behaviour of AS samples (1-4) seemed to be similar, but between the different samples from WTP2 (3 and 4) could be observed an influence due to the washing procedure used. The model community that seem to have the most overall similar catabolic profile to AS inocula was MC11. However, the model communities 17, 20 and 25 also present an

identical response to all ECO carbon sources associated to PC1 differing mainly in the utilization of the two substrates associated with PC2.

Model communities 8-10 and 13 were observed to be the most distinct from AS (Fig. 1). The differences obtained in the metabolic responses by model communities 8, 9 and 10 were due to the applied methodology, since they were prepared from the same microbial consortium but using a different procedure (see section above). These model communities were the least responsive to almost

all ECO substrates presenting the lower net areas for the substrates utilized, being however a little higher for the community grown in synthetic medium (MC9). The initial cells number was similar for MC9 and MC10 (10^8 cfu/ml) but much lower for MC8 (3×10^6 cfu/ml), which influenced the metabolic response by MC8. In the same way, the influence of the procedure for the inoculum preparation could be observed for the other commercial consortium prepared into model communities 11-13, being the inoculum grown in TSB (MC13) the least responsive, as MC8-MC10, with much lower net areas than MC11 and MC12. Despite MC13 has presented an identical initial cells number to MC12 (10^8 cfu/ml), the metabolic behaviour of MC12 was more similar to MC11, which had a lower initial cells number (4×10^6 cfu/ml) because it was a direct suspension of the

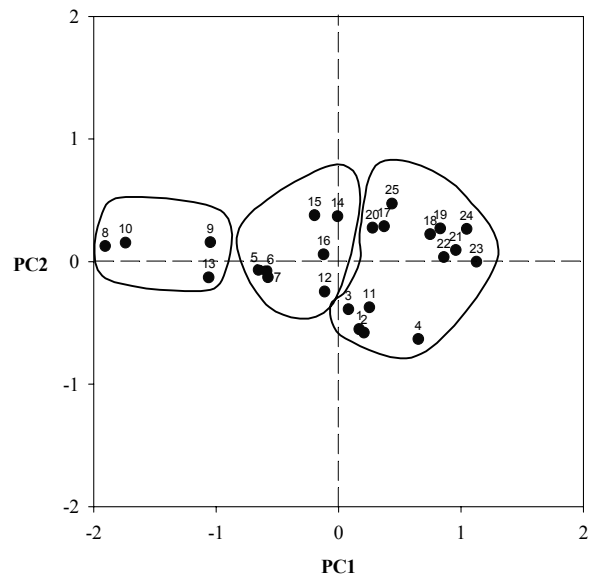


Fig. 1. Bidimensional plot of principal component analysis for BIOLOG ECO profiles (expressed as net area under the curve) of microbial communities. PC1 and PC2 refer to the first two principal components accounting for 65% and 10% of total variance, respectively.

lyophilised BI-CHEM. So, this showed that the profile of consortium grown in the synthetic medium was similar to the non-growth inoculum. On contrary, model communities 5-7 presented a similar net area response to ECO substrates, independently of the growth medium used. Despite of the OD range used (0.3-0.5) the initial cells number was identical for all (10^8 cfu/ml).

In the PCA for GN data, which results are presented in the bidimensional-plot (PC1xPC2) of Fig. 2, the PC1 explained 66% of the total variation observed while the PC2 explained only 7%. For this data set also three major clusters could be delimited. Analysis of the correlation of C source utilization on PC1 and PC2 (see Table 3) indicated that PC1 was associated to the utilization capacity of the most of GN substrates, with high correlations ($r \geq 0.85$) observed for the almost all carbon sources associated to PC1 in general, while PC2 was associated mainly to the utilization of the polymer α -cyclodextrin, the carboxylic acids: itaconic acid and D-glucosaminic acid, the amine 2-amino ethanol and the carbohydrate lactulose.

The model communities 18, 19, 21-24 (Fig. 2) were also the most responsive to the most of GN substrates associated to PC1, in opposite to MC8, MC10 and MC13 which were the least responsive to the substrates associated to this axis. AS communities (1-4) were the most responsive relatively to the utilization of the carbohydrates associated to PC2 presenting negative correlation (r : -0.49 to -0.76), as well as the model communities 11 and 12. In contrast, MC24 showed the lower utilization of these substrates, as well as MC18, MC22 and MC25.

This PCA analysis (Fig. 2), as the previous, showed also that AS communities metabolic behaviour were more similar between samples from different WTP (1 and 3) than between samples from the same WTP (1 \rightarrow 2; 3 \rightarrow 4) after subjected to a washing procedure being the difference greater for the samples of WTP2 (3 \rightarrow 4). This confirmed the influence of the procedure used for the inoculum preparation.

Table 3. Correlation of C source utilization variables on first two principal components (PC) in analysis of GN microplate data. These values correspond to the graphical analysis presented in Fig. 2.

GN substrate (Well No.)	Sub. cat. ^a	PC1	PC2	GN substrate (Well No.)	Sub. cat. ^a	PC1	PC2
3. D-melibiose	CH	0.91	^b	46. L-alanine	AA	0.93	
5. ρ -hydroxy-phenylacetic acid	CA	0.90		48. putrescine	AM	0.87	
7. L-histidine	AA	0.88		49. N-acetyl-D-galactosamine	CH	0.89	
9. α -cyclodextrin	POL		-0.76	51. sucrose	CH	0.94	
10. D-fructose	CH	0.96		52. D-gluconic acid	CA	0.90	
11. β -methyl D-glucoside	CH	0.89		53. malonic acid	CA	0.87	
12. <i>cis</i> -aconitic ac.	CA	0.91		54. L-alanyl-glycine	AA	0.90	
13. itaconic acid	CA		-0.53	55. L-pyroglutamic acid	AA	0.88	
15. hydroxy L-proline	AA	0.87		56. 2-amino ethanol	AM		-0.49
16. inosine	ARO	0.93		57. N-acetyl-D-glucosamine	CH	0.91	
17. dextrin	POL	0.89		58. α -D-lactose	CH	0.90	
18. L-fucose	CH	0.91		59. D-trenalose	CH	0.94	
19. D-psicose	CH	0.90		60. D-glucosaminic acid	CA		-0.65
20. citric acid	CA	0.90		62. L-asparagine	AA	0.92	
22. glucuronamide D	AM	0.85		63. D-serine	AA	0.64	-0.57
24. uridine	ARO	0.90		65. adonitol	CH	0.87	
25. glycogen	POL	0.62	-0.53	66. lactulose	CH	0.65	-0.65
26. D-galactose	CH	0.92		67. turanose	CH	0.90	
27. D-raffinose	CH	0.92		68. D-glucuronic ac.	CA	0.96	
29. α -ketoglutaric acid	CA	0.91		69. quinic acid	CA	0.86	
31. L-ornithine	AA	0.88		70. L-aspartic acid	AA	0.94	
34. gentiobiose	CH	0.95		71. L-serine	AA	0.92	
36. D-galactonic ac. lactone	CA	0.95		73. L-arabinose	CH	0.87	
38. D-alanine	AA	0.97		74. maltose	CH	0.93	
41. tween 80	POL	0.89		78. L-glutamic acid	AA	0.89	
42. α -D-glucose	CH	0.86		81. D-arabitol	CH	0.95	
43. D-sorbitol	CH	0.75	-0.51	82. D-mannitol	CH	0.70	-0.58
44. D-galacturonic acid	CA	0.97		83. pyruvic acid methyl ester	EST	0.95	
45. D,L-lactic acid	CA	0.93		88. glucose-1-phosphate	PHO	0.86	

^a Sub. cat.: substrate category. CA: carboxylic acid; POL: polymer; CH: carbohydrate; AA: amino acid; AM: amine; PHO: phosphorylated chemical; AMD: amide; ARO: aromatic compound; EST: ester.

^b See Table 2.

The washing procedure decreased the net areas of the most substrate utilization curves, probably due to the absence of residual organic matter existing in AS supernatant which can act as inducer.

Model communities 11 and 12 were observed to have the most overall similar catabolic profile to AS (1, 2, 3). However, the model communities 17, 20 and 25 also present an identical response to GN carbon sources associated to PC1 differing mainly in the utilization of the substrates associated with PC2. The model communities 8, 10 and 13 were observed to be the most different from AS. These results were very similar to those obtained from PCA analysis with ECO data. Once again, the influen-

ce on the community metabolic profile by the procedure used (growth medium) was confirmed for the commercial inocula (8-10 and 11-13). From PCA for GN data sets the MC9 could be considered to belong to the central delimited cluster, being closer to AS4 as model communities 5-7, 14 and 15 (see Fig. 2).

The bidimensional-plot (PC1xPC2) presented in Fig.3 shows the relationships between the microbial communities according to GP carbon source pattern utilization, which could be grouped in three major clusters (see figure). GP data were performed only for 19 out of 25 model communities, selecting the best

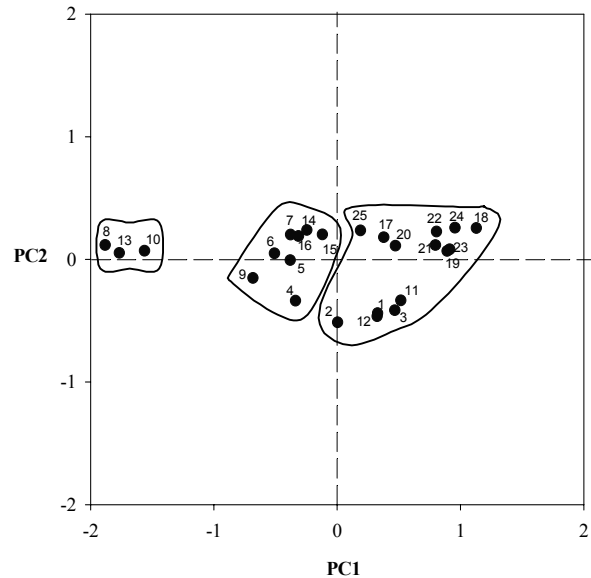


Fig. 2. Bidimensional plot of principal component analysis for BIOLOG GN profiles (expressed as net area under the curve) of microbial communities. PC1 and PC2 refer to the first two principal components accounting for 66% and 7% of total variance, respectively.

responsive model community for ECO and GN substrates from the consortia tested using different procedures (5-7, 8-10 and 11-13), i.e. selecting MC5, MC9, and MC12. In PCA for GP data, the PC1 accounted for 54% of the variation observed while the PC2 accounted for 12%, leading to a lower value of the total explained variance (66%). This may be explained by the fact that the net area under curves calculated for the substrates were under-evaluated for some community profiles, as the values of the blank net areas were higher than those observed for ECO or GN profiles. PC1 was associated to the capacity of utilization of the most of GP substrates, with high correlations ($r \geq 0.85$) observed for almost all carbon sources associated to this axis (see Table 4), while PC2 was mainly associated to the utilization of carbohydrates: α -D-lactose, lactamide, D-arabitol, lactulose, arbutin, xylitol, salicin, amygdalin and m-inositol; of the polymers α and β -cyclodextrin and the phosphorylated chemicals adenosine-5'-monophosphate and uridine-5'-monophosphate with positive correlation; and of the α -ketovaleric acid (carboxylic acid) with negative correlation (see Table 4).

From the predominant cluster (Fig. 3), the model communities 18, 19, 23 and 24 were the most responsive to the majority of GP substrates associated to the first

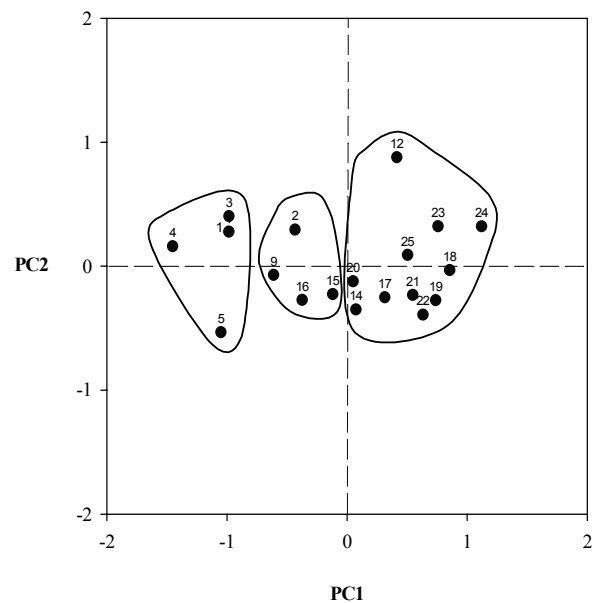


Fig. 3. Bidimensional plot of principal component analysis for BIOLOG GP profiles (expressed as net area under the curve) of microbial communities. PC1 and PC2 refer to the first two principal components accounting for 54% and 12% of total variance, respectively.

Table 4. Correlation of C source utilization variables on first two principal components (PC) in analysis of GP microplate data. These values correspond to the graphical analysis presented in Fig. 3.

GP substrate (Well No.)	Sub. cat. ^a	PC1	PC2	GP substrate (Well No.)	Sub. cat. ^a	PC1	PC2
2. L-arabinose	CH	0.90	^b	40. uridine	ARO	0.94	
3. α -D-lactose	CH		0.64	41. inulin	POL	0.71	0.51
4. β -methyl D-glucoside	CH	0.83		42. L-fucose	CH	0.88	
5. D-tagatose	CH	0.65	0.64	43. D-mannose	CH	0.88	
6. lactamide	CH		0.66	44. L-rhamnose	CH	0.94	
8. adenosine	ARO	0.86		48. adenosine-5'-monophosphate	PHO		0.71
9. α -cyclodextrin	POL		0.51	50. D-galactose	CH	0.90	
10. D-arabitol	CH		0.65	52. D-ribose	CH	0.87	
11. lactulose	CH		0.75	57. tween 40	POL	0.87	
13. D-trehalose	CH	0.90		58. D-galacturonic ac.	CA	0.92	
16. 2-deoxy adenosine	ARO	0.93		59. D-melibiose	CH	0.89	
17. β -cyclodextrin	POL		0.47	60. salicin	CH		0.77
18. arbutin	CH		0.62	61. β -hydroxybutyric ac.	CA	0.69	-0.53
19. maltose	CH	0.91		63. L-pyroglyutamic acid	AA	0.57	
21. turanose	CH	0.89		64. uridine-5'-monophosphate	PHO		0.68
23. L-alanine	AA	0.88		65. tween 80	POL	0.86	
24. inosine	ARO	0.90		72. fructose-6-phosphate	PHO	0.85	
25. dextrin	POL	0.93		73. N-acetyl-D-glucosamine	CH	0.89	
26. cellobiose	CH	0.85		74. D-gluconic acid	CA	0.90	
27. maltotriose	CH	0.87		80. glucose-1-phosphate	PHO	0.92	
28. D-psicose	CH	0.96		83. 3-methyl glucose	CH	0.88	
29. xylitol	CH		0.72	85. α -keto glutaric acid	CA	0.86	
31. L-alanyl-glycine	AA	0.85		89. amygdalin	CH		0.69
32. thymidine	ARO	0.93		90. m-inositol	CH		0.68
34. D-fructose	CH	0.85		92. sucrose	CH	0.87	
35. D-mannitol	CH	0.85		93. α -keto valeric acid	CA		-0.58
36. D-raffinose	CH	0.92					
37. D-xylose	CH	0.92					

^aSub. cat.: substrate category. CA: carboxylic acid; POL: polymer; CH: carbohydrate; AA: amino acid; AM: amine; PHO: phosphorylated chemical; AMD: amide; ARO: aromatic compound.

^bSee Table 2.

axis, in opposite to AS inocula (1, 3, 4) and MC5, which were the least responsive to those substrates. Considering the substrates associated to PC2, MC12 was the community most responsive relatively to the utilization of the substrates associated to this axis presenting positive correlation, while MC5 was the least responsive for those substrates, but present the highest response relatively to the α -ketovaleric acid ($r = -0.58$).

In contrast to PCA for ECO and GN data, the PCA for GP data (Fig. 3) separated the AS communities (1, 3, 4) and MC5 from the other model communities as the cluster that presented the lowest net areas under curves for the most of GP substrates. For AS communities these results can be explained by their bacterial composition, i.e. predominantly GN bacteria. However, this PCA also showed the influence of the washing procedure in AS metabolic profiles, being the behaviour of AS samples from different WTPs (1 and 3) more similar than the profiles of samples from the same source but subject to the washing procedure (1 \rightarrow 2, 3 \rightarrow 4). Contrary to ECO and GN data, the washing procedure increased the net areas of the GP substrate utilization curves for WTP1 samples (1 \rightarrow 2). For the samples from WTP2 (3 \rightarrow 4) was observed a decrease of substrate curves net areas with the washing procedure. This effect was due to the much higher value of the blank net area, which was subtracted to each substrate net area as a correction factor.

Cluster analysis of BIOLOG data

The dendrogram obtained by cluster analysis of grouped ECO-GN data sets (126 substrates) is presented in Fig. 4. Seven groups could be defined in this dendrogram, with an overall similarity level of less than 4%. Group I contains the AS samples (AS1-AS4) with 61% similarity and MC11 being less than 53% similar to the rest of the group. Group II contains MC5, MC9, MC10 and MC12 with 68% similarity. Group III contains the model communities 14 to 25 with 71% similarity. These three

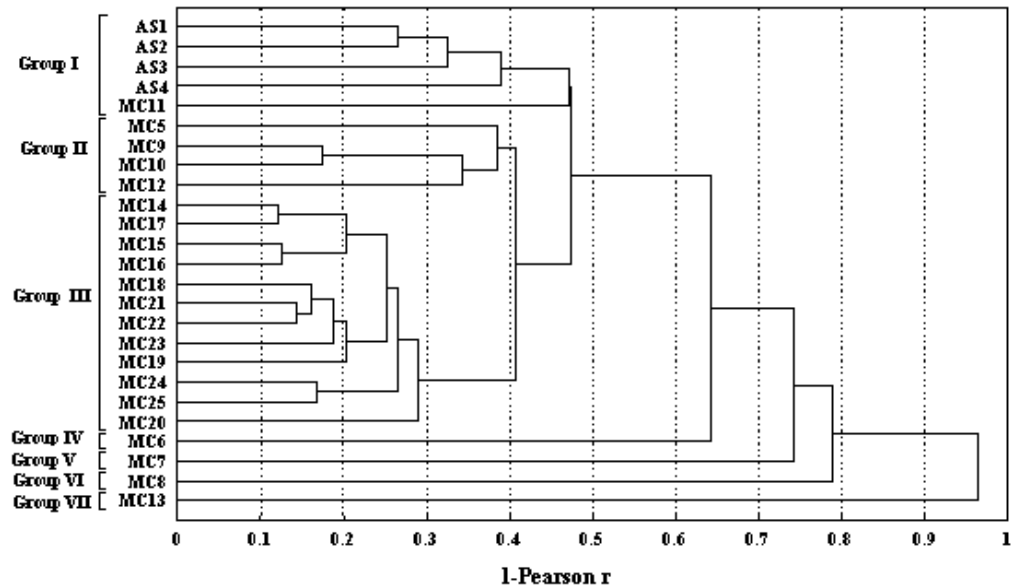


Fig. 4. Dendrogram of the metabolic fingerprints of 25 microbial communities obtained by cluster analysis of grouped ECO-GN data sets, using UPGMA and linkage distance based on Pearson correlation coefficient (AS - activated sludge; MC - model community).

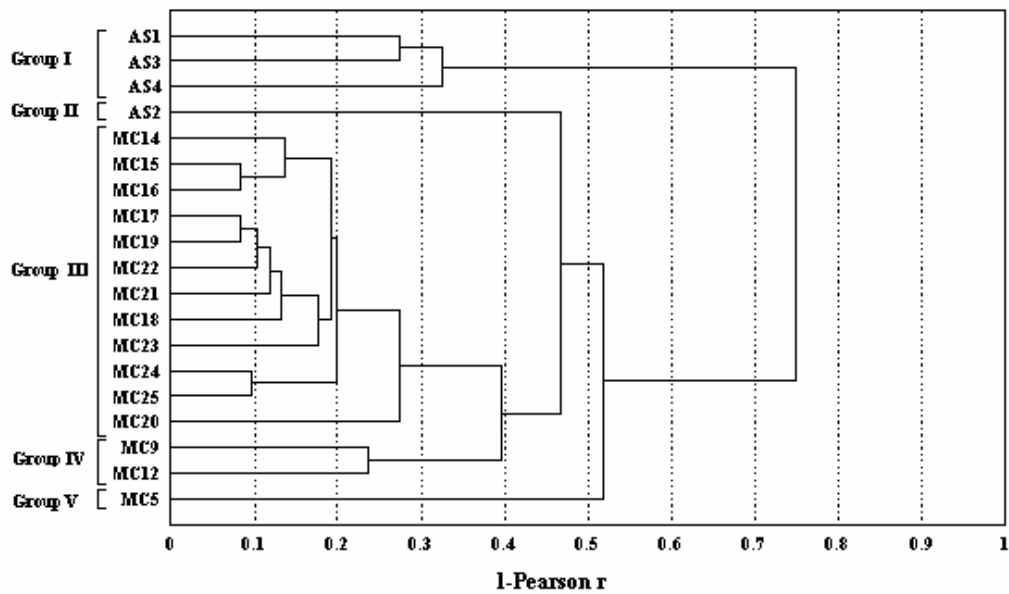


Fig. 5. Dendrogram of the metabolic fingerprints of 19 microbial communities by cluster analysis of grouped ECO-GN-GP data sets, using UPGMA and linkage distance based on Pearson correlation coefficient (AS - activated sludge; MC - model community).

groups were 53% similar. Groups IV-VII correspond to MC6, MC7, MC8 and MC13, respectively, which are linked with the first three groups at similarity levels between 36% and 4%.

The results of cluster analysis of grouped ECO-GN-GP data sets (221 substrates) are presented in the dendrogram of Fig. 5. This dendrogram, that excludes six communities (6-8, 10, 11 and 13) not tested with GP microplates, shows a different group distribution accounting the divergence of results obtained with GP data, mainly for AS samples. Five groups were defined, with an overall similarity level of about 25%. Group I contains AS1, AS3 and AS4 with 68% similarity. Group II corresponds to AS2 that was only 25% similar to the other AS samples included on group I. Group III contains the model communities 14 to 25 with 72% similarity. Group IV corresponds to MC9 and MC12 with 76% similarity and which were less than 60% similar to group III. The communities of groups III and IV were less than 53% similar to AS2. Group V corresponds to MC5 that was 48% similar to the groups II-IV.

In this data analysis, the first group, involving the major AS samples, was only 25% similar to the other group communities comparatively to the 53% similarity of all AS samples with the communities of groups II and III, observed with ECO-GN data.

Global analysis

Statistical analysis, namely PCA and cluster analysis, applied to net areas under substrate curves (ECO, GN, GP) obtained for the several model microbial communities and AS communities permitted to assess and compare the functional diversity relative to substrate utilization by each microbial community. PCA analysis for ECO, GN and GP data sets (Fig. 1 to Fig. 3) distinguished three major distinct clusters according to the similarity of the metabolic profiles presented by the microbial communities, permitting to select model communities most similar to AS

and also to evaluate the impact of different inoculum preparation on community structure.

In toxicity and biodegradability tests using AS inocula, different procedures as homogenisation, washing and settling of samples can be used for inoculum preparation (ISO, 1986; ISO, 1999; Paixão *et al.*, 2000). The procedures used modify the communities' structure and as result their metabolic profiles, influencing also their performance on toxicity and biodegradability bioassays. The washing procedure applied to AS samples led to a decrease of net areas under the curves of the substrates, as well as a decrease of the number of substrates used, in comparison with no washed samples. Therefore, these results indicate that the lack of standardization of inoculum is also a form of bias besides the different source of inoculum, as already observed by other authors (Forney *et al.*, 2001; van Ginkel *et al.*, 1995; Victorio *et al.*, 1996).

In addition, it was also demonstrated the selective effect of the culture media used for the growth of the model communities, evaluating their influence on the community level metabolic profile for ECO, GN and GP substrates utilization. The synthetic medium used in AS toxicity test (ISO, 1986) enhanced the microbial metabolic responses for the substrates utilization (higher net areas), as well as the number of substrates used. The goal of this study was to select the microbial communities with similar metabolic behaviour to AS, but also to choose the most effective methodology for the inoculum preparation since this can affect the microbial community structure, as shown in this work.

Based in PCA and cluster analysis for the net areas from BIOLOG substrates utilization, Table 5 summarizes the higher correlations obtained for the model communities more similar to AS communities. In this comparative study the correlations presented here were only relative to AS1 and AS2, because these

samples were from domestic sewage, which is usually required by internationally standardized (ISO, OECD) activated sludge - based tests (Pagga, 1997).

Table 5. Pearson linear correlations for the model communities more similar to AS from WTP1 (AS1 and AS2) based on BIOLOG data.

Microbial communities	No. of Strains	ECO	GN	GP	ECO-GN	ECO-GN-GP
		ASd ^a	ASd	ASd	ASd	ASd
AS1-AS2 ^b	ND ^c	0.81	0.74	0.47	0.73	0.53
MC5	6	0.61	0.57	0.27	0.57	0.37
MC9	≥6	0.67	0.69	0.26	0.67	0.32
MC11	≥6	0.75	0.52	ND	0.56	ND
MC12	≥6	0.68	0.63	0.28	0.62	0.29
MC14	6	0.49	0.59	0.47	0.56	0.39
MC17	5	0.41	0.58	0.45	0.54	0.41
MC18	4	0.47	0.56	0.38	0.53	0.39
MC19	5	0.33	0.61	0.47	0.55	0.44
MC21	6	0.49	0.62	0.44	0.59	0.46
MC22	6	0.42	0.59	0.49	0.55	0.45
MC23	5	0.26	0.59	0.46	0.52	0.43

^aASd: average value relative to the two AS communities from WTP1 (AS1 and AS2).

^bAS1-AS2: mean correlation value between AS1 and AS2.

^cND: not determined.

For ECO data, a high value of correlation was observed between the metabolic profiles of reference communities (AS1-AS2: $r=0.81$), being the difference obtained mainly due to the applied methodology for the inoculum preparation. The other linear correlations for ECO results (Table 5) showed that the model communities more similar to AS reference communities (AS1-AS2) were MC5, MC9, MC11 and MC12. For GN data, the best communities in terms of AS similarity were MC9, MC12, MC19 and MC21, however the remaining communities (see Table 5) presented at least 70% (0.52/0.74) of relative value of correlation in relation to AS1-AS2 internal correlation. Considering the profiles for ECO-GN data (126 substrates),

once again the communities more similar to AS were MC5, MC9, MC12 and MC21, but the other model communities presented a relative correlation to AS1-AS2 of at least 71% (0.52/0.73).

These results showed that EcoPlates were suitable for comparing the similarities and differences between the microbial communities, in terms of their metabolic profiles for 31 substrates, demonstrating an equivalent capacity to discriminate among the heterotrophic profiles as GN microplates with 95 substrates, having been observed a high percentage of concordance in the utilization of the common substrates (25) in both types of plates (84-100%). The presence of three replicate sets of substrates in the same plate also makes EcoPlates an useful choice as screening method to distinguish among communities functional diversity.

In contrast, for GP data it was observed a lower correlation between the metabolic profiles exhibit by the two AS samples (AS1-AS2: $r=0.47$), and the correlation values obtained between model communities and AS communities were related mainly to AS2, despite the values presented in Table 5 were the mean values of AS1-AS2 correlations. According to these results the communities more similar to AS were MC14, MC19, MC21, MC22 and MC23. When all metabolic data sets were combined (ECO-GN-GP), the correlations between model communities and AS communities reflected specially the GP data, being MC19, MC21, MC22 and MC23 the communities that presented the higher relative correlations ($\geq 81\%$; 0.43/0.53) once again (see Table 5).

From these data analysis, a group of model communities with different number of strains were selected as surrogate cultures of AS, based on the utilization profiles of 221 single carbon sources (ECO-GN-GP), independently of the common substrates. Fig. 6 presents the metabolic profiles of the selected communities (5, 9, 12, 17, 18, 19 and 21) in comparison with AS profiles, grouping the utilization pattern in six

substrate sets, accordingly to Preston-Mafham *et al.* (2002): carboxylic acids (CA), polymers (POL), carbohydrates (CH), amino acids (AA), amines/amides (AM/AMD) and miscellaneous (MISC = phosphorilated chemicals, alcohols, aromatics, esters, phenolic compounds).

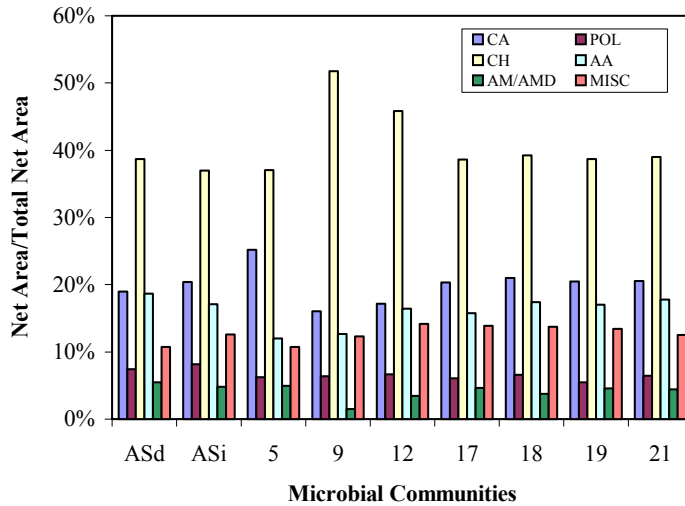


Fig. 6. Metabolic profiles for the model microbial communities (5, 9, 12, 17, 18, 19 and 21) more similar to activated sludge communities (ASd: average values for AS1 and AS2; ASi: average values for AS3 and AS4). Each bar represents the value of the percentage of net area under curves for each substrate set in relation to the total net area under curves for all of the 221 response wells over a 3 days incubation.

Some of these selected communities with a similar metabolic behaviour to AS, MC5, MC9 (BIOLEN) and MC12 (BI-CHEM), have already been tested as biological reference material in toxicity and biodegradability tests, where was demonstrated their potential to be used as alternative inocula to AS (Paixão *et al.*, 2000; 2003a,b,c). The utilization of standardized inocula, i.e. controlled mixtures of microbial strains with a specific or broad-range sensitivity, permits a more homogeneous response, the absence of sampling variability, the absence of pathogenic microorganisms and benefices from the selected strains ecological relevance. Haack *et al.* (1995), using model communities consisting of 4 or 6 isolates, as in this study, demonstrated that their substrate utilization profiles were repeatable and unique. Thus, the use of representative model communities with a

reduced number of microorganisms allow feasible its standardization, control and production.

Some commercial standardized consortia, consisting of bacteria commonly found in domestic sewage, are already used as biological material, however their test methods are not yet internationally standardized (ISO, OECD), and are only applied to determine one endpoint (e.g. Polytox™ for Polytox toxicity test, from InterLab™, Woodlands, TX, USA).

In fact, the utilization of suitable representative standardized microbial inocula as surrogate culture may act as a complement and important supplementary tool to existing activated sludge tests (OECD, ISO). The seed homogeneity and stability, as well as its easy and safe manipulation are important properties of biological reference materials, and this can be accomplished easier using harmless standardized inocula than AS samples collected from domestic sewage treatment facilities. The use of well-predefined cultures can be helpful for the standardization of biological assays, permitting to carry out controlled assays eliminating bias, reproduce test results or compare different test results, leading to an improvement of the quality of chemical and effluent testing and monitoring.

In conclusion, due to the great need of inoculum standardization in bioassays, the aiming of this work was to select model microbial communities, composed by bacteria commonly found in sewage and/or soil, which present a community level physiological profile for ECO-GN-GP substrates utilization similar to AS, together with the best methodology for the inoculum preparation.

The similar metabolic behaviour to AS is a pre-requisite necessary for a metabolic surrogate culture that is pretended to substitute AS. To validate these model communities, toxicity and biodegradability tests on specific chemicals and effluents

must be carried out to evaluate their performance and sensitivity in comparison with AS results.

Furthermore, to achieve a biological reference material able to be accepted and included in relevant standards (ISO and OECD), as alternative inoculum to AS a certification step should be prosecuted, including studies of inoculum stability and shelf life, realization of inter-laboratorial ring-tests, etc.

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Chapter 3

ALTERNATIVE INOCULA AS ACTIVATED SLUDGE SURROGATE CULTURE FOR A TOXICITY TEST

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ABSTRACT - The use of activated sludge to assess the potential toxicity and environmental impact of chemicals and wastewaters suffers from several drawbacks related with the heterogeneity, absence of standardization and health risk associated with this mixed sewage population. To search for reliable testing inoculum alternatives, the performance of two commercial inocula (BI-CHEM[®] and BIOLEN M112), a garden-soil inoculum and a pure culture of *Pseudomonas* sp., were compared with an activated sludge inoculum (AS) in the inhibition respiration test ISO 8192-1986 (E). The respiration rates of microbial inocula were assayed for the reference compound 3,5-dichlorophenol. The acute toxicity values (IC₅₀) ranged from 6.7 mg/l (*Pseudomonas* sp.) to 22.7 mg/l (garden-soil) overlapping the expected values for activated sludge microorganisms in spite of the different bacterial diversity. Assayed microbial inocula also showed higher reproducibility than AS and an overall similarity of catabolic profiles obtained with BIOLOG EcoPlates was observed between AS and some mixed inocula. These results point to the potential ability of such inocula as surrogate cultures in relevant activated sludge-based bioassays. New well-defined, standardized and safe tools will then be available for monitoring ecological impacts of hazardous substances and effluents, thus providing environmental protection.

Keywords: respiration inhibition, ISO 8192 – 1986, commercial inocula, *Pseudomonas*, soil inoculum.

INTRODUCTION

Biological assays are crucial for detection of pollution in the environment and the assessment of toxicity of wastewaters and chemical substances. The inhibitive effects of synthetic, xenobiotic organic chemicals to microorganisms are key considerations in chemical hazard management and control, because prokaryotes are ubiquitous in nature and are essential for maintaining the ecological balance.

During the past few years much effort has been developed to standardize analytical protocols (e.g. OECD, ISO, CEN) that guarantee the safety of industrial products and processes as well as the protection/control of wastewater treatment plants (Cordis, 2000). Many of those bioassays utilize activated sludge (AS) as the biological material for the study of biodegradability (Pagga, 1997; Strotmann *et al.*, 1995) and toxicity (Strotmann and Pagga, 1996), as in the case of ISO 8192 – 1986 (E), the international standard chosen for this work.

The ISO test specifies a method for assessing the potential toxicity of substances, mixtures or wastewaters to activated sludge. Information generated by this method is usually regarded as helpful in estimating the effect of a test material on mixed bacterial communities in the aquatic environment, especially in aerobic biological wastewater treatment systems (ISO, 1986). This test is a good monitor of toxic compounds that may reduce or inhibit microbial degradative activities and survival in sewage treatment plants; however, the use of activated sludge inocula is an awkward strategy, albeit available, in toxicity and biodegradability tests to assess the performance of other bioremediation systems or the health of aquatic environments. In addition to the distinct abiotic characteristics of such ecosystems, native microbial communities are functionally and structurally diverse enough to compromise the reliability of the inferred ecotoxicological effects. Furthermore, the use of a mixed microbial inoculum from an activated sludge treatment plant, which predominantly

treats domestic sewage, leads to problems as inoculum sampling, maintenance of its viability, difficulty in controlling its quantitative and qualitative characteristics and possible presence of pathogenic microorganisms.

A more appropriate strategy should be based on the real assessment of biodegradative ability of natural communities, as well as on the evaluation of toxicity responses of community members to hazardous chemicals, either isolated or in mixed cultures. The use of such microorganisms in standard toxicity and biodegradability bioassays could thus provide useful and more reliable information to be applied in the successful management of bioremediation processes and monitoring of chemical environmental impact.

In this context, the aim of the present work was to evaluate the potential of four microbial inocula: two commercial inocula (BI-CHEM[®] and BIOLEN M112), a standardized soil inoculum and a pure culture (*Pseudomonas* sp.), as reference biological material for comparison with AS (standard inoculum) in the test of the inhibition of oxygen consumption, described in the international standard mentioned above.

MATERIALS AND METHODS

Media and stock solutions

The synthetic medium (SM) stock solution was prepared by dissolving the following substances in 1 liter of distilled water: 16 g of peptone (HiMedia Laboratories, Bombay, India), 11 g of beef extract (HiMedia Laboratories), 3 g of urea, 0.7 g of NaCl, 0.4 g of CaCl₂·2H₂O, 0.2 g of MgSO₄·7H₂O and 2.8 g of K₂HPO₄. The pH was adjusted to 7.5 ± 0.5. The isotonic solution (IS) was composed of 5 g of NaCl and 0.12 g MgSO₄·7H₂O, per liter of deionized water. These stock solutions were prepared as described in ISO 8192-1986 (E). The stock solution of 3,5-

dichlorophenol was prepared by dissolving 1 g of this reference substance in 1000 ml of deionized water. Tryptone Soya Broth (TSB), from Oxoid Laboratories (Hampshire, England) and Nutrient Agar (NA), from Difco Laboratories (Detroit, USA), were prepared by adding 30 g and 23 g, respectively, to 1 liter of distilled water. These media were sterilized by autoclaving at 121° C for 15 min. All the chemicals were obtained from Merck Laboratories (Darmstadt, Germany), except when indicated otherwise.

Microbial inocula

In this study several microbial inocula were used: two commercial inocula (BI-CHEM[®] BOD Seed and BIOLEN M112), a garden-soil inoculum, a pure culture of *Pseudomonas* sp. and an activated sludge inoculum (standard inoculum).

Commercial inocula

BI-CHEM[®] BOD seed (Sybron Chemicals, Inc., Birmingham, NJ, USA) is a mixed culture inoculum tested to provide BOD results equivalent to municipal seed inoculum. As stated by the manufacturer, this product standardizes the potency and microbial population of the seed, providing improved standardization of results. BIOLEN M112 (Gamlen Industries S.A., St. Marcel-Vernon, France) is a mixed culture inoculum to be used in urban treatment plants to reduce BOD and COD and useful for degradation of foams, greases and oils. According to the manufacturer, BIOLEN cultures were developed to digest and degrade domestic-type wastes faster and much more efficiently than naturally occurring bacteria do.

For each commercial inoculum, a suspension was prepared in isotonic solution (120 mg BI-CHEM[®]/100 ml; 400 mg BIOLEN M112/100 ml) and aerated for 10 min to rehydrate the seed. 30ml of BI-CHEM[®] or 50 ml of BIOLEN M112 rehydrated suspensions were used to prepare 300 ml of inoculated diluted synthetic medium

(30-fold final dilution) in 500 ml Erlenmeyer flasks. After an overnight incubation with agitation (300 rpm) at room temperature, each culture was centrifuged (6000 rpm, 10 min) and the pellet was resuspended in isotonic medium to 0.3 absorbance units at 600 nm (10^7 - 10^8 cfu/ml), using a HACH DR/2000 Spectrophotometer. From these stock inocula, 10 ml was used in each reaction mixture of the respiration tests.

For the BIOLOG assays, 30 ml of each commercial culture grown in synthetic medium (28°C, \approx 18 h with agitation) was centrifuged (6000 rpm, 10 min) and washed twice with sterilized deionized water (SDW) or sterile 50 mM phosphate buffer (pH 7.0). The pellet was resuspended in 50 mM phosphate buffer (pH 7.0) to an OD₆₀₀ of 0.30-0.35 (10^8 cfu/ml).

Soil inoculum

Several soil samples were collected at INETI garden, from the top 10-25 cm of the surface after the removal of vegetation and rocks. The samples were homogenized, sieved (2 mm sieve) and used to prepare a suspension containing 2 g soil in 20 ml of SDW. An aliquot (5 ml) of this soil suspension was used to inoculate 250 ml of sterile synthetic medium to promote the growth of microorganisms. This mixture, after incubation at 20°C in a rotary shaker (100 rpm) over seven days, constituted the master culture broth (MCB), which was used as seed for all tests with soil. Prior to every test, 10 ml of MCB was inoculated into 250 ml sterile synthetic medium and incubated with aeration for 18 h at 20°C (King and Dutka, 1986). This culture was the stock soil inoculum (10^9 cfu/ml), and 3 ml was used in each reaction mixture for the respiration tests.

For the BIOLOG assay, 20 ml of this culture was added to 20 ml of SDW, centrifuged (6000 rpm, 10 min) and washed twice with SDW or sterile 50 mM

phosphate buffer (pH 7.0). The pellet was resuspended in 50 mM phosphate buffer (pH 7.0) to an OD₆₀₀ of ≈ 0.5 (10^7 - 10^8 cfu/ml).

Pseudomonas sp. inoculum

For the *Pseudomonas sp.* inoculum, strain NCIMB 10770 (National Collection of Industrial and Marine Bacteria, Aberdeen, UK) was grown in TSB at 28°C with rotary shaking (200 rpm). At the exponential phase, the culture was centrifuged (6000 rpm, 10 min) and washed with sterilized isotonic solution (SIS). For the respiration tests, the pellet was resuspended in SIS to 0.18 absorbance units at 600 nm (10^8 cfu/ml) and 20 ml of this stock inoculum was used in each reaction mixture. For the BIOLOG assay, the pellet was resuspended in sterile saline (NaCl 0.85%) to obtain the stock inoculum with an OD₆₀₀ of ≈ 0.16 (10^8 cfu/ml).

Standard inoculum

Activated sludge samples were collected from the aeration tank of the municipal wastewater treatment plant of S. João da Talha, near Lisbon. The concentration of activated sludge samples was 2-4 g/L (suspended solids) and the inoculum was used within 36 h of collection. For the respiration tests, 10 ml of this inoculum, which had been well homogenized by agitation (300 rpm), was used in each reaction mixture. For the BIOLOG assay the AS was allowed to settle for 10 min, after which the turbid supernatant of the sludge was adjusted to an OD₆₀₀ of ≈ 0.30 ($\approx 10^6$ cfu/ml) with sterile phosphate buffer (50 mM, pH 7.0).

Respiration assays

The respiration assays were carried out using the procedure described in the standard ISO 8192, method A, adapted (ISO, 1986). The substance tested was 3,5-dichlorophenol, which is the reference compound referred to in this standard.

For each inhibition study a series of reaction mixtures were prepared containing a defined concentration of synthetic medium and microbial inoculum with different concentrations of the test chemical. Test reaction mixtures were prepared by adding 10.7 ml of synthetic medium and the desired amount of the test chemical to a 500 ml Erlenmeyer flask. One control, without 3,5-dichlorophenol, was prepared in each test series. The mixtures were diluted to a volume of 310 ml with deionized water and aerated with an air pump. At the beginning of the test, the microbial inoculum (3, 10 or 20 ml) was aerated and added to each Erlenmeyer flask in turns at time intervals of about 5 min. The contents were transferred to the BOD bottles (\cong 310 ml), which were sealed to begin the test. Oxygen consumption was measured at 30-min intervals over 3 hours using an ORION oxygen electrode (Model 97-08-99, Orion Research, Boston, MA, USA).

Respiration data analysis

The respiration rates, defined as mgO_2/L consumed per hour, were calculated from the slopes of the regression lines obtained from the plot of the consumed oxygen against time.

The inhibitory effect of the 3,5-dichlorophenol (% inhibition) for each concentration was calculated as,

$$\% \text{ Inhibition} = (\text{Rc} - \text{Rs})/\text{Rc} \times 100$$

where Rc and Rs are the respiration rates of the control and the sample containing the test chemical, respectively. The concentration for 50% of inhibition (IC_{50}) was interpolated from the plot of the % inhibition against log of concentration by linear regression.

For each microbial inoculum, at least 10 test series to evaluate the IC_{50} of 3,5-dichlorophenol were performed. The test result was acceptable when the deviation

of the controls was within 15%. Assay repeatability (r) was calculated as $r = t\sqrt{2SD}$, where t is the two-tailed t value ($n-1$), n is the number of samples and SD the standard deviation (Kilroy and Gray, 1992).

Microbiological characterization

API identification system

Aliquots of the stock inocula suspensions (see above) were spread in nutrient agar and incubated for 24h at 30°C. Isolated microorganisms were identified by their morphological characteristics and by using the Analytical Profile Index (API) identification system: API 20 NE, API 20 E and API 50 CHB (BioMérieux S.A., France).

BIOLOG Eco assays

BIOLOG EcoPlates (BIOLOG Inc.) were used to evaluate community-level metabolic responses as well to establish the catabolic profiling of a pure culture. The EcoPlate contains three replicate wells of 31 carbon substrates (Choi and Dobbs, 1999) and a control well (A1), with no added carbon substrate. Any color development in A1 well presumably indicates utilization of carbon sources inherent to the inoculated microbial suspension.

For the BIOLOG assays with the several inocula, each suspension of bacterial cells prepared as described above was used to inoculate BIOLOG EcoPlate. Aliquots of 150 μ L were added per well using a multi-channel pipette (BIOLOG – 8 channels, BIOLOG Inc., Hayward, USA), and the EcoPlates were evaluated for absorbance changes at 590 nm using a microtiter plate reader (model MRX_{REVELATION}, Dynex Technologies Inc., Chantilly, USA). Following an initial reading (T_0), the plates were incubated at 28°C for 72 hours. The EcoPlates were read periodically at 17.5,

20, 22.5, 41, 45.5, 48.5, 66 and 72 h, during the incubation period (Guckert *et al.*, 1996). For each inoculum, the results were expressed as the mean OD₅₉₀ (OD_{T_i}) for the blank and the 31 substrates because each EcoPlate has three replicates. For each reading time (T_i), a relative value (RV) corresponding to the optical density ratio (OD_{T_i}/OD_{T₀}) was then calculated.

BIOLOG data analysis

In the analysis of BIOLOG data two different approaches were used. For a qualitative assay of each substrate, matrices of blanked relative values were obtained by subtracting the RV of the blank well (RV_{blank}) to the RV of the substrate (RV_{substrate}). These matrices were converted in boolean ones, considering positive (1) the substrates for which $(RV_{\text{substrate}} - RV_{\text{blank}}) > 0.40 \times RV_{\text{substrate}}$ and negative (0) the substrates for which $(RV_{\text{substrate}} - RV_{\text{blank}}) \leq 0.40 \times RV_{\text{substrate}}$.

A quantitative analysis was also performed by plotting the optical density ratio (OD_{T_i}/OD_{T₀}) *versus* time for the 32 wells of the BIOLOG EcoPlates. Each substrate curve was blanked (blank A1 curve subtracted) and then the curve-integration approach was used (Guckert *et al.*, 1996). Metabolic profiles of the BIOLOG results were expressed as the net area under the curve for each of the 31 response wells over a 3-day incubation.

The relationships between the BIOLOG profiles of the tested inocula were determined by principal component analysis (PCA) using ANDAD software (CVRM-IST 1989-2000, Portugal). PCA is an ordination method which projects the original set of data points into new axes or principal components (PC) so that intrinsic patterns of clustering become apparent. Each PC extracts a percentage of the variance in the original data, with the greatest variance extracted by the first axis.

PCA also allows the identification of major discriminating variables associated with a given PC (Victorio *et al.*, 1996).

RESULTS AND DISCUSSION

Respiration assays

The aim of performing the assays with several samples of the AS inoculum, obtained from a municipal treatment plant, was to get a first validation of the respiration tests with the four inocula under study. The sensitivity to 3,5-dichlorophenol and the repeatability ($n \geq 10$) of tested inocula were evaluated and compared with the AS response, as AS is the standard inoculum of the ISO test.

Table 1. Statistical analysis of the acute toxicity results (IC_{50}) obtained for the five inocula.

Microbial Inocula	Mean IC_{50} (N \geq 10)	Confidence Interval (95%)	Coefficient of Variation	Repeatability
BI-CHEM®	9.4 mg/l	8.9 - 9.9 mg/l	8.1 %	2.40
BIOLEN M112	12.8 mg/l	12.3 - 13.4 mg/l	7.2 %	2.86
Soil	22.7 mg/l	21.4 - 23.9 mg/l	7.9 %	5.72
Pure culture (<i>Pseudomonas</i> sp.)	6.7 mg/l	6.5 - 7.0 mg/l	6.3 %	1.27
Activated sludge	9.4 mg/l	8.7 - 10.2 mg/l	10.8 %	3.26

The acute toxicity values of 3,5-dichlorophenol, obtained for the tests performed with the five inocula, are presented in Table 1. Mean IC_{50} values ranged from 6.7 mg/l (*Pseudomonas* sp.) to 22.7 mg/l (soil inoculum), showing the inoculum-specific nature of toxic impact. Despite the toxicity of the reference compound to all microbial inocula, the pure culture of *Pseudomonas* sp. was more sensitive than the mixed inocula, with the soil inoculum being the least sensitive. The commercial

cultures showed a behaviour similar to AS, despite different microbial diversity, with the BI-CHEM[®] confidence interval (95%) for IC₅₀ overlapping that of AS.

The statistical analysis of the data (Table 1) shows the good reproducibility of the results obtained with the test inocula, which showed a variation of the IC₅₀ mean value from 6.3% to 8.1%. This variability was lower than that observed with the AS inoculum (10.8%), reflecting the origin and the metabolic characteristics of the microbial inoculum used in the respiration test.

Moreover, the IC₅₀ mean values obtained for 3,5-dichlorophenol with the four inocula under test were within the range referred to in the ISO standard for the AS inoculum (5-30 mg/l), validating the utilization of these microbial inocula in this respiration inhibition test as an alternative to AS inoculum.

Paixão *et al.* (2000a,b) studied these four microbial inocula in biochemical oxygen demand (BOD₅) tests. Their results showed these inocula also to be reliable as seed in those tests. The existence of important bioassays involving the activated sludge inocula, including several ISO, OECD and CEN standards, represents a potential relevant field to establish a representative and more ecologically realistic microbial inoculum that can be a surrogate culture for AS.

Microbial composition of mixed inocula

The characterization of the mixed cultures tested allowed the identification of the constituent microbial strains, providing some insight on the main groups of heterotrophic microorganisms that may be responsible for the biodegradation process.

The activated sludge inoculum contained predominantly Gram-negative bacteria, mainly those belonging to the genera *Aeromonas*, *Pseudomonas* and *Vibrio*. Beyond the well-known biodegradation abilities of such microbes, their involvement in

emerging food- and waterborne diseases is a major public health concern (Gugnani, 1999), which should be an alert to the risk associated with the increased laboratory utilization of AS inocula.

Although the actual taxonomic microbial composition of the commercial inocula must remain confidential, as required by manufacturers, the microbiological characterization carried out with BI-CHEM[®] and BIOLEN M112 showed that these inocula and the garden-soil inoculum seem to have overall similar microbial profiles, presenting predominantly Gram-positive aerobic spore-forming bacteria (*Bacillus* spp.) and some Gram-negative bacteria belonging to the genus *Enterobacter*. As this finding of similarity is in major agreement with the toxicity results, isolated strains may be regarded as potential biodegradative and suitable for ongoing studies of self-performance on acute toxicity tests using 3,5-dichlorophenol and other hazardous chemicals in order to implement safer, more reliable and well-characterized broad-range test inocula.

Metabolic characterization of inocula

BIOLOG EcoPlates were used to evaluate both qualitative and quantitatively community-level metabolic responses as well as to establish the metabolic profile of *Pseudomonas* sp.

After 72 h of incubation (Table 2), both soil and BI-CHEM[®] inocula were considered positive for 30 Eco-substrates, being negative for 2-hydroxy benzoic acid (well 19); BIOLEN M112 was positive for 22 Eco-substrates and the *Pseudomonas* culture was positive for only 19 carbon sources. The standard inoculum, AS from S.J.Talha' WTP, was positive for all the carbon sources in EcoPlate (Table 2).

Plots of BIOLOG EcoPlate responses (expressed as response curve net areas for 72 h) showing the culture metabolic profiles of the five microbial inocula used in this

work are presented in Fig. 1. Based on these quantitative metabolic profiles, a principal component analysis (PCA) was carried out to determine the relationships between the different inocula tested in comparison with AS inoculum.

Table 2. Phenotypic fingerprint for the inocula tested using EcoPlates.

Well No.	Eco-substrates	Microbial Inocula				
		AS	Soil	BIOLEN	BI-CHEM	<i>Pseudomonas</i>
2	Pyruvic acid methyl ester	1	1	1	1	1
3	Tween 40	1	1	1	1	1
4	Tween 80	1	1	1	1	1
5	Cyclodextrin	1	1	1	1	0
6	Glycogen	1	1	1	1	1
7	D-Cellobiose	1	1	1	1	1
8	α -D-Lactose	1	1	1	1	1
9	β -Methyl-D-glucoside	1	1	1	1	1
10	D-Xylose	1	1	1	1	1
11	i-Erythritol	1	1	0	1	0
12	d-Mannitol	1	1	1	1	1
13	N-Acetyl-d-glucosamine	1	1	1	1	0
14	D-Glucosaminic acid	1	1	0	1	0
15	Glucose-1-phosphate	1	1	1	1	1
16	D,L- α -Glycerol phosphate	1	1	1	1	1
17	D-Galactonic acid γ -lactone	1	1	1	1	0
18	D-Galacturonic acid	1	1	1	1	0
19	2-Hydroxybenzoic acid	1	0	0	0	0
20	4-Hydroxybenzoic acid	1	1	1	1	1
21	γ -Hydroxybutyric acid	1	1	0	1	1
22	Itaconic acid	1	1	0	1	1
23	α -Ketobutyric acid	1	1	0	1	1
24	D-Malic acid	1	1	0	1	1
25	L-Arginine	1	1	1	1	0
26	L-Asparagine	1	1	1	1	1
27	L-Phenylalanine	1	1	0	1	0
28	L-serine	1	1	1	1	0
29	L-Threonine	1	1	0	1	1
30	Glycyl-L-glutamic acid	1	1	1	1	1
31	Phenylethylamine	1	1	1	1	0
32	Putrescine	1	1	1	1	0

(1) positive: carbon source used; (0) negative: carbon source not used.

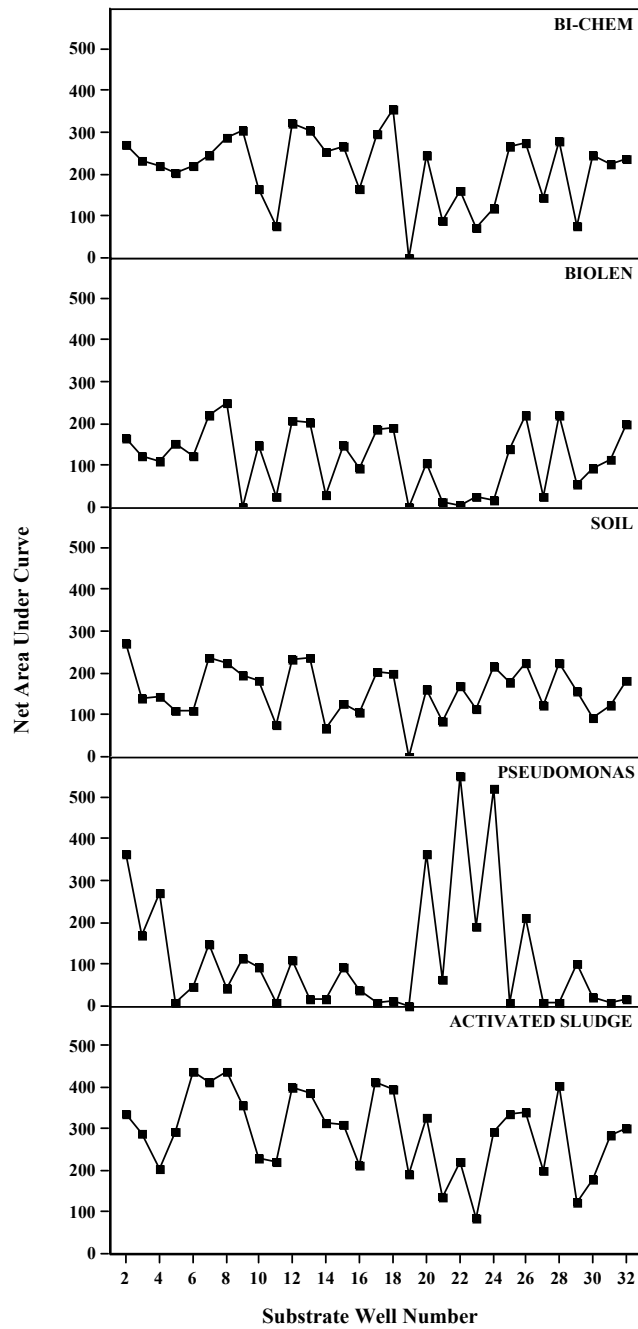


Fig. 1. Profile plots of Biolog results expressed as net area under the curve (A1 subtracted) for the several inocula tested in comparison with AS metabolic profile.

The first three principal components (PC1, PC2, PC3) explained 96.80% of the total variation observed. PC1 represents the utilization capacity of almost all Eco-substrates, being PC2 associated to Eco-substrates of wells 2, 4, 20, 22, 23 and 24, and PC3 associated to Eco-substrate 29 (see Table 2 for substrate identification).

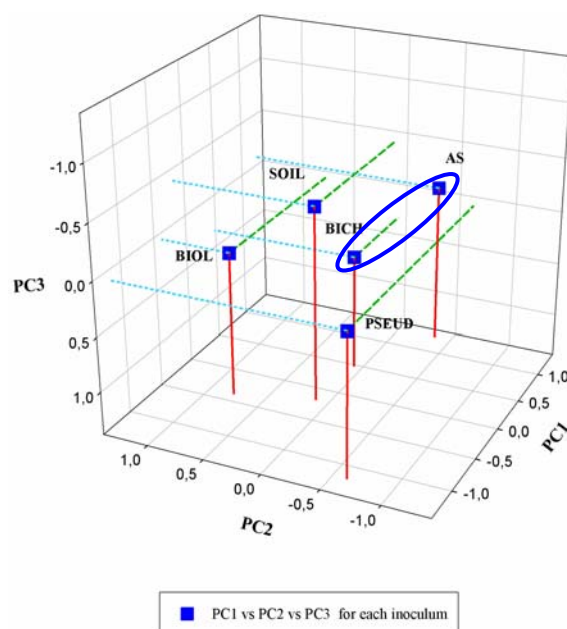


Fig. 2. Three-dimensional plot of Principal Component Analysis (PCA) of Biolog ECO profiles (expressed as net area under the curve) for the four inocula tested: soil inoculum (SOIL), BIOL M112 (BIOL), BI-CHEM[®] (BICH), and *Pseudomonas* sp. (PSEU), compared with a standard inoculum, activated sludge (AS).

The 3-D plot (PC1xPC2xPC3) presented in Fig. 2 shows the relationships between the five microbial inocula according to Eco-carbon source pattern utilization. In this context, the inoculum that seems to have the catabolic profile most similar to the standard inoculum (AS) is BI-CHEM[®], using almost all Eco-substrates and presenting higher values of net area under curve (Fig. 1) for substrates associated to PC1, as AS. The soil inoculum seems to utilize the Eco-substrates associated to the

three PCs identically, in general presenting lower values of net area under curve for Eco-substrates in relation to AS. BIOLEN M112 seems to be the mixed inoculum most different from AS, presenting the lowest values of net area under curve for almost all substrates in comparison with the other mixed inocula. As expected for a single culture inoculum, *Pseudomonas* sp. shows a reduced ability for the utilization of the major substrates, carbon sources associated with PC1 and PC3, in comparison with any of the mixed cultures used, therefore being apart; however, this culture presents the higher responses for Eco-substrates associated with PC2.

CONCLUSIONS

The results obtained in this study for the standard compound (3,5-dichlorophenol) show the potential for the utilization of the tested microbial inocula (BI-CHEM[®], BIOLEN M112, *Pseudomonas* culture and soil inoculum) as biological reference material for the ISO 8192-1986 (E) respiration inhibition test. As the performance was more precise and the constituent microorganisms are specific, well known or isolated, such inocula could be easily produced under criteria of quality control and become a more reliable alternative to the activated sludge inoculum.

The commercial inoculum BI-CHEM[®] seems to be a good choice as surrogate culture for this test once it is the most similar to AS, either in estimating 3,5-dichlorophenol IC₅₀ or for its general metabolic profile. However, further tests must be performed with this mixed microbial inoculum before this microbial community, standardized and exhibiting the same metabolic behaviour as activated sludge, can be proposed and certified as a new biological reference material (Cordis, 2000).

AS is a complex and dynamic mixture of suspended solids, various microorganisms, and extracellular material made up of polysaccharides, organic acids, proteins and lipids. The microbial population in AS depends on its waste composition and the

operation mode of the plant. Variations in microbial populations as well as on physico-chemical characteristics of activated sludge make it almost impossible to carry out controlled assays, reproduce test results or compare different test results (Cordis, 2000; Örmeci and Vesilind, 2000).

In fact, in-house inocula could provide more powerful tools for evaluating the environmental impact of hazardous chemicals or wastewaters, as controlled mixtures of microbial strains with specific or broad-range sensitivity could be prepared and used as inocula in toxicity tests. Such inocula should benefit from the standardized response, the absence of sampling variability, absence of pathogenic microorganisms and the ecological relevance of selected strains. In this context, a huge effort should be made in research studies aiming to characterize the metabolic and acute toxicity properties of new and already available biodegradative microbial strains, either in single or mixed cultures. The *Pseudomonas* sp. culture, because of its high sensitivity to 3,5-dichlorophenol and its metabolic profile, could be used in association with other complementary bacteria to prepare a new AS surrogate inoculum.

Seed homogeneity and stability as well as being easy and safe to manipulate are also important properties of these reference materials. Such controlled cultures can be helpful for the standardization of biological assays, leading to an improvement of the quality of chemical and effluent testing and monitoring.

The use of biological material as surrogate cultures may act as a complement and important supplementary tool to existing activated sludge tests (OECD, ISO, DIN). The availability of a reference material could lead to more homogeneous and comparable results and could eliminate any bias, which would be quite advantageous for inter-laboratory studies.

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Chapter 4

BIODEGRADABILITY TESTING USING STANDARDIZED MICROBIAL COMMUNITIES AS INOCULUM

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ABSTRACT - Reference materials are important tools for maintaining high quality assurance standards and this includes biological materials. A significant number of environmental international standards, including biodegradability and toxicity, involve the utilization of activated sludge (AS) inocula. The absence of inoculum standardization in these tests is a potential source of error influencing the results. In this study three defined microbial consortia, two commercial inocula and a designed bacterial inoculum, were evaluated as an AS alternative seed for the Zahn-Wellens test, using diethylene glycol as reference chemical. The results showed that to achieve diethylene glycol biodegradation with these inocula, a number of 10^5 colony forming units per millilitre of effective degrader microorganisms must be present. Moreover, the addition of AS supernatant to test mixtures improved the inocula performance being the biodegradability curves closer to those obtained with AS inocula. Among the three defined consortia, the designed inoculum replicates best the AS behaviour, for the range of tested concentrations, with degradation completed in 12-14 days. Comparisons of the community substrate utilization profiles corroborated these results, showing that the designed inoculum profile was the most similar to AS profile. The biodegradability and metabolic results obtained support the assertion that the designed inoculum can be used in the Zahn-Wellens test and as base to develop reference inocula for other biodegradability and toxicity tests.

Keywords: biological reference material, Zahn-Wellens test, standardized inocula, metabolic profile.

INTRODUCTION

Microbial degradation is the most dominant elimination mechanism of organics from the environment. Persistence of xenobiotics represents a real risk for ecosystems as well as for human beings. Therefore, the knowledge of the biodegradability of these xenobiotics is one of the most important aspects of understanding their behaviour in natural environment and during the biological treatment of wastewaters.

Various laboratory standard methods have been developed to assess the biodegradability of chemicals, and these are used to predict the influence of biodegradation on their fate and transport in the environment (Painter, 1995; Strotmann *et al.*, 1995; Pagga, 1997; Kaiser, 1998). The methods attempt to reasonably simulate the conditions that are found in a given environment or ecosystem. In this way, it is asserted that the data obtained can be extrapolated to predict what might occur in the actual environment. Many chemicals that ultimately enter the environment are largely discharged through sewage or wastewater treatment plants. Consequently, methods using activated sludge from wastewater treatment plants as inocula for tests to measure the ready or inherent biodegradability of chemicals are among the most widely used (Forney *et al.*, 2001).

In biodegradability testing, important factors which cannot be easily standardized and that greatly influences biodegradation results are the inoculum, the source of the microorganisms for the test, and their state of acclimatization and adaptation (Tabka *et al.*, 1993; Pagga, 1997).

Ideally, the activated sludge from several wastewater treatment plants, treating predominantly domestic sewage, that are used for biodegradability tests should be sufficiently similar so that the results of studies done to assess the biodegradability of chemicals could be readily compared (Forney *et al.*, 2001). However, activated

sludge is a complex and dynamic mixture of suspended solids, various microorganisms, and extracellular material made up of polysaccharides, organic acids, proteins and lipids. The characteristics of the activated sludge microbial population depend on the waste composition and operation mode of the plant. Variations in microbial population as well as on physical-chemical characteristics of activated sludge make it almost impossible to carry out controlled assays, reproduce test results or compare different test results (Cordis, 2000; Örmeci and Vesilind, 2000).

In this context, there is an obvious need to have a reference biological material, since reference materials are important tools for maintaining high quality assurance standards. In fact, the utilization of suitable representative microbial inocula can overcome some problems related with inocula collected from activated sludge plants, as sampling strategy, maintenance of viability, control of quantitative and qualitative characteristics and potential contact with pathogenic microorganisms. The seed homogeneity and stability as well as its easy and safe manipulation are also important properties of those reference materials. Thus, controlled cultures can be helpful for the standardization of biological assays leading to an improvement of the quality of chemical and effluent testing and monitoring, including more homogeneous and comparable results and to eliminate any bias.

An established biodegradation test to determine the inherent biodegradability of a chemical is the Zahn-Wellens test (ISO, 1999; OECD, 1993). This test specifies a method for the evaluation in aqueous medium of the ultimate biodegradability and, as additional information, the primary biodegradability and the total elimination from water, of organic compounds at a given concentration by activated sludge.

In this context, the aim of the present work consisted in the evaluation of the potential of three standardized microbial consortia, two commercial inocula and a designed bacterial inoculum as an alternative seed for the Zahn and Wellens test

(ISO, 1999), testing diethylene glycol (DEG) as reference compound, in comparison with a reference inoculum (AS).

MATERIALS AND METHODS

Media and stock solutions

Tryptone Soya Broth (TSB) and Nutrient Agar (NA), from Difco Laboratories (Detroit, USA), were prepared by adding 30 g and 23 g, respectively, to 1 litre of distilled water. These media were sterilized by autoclaving at 121 °C for 15 min. The test medium and all stock solutions for Zahn-Wellens test were prepared as described in ISO 9888 (1999). Saline solution (NaCl 0.85%) and 50 mM phosphate buffer (pH 7.0), sterilized, were used in the microbial inocula preparation. All chemicals (*p.a.*) were from Merck Laboratories (Darmstadt, Germany), except when referred otherwise.

Microbial inocula

In this study several microbial consortia were used: two commercial inocula, BI-CHEM[®] BOD Seed (Novozymes Biologicals Inc., Salem, VA) and BIOLEN M112 (Gamlen Industries S.A., St. Marcel-Vernon, France) (Paixão *et al.*, 2003a); a designed inoculum (DI), which is a consortium of six known non-pathogenic bacterial strains selected from environmental samples (soil, AS); and AS inocula from two different sources.

Inocula preparation

Commercial inocula

Each commercial inoculum was inoculated into TSB (150 mg BI-CHEM[®]/100 ml TSB; 400 mg BIOLEN/100 ml TSB) in 500 ml Erlenmeyer flasks, with agitation

(150 rpm) at 28 °C. After about 18 h of incubation (exponential phase), 60 ml of each culture was centrifuged (6000 rpm, 10 min) and washed twice with saline solution (NaCl 0.85%). Each pellet was resuspended in saline solution to an optical density (OD) of ≈ 0.5 at 600 nm, corresponding to $\approx 10^8$ cfu/ml; 40 ml were used to inoculate the test vessels for the biodegradability tests.

Designed inoculum (DI)

DI is a consortium composed by six selected bacterial strains belonging to species commonly found in sewage and/or soil, namely: *Acinetobacter* sp., *Enterobacter* sp., *Staphylococcus capitis*, *Pseudomonas putida*, *Bacillus subtilis* and *Bacillus cereus* (these strains are stored at the Cultures Collection of Industrial Microorganisms, from Laboratory of Industrial Microbiology in INETI, and are available if requested to the corresponding author). Each pure culture maintained on tryptic soy agar (TSA) was previously grown into tryptic soy broth (TSB) by incubating at 28 °C for about 18 hours, with agitation (150 rpm), and after the pure cultures were added in equal parts (6x10ml) to origin the DI. Then, this inoculum was centrifuged, washed and resuspended in saline solution for the tests as described above for commercial inocula.

Activated sludge inocula

AS samples were collected from the aeration tank of two municipal wastewater treatment plants, Beirolas'WTP (AS1) and S.J.Talha'WTP (AS2). The concentration of activated sludge samples was 2-4 g/l (suspended solids) and each inoculum was aerated and used within 36 h of collection. For the biodegradability tests with AS1 or AS2, the AS concentration was adjusted to 0.5 g/l of suspended solids in the final mixtures (ISO, 1999).

Microbial counting

The number of viable heterotrophic aerobic bacteria was counted by spreading 0.1 ml of sample (or its dilution in sterile 0.85% NaCl) on NA, at least in duplicate, and by incubating for 24 h at 28 °C. In the same way, the number of viable effective competent microorganisms was counted on a salt mineral medium with agar (Pettigrew *et al.*, 1990) supplemented with DEG (0.1%) as sole carbon source, and by incubating for 72 h at 28 °C. Colonies were counted and expressed as colony forming units (cfu) per millilitre.

Zahn-Wellens test procedure

The Zahn-Wellens test was performed for DEG, a reference compound, as described in the ISO Standard 9888 (1999) using the different microbial inocula. The degradation assays were carried out in 2 l Erlenmeyer flasks with a final volume of 500 ml of test mixture (mineral test medium + DEG + inoculum), at 25 °C in a rotary shaker at 150 rpm. Several assays were performed adding sterile activated sludge supernatant (80 ml supernatant from AS2 samples) into the test mixture. For each series of biodegradability assays, a blank without the test compound was set up as well as a control with the test compound (DEG) and without inoculum to determine abiotic elimination.

The biodegradation process was followed measuring the chemical oxygen demand (COD), which was determined using the Reactor Digestion Method from HACH (Hach Company, Loveland, Colorado, USA). Colorimetric readings were made with a DR/2000 spectrophotometer (HACH). At chosen time intervals through the test period (usually until 28 days), the samples taken were filtered by washed membrane filters (pore size: 0.20 µm) before the COD determination in at least in duplicates (with an error less than 5%). The data were reported as biodegradation curves based

on the COD removal (percentage of biodegradation) as a function of time. In order to interpret the COD removal, a non-linear regression model was fitted to the original data, using the Sigmaplot SPW7 for Windows® (2001- SPPS Inc., Chicago, Illinois, USA). The model chosen was the Hill sigmoid, which is usually applied to biodegradability data (Calmon *et al.*, 1999; Keursten and Groenevelt, 1996). This biodegradability test is considered valid if the % biodegradation for the reference compound is greater than 70% on the 14th day (ISO, 1999). Kaiser (1998) referred a value of 95% of DEG biodegradation in 14 days by AS in the Zahn-Wellens test.

Metabolic studies

GN and MT MicroPlates™ (BIOLOG Inc.) were used to assay carbon source utilization by the microbial inocula. GN plates contain a minimal nutritional medium with a redox dye (tetrazolium violet) along with a different organic substrate in each of the 95 wells in a microtiter plate, and a control-well with no added carbon substrate. Any colour development in control-well presumably indicates utilization of carbon sources inherent to the inoculated microbial suspension. In all the wells, MT plates have only a minimal nutritional medium with the redox dye and without any organic substrates, permitting customised plates. As a response to the metabolic activity the tetrazolium dye is reduced and turns each active well purple.

Inocula preparation

For each one of the standardized microbial inocula (BI-CHEM®, BIOLLEN or DI), 100 ml culture grown in TSB (28 °C, ≈18 h with agitation), distributed into several centrifuge tubes, was centrifuged (6000 rpm, 10 min) and washed twice with 50 mM phosphate buffer (pH 7.0). The pellets of each inoculum were respectively resuspended into phosphate buffer, and into several sterile solutions of phosphate

buffer containing different reference chemicals, namely DEG (at 500 or 1000 mg/l), sodium benzoate (500 mg/l) or sodium acetate (500 mg/l), to an $OD_{600} \approx 0.5$ ($\approx 10^8$ cfu/ml). The AS inocula (AS1 or AS2) were resuspended into the same solutions but adjusted to an $OD_{600} = 0.30 \pm 0.05$, according Guckert *et al.* (1996) and Kaiser *et al.* (1998).

Microbial community-level metabolic profile

The analysis of the metabolic utilization pattern (95 substrates) by each microbial consortium was performed inoculating GN plates, in duplicate, with 150 μ l of each microbial inoculum suspended in phosphate buffer. In addition, the potential for the biodegradation of three reference chemicals (DEG, sodium benzoate and sodium acetate), was determined inoculating MT plates with 150 μ l of each inoculum suspended in those compounds as explained above. For each inoculum, at least 16 replicate wells were inoculated per each compound tested and per the blank (inoculum resuspended in phosphate buffer without any test compound).

GN and MT plates were evaluated for OD changes at 590 nm using a microtiter plate reader (model MRX_{REVELATION}, Dynex Technologies Inc., Chantilly, USA). Following an initial reading, the plates were incubated at 28 °C for 72 or 120 h and recorded periodically during the incubation period.

BIOLOG data analysis of GN plates was done according to Paixão *et al.* (2003a,b). Biodegradability curves of the organic compounds tested in MT plates were presented by plotting the OD_{590} versus time.

Screening of inducers that enhance DEG degradation

To evaluate the effect of the presence of AS supernatant on DEG utilization by BI-CHEM[®], BIOLEN or DI, MT plates were inoculated (150 μ l per well) with each one of these inocula resuspended in diluted sterile AS2 supernatant supplemented with

500 mg/l DEG ($OD_{600} \approx 0.5$), and with the correspondent blanks. For each inoculum two blanks without DEG were used: blank 1 - inoculum resuspended into phosphate buffer, blank 2 - inoculum resuspended into diluted sterile AS2 supernatant (15-20% supernatant, sterilized by filtering through a membrane filter of pore size 0.20 μm , in phosphate buffer). At least 8 replicates were performed per each case.

Looking for other potential inducers for the DEG degradation by the standardized microbial inocula, a screening of several single organic substrates as inducers was performed by inoculating (in duplicate) GN plates with 150 μl of each one of the bacterial inocula previously suspended into phosphate buffer (control GN plates) or into 500 mg/l or 1000mg/l DEG (test GN plates).

Each inoculum requiring the presence of specific inducers to degrade the DEG showed an altered colour pattern in the GN plates with this tested chemical (test GN plates) in comparison with the pattern of the GN plates inoculated with the same microbial inoculum suspended in phosphate buffer (control GN plates). GN substrates were considered inducers to the degradation of DEG when OD values obtained in test GN plates were ≥ 0.25 (Guckert *et al.*, 1996), after blank correction (control-well subtracted) and after the subtraction of the correspondent OD value in the control GN plates.

RESULTS

Zahn-Wellens test

A first series of Zahn-Wellens tests for DEG was performed using activated sludge inocula (AS1, AS2), i.e. the ISO's reference inocula, to check the test performance. The biodegradation curves obtained are shown in Fig. 1. These sigmoid curves (r^2 : 0.992 – 0.997) have characteristics similar to those of a typical biodegradation

curve. The complete degradation was reached in 12 days for AS1 and in 10 days for AS2, presenting a lag phase of about 6 and 5 days, respectively. Thus, the biodegradation profile of AS1 and AS2 was similar with equivalent slopes (percentage of biodegradation per day), during the biodegradation phase, differing only in the lag time. In AS assays, despite the same initial biomass (0.5 g/l suspended solids) used, the real inoculum size in terms of viable bacteria (initial number of the effective competent microorganisms / initial number of total heterotrophics) in each test mixture was also quantified, being 9.8×10^4 cfu/ml / 1.5×10^6 cfu/ml for AS1, and 1.2×10^5 cfu/ml / 4.6×10^5 cfu/ml for AS2.

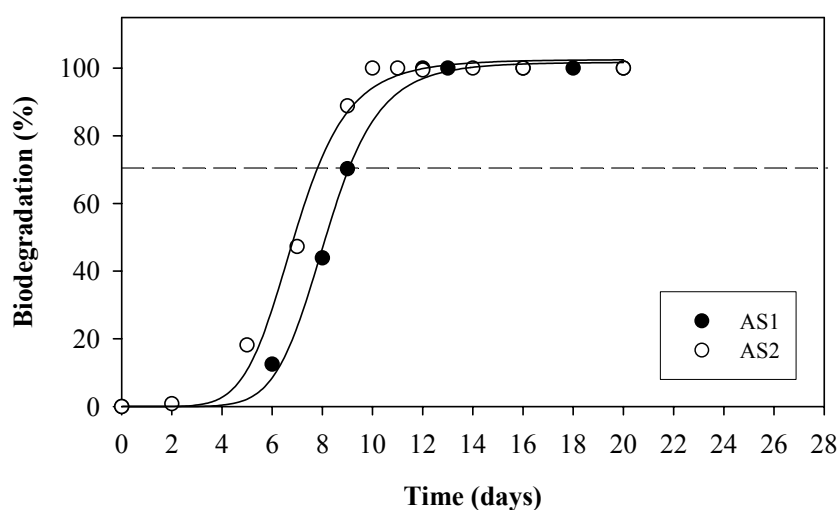


FIG. 1. Biodegradation curves of diethylene glycol ($\text{COD}_{\text{initial}} \approx 400$ mg/l) obtained by activated sludge samples, from two different sources (AS1, AS2), in Zahn-Wellens test. (The discontinuous line corresponds to the validity criterion of the test: $> 70\%$ biodegradation on the 14th day).

The results of the biodegradability assays for DEG with the three standardized microbial consortia tested are presented in Fig. 2, which show also the sigmoidal behaviour (r^2 : 0.954 – 0.996) for the substrate removal. Regarding the test validity criterion ($> 70\%$ of DEG biodegradation on 14th day) none of these inocula effectively reached it. However the DI was the inoculum which presented the

behaviour nearest to the expected for the reference inoculum, with > 70% of DEG biodegradation on 19th day due to a long lag phase of 13 days, despite only has reached a maximum biodegradation of 80%. BI-CHEM[®] and BIOLEN have presented a poor response for DEG removal. These results were obtained with an initial number of cells of $\approx 10^7$ cfu/ml, as total viable heterotrophic bacteria, in test mixtures for each one of the inocula. Results obtained in prior experiments with lower initial cell concentrations (10^4 to 10^6) showed maximum degradation plateau of less than 30% (data not shown).

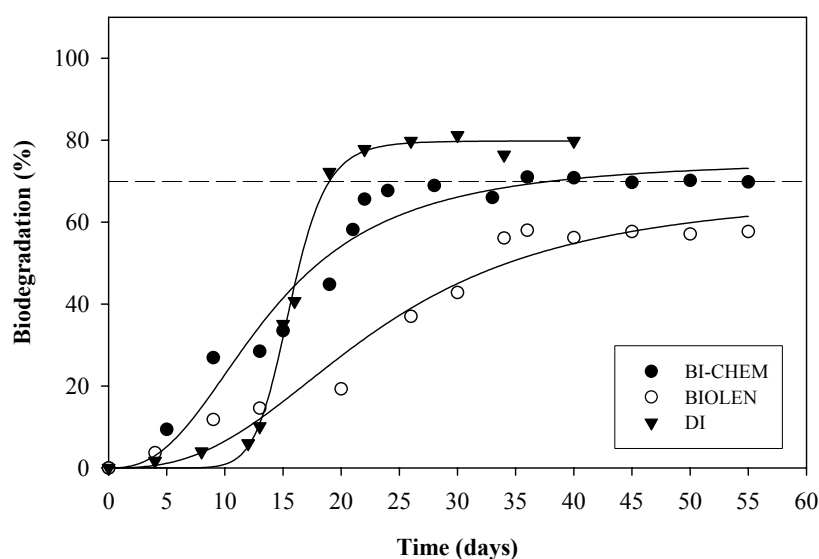


FIG. 2. Biodegradation curves of diethylene glycol ($\text{COD}_{\text{initial}} \approx 600$ mg/l) obtained by the three microbial inocula tested (BI-CHEM[®], BIOLEN, DI) in Zahn-Wellens test.

DEG biodegradation by these inocula was enhanced when sterilized AS supernatant was added to the test mixtures. Several series of biodegradability assays were performed for two concentrations of DEG (A: $\text{COD}_{\text{initial}} \approx 400$ mg/l; B: $\text{COD}_{\text{initial}} \approx 600$ mg/l) using the standardized inocula in parallel with the ISO's reference inoculum (AS2). To the test mixtures inoculated with each one of the standardized inocula was added also sterile AS supernatant, obtained from the AS sample used as

inoculum in the tests. The results obtained in these assays are presented in Fig. 3 (A and B), as biodegradation curves (sigmoid curve fitting: r^2 : 0.945 – 0.995). In the assays for both DEG concentrations tested (Fig. 3, A and B), the standardized inoculum that presented the most similar biodegradability behaviour to AS was the DI, presenting steep slopes in the biodegradation phase as AS, especially in the assays starting with 600 mg/l COD (Fig. 3B). The AS inoculum maintained the

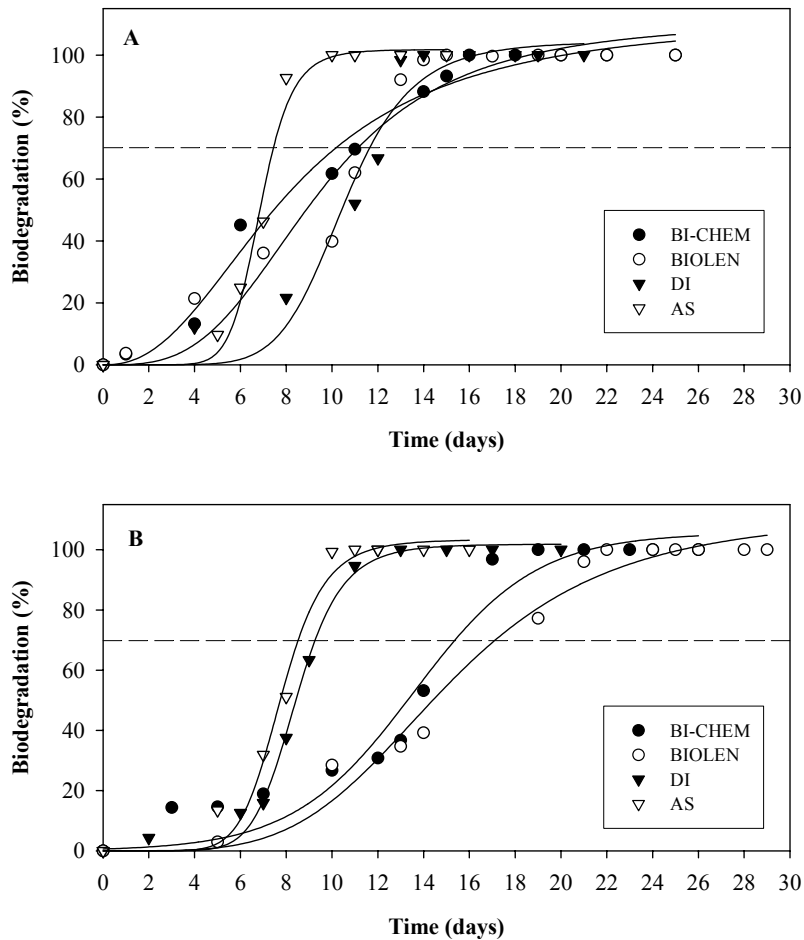


FIG. 3. Biodegradation curves of DEG obtained by BI-CHEM[®], BIOLEN and DI, after the addition of sterile AS supernatant to the test mixtures in Zahn-Wellens test, *versus* biodegradation curve obtained by AS. (A: COD_{initial} ≈ 400 mg/l; B: COD_{initial} ≈ 600 mg/l).

biodegradability profile in both assays, presenting a lag time of about 5 days (Fig. 3, A and B), and a complete DEG degradation within 10 - 11 days. In the same way, the DI also maintained a similar overall biodegradability behaviour in both assays being the complete DEG degradation observed within 12 days (Fig. 3B) - 14 days (Fig. 3A), with a lag time of 6.5 days and 7.6 days (see adjusted curves), respectively. Independently of initial DEG concentration tested, DI passed the validity criterion, since led to a complete DEG biodegradation on 14th day, as expected for AS inocula. Relatively to the commercial inocula, BI-CHEM[®] and BIOLEN, their overall biodegradation profiles were similar between them in each one of the assays (Fig. 3, A or B), and presented lower slopes during the biodegradation phases of DEG than DI and AS. The test validity criterion (>70% of DEG biodegradation on the 14th day) was only accomplished by these inocula in the assay with the lower DEG concentration (Fig. 3A). In the assay with the higher DEG concentration none of the commercial inocula reached the validity criterion due to the long lag time observed. In this assay, for BI-CHEM[®] can be observed (Fig. 3B), a initial behaviour characterized by an adaptation period of several days where the biodegradation did not crossed the 20%, which is represented as a lag phase in the adjusted sigmoid curve ($r^2 = 0.960$). Regarding the biodegradation curves adjusted for the two commercial inocula (Fig. 3, A and B), the lag phase seemed to prolong with the increase of DEG concentration, from 3 to 8 days for BI-CHEM[®] and from about 4.5 to 9 days for BIOLEN, showing an inhibition with the increase of DEG concentration tested. The complete DEG degradation was observed in 16 days (400 mg/l) and 19 days (600 mg/l) by BI-CHEM[®], and in 15 days (400 mg/l) and 22 days (600 mg/l) by BIOLEN.

In order to assess the real inoculum size, in the assays with 600 mg/l DEG, the number of total heterotrophic bacteria was evaluated as well as the number of the

effective competent microorganisms for BI-CHEM[®], BIOLEN, DI and AS, at the beginning and the end of the Zahn-Wellens tests (see Table 1).

Table 1. Quantification of the number of total heterotrophic bacteria and the number of effective competent microorganisms for BI-CHEM[®], BIOLEN, DI and AS, at the beginning and the end of the Zahn-Wellens tests.

MICROBIAL INOCULA	TOTAL HETEROTROPHIC BACTERIA (cfu/ml)		EFFECTIVE COMPETENT BACTERIA (cfu/ml)	
	Initial	Final	Initial	Final
BI-CHEM	3.1×10^7	5.2×10^7	4.0×10^5	4.0×10^7
BIOLEN	2.0×10^7	4.3×10^7	3.0×10^5	2.8×10^7
DI	3.6×10^7	5.3×10^7	7.3×10^5	5.0×10^7
AS	4.1×10^5	7.8×10^6	1.0×10^5	8.3×10^6

Metabolic studies results

Microbial community-level metabolic profile

BIOLOG GN and MT MicroPlates[™] were used to assay carbon source utilization by the several microbial inocula tested. GN plates were used to evaluate both qualitative and quantitative community-level metabolic responses.

For AS samples (ISO's reference inocula), after 72 h of incubation, AS2 was positive for all the carbon sources in GN-MicroPlate (see list in Table 2), and AS1 was positive for 94 substrates, being negative for 2,3-butanediol.

After 72 h of incubation BI-CHEM[®] was considered positive for 93 GN-substrates, being negative for 2 substrates (wells 14 and 79); BIOLEN M112 was positive for 73 GN-substrates, being negative for 22 substrates (wells 13, 14, 19, 21-23, 28, 30, 37, 39, 40, 56, 60, 61, 64, 75, 76, 79, 80, 85, 87, 92), and the DI was positive for 94 carbon sources, being negative only for 2,3-butanediol. So, it seems that BI-CHEM[®]

and DI are the most similar to the AS metabolic profiles in respect to the number and diversity of the carbon source pattern utilization.

TABLE 2. List of substrates in BIOLOG GN MicroPlate, referring the correspondent well number.

BIOLOG GN SUBSTRATES (WELL NO.)		
2. i-erythritol	34. gentiobiose	67. turanose
3. D-melibiose	35. L-rhamnose	68. D-glucuronic acid
4. acetic acid	36. D-galactonic acid lactone	69. quinic acid
5. ρ -hydroxy-phenylacetic acid	37. α -ketovaleric acid	70. L-aspartic acid
6. bromosuccinic acid	38. D-alanine	71. L-serine
7. L-histidine	39. L-phenylalanine	72. glycerol
8. urocanic acid	40. phenylethyl-amine	73. L-arabinose
9. α -cyclodextrin	41. tween 80	74. maltose
10. D-fructose	42. α -D-glucose	75. xylitol
11. β -methyl D-glucoside	43. D-sorbitol	76. α -hydroxybutyric acid
12. cis-aconitic acid	44. D-galacturonic acid	77. D-saccharic acid
13. itaconic acid	45. D,L-lactic acid	78. L-glutamic acid
14. succinamic acid	46. L-alanine	79. L-threonine
15. hydroxy-L-proline	47. L-proline	80. D,L, α -glycerol phosphate
16. inosine	48. putrescine	81. D-arabitol
17. dextrin	49. N-acetyl-D-galactosamine	82. D-mannitol
18. L-fucose	50. m-inositol	83. pyruvic acid methyl ester
19. D-psiocese	51. sucrose	84. β -hydroxybutyric acid
20. citric acid	52. D-gluconic acid	85. sebacic acid
21. α -ketobutyric acid	53. malonic acid	86. glycyl-L-aspartic acid
22. glucuronamide	54. L-alanyl-glycine	87. D,L-carnitine
23. L-leucine	55. L-pyrogutamic acid	88. α -D-glucose-1-phosphate
24. uridine	56. 2-aminoethanol	89. D-cellobiose
25. glycogen	57. N-acetyl-D-glucosamine	90. D-mannose
26. D-galactose	58. α -D-lactose	91. succinic acid mono-methyl ester
27. D-raffinose	59. D-trehalose	92. γ -hydroxybutyric acid
28. formic acid	60. D-glucosaminic acid	93. succinic acid
29. α -ketoglutaric acid	61. propionic acid	94. glycyl-L-glutamic acid
30. L-alaninamide	62. L-asparagine	95. γ -aminobutyric acid
31. L-ornithine	63. D-serine	96. D-glucose-6-phosphate
32. thymidine	64. 2,3-butanediol	
33. tween 40	65. adonitol	
	66. lactulose	

Figure 4 presents the metabolic profiles of the three microbial communities in comparison with AS profile showing the utilization pattern, grouped by six substrate families: carboxylic acids (CA), polymers (POL), carbohydrates (CH), amino acids

(AA), amines/amides (AM/AMD) and miscellaneous (MISC = phosphorilated chemicals, alcohols, aromatics, esters, phenolic compounds), in terms of percentage of net area under curves for each guild in relation to the total net area under curves for all of the 95 response wells over a 3 days incubation.

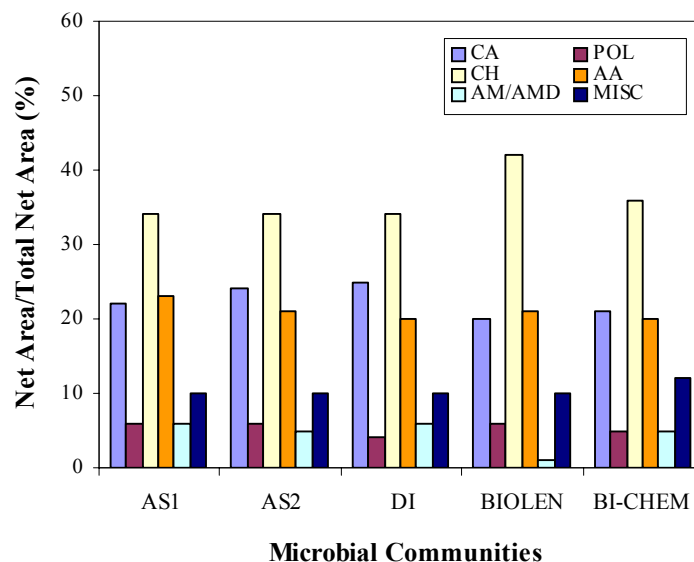


FIG. 4. Metabolic profiles for the standardized microbial consortia tested in comparison to AS communities' profiles (AS1 – Beirolas' WTP, AS2 - S.J. Talha' WTP). (CA: carboxylic acids; POL: polymers; CH: carbohydrates; AA: amino acids; AM/AMD: amines/amides; MISC: miscellaneous, namely phosphorilated chemicals, alcohols, aromatics, esters and phenolic compounds).

These results confirm the similarity of general metabolic fingerprint of BI-CHEM[®] and DI with those of AS samples. BIOLEN profile differ essentially in lower or almost null utilization of the AM/AMD guild substrates (use 1 out of 6), and by presenting the highest % net area for CH guild substrates, which are almost all utilized (26 out of 28), relatively to its total net area of colour development. Despite of non-utilization of 36% of CA substrates tested, the BIOLEN [net area (CA)/total net area] (%) for this guild is near to those observed for the other inocula. The total colour development (total net area under curves) observed for the 95 GN-substrates

was 24670 to AS1 and 25993 to AS2. For the other inocula, the net area was 24358 to BI-CHEM[®], 14926 to BIOLEN and 17351 to DI. Once more, the results showed that BI-CHEM[®] and DI were the inocula more similar to the AS samples.

The biodegradation of DEG and other reference compounds, namely sodium acetate and sodium benzoate (500 mg/l), by the different microbial consortia, was studied using MT plates. The biodegradability curves (plotted as OD₅₉₀ versus time) obtained for these reference organic chemicals by BI-CHEM[®], BIOLEN or DI in comparison with those obtained with AS inoculum, namely AS2, are presented in Fig. 5. As it can be observed, the curves are similar for BI-CHEM[®], BIOLEN and DI, presenting smaller net area under curves for AS, namely for sodium acetate and sodium benzoate. Only the AS inoculum presented positive response for the degradation of DEG within the 3 days of incubation. The negative response of the alternative inocula can be due to a longer lag phase and/or the need of inducers, and not to an intrinsic metabolic deficiency.

Screening of inducers that enhance DEG degradation

In MT plates inoculated with BI-CHEM[®], BIOLEN or DI resuspended in sterile AS supernatant with 500 mg/l DEG it was observed a significative colour development for each inoculum within the incubation period (data not shown), in contrast with controls (blank 1: inoculum resuspended into phosphate buffer, blank 2: inoculum resuspended into diluted sterile AS2 supernatant) and considering that the result for DEG biodegradation in MT plates had been negative for each one of the standardized inocula (Fig. 5); showing that AS supernatant acted as a biodegradation inducer.

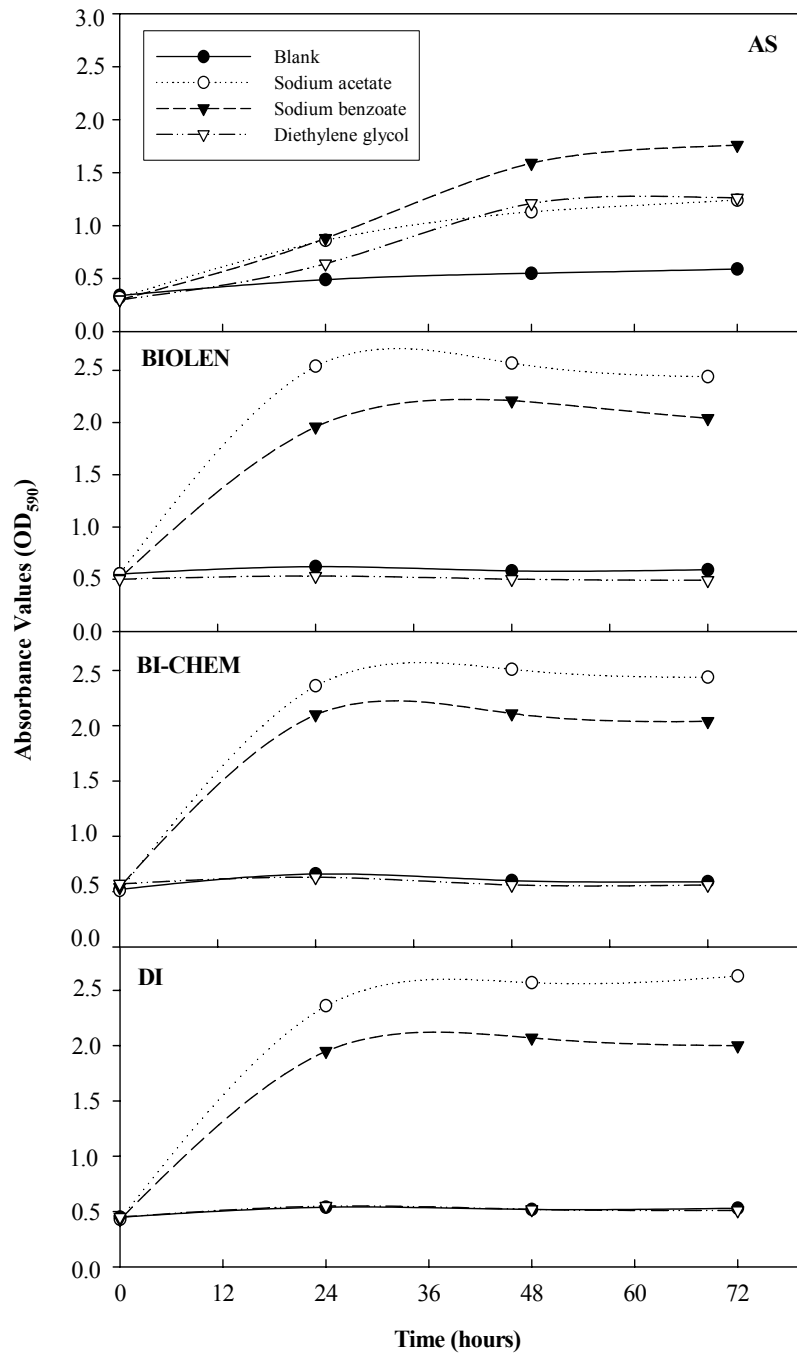


FIG. 5. Utilization patterns of three reference compounds by the microbial inocula communities tested (BI-CHEM[®], BIOLEN, DI) in comparison with activated sludge (AS).

In this context, to identify organic substrates that can act as inducers in DEG degradation, GN plates with 95 substrates were inoculated with each one of the alternative inocula previously resuspended on a known concentration of the target compound, namely 500 or 1000 mg/l DEG (test GN plates) and compared with the corresponding control GN plates, which were inoculated with each one of the inocula resuspended in phosphate buffer. After the blank correction (control-well subtracted) for each GN plate inoculated, each one of the 95 substrates tested only was considered a potential inducer when its OD value (test GN plate – control GN plate) ≥ 0.25 .

Table 3. GN substrates that serve as inducers in the biodegradation of diethylene glycol (DEG), 500 or 1000 mg/l, by the microbial inocula BI-CHEM[®], BIOLEN and DI.

		Bi-Chem		Biolen		DI	
		DEG 500 ^a	DEG 1000	DEG 500	DEG 1000	DEG 500	DEG 1000
GN SUBSTRATE INDUCERS (Well No. ^c)	24 h ^b	30, 87	12, 32, 55, 65, 68, 69, 79, 87, 91, 92	2, 4, 9, 31, 38, 66, 67, 69, 75	5, 39, 75	7, 8, 16, 32, 34, 38, 40, 45, 48, 50, 53, 58, 62, 67, 72, 77, 80, 86, 88, 89, 91, 96	---
	48 h	2, 9, 80	---	65	14, 92	20, 22, 25, 35, 36, 70	---
	72 h	8, 10, 37, 48, 56, 61, 64, 79, 85, 94, 95	9, 29, 31, 57	19, 34, 43, 45, 81, 92, 93	13	41, 44, 57, 73, 94	46
	120 h	4, 6, 7, 16, 72, 91, 92, 93	2, 16, 96	25, 33, 44, 72, 91	37, 47, 61	3, 5, 18, 24, 49	25, 72, 88, 96

^a mg/l.

^b Incubation time.

^c See list on Table 2 for the correspondent substrates.

In Table 3 are identified the GN-substrates which acted as inducers increasing the DEG degradation. Comparing the behaviour of each inocula, it was observed that for the degradation of the lowest DEG concentration (500 mg/l) the number of inducers increased from 22 (BIOLEN)→ 24 (BI-CHEM) → 38 (DI), showing this last the highest colour development for DEG degradation in the presence of some substrates. Nevertheless for 1000 mg/l DEG, DI seemed to be the most inhibited with only 5 positive responses, in contrast with BI-CHEM[®], which presented 17 positive responses. The number of positive responses varied with the inoculum used and with the incubation time. The substrates that acted as inducers in DEG degradation (Table 3) are included within the six substrate families present in GN plate.

DISCUSSION

Since chemicals that ultimately enter the environment are often discharged through sewage or wastewater treatment plants, the inoculum most used in biodegradability and toxicity screening tests is AS, a mixed culture of competitive microorganisms cultivated on organic substances present in wastewater (van Ginkel *et al.*, 1995; Forney *et al.*, 2001). In this way, microbial population in AS depends on the waste composition and operation mode of the treatment plant, which make it almost impossible to obtain comparable and reproducible results especially in inter-laboratory studies. This has undoubtedly led to the concern of the need to have a standard biological reference material.

In this context, the utilization of three well defined microbial consortia (BI-CHEM[®], BIOLEN and DI) as potential alternative seed to AS was evaluated in an inherent biodegradability test, the Zahn-Wellens test (ISO, 1999). The tests were performed for DEG as reference compound, once the test of a reference chemical is a common

practice to validate the procedure and the viability of the inoculum used (Painter, 1997).

To compare the test results obtained with these potential alternative inocula, a simultaneous study was performed using the ISO's reference inoculum (AS). The results obtained for two AS samples, from different sources (AS1, AS2), showed a similar overall behaviour in the DEG biodegradation response (Fig. 1), completing the DEG degradation in less than 14 days, as expected (ISO, 1999; Kaiser, 1998). According to van Ginkel *et al.* (1995), the variations in biodegradation curves are related to the number of competent microorganisms present in the inoculum. Despite the AS1 sample presented a higher number of total heterotrophics at the beginning of the test, the actual numbers of effective competent microorganisms in both samples used (AS1 And AS2) were similar ($\approx 10^5$ cfu/ml), thus conducting to similar biodegradation curves.

The biodegradability tests performed with the standardized consortia, BI-CHEM[®], BIOLEN and a DI, only have given acceptable results when the initial inoculum contained $\approx 10^7$ cfu/ml (total heterotrophic bacteria). However, the effective number of competent microorganisms was only $\approx 10^5$ cfu/ml, as for the AS samples, which agrees with the stipulate for biodegradability tests (Painter, 1995; Forney *et al.*, 2001). The cell density of the inoculum, and especially the percentage of specific biodegraders, is a key parameter that must be controlled (Thouand *et al.*, 1996).

The biodegradability results obtained with these three inocula (Fig. 2) were improved when sterile AS supernatant was added to the test mixtures (Fig. 3). This sterile AS supernatant can contain soluble organic compounds, inorganic nutrients and soluble microbial products (SMP) such as polysaccharides, sugars, amino sugars, organic acids, proteins, amino acids, lipids, exocellular enzymes, etc (Barker and Stuckey, 1999; Guellil *et al.*, 2001), and seemed to act as an inducer decreasing

the lag phase in the biodegradation of DEG, probably stimulating the growth of the competent biomass present in the inocula.

In the Zahn-Wellens test with the AS inoculum, the test substance is added in a relatively high concentration and is the only carbon and energy source, in addition to the small amounts of cofactors present in the medium and the minor substrate quantities transferred with the inoculum. It should be emphasized that the carbon substrates other than the test substance continuously released from sludge in small concentrations, 20-40 mg/l of dissolved organic carbon (DOC) (Kaiser, 1998), probably also favours biodegradation (Nyholm, 1991). So, the addition of sterile AS supernatant (≈ 20 mg DOC/l) to the test medium in the assays with the alternative inocula only maintained the same nutrient conditions present in the biodegradability assays with AS samples. The overall biodegradation curve profiles for BI-CHEM[®] and BIOLEN were similar between them and depended on DEG concentration, accomplishing the validity criterion only for the assay with the lower DEG concentration. For DI, the DEG biodegradation curves maintained a similar profile for the two concentrations tested (400 or 600 mg/l) with steep slopes, as those of the AS inoculum tested, presenting a complete degradation within 12 - 14 days, less than the commercial inocula and a little more than the AS used due to the longer lag time needed (Fig. 3, A and B). The results for DEG degradation by DI are in agreement with the 14 days referred by Kaiser (1998) for the reference inoculum in the Zahn-Wellens test.

The biodegradation potential for DEG and two other reference compounds (sodium benzoate and sodium acetate) was also evaluated using the BIOLOG MT plates. DEG only was used by AS inoculum, while the other two compounds were degraded by all inocula, within the 3 days of incubation (Fig. 5). This method showed that BI-CHEM[®], BIOLEN and DI only were able to use DEG when AS

supernatant was added, giving positive results in 3 days only in the presence of the sewage supernatant, suggesting a longer lag phase or the need of inducers.

In fact, inherent biodegradability tests are designed to provide experimental conditions that may promote faster and/or more complete degradation of the test compounds by various means (Kaiser, 1998). The use of inducers to enhance bioremediation has received increasing emphasis. Typically, inducers stimulate the microbial degradation of a toxic substance. Thus, the identification and subsequent use of the most effective inducer(s) may increase the degradation rate and reduce the time required for bioremediation (Gorden *et al.*, 1993).

So, to identify which organic compounds can induce DEG degradation, GN plates containing 95 carbon sources were inoculated with each one of the alternative inocula resuspended in known concentrations of DEG (500 or 1000 mg/l) and compared with the corresponding control GN plates. Several substrates seemed to act as inducers in DEG biodegradation (Table 3). However, for the inclusion of one of these inducers to the test medium, further biodegradability tests should be done in the presence of each selected inducer, instead of the AS supernatant, to confirm its ability to enhance the biodegradation potential in test medium by the activation the competent microorganisms of the inoculum used, having in account that the influence of the inducer should be negligible if lower substrate concentrations were used (20 to 40 mg DOC/l).

In addition, the results obtained with the control GN plates have also permitted to assess the functional diversity of different microbial communities based on the utilization patterns of 95 single carbon sources and compare the general metabolic profiles of the standardized inocula tested with those of the AS samples, the reference inocula (Fig. 4). The results highlight the similarity between the several inocula to what concerns their catabolic potential. BI-CHEM[®] and DI seemed to be

the most similar to AS, either in qualitative (number of substrates used) or in quantitative (substrate net areas under curves) analysis.

In this study, the results obtained showed the potential for the use of a well-defined inoculum as seed for an inherent biodegradability test (ISO, 1999), once one of the major factors which induces poor reproducibility is the lack of inoculum standardization; and highlight for the choice of some standardized microbial consortia as potential biological reference materials alternative to AS to be applied in other standardized tests, namely the respiration inhibition test and the BOD₅ test, as described by Paixão *et al.* (2000; 2003a,b).

CONCLUSION

The designed inoculum (DI) seems to be a good choice as an alternative surrogate culture to AS in the Zahn-Wellens test, once its behaviour was the most similar to AS, either in general metabolic fingerprinting or in its biodegradation response for DEG, sodium benzoate and sodium acetate. However, further tests should be performed to validate and certify this inoculum as a biological reference material (e.g. tests on xenobiotics must be carried out to evaluate its performance and sensitivity in comparison with AS results; study of the product shelf life; tests on several reference chemicals to determine reproducibility by realization of inter-laboratorial ring-tests, etc).

In fact, tailor-made inocula could provide more powerful tools to evaluate the environmental impact of hazardous chemicals or wastewaters, as predefined mixtures of microbial strains with specific and/or broad-range sensitivity could be prepared and used as inoculum in biodegradability tests, as a complement and alternative tool to existing activated sludge tests. The utilization of such inocula will permit a standardized response, absence of sampling variability, absence of

pathogenic microorganisms and ecological relevance of selected strains. Furthermore, the composition of the microbial community can be adjusted to other specifications, as for example the addition of nitrifiers.

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Chapter 5

EVALUATION OF TWO COMMERCIAL MICROBIAL INOCULA AS SEED IN A 5-DAY BIOCHEMICAL OXYGEN DEMAND TEST

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ABSTRACT - The use of two commercial microbial inocula (BI-CHEM[®] and BIOLEN M112) as reference organisms for the 5-day Biochemical Oxygen Demand test (BOD₅) was studied using both dilution and manometric methods, with the standard glucose-glutamic acid solution. A comparative study was carried out performing this test for several real effluent samples with the two commercial inocula and with a treatment plant inoculum. The microbiological characterization of these inocula was performed with the API identification system (bioMérieux S.A., France). The results obtained for the BOD₅ of the standard solution fall in the range referred for the activated sludge in the standards of the cited methods [mean values of biochemical oxygen demand obtained were BOD₅ = 199.4 mg O₂/l (VC= 4.52%) and BOD₅ = 176.8 mg O₂/l (VC = 4.66%) for the BI-CHEM[®] inoculum and BOD₅ = 209.8 mg O₂/l (VC = 7.35%) and BOD₅ = 228.6 mg O₂/l (VC = 9.37%) for the BIOLEN M112 inoculum, with the dilution and manometric methods, respectively]. Moreover, Pearson rank correlation analysis for the real effluent samples indicates that correlations between the BOD₅ values for the two commercial inocula and for the treatment plant inoculum were highly significant to 95% confidence ($r^2 = 0.922$ to $r^2 = 1.00$, $p < 0.05$).

Keywords: biochemical oxygen demand (BOD₅), dilution and manometric methods, commercial inocula.

INTRODUCTION

The environmental significance of biodegradation studies and the complexity of the phenomena, which are involved during biodegradation reactions, have initiated research since the late 1800s (Carter, 1984). Biodegradability is an useful criterion for evaluating the fate of a chemical and its environmental impact and to evaluate the effect of municipal and industrial wastes in treatment plants and receiving streams (Cailas and Gehr, 1989; Maoyu, 1990).

In any biodegradability assay it is necessary to have a microbial inoculum that is responsible for the oxidation of the soluble carbonaceous material present in the sample. Typically, a mixed microbial inoculum from an activated sludge treatment plant treating domestic sewage is used. However, this can lead to problems in terms of inoculum sampling, maintenance of its viability, difficulty in controlling their quantitative and qualitative characteristics, and the possible presence of pathogenic microorganisms.

Among standard biodegradation assays, the Biochemical Oxygen Demand (BOD) test can be considered one of the most used analytical procedures from which an important water quality indicator, the 5-day BOD (BOD₅), has been derived. In this context, the goals of this work were:

- (a) to perform a study of the potential use of commercial microbial inocula, BI-CHEM[®] and BIOLIN M112, as reference organisms for the BOD₅ test, using the dilution and the manometric methods and testing a solution of glucose and glutamic acid;
- (b) to validate the BOD₅ test with these two commercial microbial inocula, using both the dilution and the manometric methods and testing several real wastewater samples.

MATERIALS AND METHODS

Stock solutions and test mixtures

The dilution water stock solution (DW) and the standard solution of glucose-glutamic acid (150 mg/l-150 mg/l) used in the BOD₅ test were prepared as described in *Standard Methods* (APHA, 1992). Several raw wastewater samples were tested including domestic wastewaters from a municipal wastewater treatment plant and industrial effluents such as airline catering effluent, meat packing effluent, pig farm effluent, and cattle farm effluent.

Microbial inocula

Microbial inocula

Two commercial microbial inocula were used: BI-CHEM[®], BOD seed (Sybron Chimie France S.A.) and BIOLEN M112 (Gamlen Industries S.A.). For each inoculum a suspension in DW (120 mg BI-CHEM/ 500 ml DW; 400 mg BIOLEN/ 500 ml DW) was prepared and aerated for 1 hour before use.

Standard inoculum

Samples were collected from the first solid settled-out tank of a municipal wastewater treatment plant, treating predominantly domestic sewage. The supernatant from this wastewater was used as inoculum after allowing it to settle at 20°C for at least 1 hour but no longer than 36 hours after collection.

5-Day BOD procedures

The BOD₅ test was carried out using two different methodologies - dilution and manometric methods (ISO 5815, 1989; APHA, 1992). In the dilution method, the dissolved oxygen was measured using an ORION oxygen electrode (Model 97-08-

99, Orion Research, Inc., Boston, Massachusetts). For the manometric method, the WTW-OxiTop[®] Measurement System (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) was used according the instructions supplied (WTW, 1997).

Microbiological characterization

Aliquots of the commercial inoculum suspensions in DW (see above) were spread in nutrient agar plates (Difco Laboratories, Detroit, USA) and incubated for 24h at 30°C. The microorganisms isolated were identified by their morphological characteristics and the API identification system.

RESULTS AND DISCUSSION

BOD₅ test results

The BOD₅ of the standard solution was determined using the commercial inocula. The results obtained in the several replicas performed are summarized in Table 1. A statistical analysis of these data shows a good reproducibility for both manometric and dilution methods since the variation about the mean BOD₅ value is between 4.52% and 9.37%.

In addition, the mean BOD₅ values obtained are also included in the range mentioned in the operational standards of the methods used i.e. 200 ± 37 mg/l for the dilution method and 220 ± 11 mg/l for the manometric method, described for the activated sludge inoculum (see Table 1).

This suggests that these commercial microbial inocula can be used as an alternative to the activated sludge inoculum in BOD₅ tests.

Table 1. Results obtained in BOD₅ tests using the two commercial inocula.

Assay	Dilution method		Manometric method	
	BI-CHEM	BIOLEN	BI-CHEM	BIOLEN
1	199.9 ^a	173.4	232.5	252.5
2	201.9	178.4	232.5	252.5
3	206.4	176.9	207.5	240.0
4	205.9	173.4	195.0	252.5
5	202.4	169.9	220.0	240.0
6	204.4	174.9	207.5	240.0
7	180.4	198.9	210.0	200.0
8	185.9	171.4	210.0	202.0
9	199.9	177.4	185.0	217.5
10	206.9	172.9	197.5	202.5
11	---	---	---	215.0
Mean value	199.4 (VC=4.52%)	176.8 (VC=4.66%)	209.8 (VC=7.35%)	228.6 (VC=9.37%)
CI^b (95%)	193.0 – 205.8	170.9 - 182.7	198.8 – 220.0	214.2 - 243.0

^a Results are in mg O₂/l.

^b Confidence interval.

Data validation

To validate the results obtained with both BI-CHEM and BIOLEN M112 inocula, a comparative study of the commercial inocula and a treatment plant inoculum (TPI) was carried out on several wastewater samples (municipal and industrial effluents).

The results obtained in these experiments, summarized in Table 2, were compared through a Pearson rank correlation analysis (using the Statistica program, Tulsa, Oklahoma). This statistical analysis showed the existence of significant correlations

to 95% confidence ($p < 0.05$, $n=7$) between the results obtained with the three inocula (BI-CHEM, BIOLEN and TPI), using either the manometric method ($r = 0.99$) or the dilution method ($r = 0.98$ to 0.99), and the results obtained with the three inocula, independent of the method used ($r = 0.96$ to 1.00). These high correlations clearly show that the BOD tests with the commercial inocula studied are reliable, independently of the method used.

Table 2. BOD₅ values for several wastewater samples tested with the commercial inocula and a treatment plant inoculum, using the dilution and the manometric methods.

Sample Tested ^b	Dilution method			Manometric method		
	TPI	BIOLEN	BI-CHEM	TPI	BIOLEN	BI-CHEM
1	205.7 ^a	228.6	209.8	197.1	176.8	199.4
2	285.7	342.5	270.0	250.0	258.4	218.4
3	173.4	242.5	158.8	145.0	141.9	127.7
4	856.1	827.5	671.3	660.0	543.6	481.9
5	183.4	165.0	158.8	177.4	178.7	116.3
6	127.4	130.0	120.0	90.0	61.9	47.6
7	428.2	465.0	420.0	430.0	341.7	329.8

^a Results are in mg O₂/l.

^b 1: Standard solution, 2 and 3: domestic wastewaters, 4: catering alimentar effluent, 5: meet packing effluent, 6: pig farm effluent, and 7: cattle effluent.

Inocula microbiological characterization

The constituent microbial strains and the main groups of heterotrophic microorganisms responsible for the biodegradation process were identified; 13 and 14 microbial strains were isolated from the BI-CHEM and BIOLEN inocula, respectively. Both inocula seemed to have a similar microbial composition. In fact, Gram-positive aerobic sporeforming bacteria, mainly belonging to the genera

Bacillus, Gram-negative bacteria belonging to the genera *Enterobacter* and *Pseudomonas* and an yeast strain were identified in both commercial inocula. This similarity corroborates the experimental results obtained in this work and agrees with the respective application described by their producers (BI-CHEM[®], BOD seed is for standardization of BOD testing, BIOLEN M112 is an inoculum to improve the performance of municipal wastewater treatment systems).

CONCLUSIONS

The results obtained in this study point out the value of the commercial microbial inocula studied in BOD₅ tests as an alternative to an activated sludge inoculum. They have a microbial structure, specifically heterotrophic bacteria, similar to the standard inoculum.

In fact, the use of suitable commercial inocula can overcome some of the problems associated with the use of microbial inocula collected from activated sludge plants, namely, sampling, maintenance of viability, control of quantitative and qualitative characteristics and potential contact with pathogenic microorganisms.

Moreover, the comparative study with the glucose-glutamic acid standard solution and real wastewater samples performing the 5-day BOD test by the dilution and manometric methods showed the existence of high correlations between the values obtained, which strongly suggests its equivalence.

Finally, the existence of important bioassays, including several ISO standards involving the use of activated sludge inocula, represents a potential relevant field of application for the commercial inocula, which necessarily requires support by further laboratory research.

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Chapter 6

PERFORMANCE EVALUATION OF MIXED AND PURE MICROBIAL INOCULA AS SURROGATE CULTURE IN A BOD₅ TEST

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ABSTRACT - The use of activated sludge to assess the environmental impact of chemicals and wastewaters suffers from several drawbacks related to the heterogeneity, absence of standardization and health risk associated with such a mixed sewage population. To search for reliable testing inoculum alternatives, the potential of a standardized soil inoculum and a pure culture (*Pseudomonas* sp.), as reference material in a 5-day BOD test, was evaluated using the dilution and manometric methods and testing the standard glucose-glutamic acid solution. The results obtained for the BOD₅ of the standard solution fall in the range referred for the activated sludge (AS) in the standards of the cited methods, demonstrating the potential of these inocula as an alternative to AS in the BOD₅ test. Moreover, testing on real wastewater samples showed highly significant correlations ($p < 0.001$) between the BOD₅ values obtained with these two inocula and with the standard inoculum. Analysis of metabolic patterns also pointed to a similar catabolic profile between soil inoculum and AS and to the potential of a mixed *Pseudomonas*-soil inoculum as an AS surrogate culture.

Keywords: biochemical oxygen demand (BOD₅), dilution and manometric methods, microbial inocula.

INTRODUCTION

Laboratory test methods are used to determine biodegradability, an important parameter for the evaluation of the ecological behaviour of substances. Biodegradability has a key role due to the simple fact that a degradable substance will cause no long-term risk in the environment. The great variety of biodegradation processes in the natural environment and in technical plants for treating wastewater and soil wastes gave rise to a rather large number of test methods based on different principles (Pagga, 1997).

Among standard biodegradation assays, the Biochemical Oxygen Demand (BOD) test can be considered one of the most used analytical procedures from which an important water quality indicator has been derived, the 5-day BOD.

Therefore, in any biodegradability assay it is necessary to have a microbial inoculum which is responsible for the oxidation of the soluble carbonaceous material present in the sample. Generally, a mixed microbial inoculum from an activated sludge treatment plant, treating predominantly domestic sewage, is used. However, this fact can lead to problems in terms of inoculum sampling, maintenance of its viability, difficulty in controlling the quantitative and qualitative characteristics and the possible presence of pathogenic microorganisms.

A more appropriate strategy should be based on the real assessment of biodegradative ability of natural communities, as well as on the evaluation of the toxicity responses of community members to hazardous chemicals, either isolated or in mixed cultures. The use of such microorganisms in standard toxicity and biodegradability bioassays could thus provide useful and more reliable information to be applied in the successful management of bioremediation processes and monitoring of chemical environmental impact.

The main objective of this work is the development of a standard biological reference material with adequate resistance and metabolic profile similar to activated sludge (AS) that can be stored over a reasonable time period and is reliable and easily used as seed in relevant standardized bioassays using AS (ISO, OECD, CEN).

In this context, the work consisted of: (1) the study of the potential of a standardized soil inoculum (a mixed bacterial consortium with selected non-pathogenic bacteria from soil isolates well characterized) and a pure culture (*Pseudomonas* sp.) as seed in a 5-day BOD test in comparison with treatment plant inocula (AS), using the dilution and manometric methods (ISO, 1989; APHA, 1998; Wissenschaftlich-Technische Werkstätten, 1997), testing the standard glucose-glutamic acid solution, and (2) the validation of the 5-day BOD test with these two microbial inocula, using either the dilution or the manometric methods, testing several real wastewater samples.

MATERIALS AND METHODS

Stock solution

The dilution water stock solution (DW) and the standard solution of glucose-glutamic acid (150 mg/l-150 mg/l) used in the 5-day BOD test were prepared as described in APHA (1998). The synthetic medium (SM) stock solution was prepared by dissolving the following substances in 1 l of distilled water: 16 g of peptone (HiMedia Laboratories, Bombay, India), 11 g of beef extract (HiMedia Laboratories), 3 g of urea, 0.7 g of NaCl, 0.4 g of CaCl₂·2H₂O, 0.2 g of MgSO₄·7H₂O and 2.8 g of K₂HPO₄. The pH was adjusted to 7.5 ± 0.5.

Microbial inocula

In this study four microbial inocula were used: a pure culture of *Pseudomonas* sp., strain NCIMB 10770 (National Collection of Industrial and Marine Bacteria, Aberdeen, UK), a standardized soil inoculum and two treatment plant inocula.

Standardized soil inoculum

Several soil samples were collected at the INETI garden, from the top 10 to 25 cm of the surface after the removal of vegetation and rocks. The samples were homogenized, sieved (2 mm sieve) and used to prepare a suspension containing 2 g soil in 20 ml of sterilized deionized water (SDW). An aliquot (5 ml) of this soil suspension was inoculated into 250 ml of sterile synthetic medium (SM) and incubated at 20°C on a rotary shaker (100 rev/min) during seven days to promote the growth of microorganisms. The cultures obtained were used to isolate the predominant bacterial strains from soil. After a microbiological characterization of the isolated bacteria, six of them, non-pathogenic strains, were selected for a standardized soil mixture. The purified isolates are also inoculated into individual flasks containing 100 ml of SM and incubated at 20°C on a shaker (100 rev/min). After seven days, all six flasks were pooled to constitute the master culture broth (MCB) (King and Dutka, 1986), which was used as seed for all tests with this mixed bacterial consortium from soil (standardized soil inoculum).

Prior to every test, 10 ml of MCB was inoculated into 100 ml of tryptone soya broth (TSB) (Oxoid Laboratories, Hampshire, England) and incubated at 28°C with agitation (200 rev/min) for ≈20 h. This culture, 20 ml, was added to 20 ml of SDW, centrifuged (4629 g, 10 min) and washed twice with SDW or sterile 50 mM phosphate buffer (pH 7.0). The pellet was resuspended in DW for BOD₅ tests and in 50 mM phosphate buffer (pH 7.0) for BIOLOG assay, to an optical density at 600 nm (OD₆₀₀) of 0.05 (10⁷ cfu/ml) and 0.5 (10⁸ cfu/ml), respectively. For the BOD₅

tests, 1% and 25% of this soil inoculum was added to each test mixture prepared, in the dilution and the manometric methods, respectively.

Pure culture

Pseudomonas sp. inoculum was grown in TSB at 28°C with rotary shaking (200 rev/min). At exponential phase, a 30 ml aliquot was taken and centrifuged (4629 g, 10 min) being washed twice with SDW or sterile saline (NaCl 0.85%). The pellet was resuspended in DW for BOD₅ tests and in sterile saline (NaCl 0.85%) for BIOLOG assay to obtain the stock inoculum with OD₆₀₀ ≈ 0.16 (10⁸ cfu/ml). In BOD tests, the dilution 1/1000 of the stock inoculum (3x10⁵ cfu/ml) was used for the dilution method being added 2% inoculum to each test mixture prepared, and the dilution 1/2000 of the stock inoculum (5x10⁴ cfu/ml) was used for the manometric method adding 10% inoculum to each test mixture prepared.

Treatment plant inocula

Two inocula from different municipal treatment plants, treating predominantly domestic sewage, were used. A first series of samples were collected from the last solids settling tank of the Alcântara wastewater treatment plant. The supernatant was used as inoculum for the BOD₅ tests, after allowing it to settle at 20°C for at least 1h but no longer than 36h after collection (10⁶-10⁷ cfu/ml), and it was added 0.2% and 2% of this treatment plant inoculum (TPI) to each test mixture prepared, in the dilution and the manometric methods, respectively.

A second series of samples were collected from the aerated tank of the Beirolas activated sludge treatment plant, which were used within a period of 24 h of collection. The AS samples (3 – 4 g/l suspended solids) were well homogenized. For the BOD tests they were allowed to settle at 20°C for at least 1 h before the addition of the AS supernatant (≈10⁶ cfu/ml) to each test mixture prepared, being used 0.8%

and 1% of inoculum in the dilution and the manometric methods, respectively. For the BIOLOG assay, the AS was allowed to settle 10 min after which the turbid supernatant of the sludge was adjusted to an $OD_{600} \approx 0.30$ ($\approx 10^6$ cfu/ml) with sterile phosphate buffer (50 mM, pH 7.0).

BOD₅ procedures

The 5-day BOD test was carried out using two different methodologies, the dilution and the manometric methods (ISO, 1989; APHA, 1998). In the dilution method, the dissolved oxygen was measured using an ORION oxygen electrode, model 97-08-99 (Orion Research, Inc., Boston, USA). For the manometric method, the WTW-OxiTop[®] Measurement System (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) was used according to the instructions supplied (WTW, 1997; APHA, 1998).

BOD₅ data analysis

At least 10 test series of BOD₅ assays on standard glucose-glutamic acid solution were conducted to establish and compare reproducibility and variability in the experimental results for both inocula in the study with those of standard inocula (AS). Assay repeatability (r) was calculated as $r = t\sqrt{2}SD$, where t is the two-tailed t value ($n-1$), n is the number of samples and SD the standard deviation (Kilroy and Gray, 1992). The BOD₅ results obtained on several real wastewaters for soil inoculum, *Pseudomonas* sp. culture and TPI were compared through a Pearson rank correlation analysis (programme Statistica[™], Tulsa, Oklahoma).

BIOLOG EcoPlates[™] assays

BIOLOG is a microplate technique originally designed to use metabolic patterns to identify pure cultures of bacteria (Guckert *et al.*, 1986). In this study, BIOLOG

EcoPlates™ (BIOLOG Inc.) were used to evaluate community-level metabolic responses as well as to establish the catabolic profiling of a pure culture. The EcoPlate contains three replicate wells of 31 carbon substrates, which are predominantly amino acids ($n = 6$), carbohydrates ($n = 10$) and carboxylic acids ($n = 7$) (complete list in Choi and Dobbs, 1999). A control well (A1) contains no sole-carbon substrate, thus any colour development there presumably indicates utilization of carbon sources inherent in the inoculated microbial suspension.

For the BIOLOG assays with the several inocula, each suspension of bacterial cells prepared as above cited was used to inoculate BIOLOG EcoPlate. Aliquots of 150 μL were added per well using a multi-channel pipettor (BIOLOG – 8 channels, BIOLOG Inc., Hayward, USA), and the EcoPlates were evaluated for absorbance changes at 590 nm using a microtiter plate reader (model MRX_{REVELATION}, Dynex Technologies Inc., Chantilly, USA). Following an initial reading (T_0), the plates were incubated at 28°C for 72 hours. The EcoPlates were read periodically at 17.5, 20, 22.5, 41, 45.5, 48.5, 66 and 72 h, during the incubation period (Guckert *et al.*, 1996). For each inoculum, the results were expressed as the mean OD_{590} (OD_{T_i}) for the blank and the 31 substrates, since each EcoPlate has three replicates. For each reading time (T_i), a relative value (RV) corresponding to the optical density ratio ($\text{OD}_{T_i}/\text{OD}_{T_0}$) was then calculated.

BIOLOG data analysis

In the analysis of BIOLOG data two different approaches were used. For a qualitative assay of each substrate, matrices of blanked relative values were obtained by subtracting the RV of the blank well (RV_{blank}) to the RV of the substrate ($\text{RV}_{\text{substrate}}$). These matrices were converted into boolean ones, considering positive (1) the substrates for which $(\text{RV}_{\text{substrate}} - \text{RV}_{\text{blank}}) > 0.40 \times \text{RV}_{\text{substrate}}$ and negative (0) the substrates for which $(\text{RV}_{\text{substrate}} - \text{RV}_{\text{blank}}) \leq 0.40 \times \text{RV}_{\text{substrate}}$.

A quantitative analysis was also performed by plotting the optical density ratio (OD_{T_i}/OD_{T_0}) versus time for the 32 wells of BIOLOG EcoPlates. Each substrate curve was blanked (blank A1 curve subtracted) and then the curve-integration approach was used (Guckert *et al.*, 1996). Metabolic profiles of BIOLOG results were expressed as the net area under the curve for each of the 31 response wells over a three days incubation.

RESULTS AND DISCUSSION

Reproducibility and variability studies

The results obtained in the BOD₅ tests (dilution and manometric) for the standard solution, performed with the four inocula used in this study are summarized in Table 1.

The mean BOD₅ values obtained with soil and *Pseudomonas* inocula for the glucose-glutamic acid solution fall in the range described in the BOD methods standards above cited for activated sludge, i.e. 198 ± 30.5 mg O₂/l (APHA, 1998). This suggests that these microbial inocula can be used as an alternative to the activated sludge inoculum in BOD₅ tests.

Moreover, these results show that the BOD₅ test procedures with both inocula in the study are consistent, yielding mean BOD₅ values for the standard solution with coefficients of variation between 2.2% and 7.0% and the BOD₅ test procedures with standard inocula (TPI, AS) yielded slightly higher variations, with coefficients of variation between 3.1% and 9.5%. These variations showing a good reproducibility for both manometric and dilution methods are acceptable for microbial tests and comparable to those reported by Sun *et al.* (1994) for tests using activated sludge cultures.

Table 1. Results of BOD₅ tests for the standard glucose-glutamic acid solution using the soil inoculum, the *Pseudomonas* culture and two treatment plant inocula (TPI and AS).

	DILUTION METHOD	MANOMETRIC METHOD
Soil inoculum	BOD ₅ = 216.9 mg O ₂ /l (n=20) <i>SD</i> = 9.33 <i>CV</i> = 4.30% <i>IC</i> (95%): 212.5 – 221.2 r = 27.6	BOD ₅ = 209.2 mg O ₂ /l (n=30) <i>SD</i> = 14.71 <i>CV</i> = 7.03% <i>IC</i> (95%): 203.7 – 214.7 r = 42.6
Pure culture (<i>Pseudomonas</i> sp.)	BOD ₅ = 199.1 mg O ₂ /l (n=20) <i>SD</i> = 4.46 <i>CV</i> = 2.24% <i>IC</i> (95%): 197.0 – 201.2 r = 13.2	BOD ₅ = 179.9 mg O ₂ /l (n=13) <i>SD</i> = 12.32 <i>CV</i> = 6.85% <i>IC</i> (95%): 172.5 – 187.3 r = 38.0
TPI	BOD ₅ = 197.1 mg O ₂ /l (n=10) <i>SD</i> = 15.03 <i>CV</i> = 7.63% <i>IC</i> (95%): 186.4 – 207.9 r = 48.0	BOD ₅ = 213.2 mg O ₂ /l (n=10) <i>SD</i> = 13.16 <i>CV</i> = 6.17% <i>IC</i> (95%): 203.8 – 222.6 r = 42.0
AS	BOD ₅ = 196.3 mg O ₂ /l (n=10) <i>SD</i> = 18.54 <i>CV</i> = 9.45% <i>IC</i> (95%): 179.1 – 213.4 r = 65.3	BOD ₅ = 219.2 mg O ₂ /l (n=10) <i>SD</i> = 6.82 <i>CV</i> = 3.11% <i>IC</i> (95%): 214.3 – 224.1 r = 21.8

SD = Standard Deviation, *CV* = Coefficient of Variation, *IC* = Interval of Confidence,
r = Repeatability

BOD₅ data validation

To validate BOD₅ results, a comparative study of the soil inoculum, the *Pseudomonas* culture and a treatment plant inoculum (TPI), was carried out on several wastewater samples. The BOD₅ results obtained for real wastewaters, summarized in Table 2, were compared through a Pearson rank correlation analysis (Table 3).

Table 2. BOD₅ values (mg O₂/l) for several wastewater samples tested with the treatment plant inoculum, the soil inoculum and the *Pseudomonas* culture.

Sample tested ^a	Manometric method			Dilution method		
	TPI	Soil	<i>Pseudomonas</i>	TPI	Soil	<i>Pseudomonas</i>
1	213.2	209.2	179.9	197.1	216.9	199.1
2	170.0	183.3	266.7	172.8	125.9	116.3
3	346.9	130.0	255.5	370.0	134.3	148.3
4	918.4	776.7	1000.0	900.0	961.6	999.8
5	173.1	240.0	198.9	210.0	276.9	267.4
6	1118.0	933.3	1100.0	1070.0	1094.7	1009.4
7	439.0	333.3	376.7	350.0	289.8	337.2
8	387.8	306.7	365.6	185.0	205.9	305.7

^a 1: Standard glucose-glutamic acid solution, 2: Cattle effluent, 3: printing effluent, 4: cork effluent, 5 and 6: urban wastewaters, 7 and 8: domestic wastewaters.

Table 3. Correlation analysis between the BOD₅ values obtained by the two methods of BOD determination (M - manometric, D - dilution) and between the BOD₅ values obtained using different inocula in each method.

	TPI-M	<i>Pseudomonas</i> -M	Soil-M	TPI-D	<i>Pseudomonas</i> -D	Soil-D
TPI-M	-	0.98*	0.97*	0.98*	0.96*	0.96*
<i>Pseudomonas</i> -M	0.98*	-	0.98*	0.97*	0.98*	0.97*
Soil-M	0.97*	0.98*	-	0.95*	0.99*	0.99*
TPI-D	0.98*	0.97*	0.95*	-	0.95*	0.97*
<i>Pseudomonas</i> -D	0.96*	0.98*	0.99*	0.95*	-	0.99*
Soil-D	0.96*	0.97*	0.99*	0.97*	0.99*	-

* Correlations significant at $p < 0.001$, $n = 8$

This statistical analysis shows the existence of significant correlations ($p < 0.001$) between:

- the values obtained with the three inocula (*Pseudomonas*/Soil/TPI), using either the manometric method ($r = 0.97 - 0.98$) or the dilution method ($r = 0.95 - 0.99$);
- the values obtained with the three inocula, independent of the method used ($r = 0.95 - 0.99$). These high correlations clearly show that the BOD tests with these microbial inocula are reliable, independently of the method used. Similar results were obtained by Paixão *et al.* (2000) with commercial inocula as seed in BOD₅ tests.

BIOLOG EcoPlates™ assays results

BIOLOG EcoPlates were used to evaluate both qualitative and quantitatively community-level metabolic responses as well as to establish the metabolic profile of *Pseudomonas* sp.

For 72 h of incubation, the soil inoculum was considered positive for 29 Eco substrates, being negative only for two substrates, 15 (D,L- α -glycerol phosphate) and 18 (2-hydroxy benzoic acid); the *Pseudomonas* culture was positive for 19 carbon sources, being negative for the substrates 4, 10, 12, 13, 16, 17, 18, 24, 26, 27, 30 and 31 (Table 4); and the standard inoculum, AS from Beirolas treatment plant, was positive for all the carbon sources in EcoPlate (Table 4).

A scatterplot of BIOLOG EcoPlate responses comparing the soil inoculum and the *Pseudomonas* sp. culture metabolic profiles (expressed as response curve net areas) to that obtained for an activated sludge inoculum (plotted on X-axis) is presented in Figure 1. The values close to the reference line are in good agreement with AS values. Values above the reference line have an enhanced ability to utilize that carbon source and values below have a reduced ability as compared to AS.

Table 4. Phenotypic fingerprint for the inocula tested using EcoPlates.

ECO-substrates	Microbial inocula			
	Activated Sludge	Soil	<i>Pseudomonas</i> sp.	
1	Pyruvic acid methyl ester	1	1	1
2	Tween 40	1	1	1
3	Tween 80	1	1	1
4	Cyclodextrin	1	1	0
5	Glycogen	1	1	1
6	D-Cellobiose	1	1	1
7	α -D-Lactose	1	1	1
8	β -Methyl-D-glucoside	1	1	1
9	D-Xylose	1	1	1
10	i-Erythritol	1	1	0
11	d-Mannitol	1	1	1
12	N-Acetyl-d-glucosamine	1	1	0
13	D-Glucosaminic acid	1	1	0
14	Glucose-1-phosphate	1	1	1
15	D,L- α -Glycerol phosphate	1	0	1
16	D-Galactonic acid γ -lactone	1	1	0
17	D-Galacturonic acid	1	1	0
18	2-Hydroxy benzoic acid	1	0	0
19	4-Hydroxy benzoic acid	1	1	1
20	γ -Hydroxybutyric acid	1	1	1
21	Itaconic acid	1	1	1
22	α -Ketobutyric acid	1	1	1
23	D-Malic acid	1	1	1
24	L-Arginine	1	1	0
25	L-Asparagine	1	1	1
26	L-Phenylalanine	1	1	0
27	L-Serine	1	1	0
28	L-Threonine	1	1	1
29	Glycyl-L-glutamic acid	1	1	1
30	Phenylethylamine	1	1	0
31	Putrescine	1	1	0

(1) positive: carbon source used; (0) negative: carbon source not used

In this context, the soil inoculum used in this study seems to have a catabolic profile similar to the one of AS from Beirolas treatment plant, as it is also capable of utilizing almost all the Eco carbon sources (Table 4), despite presenting lower

values of net area under the curve for some substrates (Fig. 1). The *Pseudomonas* sp. culture was able to use 61% of the carbon sources in the EcoPlate (Table 4), although showing a reduced ability for the utilization of the major substrates as compared to AS (Fig. 1).

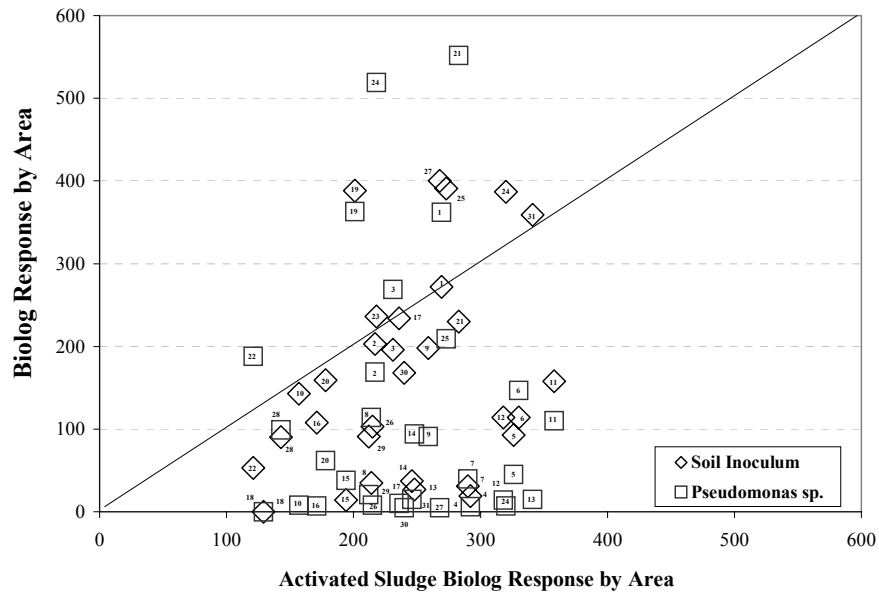


Fig. 1. Scatterplot of BIOLOG EcoPlate responses (average net area under the curve for each of the 31 carbon sources) comparing the soil inoculum and the *Pseudomonas* sp. culture metabolic profiles to that obtained for activated sludge inoculum (plotted on X-axis). The values close to the reference line are in good agreement with AS values. Values above the reference line have an enhanced ability to utilize that carbon source and values below have a reduced ability as compared to AS.

CONCLUSIONS

In biodegradability testing, important factors which cannot be easily standardized and that greatly influences biodegradation results are the inoculum, the source of the microorganisms for the test, and their state of acclimatization and adaptation (Pagga, 1997).

In this study, two inocula were tested as biological reference material for a BOD₅ test in comparison with AS inoculum: a standardized soil inoculum, which is composed by a selected and well known bacterial consortium from soil isolates and kept in laboratory constant conditions to maintain the same community catabolic profile, once soil inoculum is an optional seed described in the standards of the BOD₅ test methods performed (APHA, 1998); and a *Pseudomonas* sp. culture, since these bacteria are ubiquitous in nature and are also used as test reference organism in toxicity test methods (ISO, 1996).

The results obtained highlight the ability of the *Pseudomonas* culture and the soil inoculum to be used as seed in BOD₅ tests as an alternative to activated sludge. The comparative study carried on the standard solution (glucose-glutamic acid) and real wastewater samples, with these two inocula and an AS inoculum, allowed to verify the existence of high correlations between the values obtained, which strongly suggests their correspondence.

However, as most degradation processes in the environment strictly require a mixed population, the soil inoculum seems to be a good choice for a biological reference material, as it presents a similar catabolic profile to AS (Beirolas treatment plant), being able to utilize almost all the Eco carbon sources. The *Pseudomonas* sp. culture used as inoculum, due to the results obtained in BOD tests and its catabolic profile which complement the soil inoculum metabolic pattern, could be mixed with the soil inoculum, improving this mixed inoculum as an AS surrogate culture, once the AS inoculum used could utilize all the 31 Eco-substrates. Further tests must be performed with this mixed *Pseudomonas*-soil inoculum before we can propose and certify this mixed microbial inoculum, standardized and exhibiting the same metabolic behaviour as activated sludge, as a new biological reference material (Cordis, 2000).

In fact, the utilization of suitable representative microbial inocula can overcome some problems related to inocula collected from activated sludge plants, such as sampling strategy, maintenance of viability, control of quantitative and qualitative characteristics and potential contact with pathogenic microorganisms. The seed homogeneity and stability as well as its easy and safe manipulation are also important properties of these reference materials. These controlled cultures can be helpful for the standardization of biological assays leading to an improvement of the quality of chemical and effluent testing and monitoring.

The use of the biological material as surrogate culture may act as a complement and important supplementary tool to existing activated sludge tests (OECD, ISO, DIN). The availability of a reference material could lead to more homogeneous and comparable results and could eliminate any bias, which could be very advantageous in case of inter-laboratory studies.

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Chapter 7

GLOBAL ANALYSIS AND CONCLUSIONS

The quantity of toxicants finding their way into aquatic and terrestrial environments is alarming. To handle and treat wastes effectively, it is necessary to evaluate the ecological behaviour of the prevailing chemicals. Consequently, the evaluation of toxicity and biodegradability of organic substances and wastes is essential for their hazardous impact and risk assessment, either on a biological treatment plant or on the environment of their ultimate disposal.

Currently, health concerns are being superimposed on the objectives of wastewater treatment, particularly what concerns to toxicity and biodegradability, since chemicals that ultimately enter the environment are often discharged through sewage or WTPs. Therefore, methods that use activated sludge from WTPs as inocula for tests to measure toxicity and ready or inherent biodegradability of chemicals are among the most widely used.

Toxicity and biodegradability screening tests are standardized to ensure repeatability. However, given the enormous variability in the structure and function of microbial communities developed at different times or in different places, it is quite reasonable to question the repeatability of tests employing these communities. In the case of the AS-based tests, the microbial communities found in AS are complex assemblages of microorganisms that maintain a dynamic equilibrium by responding to changes in environmental conditions either by shifts in the structure of the community (species richness or rank abundance), or by quantitative or qualitative changes in community function. Since microorganisms within these communities are interdependent, any minor change in the environmental conditions may lead to a cascade of events that cause a great restructuring of the community and lead to

differences in community function even if the change directly influences only a limited number of populations (Forney *et al.*, 2001).

In accordance, some authors (Forney *et al.*, 2001; Cordis, 2000; Örmeci and Vesilind, 2000) refer that differences in the composition of the influent water or plant operation mode can exert a strong influence on the structure and function of an AS microbial community and this in turn could markedly influence the microbial processes responsible for the biodegradation of various chemicals during treatment. Moreover, the manipulations done in the laboratory after sludge has been collected can also cause a rapid and profound restructuring of the microbial community so that the resulting community poorly simulates that of fresh sludge (Forney *et al.*, 2001). Thus, differences in the composition of the influent wastewater, in plant operation mode, or manipulations done after collection of sludge can lead to inconsistent biodegradation or toxicity data confounding its interpretation.

The inoculum quality (composition and cell density) is therefore variable and difficult to standardize. In spite of some recommended pre-treatment for the AS inoculum used (e.g. filtration, decantation, aeration, etc), the inoculum shows huge variability and constitutes the main cause of poor reproducibility of standardized tests (Struijs *et al.*, 1995; Thouand *et al.*, 1995, 1996; Vasquez-Rodriguez *et al.*, 1999, 2000). In fact, the lack of the microbial inoculum standardization makes it almost impossible to carry out controlled assays, reproduce test results or compare different test results, indicating an obvious need to have a standard biological reference material for toxicity and biodegradability standardized tests.

Models are the most basic of scientific tools. When an entity is too large to manipulate or impossible to replicate, the relevant characteristics of the system may be reproduced in a more manageable form. Models are not exact reproductions of

the systems being studied as they incorporate only the parts needed (Cairns and Niederlehner, 1995).

In this context, the focus of this dissertation was to evaluate the ability of several well-defined microbial cultures, i.e. model communities, as biological reference materials in selected AS-based bioassays, namely: ISO 8192 - test of the inhibition of the oxygen consumption, ISO 5815 – BOD₅ test, and ISO 9888 - Zahn-Wellens test, comparing and correlating their response to pollutants, in order to estimate their potential to act as AS surrogates.

Thus, to initiate this study several AS samples from different sources were characterized microbiologically. The patterns of carbon source utilization, or community-level physiological profiles (CLPPs) for AS communities were established, based on the direct inoculation of the mixed microbial samples into BIOLOG MicroPlates™.

The results of these studies showed that when a washing step was applied to AS samples a decrease in the number of substrates used as well as a net area decrease under the respective curves was observed, in comparison with those obtained with no washed samples. Therefore, these results confirmed that inoculum preparation is also a form of bias, reinforcing the need to establish an inoculum preparation procedure with a limited and obligatory number of steps, leading to a more rigorous standardization (see chapter 2).

In the same way, individual metabolic profiles for both predominant and common non-pathogenic heterotrophic bacteria isolated from AS communities and equivalent strains selected from the CCMI of INETI, were also traced and compared with the AS'CLPPs, by cluster analysis and Pearson linear correlations between the metabolic data, to determine the relevant strains which most have contributed to AS' CLPPs. Based on these results, several model communities (MCs) described in

chapter 2 (MC14 to MC25), composed by 3 to 6 bacterial strains, were prepared and analysed as representative inocula in terms of their metabolic similarity to AS metabolic patterns. The PCA, cluster analysis and Pearson linear correlation analysis of the net areas from BIOLOG substrates utilization for these MCs, highlight for a group of model communities, composed by a different number of strains, as AS' surrogate cultures, based on the utilization profiles of 221 single carbon sources (ECO-GN-GP), independently of the common substrates, namely: MC17, MC18, MC19 and MC21 (chapter 2, Fig. 6).

Simultaneously, some other three microbial consortia were also analysed as AS surrogates for their metabolic profiles: a designed inoculum ("DI" or "standardized soil inoculum") - a well-defined consortium composed by six selected non-pathogenic bacterial strains isolated from soil, once the use of soil suspensions are also described as an optional seed to a domestic treatment plant inoculum in some biological tests (e.g. BOD₅ test), and two commercial inocula: BI-CHEM[®] and BIOLEN - standardized specialized bacterial cultures blends suitable as biological materials.

In order to identify the potential effects of the utilization of different steps in the inoculum preparation procedure on the final CLPPs of these three consortia, diverse preparation steps, commonly described in the standardized tests (e.g. growth medium, initial cell density), were applied to the referred consortia as described in chapter 2. Results demonstrated that different growth media lead to different MCs, despite of the origin on the same consortium, reflected by the different community level metabolic profile displayed by each MC. For example, when synthetic medium (ISO, 1986) was used as growth medium to prepare these consortia to test (DI: MC5, BIOLEN: MC9, BI-CHEM[®]: MC12), the number of substrates used by the respective MC was enhanced as well as the microbial metabolic responses for the

substrates utilization (higher net areas) for all consortia. The initial cell density of the microbial inoculum also was shown to influence its CLPP (see chapter 2).

The aim of this study was to select microbial communities with a similar metabolic behaviour to AS, but also to define an effective procedure for inoculum preparation since this can affect the microbial community structure (chapter 2), and so it must be also standardized beyond the inoculum composition. Based in PCA, cluster analysis and the Pearson correlation analysis of BIOLOG data for these model communities (MC5 to MC13) in comparison with the AS' CLPPs, the MC5 (DI), MC9 (BIOLEN) and MC12 (BI-CHEM[®]) have presented the higher similarity with AS' CLPPs (chapter 2, Fig. 6).

The patterns of potential carbon source utilization, or CLPPs, were analysed using multivariate statistical techniques, such as principal components analysis, to evaluate the relative degree of similarity among the microbial consortia in relation to AS samples. In conclusion, CLPP gives information about the composition of each microbial community, according to a functional profile. The CLPP is reproducible and shows discriminative power to differentiate microbial communities.

Moreover, the most similar metabolic behaviour to AS was a pre-requisite to select metabolic surrogate cultures, i.e. representative inocula, that could be an alternative to AS inocula. To assess the performance and sensitivity of these model communities selected as potential reference materials (RMs) for AS-based tests, preliminary evaluation tests were carried out on reference chemicals, to validate the procedure and the viability of the inoculum used.

In this context, three selected model communities: MC5 (DI or soil inoculum), MC9 (BIOLEN) and MC12 (BI-CHEM[®]), were evaluated as alternative seed for an acute toxicity test (ISO 8192, 1986 - a respiration inhibition test), and for inherent and ready biodegradability tests, namely the Zahn -Wellens test (ISO 9888, 1999) and

the BOD₅ test (ISO 5815, 1989; APHA, 1998), and compared with AS' results. The inhibition of the respiration rates of the microorganisms was estimated for 3,5-dichlorophenol (3,5-DCP), and the biodegradability was evaluated for diethylene glycol (DEG) (Zahn-Wellens test) and for the standard glucose-glutamic acid solution (BOD₅ test, dilution and manometric methods).

In general, for the respiration inhibition test was followed the methodology described in ISO 8192 – method A (1986), but for the preparation of each stock inoculum was imposed the condition that the cells number present (cfu/ml) should be adequate to produce a respiration rate in the range from 2 to 4 mgO₂/lh in the blank controls (King and Dutka, 1986). The test results only were considered valid when this condition was fulfilled. The acute toxicity values (IC₅₀s) obtained ranged from 9.4 to 22.7 mg/l (VC: 7.2-8.1%, $n \geq 10$), with BI-CHEM[®] being the most sensitive and presenting the most similar behaviour to AS (IC₅₀ = 9.4 mg/l, VC = 10.8%, $n \geq 10$), although the results with all the microbial consortia tested are included in the range stipulated for the 3,5-DCP (5-30 mg/l) in the standard. The variability presented by the potential RMs was lower than that observed for AS samples, reflecting the difficulty of controlling the characteristics of the different samples collected in the same WTP to obtain homogeneous inocula (chapter 3).

In the Zahn-Wellens test, the DEG biodegradation curves obtained for DI maintained a similar profile for the two concentrations tested (400 or 600 mg/l) with steep slopes, presenting a complete degradation within 12 - 14 days, being these results the most similar to those of AS tested (chapter 4).

The results obtained with the standardized microbial inocula (DI, BIOLEN and BI-CHEM[®]) for the BOD₅ of the standard solution ranged from 176.8 to 216.9 mg/l (VC: 4.3-4.7%, $n \geq 10$), by the dilution method (normalised test), and from 209.2 to 228.6 mg/l (VC: 7.0-9.4%, $n \geq 10$) by the manometric method (no normalised test),

falling in the range expected for AS, 198 ± 30.5 mg/l (APHA, 1998). Thus, all these inocula showed potential to be used as alternative seed in BOD₅ test, independently of the method used, similarly to the AS sample used (chapters 5 and 6).

The community substrate utilization pattern based on BIOLOG data showed that the CLPPs of BI-CHEM[®] and DI (soil inoculum) were the most similar to AS'CLPP (chapters 3, 4 and 6), probably because the main heterotrophic bacteria responsible for the overall metabolic activity present in these different consortia are similar, despite their different taxonomic richness.

The BOD₅ tests and the respiration inhibition test were also performed for the respective reference compounds using a pure culture of *Pseudomonas* sp. as inoculum (chapters 3 and 6). This strain was used as a representative for heterotrophic bacteria ubiquitous in all environments (water, soil, WTP), with a high metabolic potential, and because this genus is already used in toxicity tests (ISO 10712, 1995) as well as in BOD biosensors (Yoshida *et al.*, 2001). The results obtained for the standard solution by both BOD₅ test methods fall in the range described for AS inoculum, similarly to AS and the model microbial communities tested. In the respiration inhibition test, *Pseudomonas* sp. was the most sensitive inoculum to 3,5-DCP, presenting an IC₅₀ = 6.7 mg/l (VC = 6.3%, n ≥ 10). Thus, *Pseudomonas* sp. seems to show potential to be used as a biological reference material in these tests to estimate the toxic effects of a pollutant or its biodegradability, as a representative heterotrophic bacterium from AS.

With the same principle, Kelly *et al.* (1999) have constructed a bioluminescent reporter (Shk1) by addition of a plasmid containing the lux genes from *Vibrio fischeri* into an AS isolate (*Pseudomonas fluorescens*) with the goal of developing a bioluminescent bacterium compatible with the activated sludge environment. Since

then several studies with Shk1 have showed toxicity results for a wide array of toxicants that are in closer agreement with those derived from AS respirometry experiments for the same toxicants (Ren and Frymier, 2002, 2003; Lajoie *et al.*, 2003; Kelly *et al.*, 2004), contrary to observed when are used tests with bacterial strains not related to the AS environment (Gutiérrez *et al.*, 2002).

Nevertheless, pure culture testing eliminates the possibility of interspecies interaction such as synergism, commensalism, symbiosis, and antagonism that occur in the AS environment. So, for the evaluation of potential toxicity or biodegradability of a compound on a WTP, the preferred biological material to be used should be activated sludge itself. However due to all the problems associated with the use of AS, mainly to its inherent variability, the best choice toward a standardization of the microbial inoculum for AS-based toxicity and biodegradability tests highlight for the use of a feasible alternative microbial consortium representative of the AS community behaviour. The use of a surrogate microbial community well standardized, as the biological reference material (Nirmalakhandan *et al.*, 1996), can overcome the problems associated with the use of AS inocula, giving more reproducible and comparable test results, acting as a complement and important alternative tool to existing AS-based tests.

In conclusion, from the several model communities metabolically characterized only three have been evaluated in the selected biological tests for their performance as an alternative inoculum to AS, using reference compounds. In parallel, the same tests have also been performed with fresh AS samples collected from municipal WTPs (the pattern inoculum that is to be replaced), serving as a preliminary procedure validation of the test performed. A significant number of replicas (≥ 10) were performed (BOD₅ and ISO 8192 tests) to compare repeatability and variability in the results with the potential surrogate inocula (RMs) and the AS. From all

biodegradability results (BOD₅ and IB tests), DI stands out as a good choice as reference material for biodegradability tests, while from the toxicity results the BI-CHEM[®] appears as the suitable alternative to the AS, due its sensitivity to 3,5-DCP be similar to that of AS. Moreover, in terms of the overall metabolic potential, DI and BI-CHEM[®] were the most representative inocula of AS' CLPPs among the consortia tested.

In addition, beyond other microbial inocula also studied, DI and BI-CHEM[®] were validated as seed for BOD₅ test, by testing several real samples in a comparative study with a treatment plant inoculum (TPI) (chapters 5 and 6). In this validation study, a statistical analysis of the results showed the existence of significant Pearson correlations to 95% confidence ($p < 0.05$, $n \geq 7$) between the results obtained with the RMs (DI or BI-CHEM[®]) and TPI, with the manometric method ($r = 0.97-0.99$), the dilution method ($r = 0.97-0.98$), and independently of the method used ($r = 0.96-1.00$). These high correlations clearly show that the BOD₅ test, with BI-CHEM[®] or DI as the reference material, is reliable regardless of the method. The manometric method with the OxiTop[®] system (WTW, 1997) could be a good methodology to adopt with one of these RMs to determine the BOD₅ of pollutants because of the easy performance.

The ultimate purpose of this work was to permit an improvement of the effectiveness, namely in the respective reproducibility of the test performed, with impact in the normalisation procedures of analysis and biological assays. The use of well-predefined microbial consortia, with simple maintenance and manipulation, can be helpful for the normalisation of biological assays leading to an improvement of the quality of chemical and effluent testing and monitoring.

TRENDS FOR FUTURE WORK

Since the main goal is to achieve a biological reference material able to be accepted and included in relevant standards (ISO and OECD), as alternative inoculum to AS, a certification step should be prosecuted, including studies of inoculum stability and shelf life, realization of inter-laboratorial ring-tests, etc. The development of reconstitution procedures and verification of homogeneity, stability and feasibility testing are important aspects for future work. Before a new or revised test method can be used to generate information to support regulatory decisions in risk management, it must be (i) validated, its reliability and relevance for its proposed use must be determined, and (ii) accepted, one or more regulatory or research agencies must determine that fills a specific need.

Alternative tests are those that incorporate replacement, reduction or refinement of the test organism commonly used (three “R” of alternatives). Replacement refers to partial or total replacement of organisms with non-organism systems, or the replacement of an organism species with a phylogenetically lower species. Reduction means reduction of the total number of organisms required. Refinement refers to the incorporation of procedures to lessen or eliminate pain or distress to organisms, and enhance their well-being.

In this case, the goal was to replace the no standardized test organism (AS) by a standardized surrogate culture with both similar metabolic characteristics and sensitivities to pollutants, using AS-based bioassays standards, for a reliable environmental biomonitoring improving prediction of the potential fate or environmental impact of chemicals. The use of the biological material as surrogate culture may act as a complement and important supplementary tool to existing AS tests, which use no-calibration for the BOD₅ test.

Validation is a scientific process designed to characterize the operational characteristic, advantages, and limitations of a test method, and to demonstrate its reliability and relevance. The criteria for validation of a test method are, to a limited extent, a function of the purpose for which the test method will be used. When validating new or revised test methods, hypotheses are developed regarding the effects measured and their relationships to the biological effects in the biological material of concern. The relationship between the new method and the effect that is designed to predict, or the procedure that is designed to replace, must be described.

Another future approach pretended is to evaluate the DI, once is composed by 6 strains isolated from soil samples, as a biological reference material in a toxicity test directed also to predict the potential effects of the discharges of pollutants on soil, comparing its response to several toxicants with those of different soil communities, since there is a need to develop new specific test methods or adapt existing tests to assess soil quality and to assure its protection.

A rapid biological toxicity-screening test using this blend of microbial cultures may supersede the EPA recommended standard microbial test (Polytox™ test) as an inexpensive tool to test effluent toxicity. The use of a standard inoculum will add to the credibility of setting Toxicity-Based Consents for whole effluent toxicity testing. The availability of a reference material could lead to more homogeneous and comparable results and eliminate any bias, a strong advantage in the case of inter-laboratory studies.

A complementary study important to this work should be the analysis of the genetic profiles of the surrogate model communities as well as the overall genetic profile of AS, in order to establish the respective genetic patterns in addition of the metabolic patterns.

The application of molecular tools, which allow the study of microbes without prior cultivation, will give a unique insight in terms of the genetic fingerprint of the proposed microbial communities. The metabolic patterns can give information about phenotypic characteristics, as in the present study a metabolic response of each RM, under defined growth and test conditions. In fact, only the application of molecular tools can give a comprehensive information that will allow to understand the differences obtained, in the RMs' responses, and to design inocula for several applications, capable to mimetic "natural microbial consortia".

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