THE INFLUENCE OF MICROBIAL INOCULA ON BIODEGRADATION OUTCOME TOWARDS ENHANCED REGULATORY ASSESSMENTS

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Declaration

I hereby certify that this work is my own, except where otherwise acknowledged, and that it has not been submitted for a degree at this, or any other, university.

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Abstract

Chemical compounds are ubiquitous in the aquatic environment, causing numerous negative impacts and raising concern over human health and the environment. The principal elimination process is microbial degradation, the prediction of which plays an important role in risk assessment. Current biodegradation tests are notoriously variable and not effective at characterising chemical persistence. Enhancements to existing biodegradation tests have been proposed to enable a more effective prioritisation on persistence. Several of the proposed enhancements are examined in this study, including increasing total cell numbers within tests and extending test duration.

Activated sludge (AS) and marine inocula were incorporated in OCED 301B type studies with different inocula concentrations, test volumes and over extended study duration. Evolved $^{14}CO_2$ was captured as a measure of degradation, subsequently converted to a probability of degradation and used in the calculation of degradation descriptors. Culture-independent methods were employed to study the diversity within batch systems (PCR-DGGE and 454 sequencing).

AS inocula exhibited faster rates of degradation and shorter lag phases when operated at higher cell concentrations, although the greatest impact of increased cell concentration was observed in decreased inter-replicate variation ($P < 0.01$). Test volume had less effect than concentration, with inter-replicate variation again the main beneficiary of the enhancement. Typically, band richness, used as a measure of diversity, increased with increasing biomass concentration. DGGE analysis also suggested greater similarity between higher cell concentration replicates. The ideal system proposed is 300 mg SS L^{-1} at 0.5-1.0 L.

Marine systems did not show a significant concentration or volume effect ($P>0.05$). A number of systems exhibited rapid degradation rates following lengthy lag phases suggesting the importance of increasing test duration in order to accurately predict environmental behaviour and fate.

A major concern for a novel screening test would be to underestimate persistency and approve chemicals which could pose a significant threat to the environment. Chemical validation studies using a set of recognised reference compounds of varying persistence showed no false positives in the enhanced screening test.

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Abbreviations

$^{14}CO_2$	Radiolabelled carbon dioxide
4-CA	4-chloroaniline
4-FP	4-fluorophenol
4-NP	4-nitrophenol
Ani	abbreviation Aniline
AS	Activated Sludge
BAF	Bioaccumulation Factor
BCC	Bioaccumulative Chemicals of Concern
BCF	Bioconcentration Factor
BEL	Brixham Environmental Laboratory
BMF	Biomagnification Factor
BST	Biodegradation Screening Test
C	Centrifugation
CC	Glass bead (column) colonisation
ChAMP	Chemical Assessment Management Program
CO_2	Carbon dioxide
COD	Chemical Oxygen Demand
DAPI	4'6-diamidino-2-phenylindole
DDT	Dichlorodiphenyltrichloroethane
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
DOC	Dissolved Organic Carbon
EC	European Commission
ECETOC	European Centre of Ecotoxicology and Toxicology of Chemicals
ECHA	European Chemicals Agency
EDC	Endocrine Disrupting Chemical
EE2	17 α-ethinylestradiol
EEA	European Environment Agency
EEC	European Economic Community
EPA	Environmental Protection Agency
EU	European Union
FOV	Field Of View

GLP	Good Laboratory Practice
HPV	High Production Volume
HT-BST	High Throughput-Biodegradation Screening Test
IBT	Inherent Biodegradability Test
IPPC	Integrated Pollution Prevention and Control
LOD	Limit of Detection
LOQ	Limit of Quantification
LSC	Liquid Scintillation Counting
MF	Membrane Filtration
NaOH	Sodium hydroxide
NOEC	No Observed Effect Concentration
OECD	Organisation for Economic Co-Operation and Development
OSPAR	The Convention for the Protection of the Marine Environment of the North-East Atlantic
OTU	Operational Taxonomic Unit
PBT	Persistent Bioaccumulative Toxic
PCB	Polychlorinated biphenyl
PCP	Pentachlorophenol
PCR	Polymerase Chain Reaction
PEC	Predicted Environmental Concentration
PhAC	Pharmaceutically Active Compound
PNEC	Predicted No Effect Concentration
POP	Persistent Organic Pollutant
PPCP	Pharmaceuticals and Personal Care Products
PRIMER	Plymouth Routines In Multivariate Ecological Research
QIIME	Quantitative Insights Into Microbial Ecology
QSAR	Quantitative Structure Activity Relationship
RBT	Ready Biodegradability Test
REACH	Registration, Evaluation, Authorisation and restriction of CHemicals
rRNA	ribosomal ribonucleic acid
SS	Suspended Solids
SSU	Small Sub Unit
TFF	Tangential Flow Filtration

$ThCO_2$	Theoretical Carbon Dioxide Evolution
ThOD	Theoretical Oxygen Demand
TSCA	Toxic Substances Control Act
UNCED	United Nations Conference on Environment and Development
UNEP	United Nations Environment Program
USEPA	United States Environmental Protection Agency
vPvB	very Persistent very Bioaccumulative

Degradation Descriptors

t_L	Lag phase (time taken to reach 10% degradation/removal of parent compound)
$t_{1/2}$	Time taken to reach 50% degradation/removal
t_{60}	Time taken to reach OECD $ThCO_2$ pass threshold
Δt_{50}	Time difference from end of lag phase to t_{deg50}
Δt_{60}	Time difference from end of lag phase to t_{deg60}
K_{max}	Maximum degradation rate (calculated from steepest part of $^{14}CO_2$ evolution curve)

Standard Base Abbreviations

A	Adenine
C	Cytosine
G	Guanine
M	A or C
N	Any base
R	A or G purine
T	Thymine
U	Uracil
Y	C or T pyrimidine

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Chapter 1

1. Introduction

1.1 Origins of the chemical industry

The chemical industry is an essential contributor to increased living standards. Its origins are strongly tied to the industrial revolution and the need to utilise a by-product from iron production: coal tar or creosote (Jarman and Ballschmiter, 2012). Coal tar is a complex mix of organic compounds which arises from the coking of coal, primarily utilised to produce gas, for town lighting, and coke, used for its reductive properties in iron production (Kiely, 2007). Initially, coal tar held limited appeal and was disposed either to landfill or rivers but then researchers were tasked with utilising coal tar as a source for previously undiscovered compounds. August Wilhelm Hofmann is credited with the first breakthrough in his discovery of aniline, a key compound in early organic chemistry and industrial dye production (Landau, 1994). Hofmann was, subsequently, the first chemist to attempt to produce a novel synthetic compound from a compound predominantly used in industrial dye production. Although he ultimately failed to synthetically produce quinine, his research and concepts were inextricably linked to the development of compounds such as pesticides.

The German dye industry provided the impetus for pesticide formulation to counter the impact of moths on dyed cloth being produced in large quantities. Whilst there was a significant amount of research being conducted into chlorinated insecticidal compounds by a number of institutions, it was ultimately the combined efforts of the German dye industry and the Swiss chemical industry, spearheaded by Paul Müller, which can claim credit for developing arguably the most notorious pesticide, dichlorodiphenyltrichloroethane (DDT) (Jarman and Ballschmiter, 2012).

In the early twentieth century, the American chemical industry began to supersede the dominant German industry. This can be attributed to three predominant factors: firstly, the loss of World War I considerably weakened the German chemical industry, compounded by further disruption to the European industry as a whole during World War II; secondly, domination of the German dye industry led to their reluctance in finding alternative production methods more conducive to organic chemical production and finally, the American development of the discipline of chemical

engineering left them better able to design and utilise large-scale production techniques and cope with the global shift from coal to oil use (Landau, 1994).

The more recent growth of the chemical industry, particularly over the last decade, has been driven by growth in developing countries and countries with economies in transition. This coupled with resurgence in the European chemical market has led to intense global competition in the chemical industry (UNEP, 2013). North America is still currently the market leader in chemical industry output (excluding pharmaceuticals) and pharmaceutical sales (Figure 1-1Figure 1-2), but OECD forecasts predict that companies in China, India and the Middle East focusing on specialty and fine chemicals will lead to increasing numbers of new chemicals being developed in developing and transitioning countries (UNEP, 2013).

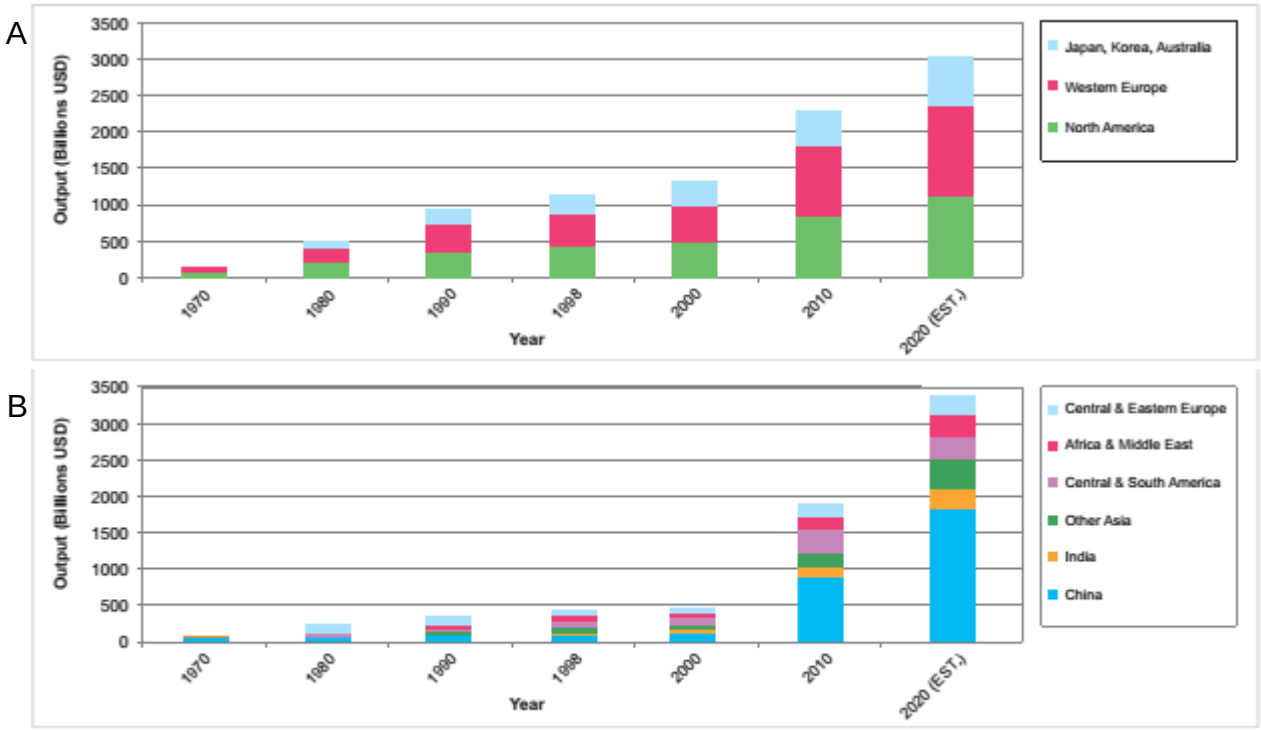


Figure 1-1 Chemical industry output for (A) developed regions and (B) developing regions and countries with economies in transition as categorised by UN statistics division. Data excludes pharmaceuticals. Industry growth is shown from the 1970s through to the estimated growth expected by 2020. Reproduced from UNEP (2013)

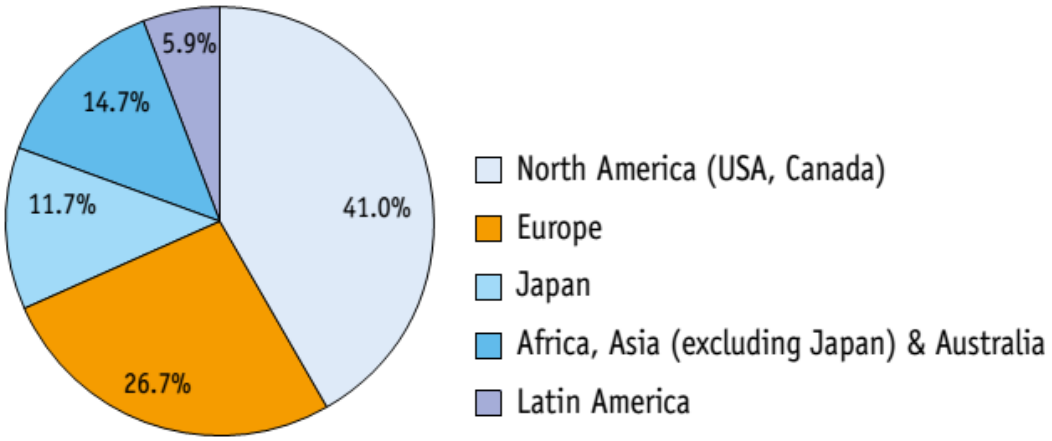


Figure 1-2 Breakdown of the global pharmaceutical market based on 2012 sales. Europe includes Turkey and Russia. Reproduced from EPFIA (2013) using data sourced from IMS MIDAS relating to 2012 audited global retail pharmaceutical market at ex-factory prices

1.1.1. *Sustaining life through chemistry*

Exponential growth of the human population has been accompanied by increased manufacture, application and disposal of chemical compounds from a range of sources, including industry, medicines and agriculture. Despite increased wastewater treatment and advancements in treatment technologies, pharmaceutically active compounds (PhAC) are thought to be present in the majority of waters receiving wastewater treatment plant effluent (Snyder *et al.*, 1999; Stackelberg *et al.*, 2004).

Medical and pharmaceutical advances have increased our ability to combat disease resulting in increased life expectancies and an age-inverted population structure with the associated problem of increased medicines consumption in a burgeoning, ageing population (Jelic *et al.*, 2011). Additionally, less expensive generic versions of compounds, following the expiration of patents, have contributed to increases in production and use (Daughton, 2003). As a result of these developments it is estimated that pharmaceutical consumption is increasing by 3-5% annually (Wennmalm, 2011).

An increasing population also encourages movement away from hunter-gatherer lifestyles towards processes capable of feeding the global population now in excess of 7 billion people. This intensification in agriculture is generally referred to as “the Green Revolution” (Matson *et al.*, 1997), harnessing the use of pesticides, fertilisers

and new crop varieties, amongst other technologies (Tilman *et al.*, 2002), to increase crop yields. In recent years there has also been a rapid growth in meat consumption (McMichael *et al.*, 2007) with an associated increase in veterinary medicines to maintain livestock production. With the global population expected to reach 10 billion by 2050 the reliance upon chemistry to combat hunger and malnutrition, particularly in developing countries, is indisputable (Cooper and Dobson, 2007). This bestows an important burden upon scientists to better understand the impact these compounds have upon recipient environments and human health and the manner in which they interact with organisms at all levels of complexity.

1.1.2. *Pathways for entry into the environment*

The presence of pharmaceuticals, hormones and other organic contaminants in aquatic environments has been identified as a significant environmental issue (Heberer, 2002). Research has shown that many of these compounds disperse following entry to the environment and can persist to a much greater extent than previously predicted (Kolpin *et al.*, 2002).

There are a number of pathways via which chemicals may enter the environment, summarised in Figure 1-3.

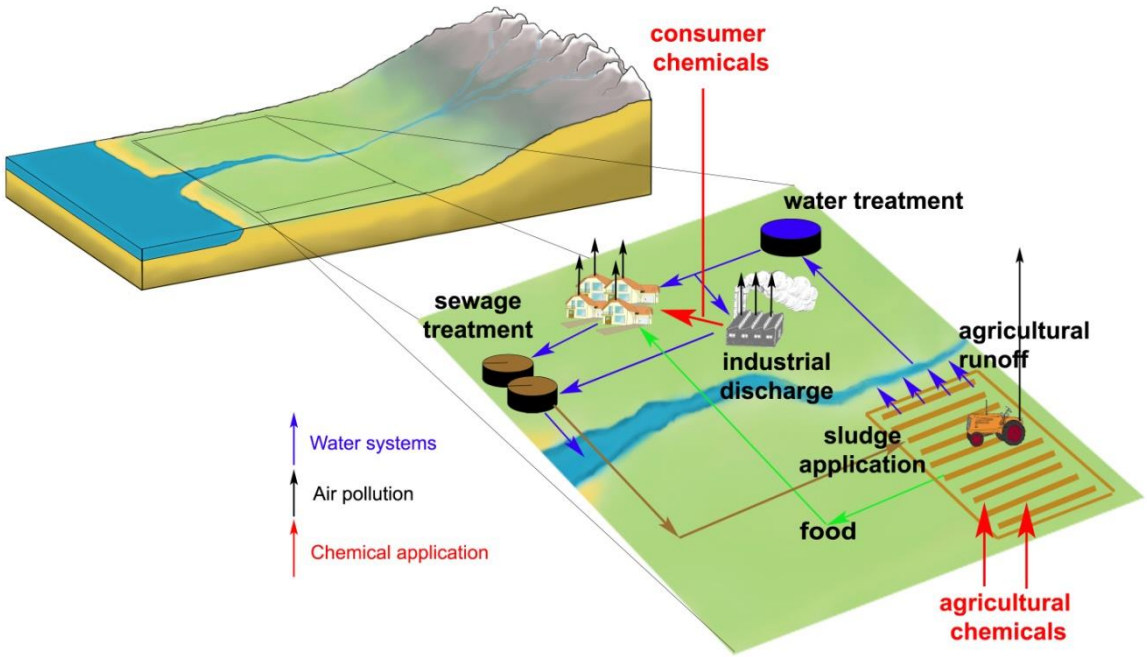


Figure 1-3 Pathways for entry of chemicals into the environment (Goodhead, 2009)

Household chemicals, pharmaceuticals and synthetic hormones enter the aquatic environment directly from wastewater treatment plants, many of which are not designed for their removal. Although disposal of compounds via the toilet, particularly for medicines, will contribute to environmental concentrations, the principal source of contamination is thought to come from compounds being excreted either as ingested or only partially broken down in the human body prior to excretion (Kümmerer, 2008; Lee *et al.*, 2011)

Veterinary pharmaceuticals enter the environment predominantly through animal waste or spillage from storage facilities. Agricultural compounds including pesticides typically enter the aquatic environment as runoff following land application.

1.1.3. *Occurrence of chemicals in the environment*

In recent years, concern has increased over the potential adverse human and ecological effects resulting from the manufacture, application and disposal of chemicals from industry, agriculture and medicines (Tyler *et al.*, 1998). The chemicals which have historically dominated research in the EU and USA since the 1970s encompass three broad groupings of chemicals, with respect to fate and effects: persistent organic pollutants (POPs); persistent bioaccumulative toxic (PBT) and bioaccumulative chemicals of concern (BCCs) (Ellis, 2006). Recent improvements in analytical techniques have led to the identification of an emerging group of compounds, typically present at trace concentrations but of considerable concern: namely the substantial number of pharmaceuticals and personal care products (PPCPs) including endocrine disrupting chemicals (EDCs).

It is widely agreed that, despite increased wastewater treatment and advancements in treatment processes, WWTP are not effective in removing PPCPs. Varying degrees of removal success have led to PPCPs being detected in the aquatic environment mostly at trace concentrations (Snyder *et al.*, 2003). The ubiquitous nature of these compounds now is such that it is becoming increasingly difficult to find unimpacted habitats, with pollutants having been found in areas as remote as the Arctic Circle, most likely as a result of chemical volatilisation and migration towards the poles (Kümmerer, 2010; Kallenborn *et al.*, 2013).

1.1.3.1. Environmental Monitoring

The past decade has seen an increased effort in measuring and regulating chemicals deemed to have tendencies towards PBT properties. This is in part due to advances in analytical techniques such as electron capture detection (Muir and Howard, 2006) and development of sampling techniques such as passive sampling (Mills *et al.*, 2011), but also because of regional monitoring programs including the Priority Pollutant list in the US, the Water Framework Directive within the EU and the Stockholm Convention administered by the United Nations Environment Program, along with nation specific legislation (Ellis, 2006; Tang, 2013).

The US EPA originally identified 129 Priority Pollutants for which water quality criteria were defined (Ellis, 2006); following the removal of three compounds there are now analytical test methods for 126 Priority Pollutants which are regulated (USEPA, 2013b). The Water Framework Directive, applicable within the EU, identified an initial list of 33 priority substances in 2000 to be subject to control measures over the subsequent 20 years, with an additional 15 priority substances recently recommended for addition as a revised (secondary) list (EC, 2013). Individual nations have also compiled additional monitoring lists, for example the UK, which developed a list of 92 chemicals expected to exceed PBT hazard criteria, in conjunction with the UK Chemicals Stakeholder Forum and the Environment Agency (Ellis, 2006).

The Stockholm Convention is a global treaty adopted in 2001 and enforced from 2004. It monitors levels of 22 POPs (12 from the original regulation and 10 recent additions) with a view to reducing, and ultimately eliminating, release of persistent, hazardous chemicals (Leslie *et al.*, 2013; Tang, 2013).

1.1.3.2. Occurrence of POPs, PBTs and BCCs

POPs were first detected in the environment in the 1960s (de Boer and Fiedler, 2013). In the 1970s many of these compounds were banned, or restricted, in industrialised countries including North America, Western Europe and Japan but are still used in Africa, South Asia, Central and South America (Kumar *et al.*, 2005). Many of these compounds are still present in the environment at significant concentrations, posing human health and environmental risks (discussed in more detail in Section 1.1.6.), despite many countries adhering to bans.

Indeed, many of these compounds, particularly organochlorine pesticides (OCPs), are still found in significant concentrations even in those countries adhering to bans e.g. North America, Western Europe and Japan (Muir and Howard, 2006; Kallenborn *et al.*, 2013). The OCP DDT is perhaps the most infamous POP, garnering notoriety through Rachel Carson's book *Silent Spring* (Carson, 2002). Despite a wealth of evidence detailing its environmental risk properties and prohibition in countries across the world certain countries, including Mexico until as recently as 1999, continued using DDT, accepting the environmental impact as an acceptable cost for eradicating disease carrying insects and protecting against illnesses such as malaria (Salazar-Garcia *et al.*, 2004). In developing areas, where malaria is a long-standing, debilitating problem, DDT is still in use, despite development of effective control measures without DDT use (Chanon *et al.*, 2003).

The persistent nature of these compounds allows them to remain in the environment for a considerable time. This stability aids long range transport in many cases, and when combined with the liberal application of the compounds following their release to market explains their ubiquity in the environment. Shen *et al.* (2004) determined the atmospheric distribution of OCPs using passive samplers, detecting DDT-related substances in 75-98% of passive air samplers based at 40 stations across North America. Weber *et al.* (2010) delivered a detailed review regarding the ubiquity of endosulfan including its occurrence in the Arctic. This ubiquity makes it difficult to avoid encountering POPs, with humans predominantly being exposed through diet. Arrebola *et al.* (2013) detected a range of POPs, including PCBs and DDT related compounds, in human adipose tissue, with frequency of detection 84-100% dependent upon the compound at concentrations ranging from 6.6 ng/g lipid to 161.5 ng/g lipid. There is a vast body of research into the impact of POPs on human and animal health and the environment (see Section 1.1.6.).

There are three main issues that hamper the accurate quantification of the true extent of pollution from persistent compounds:

1. environmental concentrations are generally decreasing due to agricultural bans and gradual degradation over the last 30 years, which may be difficult to detect due to limited analytical sensitivity (Fuoco *et al.*, 2009; Nizzetto *et al.*, 2010);
2. natural fluxes of contaminants are common with phenomena such as heavy rainfall, forest fires or glacial melts releasing compounds which are attached to solids or "stored" into lipids due to their hydrophobic, lipophilic nature (Blais *et al.*, 2001);
3. their tendency towards semi-volatility and persistence make them candidates for atmospheric long range transport and deposition globally, hampering efforts to identify pollution sources (Fernandéz and Grimalt, 2003; Weber *et al.*, 2010).

The aquatic environmental concentrations are almost certainly not indicative of the true extent of POPs in the environment and the risk posed to humans and aquatic organisms. It is generally agreed that persistence in the aquatic environment (Holm *et al.*, 2006), bioaccumulation and biomagnification (Wania, 2003) in the food chain (Arrebola *et al.*, 2013), long range transport (Shen *et al.*, 2004) and temporal fluxes in response to natural phenomena (Blais *et al.*, 2001) mean that these groups of compounds continue to pose a considerable threat to the environment, many years after production ceased in the majority of the world (Wania and Mackay, 1995; Loos *et al.*, 2009; Van Ael *et al.*, 2012; Koelmans *et al.*, 2013; Pawełczyk, 2013).

1.1.3.3. Occurrence of PPCPs

PPCPs were initially suggested as an ecological threat in the 1960s when Stumm-Zollinger and Fair (1965) predicted many of the issues we now face regarding an inverted age structure of an increasing population, chronic low-level chemical exposure and an expanding complex of synthetic chemicals. Despite research performed during the 1970s and 1980s it is only relatively recently that the warning has been heeded (Snyder *et al.*, 2003). A number of case studies detailing failures to take preventive measures where early warnings were issued are provided in two thorough European Environment Agency (EEA) reports *Late lessons from early warnings* (EEA, 2001; EEA, 2013). These reports principally relate to other pollutants but do include a number of PPCP case studies.

It is thought that a combination of their prevalent use and our inability to remove them during conventional water treatment means that pharmaceutically active compounds are now present in the majority of waters receiving wastewater treatment plant effluent (Desbrow *et al.*, 1998; Snyder *et al.*, 1999; Schwarzenbach *et al.*, 2006).

It is only relatively recently that they have been recognised as chemicals of concern primarily due to analytical limitations of detecting compounds at trace levels. Additionally, broad agreement on PPCPs as emerging pollutants has been delayed by the supposition that presence at concentrations well below those received, for example, by humans during medical treatment, means negligible detrimental human and environmental impacts (Kümmerer, 2010). In fact, the largest toxicological uncertainties surround the effects of chronic, low-dose, multi-generational exposure and simultaneous exposure to the cocktail of stressors present in the environment (Daughton, 2003). This perhaps therefore poses the greatest threat to humans and the environment.

1.1.4. *Environmental fate processes*

A combination of low volatility and the polar nature of most drugs lends them towards remaining in the aquatic compartment (Daughton and Ternes, 1999). Subsequent to entering the aquatic environment, chemicals may undergo any of a number of environmental fate and transport processes (Figure 1-4). The potential environmental implications of some of these mechanisms are discussed in more detail here.

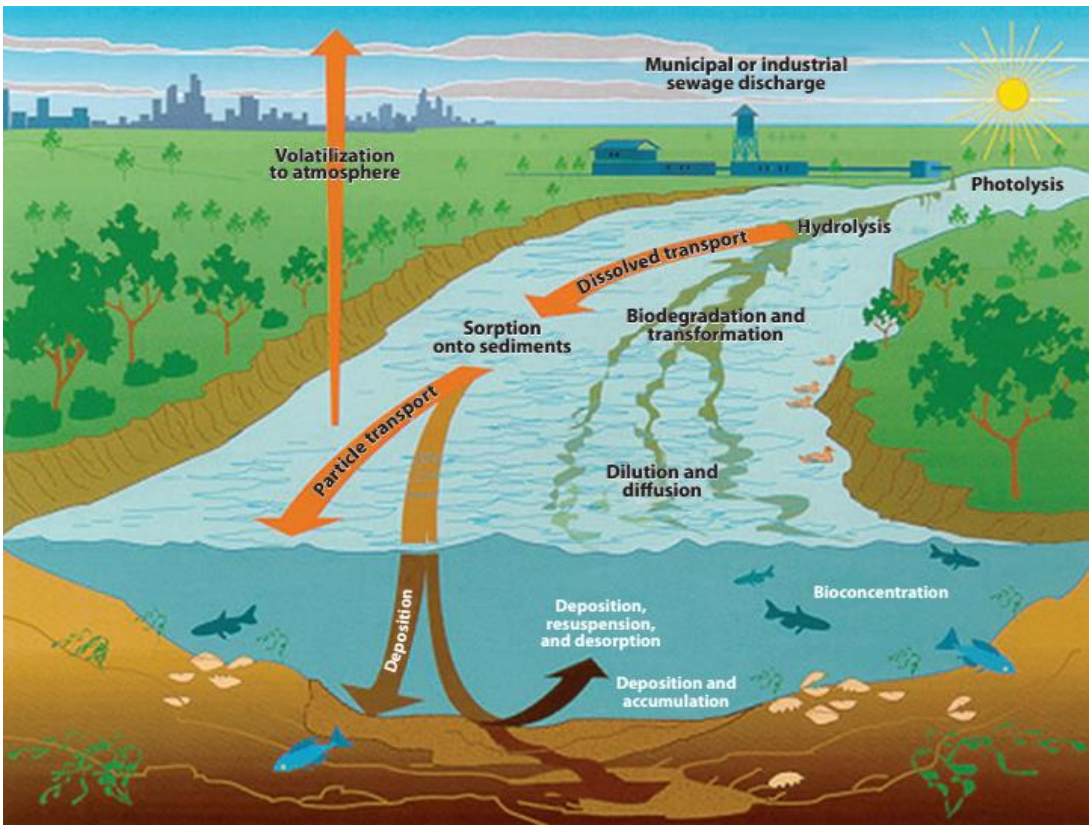


Figure 1-4 Transport and fate processes for chemicals entering the aquatic environment. Reproduced from USGS (2013).

1.1.4.1. Sorption

Compounds with a strong sorption capacity tend towards sedimentation. Their reduced bioavailability typically means that they pose a negligible direct risk to humans and animals in the environment. Sorption and deposition can, however, lead to accumulation in sediment which can impact upon fauna at the base of the food chain. There is also the possibility of sediment resuspension and desorption from the particulate, leading to flux in environmental concentrations, increasing the difficulty in mitigating risk.

This mechanism can also be exploited as a remediation tool. Materials with strong sorption characteristics, including activated carbon (Werner *et al.*, 2005) and biochar (Sopeña and Bending, 2013) are used to sorb organic pollutants, effectively reducing their bioavailability to organisms within the aquatic environment.

1.1.4.2. Volatilisation

The ubiquity of organic pollutants in the environment comes as a result of a multitude of factors. However, the specific occurrence of compounds in previously uninhabited areas at the poles can largely be attributed to one mechanism, cold condensation.

The cold condensation theory refers to the evaporation of POPs in warmer climates, their atmospheric transport toward the pole and subsequent condensation, deposition and accumulation in cooler, higher latitude environments. Colder atmospheres also slow degradation rates which results in increased persistence of pollutants in otherwise untouched areas (Wania and Mackay, 1996).

1.1.4.3. Bioaccumulation

Bioaccumulation (also referred to as bioconcentration) occurs as a result of the hydrophobic and lipophilic nature of some drugs. These compounds avoid the aqueous phase by partitioning strongly to solids in the environment and into lipids in organisms where they are stored in fatty tissues (Jones and de Voogt, 1999). As a reflection of their persistent nature, these compounds may be found within organisms at much higher concentrations than are found in the organism's environment. This is a particular issue for 'higher' organisms at the top of their respective food chains, where incremental accumulations of chemicals stored in their prey can build to potentially toxic levels, sometimes referred to as biomagnification (Mackay and Fraser, 2000; Arrebola *et al.*, 2013). Regulatory aspects of bioaccumulation are discussed in Section 1.1.5.2.

1.1.4.4. Biodegradation

Biodegradation can be considered as the microorganism induced reduction in chemical complexity (Alexander, 1999). Biodegradation is typically referred to as primary degradation or ultimate degradation. Primary degradation is the breakdown of complex chemicals into less complex transformation products which may still persist in the environment and pose a risk to human health and the environment (e.g. DDT primary degradation to the persistent DDE (Kelce *et al.*, 1995)). Ultimate biodegradation (or mineralisation) is the breakdown of complex organic chemicals and frequently, although not necessarily, the production of CO_2 or other inorganic products. This is the gold standard of chemical removal: completely removing the compound of interest without introducing any potentially harmful transformation products into the environment (Alexander, 1999).

Biodegradation is discussed in more detail in Section 1.2.

1.1.5. *Persistence Bioaccumulation Toxicity (PBT)*

Chemicals produced in certain volumes (Section 1.3) are subject to PBT assessments, which aim to identify persistent, bioaccumulative and toxic chemicals. For substances which exhibit significant persistence and bioaccumulation, even in the absence of toxicity data, the human and environmental risk posed by the unpredictable effects of long term exposure to the chemical is sufficient to warrant a higher risk assessment of vP/vB (very persistent/very bioaccumulative) (ECHA, 2008). The criteria by which PBT and vP/vB are judged have been the subject of debate, with the most comprehensive discussion taking place at a SETAC (Society for Environmental Toxicology and Chemistry) Pellston workshop to provide guidance and framework for identification of PBTs (Klečka and Muir, 2008).

1.1.5.1. Persistence and pseudo-persistence

Examining environmental fate processes, transport and contaminant release presents some interesting challenges surrounding persistence. Persistence in itself is difficult to quantify since it is not an intrinsic property of the chemical which can be measured. Rather a persistent compound is defined as having a constant and increasing presence in the environment, combined with a lack of observed degradation data in laboratory studies (Snape, 2010). This definition can be misleading due to a phenomenon termed “pseudo-persistence” (Daughton, 2003). A sufficient continual discharge of a compound will lead to that compound being detected in the environment, even with an easily degradable compound which has a short-half life. As the recalcitrance of the compound increases, and therefore the half-life, the appearance of a continued and increasing presence in the environment is detected (until half-life and discharge effectively negate each other leaving a constant concentration). However, if discharge was stopped, concentrations for all but the most recalcitrant compounds would decrease and eventually become negligible (Figure 1-5). The challenge for researchers and regulators is to design and adopt tests which recognise this phenomenon and reduce incorrect classification of compounds. Persistency is discussed in more detail in Section 1.2.

Persistency criteria defined by the European Chemicals Agency (ECHA) are presented in Figure 1-6.

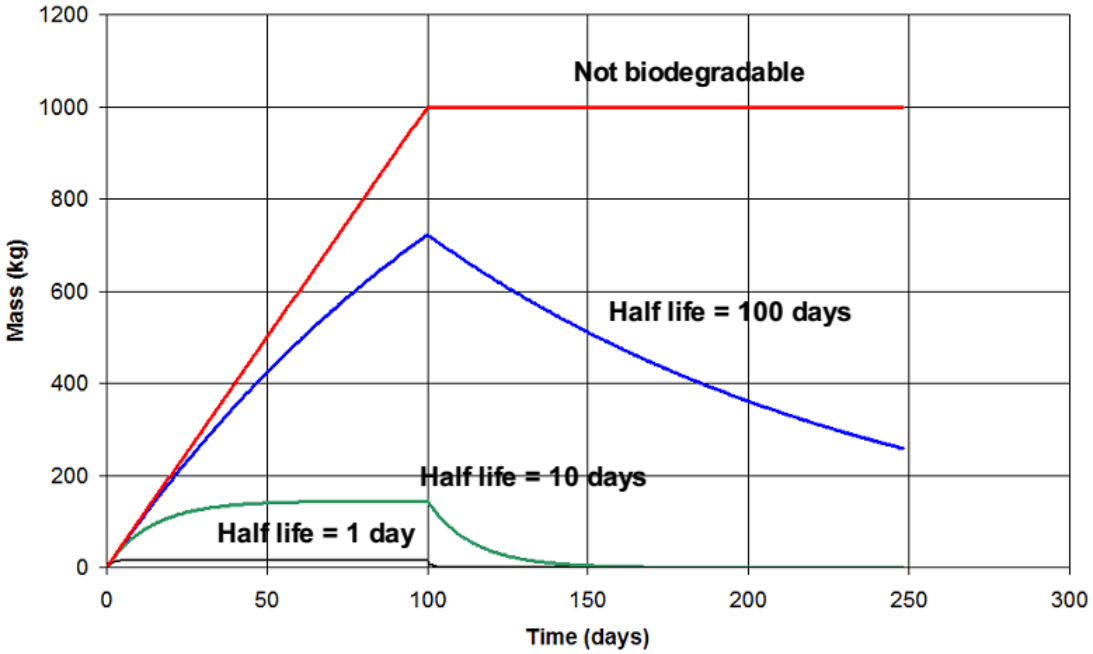


Figure 1-5 The phenomenon of pseudo-persistence highlighting the importance of correct classification of persistence for environmental risk assessment and risk management. Reproduced from (ECETOC, 2003).

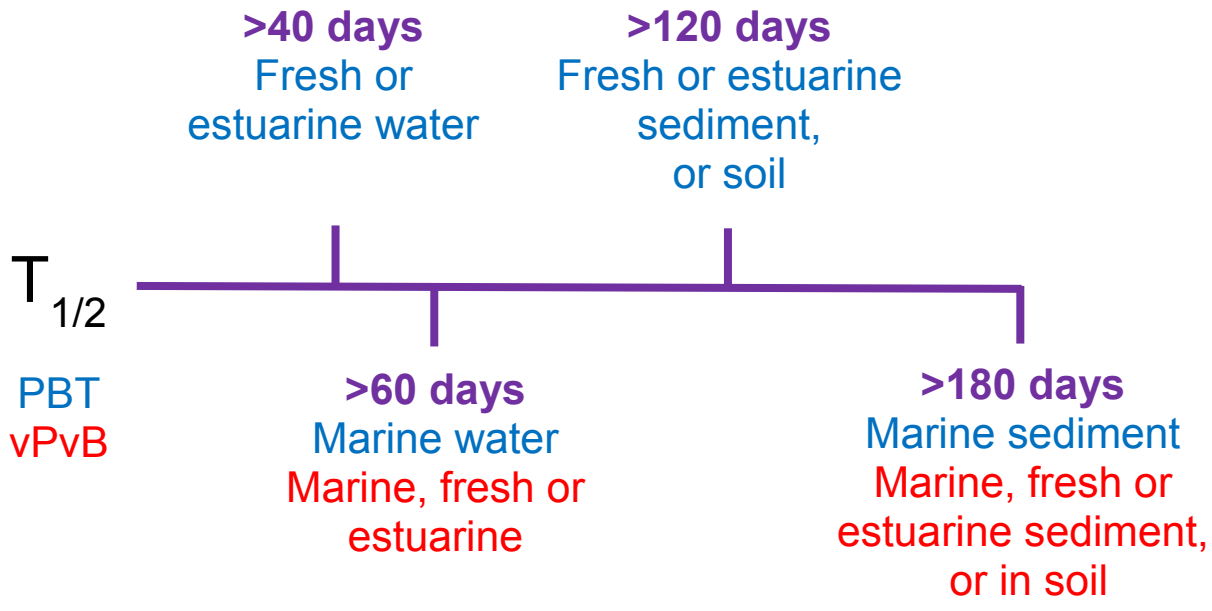


Figure 1-6 Persistency criteria defined by the ECHA to identify PBT and vP/vB chemicals (ECHA, 2008)

1.1.5.2. Bioaccumulation

Bioaccumulation is considered as being a process by which the chemical concentration within an individual organism (e.g. plant, fish, human) becomes larger than the organism's respiratory medium (e.g. water, air), diet or both (Gobas *et al.*, 2009). There are several terms associated with bioaccumulation: bioconcentration factor (BCF), the ratio of chemical concentration within a water-respiring organism to the chemical concentration in the water with exposure solely through the respiratory medium; bioaccumulation factor (BAF), the same ratio as BCF but considering exposure through both the respiratory medium and diet; biomagnification factor (BMF), the ratio of the chemical concentration in a water or air-respiring organism to the chemical concentration in the organism's diet (Gobas *et al.*, 2009).

Bioaccumulation assessments are made based on measured BCF values in aquatic species. Values of greater than 2000 L/kg identify a chemical as B and greater than 5000 L/kg as vB (ECHA, 2008).

1.1.5.3. Toxicity

Efforts are currently being made to streamline animal toxicity testing by applying the 3 R's of animal research: Replacement of animal studies or elimination of animal studies where possible; Reduction of the number of animals, either by obtaining the same information from fewer animals or more information from the same number of animals; Refinement of existing animal studies to ensure as little stress to the animals as possible (Schoeters, 2010).

A range of toxicity tests are available, based for example on skin irritation and sensitivity, mutagenicity, acute and systemic toxicity, carcinogenicity and reprotoxicity, with the type and number of tests based upon the volume of chemical produced per annum (Schoeters, 2010).

There are three principal terms applied when characterising risk through toxicity data: PEC (Predicted Environmental Concentration); NOEC (No Observed Effects Concentration) and PNEC (Predicted No Effect Concentration). PECs are calculated using predictive models typically based on a realistic worst case scenario of a number of factors including use/discharge of the chemical under consideration, removal rate of the chemical, population, volume of waste water and dilution of waste water with surface water. Predictive models have previously been shown to

produce comparable data to measured environmental concentrations but it is recommended that realistic worst case scenario inputs are used to favour overestimation of environmental concentration (Liebig *et al.*, 2006). The NOEC can be defined as the highest concentration of a chemical in a test with a mean response which does not differ significantly from the control test (Crane and Newman, 2000). NOECs can vary greatly depending upon the source of the data, principally the type of test applied. It is therefore standard practice to use the lowest NOEC value from a series to err on the side of caution. The PNEC is calculated from available data, dividing the experimentally determined NOEC by an assessment factor which is selected according to the strength of the available data (Duffus and Park, 1999).

Historically, the ratio of PEC: PNEC has been used to characterise risk, where a ratio greater than 1 (i.e. greater environmental concentrations than no effect concentrations) indicates the potential for harmful effects to occur. The ECHA currently define a PBT chemical as having a NOEC less than 0.01 mg L^{-1} for marine and freshwater organisms or possessing any of a number of carcinogenic, mutagenic, reprotoxic or chronic toxicity characteristics. As previously mentioned, there is no higher assessment of toxicity as chemicals possessing either P or B characteristics are automatically presumed to pose a significant risk to human health and the environment, even in the absence of toxicity data (ECHA, 2008).

1.1.6. *Human health and environmental implications*

There are several comprehensive reviews on the occurrence, fate and effects of chemicals in the environment (Colborn *et al.*, 1993; Halling-Sorensen *et al.*, 1998; Daughton and Ternes, 1999; Vos *et al.*, 2000; Jones *et al.*, 2004; Fent *et al.*, 2006; Kümmerer, 2008; Diamanti-Kandarakis *et al.*, 2009). There are four predominant areas of concern, identified either by recent research or by a lack of research concerning knowledge gaps: (i) the occurrence of endocrine-disrupting chemicals and hormones and their associated detrimental impact on reproduction; (ii) the occurrence of some chemotherapy drugs which may induce carcinogenic effects themselves; (iii) the prevalence of antibiotics and the development of antimicrobial resistant bacteria and (iv) the effects of long-term relatively low-level exposure to a mixture of chemicals, with potentially additive or synergistic interactions between chemicals.

Exposure to endocrine disrupting chemicals has been shown to give rise to a number of reproductive and developmental effects in exposed organisms; imposex in snails (Gibbs and Bryan, 1986), sexual disruption in fish (Jobling *et al.*, 1998), feminisation of male bird embryos (Fry and Toone, 1981) and egg shell thinning in birds following exposure to DDT and its metabolites (Ratcliffe, 1967; Wiemeyer *et al.*, 1993). Many of these effects have been reported in areas subject to man-made chemicals and were not seen prior to large scale chemical production and use (Colborn *et al.*, 1993).

Jobling *et al.* (1998) found intersexuality incidence ranging from 4-100% in riverine roach populations in the UK, with significantly increased incidence in rivers receiving sewage treatment plant effluent. Kidd *et al.* (2007) reported the collapse of a fathead minnow population in Canada in response to long-term low level exposure to the EDC, 17 α-ethinylestradiol (EE2). Adult males and juveniles exhibit increased production of the female lipoprotein, vitellogenin, causing their feminisation, as well as impaired testicular development in response to oestrogenics exposure (Harrison *et al.*, 1997).

A major mixed pesticide spill in Florida prompted investigations into alligator health in the 1980s. Population numbers fell and remain at just one tenth of those found in the late 1970s. Female alligators were found to have double the plasma 17β-estradiol double levels as found in a control group and exhibited abnormal ovarian structure. Male juvenile alligators were found to have significantly supressed testosterone concentrations, poorly organised testes and abnormally small phalli (Guillette *et al.*, 1994; Guillette *et al.*, 1996).

The human health impacts have been discussed in a thorough review authored by Diamanti-Kandarakis *et al.* (2009). Accumulations of persistent organics with the potential for endocrine disruption have been found in human adipose tissue (Arrebola *et al.*, 2013). Increased incidences of testicular, prostate and breast cancer have been observed which have been linked to exposure to EDCs (Harrison *et al.*, 1997). Over 20 years ago a review was conducted by Carlsen *et al.* (1992) detailing a reduction in male sperm count and seminal volume between 1938 and 1991, with a concurrent increase in genitourinary abnormalities including testicular cancer. Several years later, Van Waeleghem *et al.* (1996) reviewed Belgian male sperm

quality over a 19 year period. Although overall sperm count remained approximately the same, other sperm quality factors, including sperm morphology and total motility showed a significant decrease, indicating a general reduction in sperm quality. A decrease in sperm quality is generally considered a response to a number of factors, including for example obesity (Reis and Dias, 2012), the concept of increased prevalence of EDCs as a contributing factor is still relatively controversial. An investigation into reproductive effects observed in Mexican vector control workers exposed to high levels of DDT saw increased incidence of abnormalities with workers almost 4 times more likely to experience birth defects following the first DDT exposure (Salazar-Garcia *et al.*, 2004).

Neu (1992) described the emerging crisis regarding antibiotic resistance due to significant production, use and discharge of antibiotics into the environment. Exposure to considerable quantities of antibiotics has allowed bacteria to evolve and develop resistance to antimicrobial agents. A review of antibiotic use and antimicrobial resistance in Europe found higher rates of antimicrobial resistance in countries with higher antibiotic use (Goossens *et al.*, 2005). A trend for shifts away from narrow spectrum antibiotics to new broad-spectrum antibiotics was also reported, which may be indicative of a shotgun approach towards antibiotic use rather than focused application only where it is necessary. The repercussions of antimicrobial resistance are vast, highlighted by the development of meticillin-resistant *Staphylococcus aureus* (MRSA) which are responsible for a number of diseases in man and are resistant to the most commonly prescribed antibiotics (Grundmann *et al.*, 2006).

Assessing the impact of chronic exposures to low level chemical mixtures is, by its very definition, difficult and a focal point for toxicity researchers (SCHER *et al.*, 2012). It is not possible to simulate chronic low-level effects in short-term experiments which would allow us to make recommendations now. It has been stated that during a lifetime of 70 years consuming two litres of water per day, the low levels of chemicals present in the environment would still be less than a typical therapeutic dose (Kümmerer, 2010). This however, does not consider that: chronic and acute exposures typically induce very different responses; chemical mixtures will behave differently in the environment to single doses with potentially additive or synergistic

interactions and the potential effect on small children and foetal development during pregnancy (Kümmerer, 2010).

1.2. Biodegradation and risk assessment

1.2.1. *Biodegradation, biodegradability and tests for their assessment*

Biodegradation, biodegradability and the factors affecting their outcome in biodegradation tests are relatively poorly understood and receive little scientific attention. Typically biodegradation is viewed with respect to the intrinsic properties of the chemical and rarely from the perspective of the bacteria, namely the probability of encountering specific degraders of the test compound within the test inoculum.

Aquatic environments are subject to a host of chemical compounds, both natural and synthetic. The principal elimination process for the removal of these compounds is microbial degradation. The ability to predict biodegradation plays an important role in determining eventual environmental concentrations, exposure risks and ultimately the long term effects of chemicals.

1.2.2. *Assessing biodegradability*

In recent regulatory history Ready Biodegradability Tests (RBTs) have formed the core protocol for developing regulatory guidelines. This is most likely due to their ease of use, relatively straightforward interpretation and the sheer volume of data collated since their introduction in the 1970s (Aronson *et al.*, 2006). RBTs are stringent tests aimed to screen out compounds which undergo rapid mineralisation in all environments through routine use. Recently, however, regulatory concerns have developed at a faster rate than the international standards that govern them, with an increased emphasis placed on identifying compounds that show tendencies towards persistence, bioaccumulation and toxicity (PBT). In their current guise, RBTs are not effective as screens for persistence. They are notoriously highly variable, producing a large number of false negatives, whereby a chemical fails a test not necessarily because it is poorly biodegradable but rather because the test itself has failed (false negatives estimated to account for 20-80% of RBT fails (ECETOC, 2007)). This failure may occur for a number of reasons, only one of which relates to the inherent persistency of the compound in question (Figure 1-7).

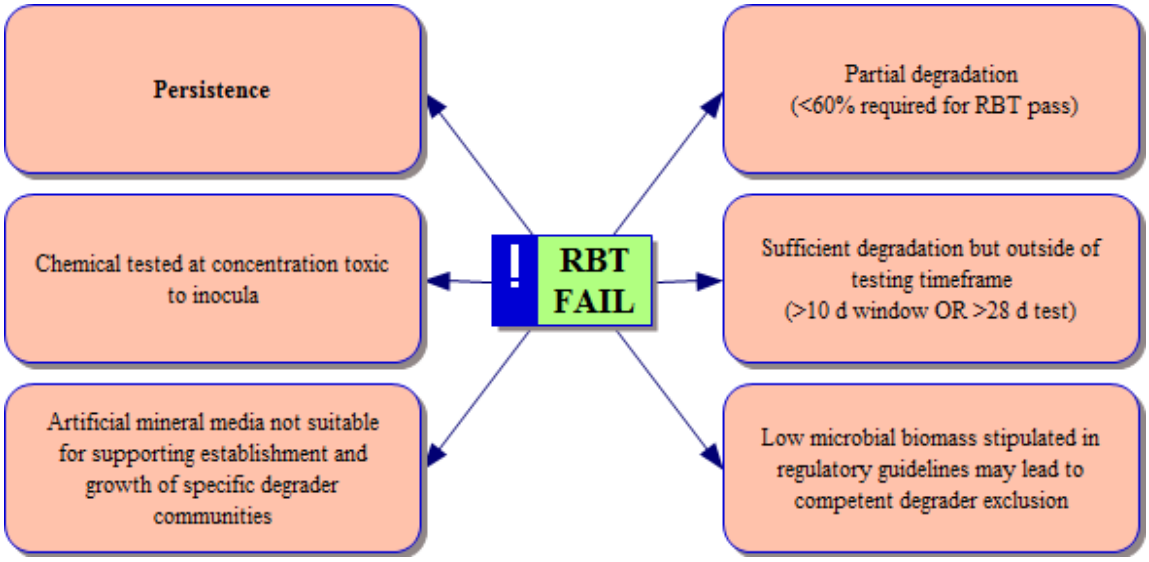
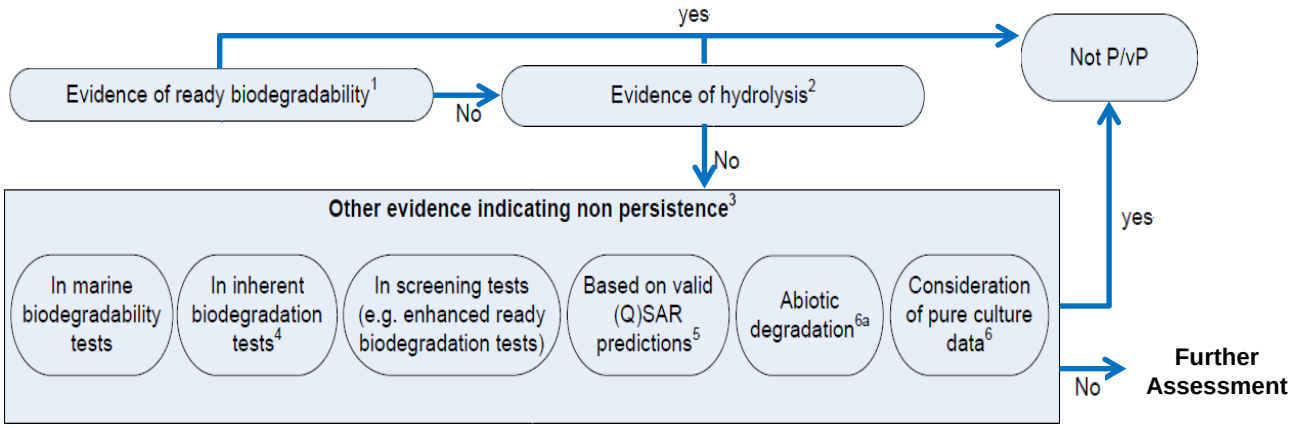


Figure 1-7 Possible reasons for RBT failure, only one of which being that the test compound is persistent. Approximately 20-80% of RBT fails are attributed to false nagatives (ECETOC, 2007), which may occur as a result of the factors above

The stringency within RBTs leaves no doubt as to ready biodegradability when a compound passes. The problem lies in the considerable expense of retesting and/or performing higher-level tests associated (Section 1.3.2) with the high number of RBT fails and the paucity of additional information obtained from RBTs with respect to persistence. Biodegradation and persistence workshops sponsored by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) have suggested that new methods prioritising chemicals based on persistence should focus more on biological criteria including increasing the total bacterial cell numbers and increasing the opportunity and time for the establishment of specific degrader communities (ECETOC, 2003; ECETOC, 2007). Proposed methods for increasing environmental relevance in the inoculum have since been published (EC, 2009) as part of REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), new legislation introduced in 2007 to streamline chemical regulation and risk assessment (Williams *et al.*, 2009; ECHA, 2013). REACH is discussed in more detail in Section 1.3.4.

In addition to the inoculum, there are other environmental realism issues which impact upon biodegradation assessments. There are a myriad of factors which can reduce the environmental realism of tests further, including pH (Lydy *et al.*, 1990),

light (Thomas and Hand, 2011) and temperature (Manzano *et al.*, 1999). These issues have been reviewed elsewhere (Kowalczyk *et al.*, 2013) and are not the focus of the present study, which considers the importance of the inoculum on degradation.



PBT Assessment, ECHA, 2008

Figure 1-8 The first steps in persistence assessment under REACH (adapted from ECHA (2008)). P/vP = persistent/very Persistent

1.2.3. *The Biodegradation Lottery*

In the course of performing degradation testing, there occurs a phenomenon termed the 'biodegradation lottery' (Goodhead *et al.*, 2008) whereby the probability of a positive outcome is dependent upon the chance inclusion of specific degraders of the test compound within the test inoculum. Proposals for enhanced biodegradation screening tests within REACH include increasing the total number of cells within tests, either by increasing the inoculum cell concentration or by increasing the test volume. Low cell numbers typified in small volume tests have been shown to result in high variability in biodegradation outcome (Nyholm *et al.*, 1984; Nyholm and Kristensen, 1992), potentially due to reduced inoculum diversity. Increasing cell number has been shown to correlate with an increase in diversity (Davenport *et al.*, 2010; Goodhead *et al.*, 2013). Increasing the diversity of the test inoculum should increase the likelihood of incorporating competent degraders of the test compound within the test, in turn decreasing the variability and increasing the reliability (or probability) of their detection. This would be observed as a positive outcome in a biodegradation test. An absence of degraders would still be observed as a negative outcome in a test, thereby protecting the stringency of the test.

Efforts to improve chances of 'winning' the biodegradation lottery may be seen as biasing the test towards biodegradation by providing an unrealistic cell number within test inocula, particularly when compared to the stringent, low microbial biomass stipulated for OECD screening tests (Table 1-2). Actually, the concentrations within OECD tests may be considered as unrealistically low, with the inoculum used in RBTs providing a worst case scenario with respect to concentration (Painter, 1995) approximately 1000-fold lower than the original samples (Goodhead *et al*., 2013). In using total cell numbers (e.g. 10^8 cells mL^{-1} in activated sludge (Goodhead *et al*., 2013), 10^4-10^6 cells mL^{-1} in surface water (Thouand *et al.*, 1995; Gilbert *et al.*, 2009)) more akin to those naturally found in the environment, the ultimate goal is to provide a more environmentally realistic inoculum for use in robust screening studies which allow better predictions to be made concerning the environmental fate of chemicals.

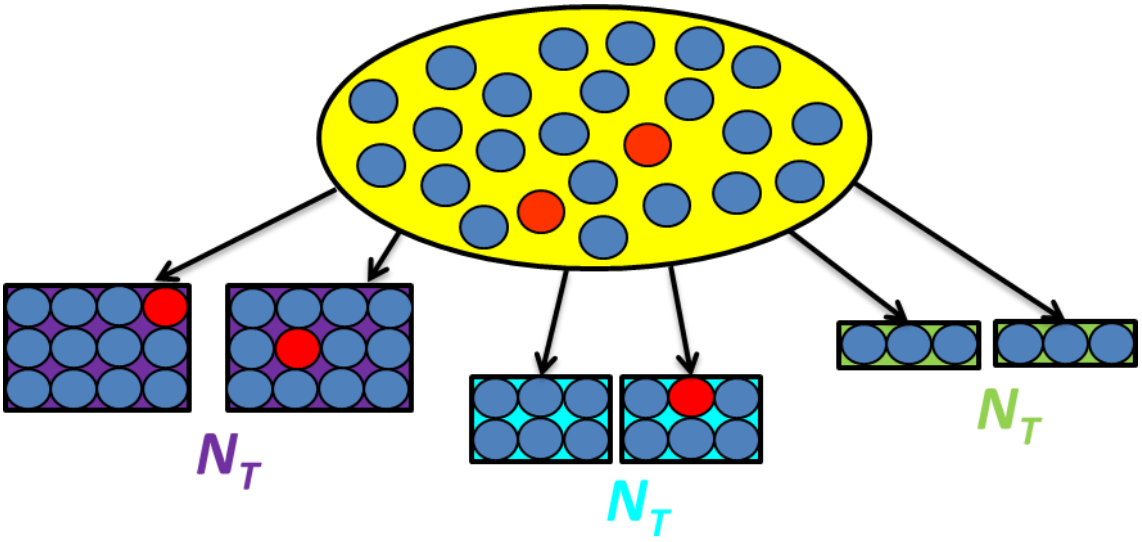


Figure 1-9 Biodegradation lottery where specific degraders are represented by red circles and N_T refers to the number of individuals in a sample. As N_T decreases, the likelihood of specific degrader inclusion also decreases. Smaller N_T values may lead to less representataive inoculum samples and correspond with increased variation in biodegradation outcome and kinetics

1.2.4. *Kinetics of biodegradation*

The kinetics of biodegradation in the aquatic environment have been discussed to a great extent, summarised in an excellent review by Battersby (1990). There is the potential to use kinetic data in predictive models which would allow determinations of persistence for prioritising chemicals for assessment, which would in turn allow more focused, and potentially, less laboratory testing.

First order kinetics refers to degradation being proportional to a single factor, typically the chemical concentration. Although this has commonly been applied to describe the degradation of organic chemicals in aquatic environments it is not strictly an accurate representation, in that there may be one or a number of different mechanisms occurring at the same time. Including the role that bacteria play in biodegradation leads to pseudo-first order, where a second-order reaction occurs but exhibits first-order rates, or second-order, where the rate of biodegradation is a function of both the concentration of the chemical and the microbial biomass (Battersby, 1990). The most commonly referred to measure within biodegradation kinetics is half-life (also referred to as t_{deg50}, t_{50} and $t_{1/2}$). If first-order rates apply and degradation rate is a constant then half-life may be calculated as described in Equation 1-1, where k refers to the rate of degradation:

Equation 1-1 Calculation of half-life in first order reactions, where *t* represents time and *k* represents the reaction rate constant

$$t_{\frac{1}{2}} = \frac{ln2}{k}$$

Blok and Booy (1984) used Monod growth kinetics to reflect the theory that microbial growth will occur with substrate utilisation, reaching a maximum at high concentrations. This can be considered as true for laboratory screening studies, where the test chemical acts as the sole carbon source, but may not be true in the actual environment where alternative energy sources are available (Battersby, 1990). Microbial growth (μ) will increase to a maximum growth rate (μ_{max}) where another parameter becomes a limiting factor towards cell growth. Monod type reactions are dependent upon microbial growth and substrate concentration, considering multi-enzyme systems. At low substrate concentrations, degradation progresses as a first order reaction; increasing substrate concentration results in second order rates as

microbial growth slows; finally becoming zero-order reactions, where the reaction rate remains constant with an increasing substrate concentration (Figure 1-10). Michaelis-Menten reactions appear to be similar to Monod reactions; however Michaelis-Menten reactions consider a single-enzyme as opposed to the multiple enzyme systems in Monod kinetics (Kovárová-Kovar and Egli, 1998). These terms are the most commonly used expressions regarding biodegradation kinetics. The selection of the appropriate model to describe degradation data depends upon several factors including: (i) whether a single substrate or mixture is applied, (ii) whether an isolated bacterium or mixed bacterial system is used and (iii) the presence of other limiting factors, such as chemical concentration, bacterial concentration or the absence of requisite nutrients for cell growth (Simkins and Alexander, 1984; Simkins and Alexander, 1985).

The nature and the quality of data generated by RBTs leads to limitations in their use to assess biodegradation kinetics. Some of these limitations are of the same nature as those associated with predictive modelling and Quantitative Structure Activity Relationships (QSARs; Section 1.3.2.7). These limitations include: (i) the data generated by RBTs is typically not sufficiently reliable or suitable to allow the successful determination of kinetics, (ii) the analysis methods used may not be sensitive enough, (iii) data may not be collected often enough to accurately calculate degradation kinetics and (iv) modelling kinetics typically uses a half-life measure, which is not a regulatory test endpoint, and is therefore often calculated using read-across methods. As with predictive modelling, more reliable data generated in GLP facilities from environmentally-relevant tests using sufficiently sensitive analytical methods is needed to produce more robust degradation kinetic data.

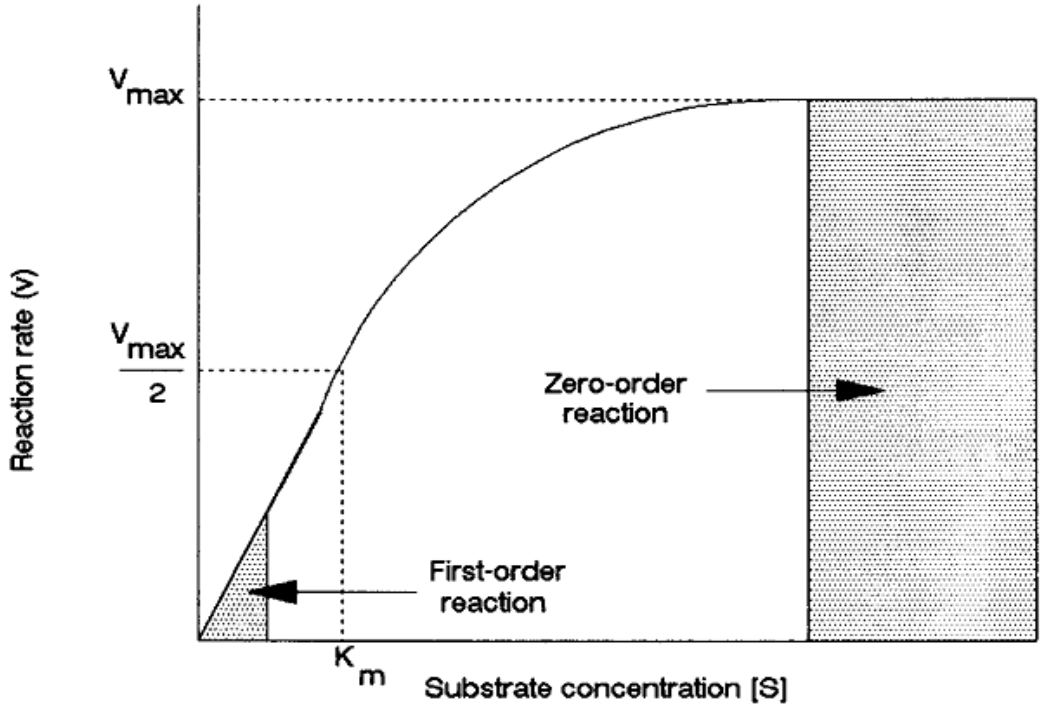


Figure 1-10 Monod growth kinetics suggested by Blok and Booy (1984) as representing the theory that microbial growth will occur with substrate utilisation until a maximum is reached at high substrate concentration

1.2.4.1. Degradation descriptors

There are several other measurable parameters which are not strictly kinetics of degradation but instead may be related to different forms of degradation. Nyholm *et al.* (1992) detailed several of these degradation descriptors, summarised in Table 1-1, with several additional measures based on OECD test guidelines and technical reviews (ECETOC, 2003; ECETOC, 2007; Comber and Holt, 2010; Snape, 2010).

These measures of degradation can be used to assess intra- and inter-test variability when assessing biodegradability. They allow for greater analysis between tests than simply a pass or fail. They are often related to half-life and may be used in place of half-life for a more descriptive assessment of degradation but are best used alongside actual measured half-lives.

Table 1-1 Degradation descriptors, in line with existing (OECD) and proposed (REACH) biodegradation/persistency assessments. Shaded descriptors have previously been described (e.g. Nyholm *et al.*, 1992). Non shaded descriptors have been created to facilitate the assessment of variation .

Expression	Unit of measurement	Description
t_L	Days	Lag phase (time taken to reach 10% degradation/removal of parent compound)
$T_{1/2}$	Days	Time taken to reach 50% degradation/removal
t_{60}	Days	Time taken to reach OECD $ThCO_2$ pass threshold
$\Delta\ t_{50}$	Days	Time difference from end of lag phase to t_{deg50}
$\Delta\ t_{60}$	Days	Time difference from end of lag phase to t_{deg60}
K_{max}	$\%^{14}CO_2$ evolution / day	Maximum degradation rate (calculated from steepest part of $^{14}CO_2$ evolution curve)

1.3. Chemical regulation and testing

1.3.1. *Global historical perspective*

Chemical regulatory assessment in its current guise developed in an ad hoc manner in separate regions from different, but simultaneous, seminal events. These events arose from the realisation that the unregulated manufacture, application and disposal of chemicals can result in long term human health and environmental risks and the desire to increase scientific knowledge of their impact.

1.3.1.1. United States of America

DDT (dichlorodiphenyltrichloroethane) was the compound of interest in *Silent Spring* (Carson, 2002), a critique of pesticide practices in the United States following on from the Second World War. Carson's publication detailing the link between excessive, unregulated DDT application and a decline in bird of prey populations, directly led to the President's Science Advisory Committee investigation into the risk posed to human health and the environment by pesticides. This ultimately led to the ban of DDT as a general pesticide within the United States, employing the precautionary principle, which became a universal theme in global chemical regulation. The precautionary principle gained prominence in the 1992 Rio Declaration (UNCED, 1992); it reasons that anticipatory action should be taken where there is a potential for harm to human health or the environment even when

there is not comprehensive scientific evidence of harm (Raffensperger and Tickner, 1999). *Silent Spring* (Carson, 2002) was arguably the springboard for the environmental movement throughout the 1960s and 1970s which included the founding of the United States Environmental Protection Agency (USEPA) and the conception of organisations such as Greenpeace. This was followed in 1976 by Toxic Substances Control Act (TSCA), which required producers and polluters to record and make data available to the USEPA about their chemical uses, including their potential risks (Raffensperger and Tickner, 1999). In 2007, the Chemical Assessment and Management Program (ChAMP) was implemented to develop screening level characterisation for High Production Volume (HPV) chemicals. This was replaced in 2009 with a comprehensive initiative to enhance the current chemicals management program and the development in 2012 of the Existing Chemicals Program Strategy to assess existing chemicals that have been prioritised for characterisation (EPA, 2013).

1.3.1.2. Japan

Chemical regulation in Japan arose in response to public concern over PCB (polychlorinated biphenyls) contamination (Ikeda *et al.*, 2001). The Chemical Substances Control Act, known as 'Kashinho', implemented in 1973 and amended in 1986, introduced a notification and prior assessment system for substances new to the market (Naiki, 2010). As government authorities, the Ministry of International Trade and Industry (MITI) and the Ministry of Health and Welfare (MHW) enforced the Act. The requirement for all substances to have undergone standardised biodegradation testing led to the development of the MITI(I) and MITI(II) biodegradation screening tests which were later adopted by the OECD as ready biodegradability and inherent biodegradability screening tests respectively (OECD, 1981b; OECD, 1992a; Ikeda *et al.*, 2001).

1.3.1.3. European Union

The beginning of the regulatory era within the EU can be traced back to the demise of the 'principle of microbial infallibility' (Painter, 1974). Ironically, more complex synthetic molecules produced in the 1950s under the concept of 'better living through chemistry' (Copley *et al.*, 2012) were found to produce unsightly foaming in conventional wastewater treatment plants, leading to the realisation that compounds were not being degraded during treatment. Prior to this, there was a commonly held

belief that given the opportunity and favourable conditions, any organic compound would biodegrade: the principle of microbial infallibility. The 1950s and 1960s heralded an era of biodegradability testing development developed predominantly around the aerobic biodegradability of synthetic detergents (Allred *et al.*, 1964; Bunch and Chambers, 1967). In 1967, the dangerous substances directive was introduced by the European Economic Community (EEC) for the classification, packaging and labelling of dangerous substances. Notification of new substances was introduced via an amendment in 1981. In 1996, the Integrated Pollution Prevention and Control (IPPC) directive was implemented, placing responsibility on pollution producers to control their wastes. Most recently, the EU has implemented the Registration, Evaluation, Authorisation and restriction of CHemicals (REACH) to streamline previous legislation and place an increased emphasis on intelligent approaches to persistency, bioaccumulation and toxicity (PBT) testing (EC, 2009). REACH was introduced to encourage the development of newer, safer chemicals as opposed to reliance on existing chemicals, which required no hazard information. Previous regulation was characterised by low throughput methods which led to negligible information on chemical safety. REACH shifted the onus from regulators to industry, proposing high throughput methods and a tiered assessment system based on production volume (Section 1.3.4.). Whereas the EU has previously taken their lead from the US with regard to chemical regulation, by adopting the precautionary principle amongst other concepts (Løkke, 2006), they now appear to be leading the way for other regions to follow, with the US and Japan looking to adopt ideas from REACH.

1.3.2. *OECD and laboratory testing*

The Organisation for Economic Co-operation and Development (OECD) is an inter-governmental organisation which has grown from 18 member countries at its inception in 1960 to 34 countries today from North and South America, Europe, Asia and the Pacific region (OECD, 2013b). It works as a collaborative effort between countries to respond to international problems, including the co-ordination and harmonisation of policies.

Their harmonisation of chemical regulation resulted in internationally accepted regulatory testing methods for a range of chemicals including PPCPs, pesticides and industrial chemicals. They are responsible for more than 100 testing guidelines

which are performed throughout laboratories worldwide in the fields of environmental fate and effects, ecotoxicology, and ultimately environmental risk assessment.

In 1980, the OECD introduced a series of internationally accepted guidelines for testing the biodegradability of chemicals, with several amendments issued in 1992 (OECD).

OECD guidelines distinguish between three types of biodegradation testing: ready biodegradability testing, inherent biodegradability testing, and lab-scale or simulation studies with increasing precision, complexity and resource requirement from the lower tier ready biodegradability tests (RBTs) to the higher tier simulations or environmental observations (Figure 1-11).

1.3.2.1. Role of biodegradation testing

Predicting the removal of compounds from the environment plays an important role in risk assessment. Bioaccumulation and chronic exposure to mixtures of pollutants poses a significant threat to human health and the environment (Section 1.1.6).

Data derived from biodegradation testing is presently being used in the development and validation of predictive models, which may ultimately be used to prioritise testing towards chemicals more likely to pose an environmental and human health risk.

1.3.2.2. *Overview of current tests*

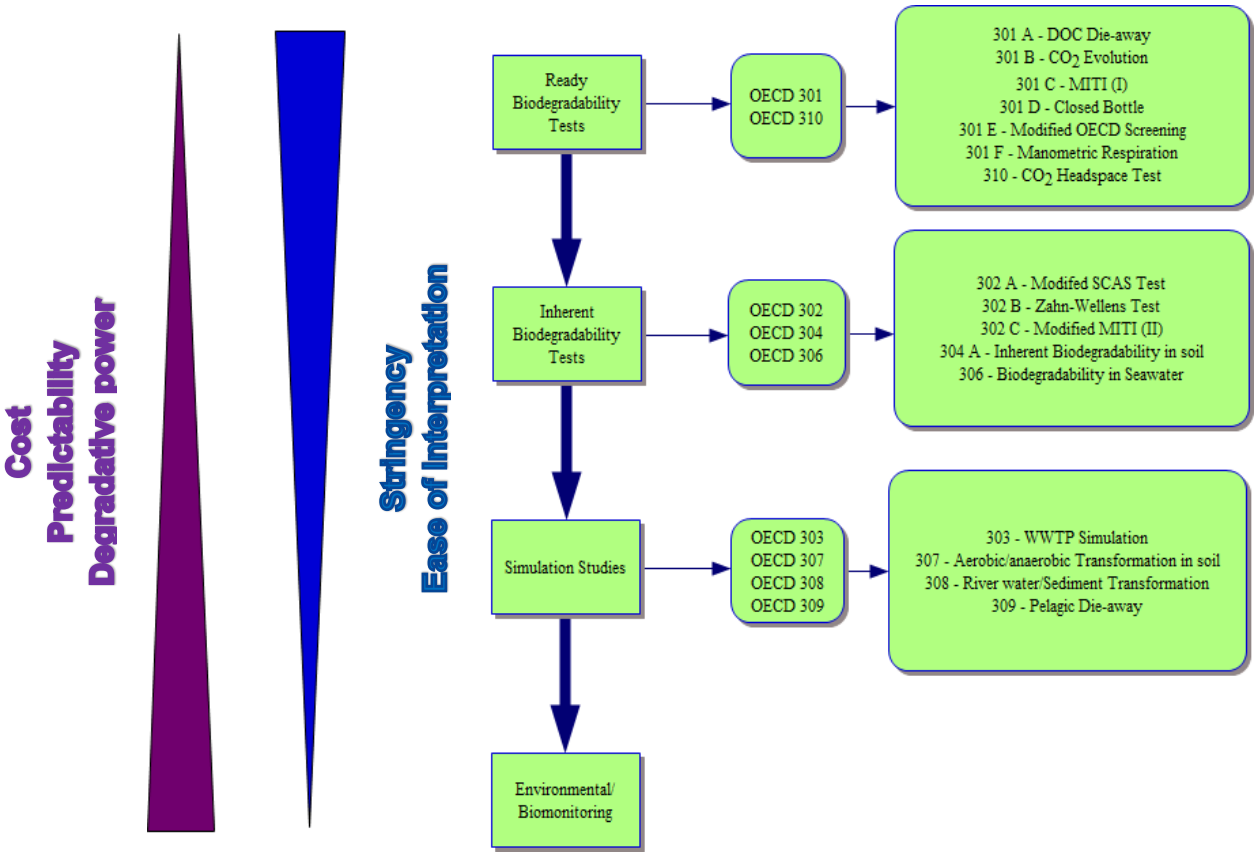


Figure 1-11 Overview of the standard biodegradability tests published by OECD, the testing hierarchy and the current testing paradigm; displaying characteristics of the hierarchy in terms of quality, relevance and resource requirements. Adapted from Comber and Holt (2010)

1.3.2.3. *Ready biodegradability tests (RBTs)*

RBTs are designed to screen out chemicals that undergo rapid mineralisation in all environments through routine use (Section 1.2). From the outset these tests were intended to be stringent, eschewing environmental realism for a worst case scenario assessment of chemical fate (Painter, 1995). Ready biodegradability assessment is covered predominantly by the OECD 301 series, comprising six tests termed 301 A through to 301 F, with the more recently introduced OECD 310 test. These tests share the same basic principle whereby a test chemical, environmental inoculum and mineral media are combined to assess the chemical's aerobic biodegradability. The differences between the tests lie predominantly in the biological criteria, namely the source and concentration of the environmental inoculum, in addition to the manner in which biodegradation is measured. A summary of the criteria used within the tests is given in (Table 1-2). Chemicals which pass these tests can be regarded as being

biodegradable and have no need for further testing. A fail in these tests is not necessarily an indication that the chemical is not biodegradable, as discussed in Section 1.2, but signifies the requirement for higher tier testing (OECD, 1992a).

RBTs are the first level of chemical degradability screening. A test chemical is applied as the sole carbon and energy source to an environmental inoculum, which has not knowingly been exposed or pre-adapted to the test chemical in question, at an artificially high ratio of chemical to microbial biomass. The disappearance, or otherwise, of the test compound is monitored over a period of 28 days. These tests are not designed to be environmentally relevant; instead they act as a worst case scenario such that any compound which passes a test can be definitively regarded as a readily biodegradable compound. The stringent nature of the tests naturally leads to a considerable number of false-negatives. To reflect this, an RBT failure is not seen as a definitive assessment that a compound is not biodegradable; rather that more testing is needed in order to make a fully informed decision.

Table 1-2 Summary of OECD RBT test parameters with respect to the test methods, OECD 301 A-F (OECD, 1992a). (***AS*** Activated Sludge; ***COD*** Chemical Oxygen Demand; ***DOC*** Dissolved Organic Carbon; ***SS*** Suspended Solids; ***$ThCO_2$*** Theoretical Carbon Dioxide Evolution; ***ThOD*** Theoretical Oxygen Demand)

Test Duration	Inoculum Source	Inoculum Concentration	Endpoint Analysis	Chemical Concentration
28 Days	Composite from ≥ 10 sites (e.g. sludge, surface soil, water from treatment plant, river, sea, lake)	≤ 30 mg SS L^{-1}; ≤ 100 mL effluent L^{-1}; 10^7-10^8 cells L^{-1}	DOC	10-40 mg DOC L^{-1}
	AS; secondary effluent; surface waters or soil	30 mg SS L^{-1}; 10^7-10^8 cells L^{-1}	$ThCO_2$	10-20 mg DOC L^{-1}
	Derived from secondary effluent of treatment plant/lab unit predominantly receiving domestic sewage	≤ 5 mL effluent L^{-1}; 10^4-10^6 cells L^{-1}	ThOD	100 mg chemical L^{-1}
	AS; sewage effluent; surface waters; soil or MIXTURE	0.5 mL effluent L^{-1}; 10^5 cells L^{-1}	COD	2-10 mg chemical L^{-1}
		4-30 mg SS L^{-1} or 10% v/v secondary effluent	CO_2	50-100 mg ThOD L^{-1}
				2-40 mg C L^{-1}

301 A
301 B
301 C
301 D
301 E
301 F
310

1.3.2.4. Inherent biodegradability tests (IBTs)

As with RBTs, there are a range of IBT guidelines available within the OECD, which share common themes. These screening tests are less stringent than RBTs, typically having a more relaxed testing duration and test chemical concentrations more favourable towards biodegradation. A more detailed summary is given in Figure 1-11 and 1.3.3.7. A pass within an IBT is a definitive assessment of biodegradability whereas a fail does not constitute a definitive assessment of non-biodegradability (persistence) but indicates the need for further testing.

1.3.2.5. Simulation tests

Simulation tests are the most environmentally relevant laboratory test currently offered within the OECD testing framework. These tests use more environmentally realistic inocula and test chemical concentrations, at ratios more likely to be found in the aquatic environment, with degradation observed over a prolonged period of time to obtain a full understanding of the chemical's behaviour within an environmental compartment. Beyond simulation tests are biomonitoring studies performed *in situ*. Simulation tests are definitive assessments whereby a pass identifies the chemical as biodegradable, and a fail is considered sufficient evidence for the test compound to be classified as not readily biodegradable. Modelling data may be used alongside simulation data but is not suitable as a stand-alone assessment.

1.3.2.6. The testing paradox

Degradative power and predictability within biodegradation tests come at a price. In an ideal world, simulation tests or environmental observation data would be available for all compounds, providing a true representation of how a given compound will behave following release into the environment, thereby allowing regulators to make fully informed decisions regarding their potential impact on human health and the environment. These tests, however, are the most costly, both with respect to resources and the required manpower for generating and analysing the considerable data garnered. As such they are only used when other tests have failed to provide a definitive assessment of the compounds behaviour. The sheer volume of chemicals awaiting assessment also necessitates less intensive, high throughput methods.

1.3.2.7. Predictive modelling

The reliable prediction of chemical behaviour using a model would allow prioritisation of chemicals that pose greatest risk to the environment and humans. Quantitative structure activity relationship (QSAR) models typically link predictor variables, predominantly physiochemical properties, to response variables, which may include biological activity of the compound (Arnot *et al.*, 2006; Pavan and Worth, 2006). Their use in ecotoxicology is particularly encouraged due to the potential for reducing animal testing (Hofer *et al.*, 2004; Benfenati, 2011).

The principal area of concern regarding the use of predictive modelling is the quality of existing data. It is widely agreed that a model is only as good as its input data, inextricably linking the model and experimental work. Where experimental data is available, it has not necessarily been obtained from a GLP (Good Laboratory Practice) facility (OECD, 1998). It therefore lacks the quality assurance guarantee enjoyed by GLP-generated data. According to eChemportal, a collaborative effort between the OECD and the ECHA to catalogue existing chemical data, less than 20% of screening test data has been obtained from GLP facilities and can be considered reliable (OECD, 2013a). The lack of regulatory data is combined with circumstances where data is either not reliably produced or is not replicated. A considerable amount of the experimental data is also obtained by read across methods since models typically input half-life data (or the time taken for 50% of the applied compound to be removed), a measure not presently used within biodegradation screening tests (OECD, 1992a).

At present, modelling results cannot be used as evidence of biodegradability but may be submitted alongside simulation test data. The focus, in the short term, should reside with improving biodegradation studies to provide better quality, more reliable input data so that more faith can be placed in modelling outputs.

1.3.3. *Critique of existing biodegradation screening tests*

Biodegradation tests should aim to mimic real environmental impacts and represent small-scale representations of real environments in order to provide a realistic test of the environmental fate of chemicals. Reliable extrapolation from the small scale systems to predict local and regional environmental impacts depends on the test systems truly being representative of the real environment both in terms of the

physico-chemical conditions and the nature of the microbial populations present. These criteria are currently not met within OECD testing guidelines, are difficult to achieve and may never be truly realised, particularly given the probability of adequately sampling relatively rare organisms (Curtis *et al.*, 2006).

As previously mentioned, there are other biodegradation screening test parameters (e.g. pH, light, temperature) whose environmental relevance has been questioned. The focus of the present study, however, was on the impact of the microbial inoculum, with parameters such as light, temperature and pH maintained within and between tests.

1.3.3.1. RBT parameters

Concerns have been raised about RBTs since their introduction, particularly with respect to the large variation observed between the different types of RBT (ECETOC, 1983) which has led to a number of reports, ring tests and reviews with a view to standardising tests where possible (Blok *et al.*, 1985) and elucidating the role and origins of important testing criteria.

RBTs have historically formed the central foundation for assessing biodegradation in regulatory frameworks. This is most likely due to their relative low cost, ease of interpretation and the sheer volume of data collated since their introduction (Aronson *et al.*, 2006). As previously discussed (Section 1.3.2.3), these stringent screening tests are notoriously hindered by high levels of variation including inter-replicate, inter-test, inter-facility and temporal variation (Nyholm *et al.*, 1984; Painter, 1995). In reality, the majority of chemicals fail to pass RBTs, leading to more intensive, expensive testing without imparting any real knowledge gains. This is because a fail is not indicative of poor biodegradability but is more representative of a failure within the test itself (Figure 1-7).

In contrast with some of the more rigid test variables such as test duration, the biological criteria suffer from a lack of adequate definition which can reasonably be presumed the main source of variability and responsible for the large number of false negatives encountered (Vázquez-Rodríguez *et al.*, 2007). Inoculum source, concentration and preparation are thought to be the main parameters influencing variability in RBTs. The reliability of the tests is compromised by additional factors,

typically due to their excessive stringency, including test duration, test volume and pass thresholds.

1.3.3.2. Inoculum source, concentration and preparation

A central assumption in all biodegradability tests is that a randomly selected sample from an environmental compartment will exhibit a diversity representative of the sampled compartment, providing the requisite range of microorganisms to assess the biodegradation potential of a particular compound. Despite a historical understanding of the important role microbes play (Sweeney and Foote, 1964), biodegradation outcome is presently dependent upon the potential of an arbitrary inoculum of a relatively low concentration mixed microbial community, to degrade a chemical under standardised conditions. Too rarely is biodegradation considered from the perspective of the bacteria, with little consideration given to the probability of encountering specific degraders of the test compound in the test inoculum.

OECD guidelines stipulate inocula may be sourced from: activated sludge, surface waters, soils, a mixture of the aforementioned, a 10 site composite sample, or derived from secondary effluent at varying concentrations. The maximum concentration of these inocula is fixed at 30 mg Suspended Solids (SS) L^{-1} or 10^5 cells mL^{-1}, this is considerably lower than might be expected in natural environments (3000 mg SS L^{-1} and 10^{10} cells mL^{-1} are common (Andreottola *et al.*, 2002; Goodhead, 2009)), and varies by four orders of magnitude between tests. In their current form, screening tests can be considered as much a test of the inoculum as a test of the chemical biodegradability.

Pre-treatment or preadaptation of the inocula is not permitted under OECD guidelines. Inocula should not have been knowingly exposed to the test compound prior to the test. Preconditioning, however, is permitted, including filtration, centrifugation, settling and decantation and aeration. These treatments have been shown to normalise observed degradation potential characteristics but reduce the efficacy of the inocula to degrade even readily biodegradable test compounds (Vázquez-Rodríguez *et al.*, 2007). This is probably due to the removal of selected members of the microbial communities themselves, reducing overall diversity and leading to the exclusion of relatively rare specific degrader organisms (Goodhead *et al.*, 2013).

Biodegradability and persistence are often thought of as intrinsic properties of a chemical. In fact, they are complex properties influenced by a number of factors, of which biodegradation potential is only one, albeit the most important and least understood. Understanding the relationship between microbial diversity and biodegradation outcome, the occurrence and abundance of competent degraders and the variation between inocula will ultimately facilitate the design of more robust screening studies and enable better predictions on biodegradation.

1.3.3.3. Test volume

When biodegradation does occur, it is generally presumed that the bacteria responsible are minority organisms with a minimum abundance of one in the initial inoculum. In using low concentration inocula and relatively low test volumes, the probability of encountering rare organisms, even in replicate samples, is reduced and their inclusion is a matter of chance (Thouand *et al.*, 1995; Ingerslev and Nyholm, 2000). RBTs are performed typically at low volumes, with some variation between tests, ensuring variations in diversity between tests and ultimately affecting the reproducibility of biodegradation outcome, within and between testing facility. This phenomenon refers to the decreasing probability of encountering specific degraders of the test compound within the test inocula, with decreasing test volume or inocula concentration (Nyholm *et al.*, 1992).

1.3.3.4. Test duration

RBTs proceed for 28 days, with a “readily biodegradable” compound also adhering to the requirement of sufficient degradation within a ten day window. Even in early development, there were concerns over the robustness of RBTs with respect to the arbitrary test duration assignments (Painter, 1995).The test period was adopted by the OECD to encourage adaptation of the bacteria to the test compound. There does not appear to be any evidence that presence of compounds for greater than 28 days lead to harmful exposures. The 28 day test duration has been utilised since biodegradability testing in the 1960s and it is possible that this arbitrary time period has simply been retained because it has been used previously thereby accumulating a plethora of data testing at this time point. One of the earliest references to a 28 day test suggests the time period simply because it would negate the need for weekend work (Bunch and Chambers, 1967). An argument for maintaining the test duration, as opposed to extending beyond 28 days, lies in the suggestion that beyond 28 days,

interference, alluding to reductions in analytical sensitivity, renders the data unreliable for some analytical methods (Painter, 1995). There does not appear to be any evidence suggesting that a compound which degrades, for example, in 60 days poses any more of an exposure risk than a compound which degrades within a 28 day period. To date, there is no evidence to support the idea that a compound which undergoes complete mineralisation in a longer test is more likely to present a persistent threat to the environment than a compound which fulfils current RBT pass criteria. However, as previously mentioned, the human health and environmental impacts associated with prolonged exposure to PPCPs are not clear (Section 1.1.6).

The “10 day window” opens at 10% degradation, thought to indicate the cessation of the lag phase. Painter (1995) suggested the window may exist in reference to the 7-10 days typically required for primary standard degradation in the original OECD guidelines for assessing biodegradability of anionic surfactants. Following its introduction, little research was conducted regarding ten day windows and few studies utilised them during their analysis at the time, making it difficult to assess their necessity. The decision to preserve the ten day window is thought to be predominantly based on a ring study, the data of which is questioned by the two largest contributors, where a greater proportion of test passes was observed without a ten day window than when a post lag phase window was incorporated (52% and 47% respectively (Painter, 1995)). Whilst the requirement for stringency, particularly in primary screening studies, is understandable, the inclusion of a test parameter simply because non-inclusion would lead to a greater number of passes, with no other apparent underlying scientific explanation, is hard to justify.

Ultimately, the arbitrary test durations seem to lend themselves poorly to environmental realism, particularly with respect to the ten day window. It is counter-intuitive that a compound may potentially degrade within 11 days but necessitate further, costly assessments of biodegradability, whereas a compound requiring 28 days to achieve sufficient mineralisation but fulfilling the ten day window, may be classed as readily biodegradable.

1.3.3.5. Pass threshold

A compound may be classed as readily biodegradable if one of the following requirements is fulfilled: a 60% reduction in ThOD (theoretical oxygen demand) or $ThCO_2$ (theoretical CO_2), or a 70% reduction in DOC. A ring test performed shortly after the initial introduction of the 301 series (Painter and King, 1985) suggested that whilst the high DOC removal rate was justified, there was evidence to support a reduction in ThOD and $ThCO_2$ to 50% to reflect that a proportion of the test compound carbon will be utilised in new cell synthesis. The study found in some cases that a ThOD of less than 60% correlated with a DOC removal of greater than 90% (Painter and King, 1985). The ring test led to some modifications which were implemented in the modified OECD guidelines (OECD, 1992a) but the initial pass thresholds were maintained, presumably to maintain the stringent integrity of the tests (Weytjens *et al.*, 1994).

The selected values are arbitrary, but may be suitable for relative biodegradability classification purposes regarding ready biodegradability and inherent biodegradability. Arguably the more important value is the rate at which 50% degradation is reached. Recently, environmental concerns have developed at a faster rate than these international standards, with a regulatory shift from screening out chemicals in routine use which undergo rapid mineralisation in all environments, to identifying chemicals which persist in the environment, a quality typically assigned based on biodegradation half-lives. Although screening tests are not effective as screens for persistence and were not originally designed to assign half-lives for chemicals, they are increasingly being used to do so in light of this shift (Aronson *et al.*, 2006).

A 50% degradation rate value holds importance not only in evaluating persistence, but also for modelling purposes. Presently, predictive models calculate half-life values based on degradation data and read across methods. Utilising a 50% degradation end point would remove the need for data manipulation, facilitating the predictive modelling framework.

1.3.3.6. Endpoint analysis

The OECD 301 series incorporates four different endpoint analyses: DOC removal, CO_2 evolution, BOD and COD. Typically DOC, CO_2 and BOD are utilised for degradation analysis, however, in situations where it is not possible to measure BOD or calculate ThOD from BOD measurements, it is acceptable to use COD values as a measure of degradation. COD values are notoriously prone to high degradation measures if the test substance is incompletely oxidised during the COD test, a flaw recognised in the OECD guidelines.

Even if COD is disregarded, it has been shown that there is poor correlation between DOC removal and ThOD, as reported by Painter and King (1985), making it difficult to draw direct comparisons between tests.

1.3.3.7. Inherent biodegradability test concerns

The predominant focus of this critique, and of proposed enhancements to existing studies, is on screening tests and particularly RBTs. Notwithstanding the reduced complexity of RBTs in comparison with inherent biodegradability studies and simulation tests, and their ease of interpretation; RBTs are also the test which most testing facilities will ultimately fall back on. The majority of experimental data available for ‘tested’ chemicals is in the form of RBT assessments. According to eChem portal (OECD, 2013a), more than four times as many RBTs have been performed as inherent tests when assessing chemical biodegradability (4604 and 1013 respectively).

IBTs are less stringent than RBTs and overall give more opportunity towards biodegradation. They are designed to select for chemicals which, given the opportunity, show an inherent potential for degradation. Despite this difference, many of the drawbacks associated with RBTs are still applicable to inherent tests, particularly with respect to the inoculum and the test duration.

The inoculum is still subject to preparation methods which are thought to reduce diversity. Inocula concentrations remain variable between tests with no characterisation of the inoculum or concentration in the majority of inherent tests. The positive aspect with regards to the inoculum in the 302 A guideline is the increased environmental relevance gained in some tests by using inocula directly from source, rather than using unrealistically low microbial concentrations in diluted

inocula or artificially amended systems. The ratio of chemical compound to biomass is also more environmentally realistic.

The 302 B Zahn-Wellens inherent test is subject to the arbitrarily assigned 28 day test duration. However, the 302 A and 302 C are given varying test durations dependent upon experimental observations, which are more reflective, and representative of behaviour in the environment.

There also remain concerns, for IBTs and RBTs alike, over issues such as the varying manner of endpoint analysis and the suitability of the artificial mineral media for supporting the establishment and growth of competent degrader communities.

1.3.4. *REACH*

1.3.4.1. Regulatory drivers for REACH

The growth of the chemical industry led to a proliferation in the number of chemical compounds available for purchase. At present, there are estimated to be more than 100, 000 commercially active substances in the EU, with similar numbers registered in the US and Japan (Muir and Howard, 2006). The existing legislation within the EU, prior to the introduction of REACH, placed a burden of proof upon producers and importers of chemicals, to provide evidence detailing the ecotoxicological and environmental fate effects of their chemicals. This legislation, however, only applied to 'new' chemicals, defined as those introduced to market after 1981, approximately 3,000 or less than 3% of all commercially available chemicals. This legislation ultimately negatively impacted the chemical industry in two interconnected ways. Firstly, the emphasis was drawn away from research and development of novel compounds due to the legislative difficulties associated with new compounds. This in turn, led to the associated behaviour of companies falling back on compounds produced before the 'new' compound watershed, which had little or no toxicological and environmental fate data. The legislation effectively encouraged the use of compounds with unknown environmental effects and discouraged the development of novel compounds with both more desirable environmental qualities and more targeted towards the required purpose.

REACH imposes a burden of proof upon producers and importers of all chemicals for quantities greater than 100 tonnes per annum. This places the same data

requirements upon old and new compounds if they are to be used in volumes deemed to be liable to affect the environment.

1.3.5. *REACH higher tier testing*

REACH legislation aims to enable better prioritisation of persistence; to this end several modifications and enhancements to existing screening studies have been identified.

1.3.5.1. Modified biodegradation screening tests

Modified biodegradation screening tests are intended to deal with difficult substances for example by utilising surfactants with compounds exhibiting poor water solubility. Compounds which pass a modified biodegradation screening test may still be classified as readily biodegradable, dependent upon their adherence to the other RBT requirements of pass threshold and degradation period.

1.3.5.2. Enhanced biodegradation screening tests

Several enhancements have been suggested under REACH, in order to develop an enhanced tier test as a compromise between the stringent RBTs and the more expensive higher tier tests. The enhancements are designed to circumvent some of the commonly agreed reasons for failure (Figure 1-7).They include:

i. Increased test duration beyond 28 days
ii. Semi-continuous test systems with environmental samples
iii. Increased vessel size or inoculum concentration derived from environmental samples not previously exposed
iv. Running two ready tests in series

A pass in an enhanced screening test is not evidence of ready biodegradability, even if all RBT pass criteria are met, due to the reduced stringency associated with the enhancements. It is, however, an indication of ultimate biodegradability and would negate the need for further testing.

1.4. High throughput biodegradation assays

The requirement within REACH for industry to provide risk data for all new and existing chemicals produced in volumes greater than 100 tonnes per annum has created a requirement for rapid, reliable, high throughput methods. These methods need to be capable of rapidly and reliably characterising chemicals based on risk and identifying those chemicals which are likely to persist in the environment and pose a threat to human health and the environment. There has recently been a call for these types of high throughput methods in ecotoxicology to reduce the number of animal tests required for the considerable number of chemicals awaiting assessment under REACH (Gray and Cohen, 2012). An analogous rationale can be used for environmental fate assessments, which would contribute towards more environmentally friendly drug development and testing.

1.4.1. *Green drug design*

Green chemistry considers the development of products and processes that reduce or eliminate the generation and application of hazardous wastes (USEPA, 2013a). The principles of green chemistry can be applied to drug development during both the design and the assessment stages. High throughput, rapid, reliable characterisation of the risks posed by a chemical to human health and the environment can reduce potential impact on the environment by identifying chemicals which are liable to persist earlier in their development process, minimising their continued exposure to the environment. More reliable screening level tests will also reduce the need for further, more intensive testing.

1.4.2. *Colorimetric endpoint analysis*

The use of colorimetric endpoints has previously been used to observe degradation in high throughput tests (Thouand *et al.*, 1995; Goodhead *et al.*, 2013). Goodhead (2009) developed a novel colorimetric assay based on an azo-coupling reaction used in 96-well plate high throughput degradation assays, with colour intensity linked to chemical concentration, allowing for the rapid assessment of biodegradation outcome of a range of phenolic compounds. Thouand *et al.* (1995) and Goodhead (2009) have previously measured 4-nitrophenol degradation based on the intensity, or absence, of the chemical's natural yellow colour. These simple colour change endpoints can be used as a rapid tool for assessing chemical degradation.

1.5. The molecular revolution

It is estimated that the earth comprises in excess of 10^{30} prokaryotes (Whitman *et al.*, 1998) with prokaryotic diversity the subject of great discussion. Although it is beyond our current capabilities to accurately define, global diversity (Curtis *et al.*, 2006) estimates range from 10, 000 (Finlay and Clarke, 1999) microbes at the moderate end to 10 million (Curtis *et al.*, 2002) at the high end of speculation, depending on your views regarding the extent of microbial diversity. Until the end of the twentieth century, microbial ecology was dominated by culture dependent techniques which are now known to be suitable for only a small proportion (0.1-10%) of bacteria (Head *et al.*, 1998). The work of scientists including Woese (1987) towards identifying the 99% of bacteria which are not capable of being cultivated has helped shape the discipline of microbial ecology as it is known today (Muyzer, 2000). Although microbes have been observed since the 17th century (Hooke, 1665) and ecological oriented research has been performed since their discovery, the discipline only became an area of specialisation in the 1970s. It is not merely a coincidence that this was also the era in which the scientific community became widely aware of the environmental concerns associated with the chemical industry in all its guises. It is rather a reflection of the inextricable link between the microbial world, human health and the environment (Atlas and Bartha, 1993).

1.5.1. *16S ribosomal RNA*

The 1990s saw the emergence of a new area of specialisation, termed ‘molecular microbial ecology’ with work on small subunit ribosomal RNA (SSU rRNA) by Woese (1987). Previously microbiology had focused on pure culture work, spearheaded by influential scientists including Louis Pasteur and Robert Koch. As environmental issues developed it became clear that the answers did not lie in pure culture work and advancements beyond culture dependent techniques were required to provide solutions. The ubiquitous nature of SSU rRNA allowed scientists to begin inferring relationships between organisms based on sequence data. Essentially, ‘universal’ domains within the 16S region are conserved across all phylogenetic domains. By carefully aligning these conserved regions for two sequences, base substitutions in variable regions can be attributed to evolutionary change (Head *et al.*, 1998). In theory any gene may be used, but the ubiquity of the 16S gene and its combination of highly conserved and variable regions make it ideal for comparative analysis.

Additionally, it is considered as evolutionarily neutral and is associated with an essential function in all living cells.

Identifying a target gene for sequence analysis was only the starting point for the molecular evolution. The development of tools to exploit this advancement in knowledge was then required.

1.5.2. *Nucleic acid extraction and purification*

The first step in molecular work is extracting nucleic acids from the environmental sample of interest. The first protocol was published in 1980 (Torsvik, 1980) building on work by Marmur (1961) who used a combination of chemical lysis and temperature to attempt to isolate DNA from microorganisms. The development of nucleic acid extraction techniques removes the reliance on culture dependent methods, in theory providing a more representative view of the microbial community (Milling *et al.*, 2005). Methods developed over the years have typically used chemical lysis, physical lysis, heat or any combination (Zhou *et al.*, 1996), with the chosen method depending on the source of the environmental sample and the expected quality of the DNA. Presently, whilst more labour intensive methods are still used, extraction kits are typically used with reagents, specialised tubes and detailed protocols provided, for example the FastDNA SPIN kit for soil (MP Biomedicals, Cambridge, UK) which combines physical and chemical lysis to yield DNA for use in molecular analyses. These kits typically incorporate purification steps which remove impurities, such as humic material, which hinder downstream processes (Tsai and Olson, 1992).

1.5.3. *Polymerase Chain Reaction (PCR)*

The real benefits of extracting pure DNA products relatively free of the contamination seen in earlier attempts (Torsvik, 1980) was not seen until almost a decade later when molecular tools including PCR became more widely available. PCR provides a rapid, simple method for the amplification of specific nucleic acids (Mullis *et al.*, 1986), only requiring small amounts of DNA, as long as they are of a sufficient quality to prevent amplification of non-target DNA. PCR is the primary step in culture independent techniques such as DGGE and sequencing, typically targeting the 16S rRNA gene, which contains highly conserved regions between bacteria, in addition to variable regions allowing for distinctions to be made between taxa (Head *et al.*, 1998).

PCR involves three stages: denaturation, annealing and extension. DNA is subjected to temperatures of 93-95ºC for several minutes to denature the hydrogen bonds maintaining the DNA double-helix structure, yielding two strands of complementary sequence. The temperature is then lowered to an experimentally determined optimum temperature (typically 40-72ºC) typically for one minute, to allow specific primers to anneal to the target sites. New DNA strands are synthesised between the specific primers. During the extension or elongation step, the temperature is increased to be optimal for the enzyme DNA polymerase (typically 72ºC); this results in elongation of the DNA fragments which are annealed to the primers. Successive cycles result in an exponential increase in fragments identical to the original template DNA.

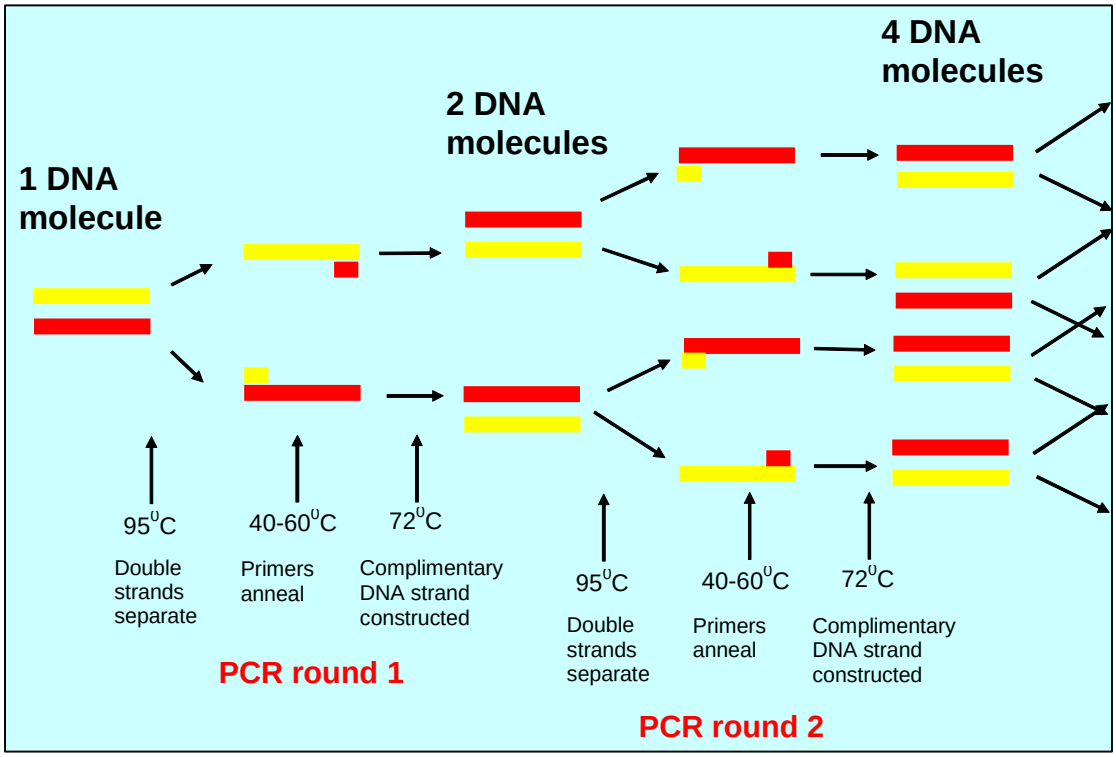


Figure 1-12 Summary of the polymerase chain reaction (PCR) identifying the cycle of denaturation, annealing and extension steps which yield multiple fragments identical to the initial template DNA. Primers are designed to target specific areas such as the 16S rRNA gene which has conserved regions universal to all bacteria and variable regions which allow for distinction between taxa. Reproduced from Davenport (2008)

1.5.3.1. Pitfalls of PCR

PCR-mediated analysis of 16S rRNA facilitates culture independent methods for assessing microbial diversity (Head *et al.*, 1998). There are, however, limitations to PCR which may result in misleading descriptions of diversity within investigated environments (von Wintzingerode *et al.*, 1997).

The principal issues which must be considered include: contaminated DNA samples; inhibition of PCR by co-extracted contaminants such as humic substances; amplification bias and formation of PCR artefacts (von Wintzingerode *et al.*, 1997; Davenport, 2008).

PCR is capable of amplifying very small concentrations of DNA (Steffan and Atlas, 1988), which facilitates the introduction of contamination even at very low levels. Contamination may be introduced for example by contaminated reagents or from airborne contamination and is particularly prone to occur when using universal bacterial primers which target conserved regions occurring in most bacteria. Contamination can be assessed by the use of false negatives, where PCRs are simultaneously performed containing no DNA template: occurrence of a PCR product would indicate a source of contamination.

Humic substances, or organic matter, may be co-extracted with nucleic acids during chemical and physical lysis processes. High concentrations of humic substances are known to inhibit the polymerase enzyme, making it important to remove impurities during the extraction procedure. Dilution can reduce the impact of inhibition but it is important to consider that overly low DNA concentrations may reduce PCR efficiency (von Wintzingerode *et al.*, 1997)

Differential PCR amplification exhibits the potential for introducing selective bias and altering the apparent microbial diversity of environmental samples, with some concern that abundant sequences may be preferentially amplified and rare or relatively low level sequences being discriminated against. This potential bias is important to bear in mind when analysing culture independent data (Head *et al.*, 1998).

PCR artefacts suggest the presence of organisms that are not truly present and are a particular problem associated with complex samples. They may be formed as:

chimeras between homologous molecules; deletion mutants as a result of stable secondary structures and point mutants due to polymerase errors (von Wintzingerode *et al.*, 1997).

1.5.4. *Community fingerprinting techniques*

The development of culture independent techniques has allowed the study of microbial diversity at the genetic level. The two principal applications of 16S rRNA amplification have been sequencing of clones within environmental samples and community fingerprinting techniques (Muyzer, 1999). The creation of clone libraries for the 16S rRNA gene facilitated early attempts to characterise diversity within the microbial world (Head *et al.*, 1998). The process of cloning and sequencing can prove to be laborious, time consuming and expensive depending on the research questions being asked. A desire to look at shifts in microbial communities, for example to look at changes in populations over time or in response to an external stimulus, drove researchers to look at alternative methods (Muyzer, 1999).

One such approach involves community fingerprinting techniques. Muyzer (2000) provides a comprehensive summary of the different genetic fingerprinting techniques available, one of which is discussed in more detail here: denaturing gradient gel electrophoresis (DGGE). DGGE is a method for separating double stranded DNA fragments of the same length but different sequence composition (Muyzer *et al.*, 1993). The method exploits the difference in bond strength between Adenine-Thymine (AT: two hydrogen bonds) and Guanine-Cytosine (GC: three hydrogen bonds), which results in increasing stability of DNA fragments to denaturants with increasing GC content (Green *et al.*, 2009). These differences can be resolved electrophoretically using a polyacrylamide gel containing an increasing concentration of denaturants. A GC-rich clamp is attached to the forward primer and is incorporated fragments amplified during PCR which prevents them from subsequently denaturing completely during DGGE (Sheffield *et al.*, 1989). The output is a distinct banding pattern characterising the genetic diversity within an environmental sample (Muyzer and Smalla, 1998). Each band is given to represent a distinct operational taxonomic unit (OTU). The number of bands can be given as an estimate of species richness and the presence and absence of these bands can be interpreted to make comparisons of similarity between two or more environmental samples.

There are several limitations in using DGGE for diversity assessments and similarity comparisons. Initially, there are the inherent limitations with PCR to consider which have been discussed previously (Section 1.5.3.1). The DGGE banding pattern may not provide an assessment of the active community but rather identify dormant cells or the residual DNA of dead cells (Ben-Amor *et al.*, 2005). This may prove particularly misleading when looking at shifts in microbial communities in response to external stimuli and trying to deduce mechanisms and pathways. Additionally, Muyzer *et al.* (1993) suggests that DGGE may only be suitable for identifying the most abundant OTUs within a sample (>1% of the total population). It is important to consider these limitations when interpreting DGGE outputs.

1.5.5. *The -omics*

The inherent limitations of existing methods and the constant desire for more rapid generation of increasing volumes of data have encouraged the development of high throughput molecular analysis methods. These high throughput methods are grouped together as the '*–omics'*, including genomics, metagenomics and metabolomics amongst others. These methods offer a powerful tool for investigating microbial diversity and making comparisons of similarity between communities but their predominant utility lies in their capacity to better identify the mechanisms and pathways involved in adaptive responses (Snape *et al.*, 2004) and the additional information they might provide pertaining to erratic environmental fate assessments of chemicals and identifying the occurrence and abundance of competent degraders within environmental samples (Kowalczyk *et al.*, 2013).

In recent years, there has been a shift away from traditional sequencing techniques towards high throughout platforms including Illumina (Illumina, 2013) and 454 (Roche, 2013) sequencing (Mamanova *et al.*, 2010; Metzker, 2010). These platforms share a common *modus operandi* of massively parallel sequencing of DNA molecules separated in a flow cell (Voelkerding *et al.*, 2009). 454 and Illumina sequencing mechanisms are discussed in more detail in comprehensive reviews by Voelkerding *et al.* (2009) and Metzker (2010), incorporating emulsion PCR and solid-phase amplification respectively. Despite the different methods and substantial differences in read length, Luo *et al.* (2012) reported a comparable view on the platforms when analysing the same microbial community DNA sample.

The 454 technology has been applied to answer questions about temporal shifts in microbial diversity in the English Western Channel (Gilbert *et al.*, 2009; Gilbert *et al.*, 2010). McLellan *et al.* (2010) used 454 sequences to assess temporal and loci differences in untreated sewage populations and to detect specific microbial enrichment within sewage influent. Palenik *et al.* (2009) have utilised the 454 platform to investigate coastal microbial diversity in California and the proposed mechanisms behind shifts in population structure and diversity.

454 sequencing involves the reading of a single-stranded DNA molecule, to which single matching nucleotides are sequentially added. A chemi-luminescent reaction, which is of a distinct intensity for each nucleotide, occurs when nucleotides are incorporated into the sequence. The reaction, and its intensity, is recorded with a proportionally increasing intensity of light observed with consecutive complementary nucleotides (e.g. AAAA) in the single stranded DNA molecule. These light reactions are analysed to generate millions of sequence bases. Illumina sequencing was not used in the present study; more detail regarding the Illumina mechanism has been reported by Voelderking *et al*. (2009).

The sheer volume of data created by next generation sequencing methods necessitates a clear understanding of which questions are being asked during interpretation in order to obtain meaningful answers.

1.6. Aims

The main aims of this thesis are:

1. To evaluate cell concentration methods for the concentration of bacteria in aqueous samples assessed against a framework of scientific and practical criteria.
2. To investigate the effect of inoculum concentration and test volume on biodegradation outcome and degradation descriptors in enhanced biodegradation screening tests.
3. To develop and validate an enhanced biodegradation screening test capable of characterising chemicals based on their persistence, using a reference set of chemicals with a range of known persistence.

Chapter 2

2. Materials and Methods

General methods are described here, whilst more detailed information is presented in the relevant chapters, where necessary.

2.1. Environmental Compartment Sampling

2.1.1. *Sample site selection*

Samples were collected from the sites given in Figure 2-1.

Figure 2-1 Sample locations from around the United Kingdom. Inocula used in experiments discussed in Chapter 3 were collected from Tees Estuary (estuarine), Temple Sowerby (river) and Plymouth (marine). Inocula used in experiments discussed in Chapters 4 and 5 were sampled from Buckland sewage treatment works in Newton Abbot (AS) and Brixham (marine)

2.1.2. *Activated sludge sampling*

Activated sludge (AS) was collected from wastewater treatment plants receiving predominantly domestic wastewater. Samples were stored in sterile plastic carboys (Thermo Fisher Scientific, Waltham, MA, USA) with sufficient headspace to remain aerobic during transportation. Subsequently, AS was kept aerated at room temperature until use, which was usually on the same, but not later than the subsequent day. More details regarding sample collection are provided in subsequent chapters, where relevant.

2.1.3. *Surface water sampling*

Marine samples were collected from locations off the coast of the United Kingdom (UK). Marine samples were collected a sufficient distance from the coast to mitigate the risk of run off contamination from surrounding land (typically 1-2km). River and estuarine samples were also collected from UK locations. Samples were collected from as close to the middle of the river and estuary sample site as feasible, avoiding sediment dredging. Samples were stored in sterile plastic carboys with sufficient headspace to remain aerobic during transportation, following which they were kept aerated at room temperature until use, usually on the same, or subsequent, day. More details regarding sample collection are provided in subsequent chapters.

2.1.4. *Inoculum treatment and pre-conditioning*

Inocula were not pre-treated by washing with mineral media, a common practice in OECD regulatory tests, due to associated observed decreases in diversity and biodegradation potential (Goodhead, 2009; Vazquez-Rodriguez *et al.*, 2011; Goodhead *et al.*, 2013).

2.1.4.1. Activated sludge pre-treatment

Prior to use as inocula in high throughout biodegradation screening tests, AS samples were serially diluted with sterile OECD defined mineral media (OECD, 1992a) to deliver a range of AS concentrations from 10^8 to 10^3 cells mL^{-1} (also referred to as 10^0 to 10^{-5} dilutions).

Prior to use as test inocula in enhanced biodegradation screening tests, total suspended solids was determined (Section 2.2.1). AS samples were concentrated to increase the biomass concentration. AS samples were left to settle for 1 hour, following which approximately 5 L of supernatant was decanted from the plastic

carboy. The settled sludge was then resuspended, subsequently 500 mL samples were transferred to sterile 500 mL Nalgene bottles (Thermo Fisher Scientific Inc., Waltham, MA, USA) and centrifuged at 4,000 x *g* for 10 minutes using a Beckman Coulter Avanti J26 with JA-14 rotor (Beckman Coulter, Brea, CA, USA). Approximately 200 mL supernatant was decanted and the sludge was resuspended following which total suspended solids were determined again. AS was then diluted with sterile OECD defined mineral media (Section 2.5.1) to achieve the desired biomass concentrations. Total cell counts were performed as detailed in Section 2.2.2.

2.1.4.2. Surface water pre-treatment

Prior to use as inocula, cell concentrations were determined via total cell counts (Section 2.2.2). Surface water samples were then concentrated by the selected method (2.4.2) and total cell counts were repeated to confirm success of the concentration method.

2.2. Test inoculum characteristics

2.2.1. *Suspended solids calculations*

Total suspended solids were determined in triplicate according to *Standard Methods* (APHA, 2005). A 25 mL sample was passed through a pre-weighed pre-ignited and dried glass fibre filter (GE healthcare, Maidstone, Kent, UK) using a Buchner flask and vacuum pump. The filter, containing the sample solids, was subsequently transferred to a 105ºC oven for one hour prior to being weighed, to allow water to evaporate. The difference between the processed filter weight and initial filter weight is referred to as total suspended solids (TSS), given as mg L^{-1}. The filter may then be ignited at 550ºC for ten minutes to burn off all volatile organic carbon yielding the volatile suspended solids (VSS) and ash.

2.2.2. *Total cell counts*

Total cell counts were determined by epifluorescence microscopy following staining with the fluorescent DNA stain 4', 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO, USA). Samples were serially diluted with sterile MilliQ (18 MΩ-cm) water, which had been filter sterilised using 0.1 µm pore filters (Sartorius, Göttingen, Germany) attached to sterile 20 mL syringes (BD Plastipak, Franklin Lakes, NJ,

USA). Solutions were incubated at room temperature in the dark for 30 minutes, following which 30 µL of sample was mixed with 70 µ sterile MilliQ water before filtering through a 0.2 µm polycarbonate nucleopore filter (Millipore, Billerica, MA, USA). A drop of anti-fadant (Citifluor Ltd., Canterbury, UK) was placed on a standard glass microscope slide on to which the air dried filter was then placed. A further drop of anti-fadant was added to the filter, atop which a glass cover slip was placed. Slides were viewed under UV light with an epi-fluorescence microscope (Olympus BX-40) at 100X magnification using an oil immersion lens. Cell numbers were counted in 20 randomly selected fields of view (FOV), and the average number of cells per mL was calculated as shown in Equation 2-1.

Equation 2-1 Enumeration of total cells following staining with DAPI

$$Total\ cells\ per\ mL = \frac{Average\ cells\ per\ field\ of\ view\ (FOV)(cells) \times Filter\ area(mm^2)}{FOV\ area\ (mm^2)\ \times Subsample\ Volume\ (mL)}$$

2.2.3. *Bacterial community analysis*

Typically, samples were collected and stored prior to microbial analysis at a later date (Section 2.6). Samples were either stored directly in sterile 50 mL centrifuge tubes (Sigma-Aldrich, St. Louis, MO, USA) or fixed in a 1:1 ratio with a suitable fixative (ethanol for AS, river and estuarine sources; formalin for marine samples (Sigma-Aldrich, St. Louis, MO, USA) and stored in a sterile 50 mL centrifuge tube. Samples were then stored at -20ºC until use.

2.3. Test chemical selection

Test chemical selection was predominantly based on two main criteria: the necessity to represent a group of reference chemicals with varying degrees of persistence (Section 5.1.1) and the desire to compare chemical degradation data with previous research performed into biodegradation, persistence and the effects of inocula (Goodhead, 2009).

2.3.1. *Selection of chemicals of interest*

Comber and Holt (2010) developed a set of reference chemicals based on rigorous selection criteria (Section 5.1.1), encompassing varying degrees of persistence and non-persistence, with a view to the list being applied for the validation of new biodegradation test methods under REACH. Chemicals were assigned a Bin number based on their persistence ranging from readily degradable Bin 1 chemicals to recalcitrant Bin 4 compounds.

Compounds from Bin 1-3 were selected for testing with preference given to chemicals where previous research had been conducted for comparison and where ^{14}C labelled compounds were readily available.

The chemicals selected for this study are shown in Table 2-1.

Table 2-1 Chemicals selected to be used for validation of the enhanced screen for persistency. [a] according to Comber and Holt (2010).

Chemical	Bin	Reference List[a]	Previous Research	^{14}C Available	Comments
Aniline	1	Y	Y	Y	
4-Nitrophenol	2	N	Y	Y	Wealth of data (Goodhead, 2009; Thouand *et al*., 1995)
4-Fluorophenol	2	Y	Y	Y	
4-Chloroaniline	2	Y	Y	Y	
Pentachlorophenol	3	N	Y	Y	Included in reference report but ultimately not selected

The Bin 1 chemical selected was aniline taken from the reference list of chemicals (Comber and Holt, 2010). 4-chloroaniline and 4-fluorophenol were selected from the reference list as Bin 2 chemicals. 4-nitrophenol was also selected as a Bin 2 chemical due to a wealth of data from previous studies including those by Goodhead (2009) and Thouand *et al.* (1995) but was excluded from the list of reference chemicals due to variable historical performance in biodegradation studies. Pentachlorophenol was selected as a Bin 3 chemical due to previous research

conducted. It was included in the report issued by Comber and Holt (2010) but was ultimately not selected as a compound on the list of reference chemicals.

2.4. High throughput biodegradation screening tests (HS-BSTs)

High throughput biodegradation screening tests (HT-BSTs) were performed in 96-well microtitre plates (BD Biosciences, San Jose, CA, USA) with a working volume of 300 µL per well, allowing sufficient headspace for the systems to remain aerobic, as previously described by Goodhead (2009).

2.4.1. *Test chemical preparation*

Test chemicals were prepared in sterile mineral media concocted from stock solutions of mineral salts and trace elements added to sterile distilled water as detailed in OECD 301 guidelines for the testing of chemicals (OECD, 1992a) (All chemicals sourced from Sigma-Aldrich, St. Louis, MO, USA).

Concentrated stock solutions of test chemicals were prepared at an initial concentration of 1 g C L^{-1} equating to 1 mg C mL^{-1}. Thus 10 mL stock solution added to 1 L sterile mineral media would give a 10 mg C L^{-1} test solution. Carbon concentrations were calculated as shown in Equation 2-2.

Equation 2-2 Equation for calculating carbon concentrations for stock chemical solutions

$$Chemical\ mass\ required\ to\ give\ 1\ g\ C = Molecular\ mass / Carbon\ mass$$

2.4.2. *Inocula concentration methods*

2.4.2.1. Membrane filtration (MF)

5 L of each sample were concentrated by filtration through sterile 0.22 µm filters (Scientific Laboratory Supplies, Hessle, UK). Filters were replaced when they reached their capacity and were placed in sterile 50 mL centrifuge tubes containing 50 mL filtrate. The filters were then agitated vigorously using a sterile glass rod, to aid the resuspension of biomass in the filtrate. Following settling of the filter remnants, the concentrated sample was then decanted into a new, sterile 50 mL centrifuge tube to produce 50 mL concentrated test inoculum.

2.4.2.2. Tangential flow filtration (TFF)

5 L environmental sample were filtered through a 0.22 µm TFF unit (Millipore, Billerica, MA, USA); the permeate was collected and the retentate recycled to the feed tank until the entire sample had been processed as permeate, leaving a suspension of cells on the surface of the filter which were collected by reversing the flow and backwashing the filter with 50 mL permeate collected in a sterile 50 mL centrifuge tube. A schematic of the setup is shown in Figure 2-2.

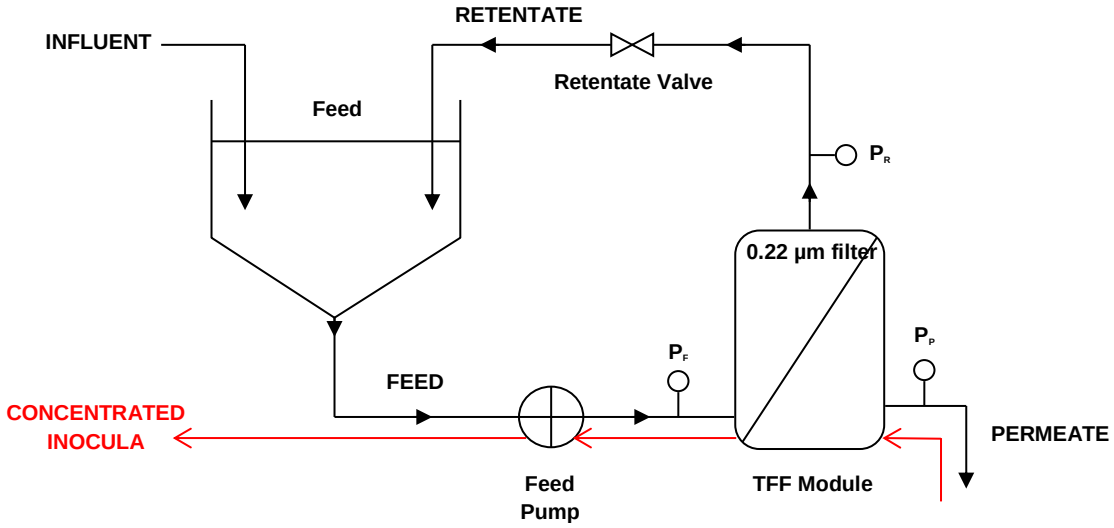


Figure 2-2 Schematic diagram showing the setup of a TFF manifold for concentrating bacteria within an aqueous environmental sample

2.4.2.3. Centrifugation (C)

Environmental samples were concentrated by adding 250 mL sample to a sterile centrifuge tube. The sample was centrifuged at 3400 × *g* for 30 minutes. The supernatant was discarded leaving a pellet, further environmental sample was added to the centrifuge tube and the process repeated until 5 L sample had been processed. The pellet was subsequently resuspended in 50 mL supernatant.

2.4.2.4. Glass bead colonisation (CC)

A method previously described by Mauffret *et al.* (2009) for enrichment of biofilms onto glass beads was followed with some modifications. Sterile 0.4 mm glass beads were packed into a 50 mL syringe sealed with 5 µm cloth and cotton wool at the bottom and a rubber bung at the top, which the environmental sample was fed through via plastic tubing using a peristaltic pump. Sample was passed through the

column at a rate of 15 mL min^{-1} and recirculated to the feed tank over a period of 10 days. The period of colonisation was reduced from 72 days based on a 30 day study indicating that a stable concentration of cells was established on the beads after a 10 day period. At the end of the colonisation period, the glass beads were sonicated in 50 mL of the respective environmental sample, encouraging the resuspension of biomass, yielding a concentrated test inoculum (Mauffret *et al.*, 2009).

2.4.3. *Test inoculum preparation*

Serial dilutions of the concentrated inocula were prepared by adding 3 mL of inoculum to 27 mL sterile mineral medium in sterile 50 mL centrifuge tubes (Sigma-Aldrich, St. Louis, MO, USA). This volume was made up to 30 mL with the addition of 3 mL of the inoculum. The solution was mixed with the mineral media by drawing up and down the pipette several times before 3mL of the mixture was transferred to a new sterile tube containing 27 mL sterile mineral media. This process was repeated to give five dilutions, referred to hereafter as 10^{-1} – 10^{-5}. In addition, a sample of concentrated inoculum was also used, referred to as 10^{0}. Actual cell concentrations ranged from 10^{3} - 10^{8} cells mL^{-1} where 10^{0} refers to 10^{8} cells mL^{-1}.

2.4.4. *HT-BST*

A multistep multi-channel pipette (Thermo Fisher Scientific, Waltham, MA, USA) was used to pipette 250 µL of a 10 mg C L^{-1} 4-nitrophenol solution, made up in mineral media as previously detailed (Section 2.4.1), into each well of a 96-well plate. The multichannel pipette was again used to inoculate the plates with 50 µL of the desired inoculum concentration. The most dilute inocula were prepared first working back to the original neat sample concentration to limit significant carryover of cells.

Following inoculation of the plates, a lid was placed on the plates which were then sealed with Parafilm (Pechiney Plastic Packaging Company, Chicago, IL, USA). The sealed plates were placed on top of water saturated filter papers lining a plastic box. The box was then wrapped in cling film to create a moisture chamber and reduce the risk of evaporation from the wells. The unit was subsequently sealed in tin foil to prevent photo interference. The HS-BSTs were incubated at 20ºC either in an incubator or in a temperature controlled room.

Control plates were constructed by the addition of glucose to the mineral medium to give a final glucose concentration of 500 mg L^{-1}. Complete degradation of glucose

indicated that chemicals were not toxic at the test chemical concentrations used. Abiotic controls were prepared by addition of 100 mg L^{-1} mercuric chloride (Sigma-Aldrich, St. Louis, MO, USA), to observe whether physico-chemical removal of the test chemical occurs, such as sorption.

2.4.5. *Endpoint analysis*

At the end of the incubation period, plates were removed from the moisture chamber, 100 µL supernatant was extracted from each well and transferred to a new sterile 96-well plate to prevent interference from settled solid particles during spectrophotometric analysis.

Absorbance was measured using a plate-reading spectrophotometer (MultiSkan Spectrum, ThermoFisher Scientific, Waltham, MA, USA) at an optimum wavelength of 400 nm. Values were compared to standard concentration curves generated from triplicate absorbance readings of standard concentrations ranging from 0-35 mg L^{-1}.

A positive biodegradation outcome was defined as 70% removal of test compound, akin to a 70% DOC removal in an OECD 301 series test. The wells were assigned a score of one, if sufficient degradation had occurred, or zero if not, and the probability of degradation was calculated as an average of the 96 wells.

2.5. Enhanced screening tests

2.5.1. *Activated sludge inoculum preparation*

Following concentration, if required, and determination of TSS and total cell counts, the AS was amended with sterile OECD mineral media to reach the desired suspended solids concentrations of: 3000, 300, 30, 3 or 0.3 mg SS L^{-1}. The 30 and 3 mg SS L^{-1} systems are typically used in OECD 301 standard tests, with the other concentrations selected to give concentrations orders of magnitude above and below those typically utilised. The volumes of AS and sterile mineral media required were calculated using Equation 2-3, given that the initial and final AS concentrations were known, as was the final required test volume.

Equation 2-3 Equation for calculating the ratios of AS to mineral media to achieve the desired cell concentrations

$$Volume_1 \times Concentration_1 = Volume_2 \times Concentration_2$$

2.5.2. *Marine inocula preparation*

Following the assessment of various cell concentration methods (Chapter 3), tangential flow filtration was determined to be the most suitable method for large-scale biodegradation tests requiring the processing of tens to hundreds of litres of environmental sample to yield a test inoculum.

Marine water was sampled from a 35,000 L on-site storage tank at Brixham Environmental Laboratory held at ambient seawater temperature (subject to seasonal variations but typically 6-16ºC). The seawater was drawn through a 25 µm screen upon entering the tank but all further on-site treatment processes occur downstream of the tank. The sample was collected prior to these treatment processes. The site has an average flow through of approximately 10,000 L per day meaning a constant flux of seawater and an estimated residence time within the tank of 3-4 days.

The marine sample was pre-screened through a 10 µm nominal pore size cartridge filter (Millipore, Billerica, MA, USA). The sample was then processed by TFF, essentially as described in Section 2.4.2.2. Four nominal concentrations of inocula were used in marine enhanced biodegradation screening tests: 100 ×, whereby 100 L pre-screened marine sample were processed through a TFF unit and the filtered bacteria were reconstituted in 1 L permeate; 10 × (10 L into 1 L permeate); filtered (pre-screened through a 10 µm filter without concentration) and direct collection from the tanks, termed raw.

2.5.3. *Test chemical preparation*

Enhanced biodegradation test vessels were dosed with a mixture containing stock solutions of radiolabelled and non-radiolabelled test compounds. Radiolabelled compounds were used to increase the sensitivity of the analysis allowing the accurate observation of degradation below 1% of the originally applied concentration. A mixture of radiolabelled and non-radiolabelled solutions were used as the specific

activity of the radiolabelled test compounds typically meant that the required levels of radioactivity were equivalent to negligible chemical concentrations in comparison with the 10 mg C L^{-1} required in OECD regulatory studies.

2.5.3.1. Non-radiolabelled chemical preparation

A concentrated 1 g C L^{-1} stock solution of the test chemical was prepared in sterile mineral media according to OECD 301 B guidelines (Section 2.4.1) (OECD, 1992a).

2.5.3.2. Radiolabelled chemical preparation

The radiolabelled test compounds were dispensed by a radiation officer at Brixham Environmental Laboratory based on the amount of radioactivity requested (Section 2.5.5.1). Radiolabelled solutions were made up to 5 mL using sterile mineral media in a volumetric flask. Triplicate 0.1 mL subsamples of the radiolabelled solution were added to 7 mL glass LSC vials (Perkin Elmer, Waltham, MA, USA) and mixed with Gold Star scintillation cocktail (Meridian, Surrey, UK). Radioactivity was determined via liquid scintillation counting (LSC) using a Tri-Carb 2800-TR liquid scintillation counter (Perkin Elmer, Waltham, MA, USA).

2.5.3.3. Preparation of chemical dosing solution

Having prepared the non-radiolabelled and radiolabelled solutions and having ensured the correct level of radioactivity, the test dosing solution was prepared by transferring 10 mL of non-radiolabelled solution per litre of test inoculum to be dosed, in to a sterile 500 mL glass bottle. The entire radiolabelled solution (5 mL) was added to this using glass Pasteur pipettes (Sigma Aldrich, St. Louis, MO, USA). The carbon concentration of the radiolabelled solution was negligible compared to the 1 g C L^{-1} present in the non-radiolabelled solution. Triplicate 0.1 mL subsamples of the test dosing solution were taken and analysed by LSC as previously described.

The requisite amount of activity was based on the limit of quantification (LOQ) of 3.5 Bq when using a TriCarb liquid scintillation counter. The calculations ensured at least 3.5 Bq would be present in the NaOH subsample (5 mL of the sampled 50 mL, see Section 2.5.5.1) if 1% of the test compound had been degraded, allowing low levels of degradation to be observed.

2.5.4. *Enhanced OECD 301 B*

Triplicate batch biodegradation screening test systems were constructed, containing an inoculum at the desired cell concentration and volume. Sufficient headspace in the vessels was allowed for the systems to remain aerobic in the event of a failure in the aeration system. Where different volume systems were used, similar bottle geometries and head space were maintained between different systems as far as possible. Test chemical solution was added to each system at a ratio of 10 mL to 1 L of test inoculum to give a test chemical concentration of 10 mg C L^{-1}. The applied radioactivity was calculated based on the testing dose volume and the subsample scintillation counts.

The test vessel was preceded by an influent scrubber containing 2M NaOH (Sigma Aldrich, St. Louis, MO, USA) and a humidifier containing RO water. Following the test vessel was an empty 100 mL glass bubbler and tube (termed a trap), two traps containing 50 mL 2M NaOH and a final empty trap connected to a central extraction manifold which maintained a flow of air through the system, allowing the capture of evolved carbon dioxide (CO_2) and preserving the aerobic nature of the test inoculum (Figure 2-3). All components were airtight and connected by butyl rubber tubing. Activated charcoal embedded devices, Orbo tubes (Sigma Aldrich, St. Louis, MO, USA), were positioned between the test vessel and the subsequent empty trap to capture volatile compounds.

Systems were kept aerobic, in the dark or diffuse light, in a temperature controlled room (22ºC ± 2ºC) and sampled periodically throughout the test duration.

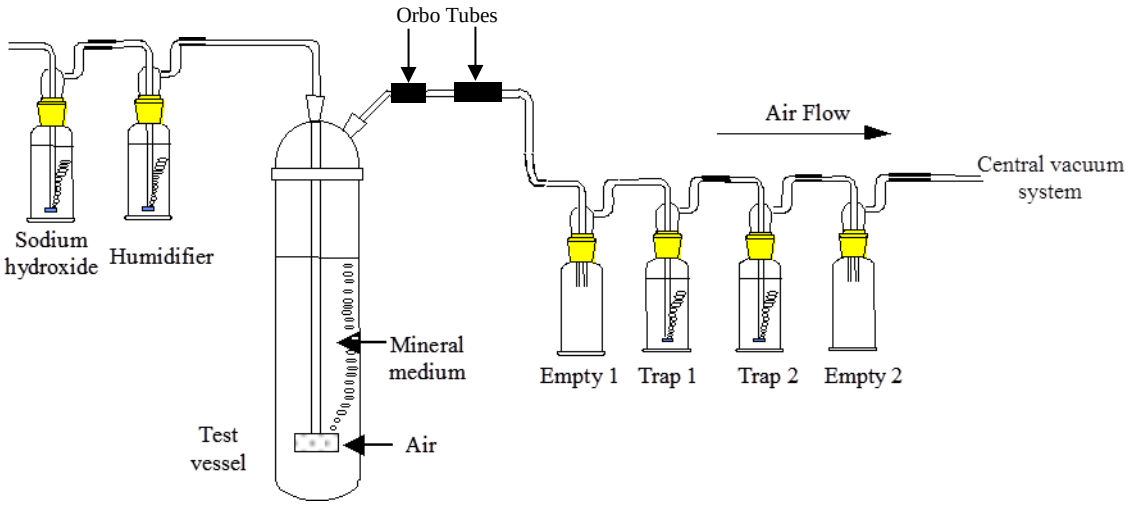


Figure 2-3 Modified OECD 301B experimental apparatus. An influent 2M NaOH scrubber and humidifier are positioned prior to the test vessel which is followed by two NaOH traps used to capture evolved $^{14}CO_2$ as a measure of test compound degradation. Reproduced from BEL (2013)

2.5.5. *Determination of biodegradation*

2.5.5.1. $^{14}CO_2$ evolution

Biodegradation was determined by capturing evolved $^{14}CO_2$ in 2M NaOH (Figure 2-3). During periodic sampling, the NaOH from Trap 1 in each system was collected in a pre-weighed Nalgene bottle (Thermo Fisher Scientific, Waltham, MA, USA) which was subsequently re-weighed. Trap 2 was transferred to the Trap 1 position and Trap 1 was refilled with 50 mL 2M NaOH and returned to the Trap 2 position. Triplicate 5 mL subsamples of NaOH from each system were transferred to 20 mL glass LSC vials (Perkin Elmer, Waltham, MA, USA), mixed with Gold Star scintillation cocktail and analysed by LSC. Radioactivity was calculated as shown in Equation 2-4.

Equation 2-4 Equation for calculation of radioactivity based on NaOH LSC data

$$Radioacivity\ (Bq) = \frac{Mean\ subsample\ activity\ (Bq)}{Sample\ volume\ (mL)} \times \frac{Total\ NaOH\ weight\ (g)}{NaOH\ density\ (1.08\ g\ mL^{-1})}$$

The accumulated radioactivity was then determined by adding the radioactivity calculated at the sample point to the radioactivity in previous subsampling (i.e. the mean radioactivity from the triplicate subsamples of the respective system at each previous sample point). $^{14}CO_2$ evolution was then calculated as shown in Equation 2-5 , given to directly represent the test chemical degradation.

Equation 2-5 Equation for the calculation of degradation based on evolved $^{14}CO_2$

$$Degradation\ (\%) = \frac{Accumulated\ radioactivity\ (Bq)}{Originally\ applied\ radioactivity\ (Bq)} \times 100$$

A positive biodegradation outcome in the OECD 301 series testing for ready biodegradability was defined as 60% degradation within a 28 day test and furthermore within a ten-day window beginning once degradation exceeded 10%, given to represent the end of the lag phase. Typically the 28 day test duration and ten-day window were disregarded for calculating probability of degradation and vessels were assigned a score of one if degraded (≥ 60%), or zero if not (< 60%), from which the average value was used as the probability of degradation.

2.5.5.2. Residual ^{14}C activity

The remaining radioactivity, and thus presumed remaining test chemical, was determined by periodically sampling 5 mL from each test vessel. In high biomass concentration tests, samples were filtered to allow differentiation between radioactivity remaining in solution and radioactivity bound to biomass, and to prevent interference from the biomass during LSC analysis. All samples were acidified by addition of two drops concentrated hydrochloric acid and aerated with compressed air for approximately twenty minutes to drive off inorganic CO_2 (Ingerslev and Nyholm, 2000). Samples were subsequently made up to 20 mL with scintillation cocktail and analysed via LSC. Where applicable filter papers were combusted (A307 Sample Oxidiser: Perkin Elmer, Waltham, MA, USA), the resultant solvent was made up to 20 mL with scintillation cocktail and analysed via LSC.

2.5.5.3. Mass balance determination

Upon completion of the study, a mass balance was performed to account for the applied radioactivity. The contents of the vessel were filtered (GE Healthcare, Maidstone, Kent, UK) or centrifuged to remove solid particles. The filters or solid pellet were then combusted. The filtrate was stored in a pre-weighed Nalgene bottle and triplicate 5 mL subsamples were taken and analysed via LSC. Orbo tubes were rinsed with 10 mL of methanol which was collected in 20 mL glass LSC vials, mixed with scintillation cocktail and analysed by LSC. If necessary, the test vessels were then rinsed with methanol or an appropriate solvent to ensure the test compound had not sorbed to glass surfaces.

2.5.5.4. Biological sampling

A sample was taken from each vessel at the end of the study, transferred into a sterile plastic centrifuge tube and stored at -20ºC for molecular analysis.

2.6. Bacterial community analysis

2.6.1. *DNA extraction*

Total DNA was extracted from 250 µL each sample. As each condition was carried out in triplicate test vessels, giving true biological replicates, only one extraction was performed per vessel. Total DNA was extracted using the FastDNA SPIN kit for soil (MP Biomedicals, Cambridge, UK) via a combination of chemical and physical lysis as previously described (Goodhead *et al.*, 2013). DNA extracts were stored at -20ºC until required.

2.6.2. *PCR*

16S rRNA gene fragments were amplified by PCR using primers and conditions specified by Muyzer *et al.* (1993), targeting conserved regions of the gene (Primer 2: 5'-ATTACCGCGGCTGCTGG-3'; Primer 3: 5'-*CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG*CCTACGGGAGGCAGCAG-3'). Primer 3 contains an additional 40-nucleotide GC-rich sequence (GC clamp) (Muyzer *et al.*, 1993). PCR reactions were carried out in 200 µL PCR tubes, containing 1 µL template DNA, 10 pmol (in 1 µL) of each primer and 47 µL of MegaMix-Blue PCR-ready mix (containing *Taq* polymerase in 1.1 × reaction buffer (2.75 mM $MgCl_2$) with 220 µM dNTPs, loading dye and stabiliser: Microzone,

HaywardsHeath, UK). PCR was performed using a Thermo-cycler (Thermo Fisher Scientific, Waltham, MA, USA), with the following amplification conditions: an initial denaturation phase at 95ºC at 3 minutes prior to a one minute denaturation step at 95ºC, followed by one minute cycle at 65º, reducing by 1ºC every second cycle until 53ºC, at which stage 15 additional cycles were performed and a final extension step of 72ºC for ten minutes. Positive controls of a known bacterial isolate and negative controls containing filter sterilised deionised (18 M Ω) water were used to corroborate that PCR was working correctly and that reagents or consumables (e.g. pipette tips) were not contaminated.

2.6.3. *Agarose gel electrophoresis*

The presence of the correct size PCR product was checked by analysis on a 1% agarose gel. 100 mL of 1 × TAE buffer (40 mM Tris-Acetate, 1mM EDTA, pH 8.3: Eppendorf, NY, USA) was added to 1 g of agarose powder (Sigma Aldrich, St. Louis, MO, USA). The solution was heated until it became clear, indicating the agarose had melted. 20 µL Nancy-250 (5 mg mL^{-1}: Sigma Aldrich, St. Louis, MO, USA) was added and the solution thoroughly mixed before being carefully poured into a gel cast containing 20 well ladders. After 20 minutes of cooling, the ladders were removed to leave 20 wells per ladder. Subsequently, 5 µL of each PCR product were added to the wells (PCR samples not containing MegaMix-Blue were prepared by combining 5 µL PCR product with 2µL loading buffer which was then pipetted into the appropriate well) and run against a standard PCR ladder containing products of known size (Sigma Aldrich, St. Louis, MO, USA). Electrophoresis was performed at 100V for 45 minutes using a PowerPac 3000 (Bio-Rad, Hemel Hempstead, UK). The gel was subsequently analysed on a UV transilluminator.

2.6.4. *Denaturing gradient gel electrophoresis (DGGE)*

DGGE was performed as described previously (Green *et al.*, 2009; Goodhead *et al.*, 2013) using the D-code system (Bio-Rad, Hemel Hempstead, UK). 10% polyacrylamide gels (0.75-mm thick, 16×16 cm) were run in 1 × TAE buffer (40 mM Tris-Acetate, 1mM EDTA, pH 8.3). A gradient of 30-60% denaturant was used (where 100% denaturant contains 7 M urea plus 40% v/v formamide in 1 × TAE). Gels were run at 60ºC for 900 volt hours (approximately 4.5 hours at a constant voltage of 200 V). Gels were subsequently stained for 30 minutes using SYBR Gold

(diluted to 1:10000 in 1 × TAE: Sigma Aldrich, St. Louis, MO, USA) and imaged using a UV transilluminator.

2.6.4.1. DGGE analysis

DGGE images were normalised utilising standard marker lanes of known banding pattern and BioNumerics software (Applied Maths, Austin, USA). Band presence and absence data was exported from BioNumerics, allowing the calculation of richness (band number) and evenness (Pielou's evenness index).

A band quantification matrix was exported from BioNumerics to PRIMER v6 (Plymouth Routines In Multivariate Ecological Research version 6: PRIMER-E Ltd., Ivybridge, UK) for community similarity analysis using non multi-dimensional scaling (NMDS) analysis.

Similarity between bacterial communities was calculated using the Bray-Curtis algorithm (Equation 2-6). These similarities were used to perform cluster analyses (Unweighted Pair Group Method with Arithmetic mean; UPGMA) and non-metric multi-dimensional scaling analysis (NMDS) (Hale *et al.*, 2010). Each band was given to represent an operational taxonomic unit (OTU) and the number of bands was given to represent the species richness of a sample

Equation 2-6 Calculation of Bray-Curtis similarity index using band presence/absence data exported from BioNumerics

$$Bray - Curtis\ similarity = \frac{2 \times Number\ of\ shared\ taxa\ (bands)}{[Number\ of\ taxa\ (bands) in\ A + Number\ of\ taxa\ (bands) in\ B]}$$

2.6.5. *454 sequencing*

2.6.5.1. Amplicon library preparation for 454 sequencing

16S rRNA gene fragments were amplified by PCR using barcoded MID (multiplex identifier) primers allowing for identification of samples downstream. The F515 (5'-GTGNCAGCMGCCGCGGTAA-3') and R926 (5'-CCGYCAATTYMTTTRAGTTT-3) primers were attached to an adaptor sequence (CGTATCGCCTCCCTCGCGCCATCAG and CTATGCGCCTTGCCAGCCCGCTCAG respectively) and a unique 8 base barcode

sequence to allow distinction between samples in downstream processes. Full primer sequences are presented in Appendix A. PCR reactions were carried out in 200 µL PCR tubes, containing 1 µL template DNA, 1 µL of each primer (with respective barcode attached), 5 µL Fast Start High Fidelity buffer (Roche Applied Science, Penzberg, Germany), 0.5 µL Fast Start High Fidelity enzyme blend (Roche Applied Science, Penzberg, Germany), 0.5 µL PCR nucleotide mix (Roche Applied Science, Penzberg, Germany), and made up to a 50 µL reaction volume with sterile Milli-Q water. PCR was performed using a Thermo-cycler (Thermo Fisher Scientific, Waltham, MA, USA), with the following amplification conditions: an initial denaturation phase at 95ºC at 4 minutes prior to 25 cycles of one minute at 95ºC, 45 seconds at 55º, one minute at 72ºC and a final extension step of 72ºC for seven minutes.

PCR products were purified using the MinElute PCR purification kit (Qiagen, Limburg, Netherlands) as per the manufacturer's instructions, with elution into a final volume of 20 µL. Where DNA content was not sufficiently high, multiple PCRs were performed and the PCRs were bulked together during this purification step i.e. several PCR products were combined into one bulk and processed through a single MinElute spin column.

DNA content was analysed by fluorometric quantitation using the Qubit 2.0 fluorometer (Life Technologies, Paisley, UK) by addition of 1 µL PCR product to 199 µL working solution, 2 minute incubation at room temperature and subsequent analysis on the fluorometer.

The presence of DNA was analysed on a 1% agarose gel as previously described. Samples were prepared in equimolar amplicon pools (based on the measured DNA concentrations) containing 24 samples per pool (corresponding to the 24 unique 8 base bar codes available).

2.6.5.2. 454 sequencing analysis

Amplicon pools were sent to the Centre for Genome Research at Liverpool University for next generation sequencing which was performed on a 454 Genome Sequencer (GS) FLX platform with titanium chemistry.

Sequencing data was not denoised prior to analysis with the open source software package, Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso *et al.*, 2010). Operational taxonomic units (OTUs) were selected against the 16S rRNA gene database, Greengenes (McDonald *et al.*, 2012), based on a 97% similarity to sequences contained within the Greengenes database. Sequences which did not match a sequence contained within the database were not included in subsequent analysis. OTUs were assigned at the genus level. The data were analysed using QIIME to give measures of alpha and beta diversity.

The use of a closed reference OTU picking approach (only including those sequences which match a sequence contained within the reference database), was suitable for analysing data which had not been denoised but will lead to the omission of sequences from the raw data which have not yet been added to the reference database which may affect the overall number of observed OTUs and the analysis of community composition. Approximately 30% of the inputted reads were discarded as a result of failing to match sequences contained within the Greengenes database. It is important to note that there is no distinction here between which reads may have related to new OTUs not yet contained within the database and which may have been noise or chimeras.

Complete analysis would begin with denoising of the 454 data to remove sequencing errors which may identity rare OTUs not actually present and chimera checking to remove chimeras. The data would then be assigned by open reference OTU picking, whereby sequences matching a sequence within the database are assigned to the relevant OTU and those sequences which do not match a sequence within the database are reported as new sequences, allowing subsequent analysis of a complete dataset.

Chapter 3

3. Evaluation of bacterial cell concentration methods for aqueous inocula

3.1. Introduction

The extent of bacterial diversity has been the subject of great discussion, with estimates ranging from 10, 000 (Finlay and Clarke, 1999) to 10, 000, 000 (Curtis *et al.*, 2002) “species”. Although the advent of culture independent techniques broadened our horizons (Section 1.5), it is suggested that the true diversity of a given environment is unknowable and far beyond our sampling capabilities (Curtis *et al.*, 2002). Assuming that prokaryotic organisms are ubiquitous in all environments, it has been estimated that a 10 km^3 marine sample would be required to represent at least one individual of the least abundant species. This sample requirement increases to a 27 km^2 area for more complex soil environments (Curtis *et al.*, 2006). In order to gain a more realistic aqueous environmental sample for use in laboratory and pilot scale studies, the bacteria in large environmental samples may be concentrated, preserving the diversity of a larger sample, but in a smaller manageable test volume. The concentration of aqueous samples has been shown to be useful in assessing the diversity of bacterial communities within environmental samples (Goodhead *et al.*, 2013); investigating the impact of biomass concentrations in small scale biodegradation assays (Thouand *et al.*, 1995) and looking at relatively rare organisms in the environment (Goyal and Gerba, 1980).

A central assumption in all biodegradability tests is that a randomly selected sample from an environmental compartment will exhibit a diversity representative of that compartment, in order to determine the true fate of a compound once it enters the aquatic environment. Standard biodegradation tests (OECD, 1992a) use inocula from a range of sources, with concentrations ranging over four orders of magnitude (Table 1-2). These variable, and environmentally unrealistic, low bacterial cell concentrations result in high variability observed within standard tests and a high incidence of false negative results, incorrectly identifying biodegradable compounds as non-biodegradable (Figure 1-7). The potential to produce more reliable, less variable, tests by increasing research efforts on the test inoculum has been identified in workshops sponsored by the European Centre for Ecotoxicology and Toxicology

of Chemicals (ECETOC) (ECETOC, 2003; ECETOC, 2007; Snape, 2010). The outcome of these workshops was a proposal for enhanced biodegradation screening test methods with a focus on increasing the total bacterial cell count within tests and allowing adaptation of microbial communities to the test chemical. These proposals were adopted by the European Chemicals Agency (ECHA) as part of REACH, discussed in more detail in Section 1.3.4 (EC, 2009).

In order to consider the true biodegradability of a chemical, many of the microorganisms it is likely to come into contact with in the environment should be present in the test inoculum. Whilst this would obviously represent an unrealistic inoculum and potentially promote the occurrence of false positives, the sample sizes required for this would also be prohibitive and unrealistic in laboratory tests. The best available solution would be to concentrate bacteria from environmental samples in to manageable inocula volumes whilst preserving a realistic community diversity, which a chemical may be expected to encounter in the environment.

The need for testing at increased inocula concentrations is not a new concept. Thouand *et al.* (1995) suggested that a biomass concentration of 52 mg SS/L was necessary to give very high probability of biodegradation for inherently biodegradable compounds in small scale biodegradation tests. This is almost twice the maximum concentration typically used within OECD screening tests (OECD, 1992a). Nyholm *et al.* (1984) and Nyholm and Kristensen (1992) discussed the increased exclusion of competent degraders and high variability in biodegradation outcome when working with small sample sizes and thus low cell numbers. The current screening tests for assessing biodegradability of chemicals are critiqued in Section 1.3.3, with poor environmental realism being a principal concern.

One of the focuses within REACH is to increase the emphasis on intelligent approaches to PBT testing and implement modified and enhanced screening tests to enable a more effective prioritisation on persistence. One approach is to use environmental samples as inocula at more environmentally realistic concentrations within enhanced biodegradation screening tests (Comber and Holt, 2010). It is thought that increasing the biomass concentration will in turn increase the bacterial diversity within the inoculum and thus increase the likelihood of incorporating organisms which are capable of degrading the chemical in question (Nyholm *et al.*,

1992). The ultimate aim however, is not for chemicals to pass a biodegradation assessment, but for the high variability associated with biodegradation screening tests to be reduced for the generation of reliable and reproducible biodegradation data.

Many different cell concentration methods have been proposed. The selection and critical evaluation of selected concentration methods and a framework for assessing method suitability is discussed below.

3.1.1. *Membrane filtration*

Membrane filtration in this context constitutes the dead end filtration of an environmental sample and the subsequent resuspension of bacterial cells collected on the membrane surface to yield a concentrated inoculum. Filtration and membrane filtration techniques are typically used as Standard Methods (APHA, 2005) for the determination of total (mixed liquor) suspended solids and faecal coliform counts respectively. Membrane filtration is a commonly used practice in the sterilization of liquids which cannot be autoclaved, in addition to being used for the enumeration of phyto- and bacterio-plankton in aqueous samples by microscopy (Hobbie *et al.*, 1977; Glockner *et al.*, 1996; Porter *et al.*, 2004) and for their concentration prior to assessing microbial diversity and activity (Yannarell and Tripplett, 2004). The method has previously been used to concentrate bacteria from environmental samples for inocula in 96-well plate high throughput biodegradation screening tests (Goodhead, 2009) in the preparation of inocula for use in 96-well plate high throughput screening tests.

3.1.2. *Tangential flow filtration*

Cross flow or tangential flow filtration is typically used as a means of purification in the pharmaceutical industry, for domestic potable water treatment and supply, and for the concentration and study of planktonic cells from aqueous samples (Porter *et al.*, 2004). Its application has been advocated as a means of concentrating microbial biomass for use in biodegradation testing (ECHA, 2012). The advantage over dead end filtration is the continual washing of the filter surface by the cross-flow system, preventing filter fouling and encouraging biomass resuspension (Tanny *et al.*, 1980) thereby allowing filtration of greater quantities of aqueous environmental samples than conventional filtration methods. Tangential flow filtration units are available in a

range of sizes, capable of processing from tens to hundreds to thousands of litres at the largest scale (Millipore, Billerica, MA, USA; Novasep, Pompey, France).

3.1.3. *Centrifugation*

Centrifugation involves the separation of mixtures based on their relative density with more dense particles moving away from the axis of the centrifuge. It is a standard method for the preparation of inocula within regulatory tests (OECD, 1992a) and has previously been utilised as an inoculum preparation method in modified screening tests (O'Malley, 2006). It is typically employed either to remove coarse organic matter particles, leaving a supernatant for use as test inocula, or to concentrate bacterial cells within an environmental sample, yielding a pellet which is resuspended to use as an inoculum (Thouand *et al.*, 1996; O'Malley, 2006).

3.1.4. *Glass bead (column) colonisation*

Surface attached bacterial communities and biofilms have previously been used for biodegradation and bioremediation purposes due to their high microbial biomass (Singh *et al.*, 2006). Mauffret *et al.* (2009) colonized glass beads by passing seawater through a packed column of beads over a 72 day period. Cell concentrations were increased by approximately three orders of magnitude and utilization of the final inocula resulted in a reduced lag phase and increased mineralization of linear alkyl sulfonate (LAS). This method required glass beads to be exposed for a prolonged period to the environmental sample with either a constant supply of the sample or a recirculationary system. Syringes can be adapted to act as columns and the glass beads are readily available from major laboratory suppliers. Although it is one of the few methods specifically designed to enhance microbial biomass for biodegradation tests, its effects on changing the microbial community composition of aqueous samples has never been addressed.

3.2. Objectives:

- *Evaluate different existing cell concentration methods for concentrating bacteria in aqueous samples*
- *Develop a scientific and practical criteria framework for assessing the suitability of cell concentration methods*
- *Determine the impact increased bacterial concentration has upon key variables including biodegradation outcome and community composition*
- *Make recommendations for the use of cell concentration methods to deliver concentrated inocula for use in enhanced biodegradation screening tests*

3.3. Hypotheses:

- *Increasing bacterial concentrations will lead to increased bacterial diversity and produce communities enriched from the original community*
- *Increased inocula concentrations will lead to a greater probability of degradation in high throughout biodegradation screening tests*

3.4. Materials and methods

3.4.1. *Sampling sites*

River, estuarine and marine samples were collected from environmental compartments in the United Kingdom: the River Eden at Temple Sowerby, the Tees Estuary at Teesmouth and the English Channel at Plymouth respectively (Figure 2-1). Sample sites were selected based on the probability of biodegradation for three substituted phenols (4-hydroxybenzoic acid (4-HBA), 4-nitrophenol (4-NP) and 4-fluorophenol (4-FP)) at inocula concentrations ranging from 10^3-10^9 cells mL^{-1}; showing an increasing probability of degradation with increasing concentration and a probability of 1 for most compounds at the highest concentration. Activated sludge samples were not included as their high cell concentration precludes them from filtration techniques. Furthermore, their high specific density (1.02 g mL^{-1}) and floc formation naturally lends itself to gravity based methods such as centrifugation rather than size exclusion as a means of cell concentration.

3.4.2. *Sampling*

Disinfected and rinsed carboys were used to collect 10 L of environmental sample from each sample site. Marine samples were collected using a converted manual bilge pump, from approximately 2-4 metres below the surface. River water samples were collected using a sterile collection bucket secured with a length of rope. Samples were collected from as close to the centre of the river as possible by lowering the collection bucket from a bridge spanning the river. Estuary samples were collected using a sterile collection bucket secured with a length of rope, by wading into the estuary and sampling from as close to the centre of the estuary as feasible. All environmental compartments were sampled in April within 24 hours of each other. Samples were kept aerobic at 4^0C until processing within 24 hours of collection. 5L of each surface water sample were concentrated to give a nominal 10 × cell concentrate using either: centrifugation, membrane filtration, tangential flow filtration or glass bead colonisation, in a final volume of 50 mL of filtrate. Total cell counts of all samples, pre- and post-concentration, were determined via epifluorescence microscopy using the fluorescent DNA stain, 4', 6-diamidino-2-phenylindole (DAPI) (Davenport and Curtis, 2004). For molecular analyses, 25 mL of each original and concentrated sample were stored at -20^0C in 1:1 ethanol (v/v) prior to analysis.

3.4.3. *High throughput biodegradation screening test (HT-BST) preparation*

River, estuarine and marine environmental samples were concentrated by the selected cell concentration methods: membrane filtration (Section 2.4.2.1); tangential flow filtration (TFF: Section 2.4.2.2); centrifugation (C: Section 2.4.2.3) and glass bead (column) colonisation (CC: Section 2.4.2.4), as previously described.

High throughput biodegradation screening tests (HT-BSTs) were prepared as previously described (Section 2.4.4) using the concentrated environmental samples as inocula. HT-BSTs were dosed with 4-nitrophenol as the sole carbon source at a test concentration of 10 mg C L^{-1} (Section 2.4.4).

3.4.4. *Determination of biodegradation*

4-NP transforms from a yellow to a colourless solution on degradation, which was determined against a calibration curve of 4-NP standard concentrations (1-35 mg L-1) at an optimum wavelength determined as 400 nm. After 60 days, biodegradation was determined by colorimetric detection of residual parent compound in each microtitre

well using a Multiskan® Spectrum spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). A positive response was defined as 30% or less of the initial chemical remaining, which equates to the 70% DOC removal level demarcated in OECD 301 guidelines for the testing of chemical ready biodegradability (OECD, 1992a). Probability of degradation was then calculated based on the frequency of positive wells for each inocula concentration within each environmental compartment.

3.4.5. *Bacterial community analysis*

Samples were collected for bacterial community analysis at the beginning of the experiment.

Total DNA was extracted from triplicate 250 µL subsamples of each sample using FastDNA SPIN kit for soil (BP Biomedicals, Solon, Ohio, USA) by a combination of chemical and physical lysis, as detailed by Baptista *et al.* (2008). DNA extracts were stored at -20ºC until required for subsequent analysis, upon which it was transferred to 4ºC storage until use.

3.4.5.1. DGGE

PCR was performed as described previously in Section 2.6.2 to prepare extracted DNA for DGGE analysis. DGGE was performed with the Bio-Rad D-CODE system as detailed in Section 2.6.2. DGGE images were analysed as detailed in Section 2.6.4.1.

3.4.5.2. 454 sequencing

Amplicon libraries were prepared for 454 sequencing as described previously in Section 2.6.5. Sequencing was performed at the Centre for Genome Research at Liverpool University using the 454 GS-FLX platform with titanium chemistry.

454 sequencing data was analysed as detailed in Section 2.6.5.2.

Additional statistical analyses were performed within Minitab (Minitab Ltd., Coventry, UK).

3.4.6. *Method ranking*

The different cell concentration methods were ranked based on scientific and practical criteria. The scientific criteria were: final cell concentration achieved, similarity of the concentrated communities to those in the original sample, the diversity of the concentrated community and the greatest probability of degradation

at lowest cell concentration. The practical criteria were: equipment cost, operational and maintenance costs, potential sample throughput and the level of skill required to implement the procedure. The highest ranked method in each category was awarded a score of 1, and the lowest-ranking method was given a score of 4. Average ranks were calculated based on the cumulative rank scores for scientific and practical criteria separately and for their combined scores overall. No preferential weighting was given to any of the criteria.

3.5. Results and Discussion

3.5.1. *Scientific criteria*

3.5.1.1. Cell concentration

Cell concentration was successfully increased for all methods, by as little as 10-fold (centrifugation), to as much as 1000-fold (membrane filtration), in seawater. Starting concentrations were typically 10^5-10^6 cells mL^{-1} (Table 3-1). Membrane filtration consistently ranked highest with respect to cell concentration, regardless of the aqueous sample used. Membrane filtration, tangential flow filtration and glass bead colonization produced maximum inocula concentrations of 10^8 cells mL^{-1}; centrifugation produced a maximum inocula concentration of 10^7 cells mL^{-1} (Table 3-1). Both membrane filtration and centrifugation produced final cell concentrations with least variation between environmental compartments. Membrane filtration and tangential flow filtration both concentrate samples based on the principle of size exclusion, although in the former exclusion is in the direction of the flow, while the latter occurs perpendicular to the flow. Glass bead colonisation also gave high cell concentrations in contrast to centrifugation which concentrated fewer cells than any other method (Table 3-1), probably because many bacterioplankton are present as small dispersed unicells possessing a low specific gravity. According to Stoke's law such samples would require high centrifugal forces and long centrifugal times to overcome buoyancy and viscosity forces. In contrast to the method here, ultracentrifugation may be successful in concentrating such cells , but is more costly than conventional centrifugation and may impact upon cell viability (Børsheim *et al.*, 1990). Moreover, in aquatic microbiology, centrifugation is seldom used and often

criticized for its impracticality and inability to capture small cells (Šimek and Straškrabová, 1992; Lemke *et al.*, 1997).

Table 3-1 Summary of the cell concentrations and OTU richness achieved using different cell concentration methods for different environmental samples. For DGGE, OTU richness refers to the number of bands in each sample, where each band is given to represent a taxon. For 454, richness refers to the number of observed OTU based on sequences contained within the 16S rRNA reference library 'Greengenes' (McDonald *et al*., 2012). Values in parentheses represent one order of standard deviation. (-) indicates it was not possible to calculate standard deviation.

	River			**Estuarine**			**Marine**		
	Cells/mL	Richness		Cells/mL	Richness		Cells/mL	Richness	
		DGGE	454	DGGE	DGGE	454		DGGE	454
Glass bead colonisation	10^8	7 *(±0.0)*	584 (-)	10^8	19 *(±0.6)*	363 (±7)	10^7	5 *(±0.6)*	222 (-)
TFF	10^7	29 *(±0.6)*	517 (-)	10^8	19 *(±1.2)*	398 (±18)	10^7	26 *(±1.5)*	460 (±14)
Centrifugation	10^7	30 *(±2.0)*	405 (±79)	10^7	19 *(±1.2)*	324 (±2)	10^7	10 *(±0.6)*	293 (-)
Membrane filtration	10^8	22 *(±1.2)*	645 (-)	10^8	24 *(±1.0)*	356 (±23)	10^8	16 *(±0.0)*	213 (±53)
Original	10^6		184 (-)	10^6	18 *(±4.5)*	423 (-)	10^5	23 *(±0.6)*	417 (-)

3.5.1.2. Bacterial community similarity

One of the most important scientific criteria is the ability to increase bacterial diversity without adversely changing the community structure of the predominant taxa present in the original sample. Inocula would therefore contain the same taxa in approximately the same proportions as in the original samples, but also include additional rarer taxa. Such bacterial communities were expected to retain a large number of taxa present in the original samples, and have greater taxa richness than the original community.

The similarity of bacterial communities within triplicate samples derived from the same cell concentration method were significantly greater than those similarities between different cell concentration methods (ANOSIM, $P < 0.001$), with the majority of triplicates (13 out of 14) showing more than 60% similarity in bacterial community composition (Figure 3-1). It is perhaps unsurprising that those methods based on size exclusion gave bacterial community fingerprints that were most similar to the

original communities. Membrane filtration consistently produced communities with the highest similarity to the original sample, followed by tangential flow filtration (Figure 3-1). Centrifugation did not produce samples showing high similarity to the original samples, most noticeably in marine samples. This is possibly due to the inability of centrifugation to adequately capture smaller cells, which are more abundant in the marine environment than in estuarine or river waters (Palumbo *et al.*, 1984; Bernard *et al.*, 2000). Glass bead colonisation regularly resulted in the lowest bacterial community similarity with the original sample.

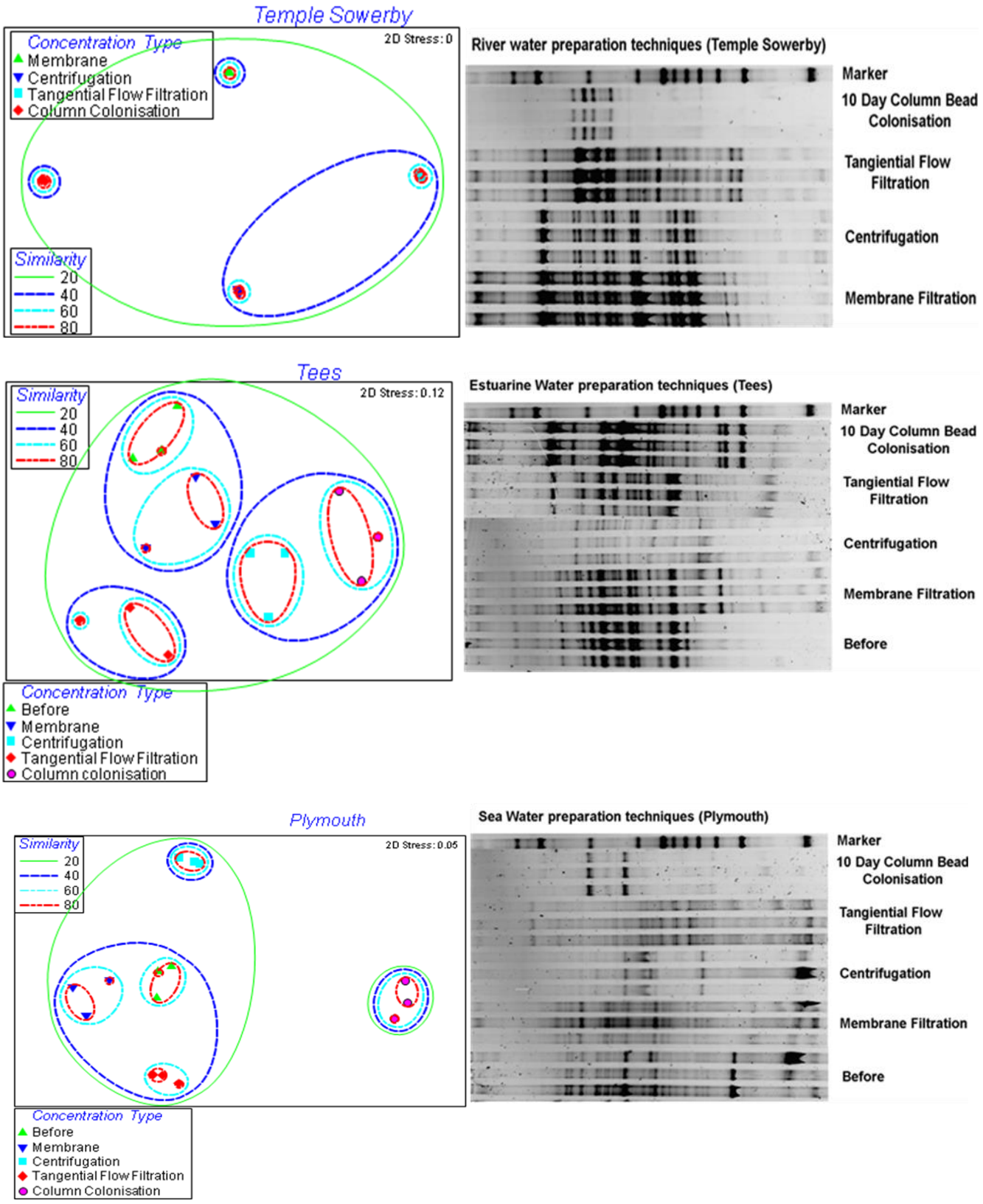


Figure 3-1 Non-metric multidimensional scaling plots overlaid with cluster analysis threshold similarities (shown on the left of the figure). Based on the fingerprint gels (DGGE: shown on the right of the figure) for river samples at the top, estuarine samples in the middle, and marine samples at the bottom. The marker is a composite of clones selected to give a distinctive banding pattern when run on a DGGE gel, prepared by Fiona Read at Newcastle University.

3.5.1.3. Operational Taxonomic Unit (OTU) richness

In DGGE gels, a band is given to represent a taxon and the number of bands is given to represent the operational taxonomic unit (OTU) richness for the most abundant taxa. Generally, membrane filtration, tangential flow filtration and centrifugation produced bacterial communities with OTU richness values similar to, and often greater than, the original native sample (Figure 3-1; Table 3-1). Following data aggregation, only membrane filtration and tangential flow filtration had mean values (20.6 and 24.8 respectively) greater than the original sample (20.5) whilst centrifugation averaged 19.7. All four samples were statistically indistinguishable from each other but significantly different from glass bead colonisation, which had an OTU richness of only 10.6 (ANOVA, $P < 0.001$).

Whilst membrane filtration consistently produced communities with the highest similarity to the original sample, depending on the sample type, it did not always appear to capture the most additional taxa. Indeed this method sometimes resulted in less detectable taxa than the original sample (Figure 3-1; Table 3-1). However, it must be noted that DGGE has a relatively high detection limit based on abundance (1%) (Woodcock *et al.*, 2006; Akarsubasi *et al.*, 2009) and bacterial communities are thought to be log-normally distributed (Quince *et al.*, 2008). Therefore, relatively small increases in cell concentration may be insufficient to substantially increase the number of detectable bacteria. Glass bead colonization often had the lowest OTU richness, where some of the resulting dominant taxa were not those that dominated, or were even present in the original sample (Figure 3-1; Table 3-1). This is consistent with a method selectively enriching only a proportion of the original community, which are likely to be only those taxa capable of forming biofilms. Such communities are misrepresentative of the majority of the natural aqueous environment, which is understandably an undesirable trait when the aim is to study the biodegradation potential of communities from the *in situ* environment.

The use of another diversity index based on the abundance (band intensity) distribution across taxa, Pielou's evenness index (*J'*) was unable to distinguish differences between different treatment methods (data not shown).

For marine samples, tangential flow filtration was the only method which increased OTU richness beyond that exhibited in the original sample; the other methods

reported reductions in OTU richness (Table 3-1). For estuarine samples, all methods increased the OTU richness compared to the original sample. For river samples, centrifugation and tangential flow filtration gave the highest OTU richness values followed by membrane filtration (30, 28.7, and 21.7, respectively), and glass bead column colonisation had a significantly 3-fold lower OTU richness (7) than membrane filtration (t-test, $P < 0.01$). PCR failure prevented a comparison of the concentration methods with the original sample from the river in this case. When a sample from the same location, but taken at a different time to the others, was used as a proxy for the original sample, centrifugation, tangential flow filtration and membrane filtration gave higher taxa richness values than the proxy (data not shown).

Richness estimate produced from 454 data (Table 3-1) did not appear to show significant differences in number of observed species, with the exception of the tangential flow filtration concentrated marine sample which showed approximately twice the diversity in marine samples compared to alternative cell concentration methods.

3.5.1.4. Impact on biodegradation outcome

The lowest cell concentration resulting in a high probability of degradation (100%) was observed in inocula derived from concentrating cells by centrifugation (10^7 cells mL^{-1} with river and estuarine inocula), followed by tangential flow filtration (90% probability of degradation at 10^7 cells mL^{-1} with river inocula) and membrane filtration (100% probability of degradation at 10^8 cells mL^{-1} with river and estuarine inocula) (Figure 3-2). Glass bead colonisation showed a relatively high probability of degradation at higher inoculum concentration in river water HT-BSTs, but very low probability of degradation for concentrated estuarine samples. The poor probability of degradation was probably a result of the reduced diversity observed due to the preparation bias, selectively enhancing certain bacteria and potentially excluding competent degraders of the test compound from the original sample

The HT-BSTs showed no degradation of the test compound, 4-NP, at any concentration of marine inocula (Figure 3-2). It is suggested that this is not necessarily an indication of the biodegradation potential of 4-NP but may be due to a confounding factor, for example the 250 µL sterile mineral media may be inducing an

osmotic shock in the marine microorganisms. In addition, enhanced marine biodegradation tests for 4-NP have shown a tendency towards long lag phases, extending beyond a typical 28 day test and, in some cases, beyond a 60 day period (Chapter 4).

A summary of the ranked outcomes for scientific criteria can be seen in Table 3-2.

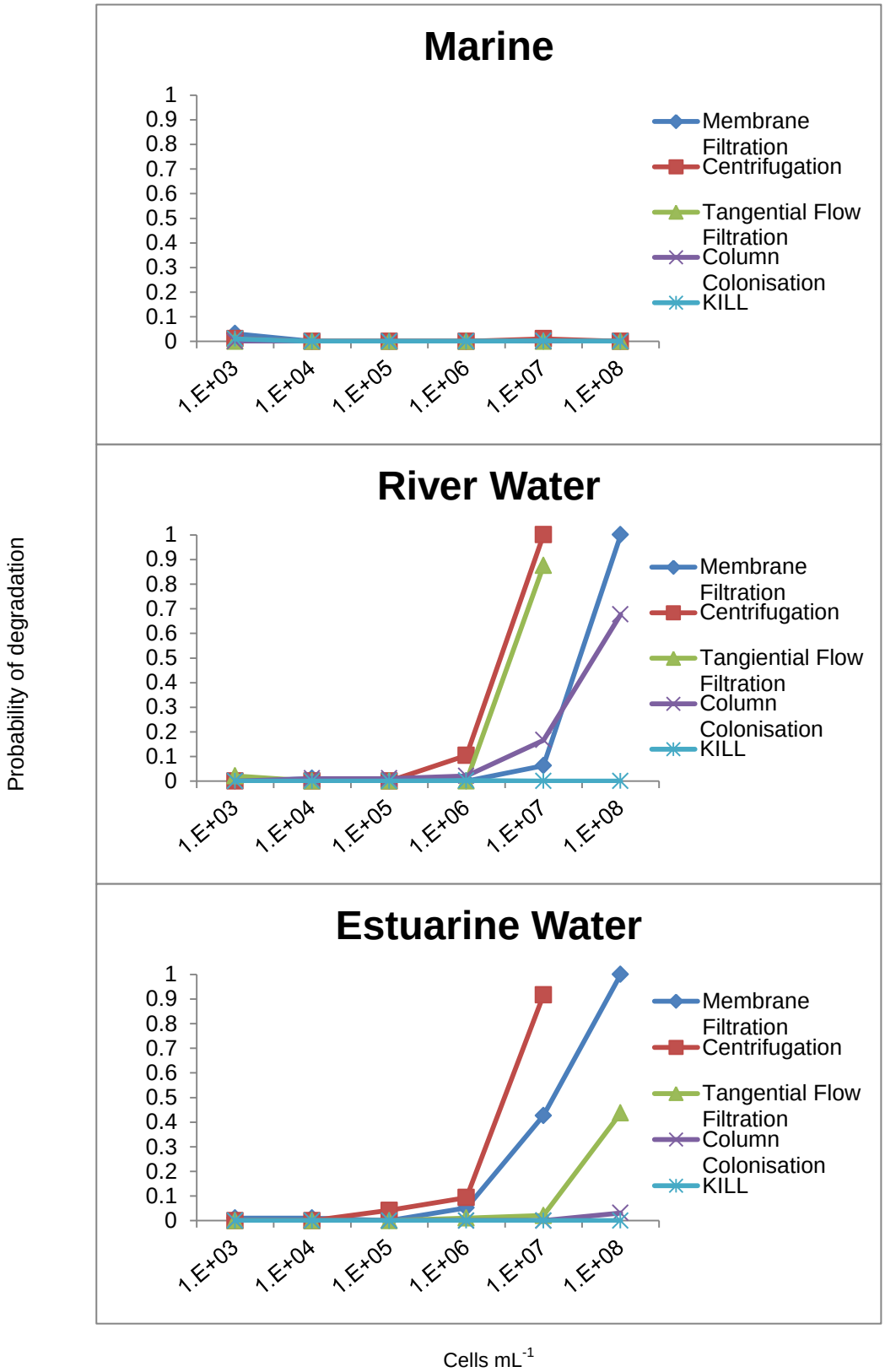


Figure 3-2 The probability of 4-NP biodegradation for concentrated environmental inocula prepared using various cell concentration techniques.

Table 3-2 Summary of ranked outcomes for cell concentration methods using the stated scientific criteria

Sample	Bacterial community similarity	Cell concentration	Band richness	Greatest probability at lowest [cell]
River	PCR Fail	MF=CC>TFF=C	C>TFF>*MF*>CC	C>TFF>MF>CC
Estuarine	MF>*TFF*>C>*CC*	MF=TFF=CC>C	MF>TFF=C=CC	C>MF>TFF>CC
Marine	MF>*TFF*>C>*CC*	MF>TFF=C=CC	TFF>*MF*>C>*CC*	C=TFF=MF=CC

C=centrifugation; MF=membrane filtration; TFF=tangential flow filtration; CC=glass bead colonization. Differences in font colour show treatments which gave statistically significant differences

3.5.2. *Practical criteria*

A summary of the practical considerations can be found in Table 3-3.

3.5.2.1. Equipment cost and availability

Of the methods evaluated, equipment for membrane filtration equipment is probably that most commonly found in environmental laboratories, as it is required for Standard Methods (APHA, 2005). The glass bead colonisation method was adapted to utilise readily available syringes, thus the glass beads would be the only piece of equipment not expected to already be part of an environmental laboratory. Although a centrifuge would be expected as a component of most laboratories, not all facilities are likely to have a centrifuge of the correct centrifugal force and capacity required for concentrating large aqueous samples. TFF units are widely used in the pharmaceutical industry; however they are not as yet widely employed in the majority of other types of laboratory. Tangential flow filtration units are available in a range of sizes dependent upon the process requirements with a price range to reflect the varying process capacities.

3.5.2.2. Operational and maintenance costs

The most considerable cost pertains to the staff hours required to process the samples which will ultimately approximate closely to the time required for the method to be performed. For tangential flow filtration, membrane filtration and glass bead

colonisation there are electricity and maintenance costs associated with requisite pumps, but the most significant outlay will be on consumables. Membrane filtration requires new filters; glass beads and tubing for glass bead colonisation; and whilst tangential flow filtration filters are reusable, cleaning and sanitising solutions are required to maintain the filter integrity.

Centrifugation is the most expensive method, expending considerable energy depending on the centrifuge capacity. Yearly service contracts are required and replacement parts can be costly. The broad range of procedures requiring the use of centrifugation, however, could arguably be considered a general laboratory expenditure rather than protocol specific.

3.5.2.3. Sample throughput

Sample throughout is highest in tangential flow filtration, followed by centrifugation, membrane filtration and glass bead colonisation respectively. Tangential flow filtration and centrifugation throughput are dependent upon the size of the system used, with tangential flow filtration manifolds and centrifuges being available with a range of process capacities. Glass bead colonisation requires sufficient time for the beads to be colonised making it the most time intensive method.

3.5.3. *Overall method ranking*

Concentration methodologies were ranked according to their performance for all of the scientific and practical criteria described above. A score of 1 was assigned to the best performing procedure(s) and protocols were ranked based on their average rank, with a lower overall average being the most desirable outcome (Figure 3-3).

Based on the scientific criteria, membrane filtration would be the best concentration method, scoring the highest average rank (Table 3-2). The highest average rank with respect to practical criteria was attributed to tangential flow filtration. Overall, as an average of the two main groups of criteria, tangential flow filtration was designated as the highest ranked method, closely followed by membrane filtration. Column colonisation was the lowest ranked method overall, performing particularly poorly with respect to the scientific criteria (Table 3-2). It is worth noting that, whilst no preferential weightings were given to any of the criteria selected, if the main criterion is considered to be the community similarity of the concentrated sample to the

original unprocessed sample, and the sample throughput, those methods ranked highest would still be the best performing, highest ranking systems.

The scientific criteria were applied to the inoculum sources as well as the concentration methods to determine any differences between environmental compartments. Practical rankings were deemed to be the same for all environmental compartments. Despite some subtle differences in the scientific rankings between compartments, the outcome of the overall ranking order was not affected (Figure 3-3).

Although the main practical considerations included costs of equipment, operation and training, it also included the throughput for each method. Throughput has a bearing on the number of unit tests which can be performed over a given time period, therefore also influencing the overall cost of biodegradation tests. Although membrane filtration and glass bead colonization were estimated to be the least expensive in terms of capital, they were also the two methods with the lowest throughput. Notwithstanding the poor rankings of the latter method in terms of the scientific criteria (Table 3-2), these methods would be logistically difficult to implement if large sample volumes are required (Figure 3-3).

3.6. Conclusions and Recommendations

- DGGE analysis indicated no significant difference in OTU richness between the original sample and samples concentrated by centrifugation, membrane filtration and tangential flow filtration. Glass bead colonisation, however, had a significantly lower OTU richness (P<0.001). 454 sequencing analysis is ongoing, but preliminary analysis suggests no significant difference between the original and concentrated samples.
- Increased inocula concentrations typically produced a greater probability of degradation in high-throughput biodegradation screening tests.

Based on the chosen selection criteria, it is recommended that there is no single concentration method fit for all purposes but rather the appropriate method should be selected on a case-by-case basis (Figure 3-3). The poor ranking of glass bead colonisation with respect to the scientific criteria (Table 3-2), in addition to the logistical unfeasibility of implementing on a laboratory scale, preclude this method from selection. The remaining methods, however, have their own role within cell

concentration. Membrane filtration would be the best method when biomass concentrations are not excessively high and relatively small volumes (up to 5 L) are being processed. The increased throughput capability of tangential flow filtration render it the most suitable method when processing large sample volumes, and centrifugation would be the most appropriate method for samples with high biomass concentrations, such as activated sludge.

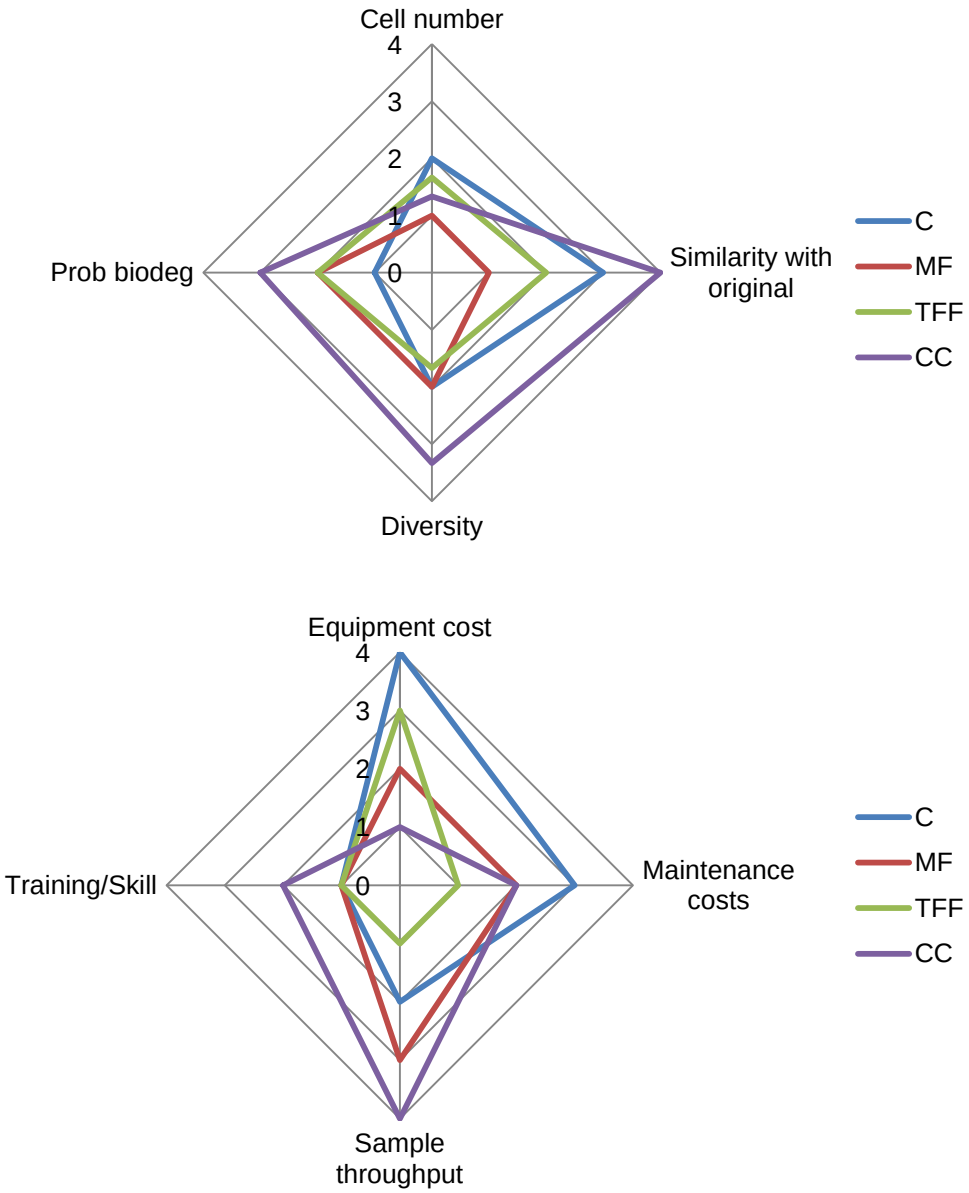


Figure 3-3 The average rankings for the cell concentration methods, with respect to scientific criteria (top) and practical criteria (bottom). Proximity to the centre of the radar graph indicates the performance of the technique, with the closest co-ordinates being the most desirable ranking. Where, TFF; tangential flow filtration, MF; membrane filtration, C; centrifugation, CC; column colonisation.

3.7. Acknowledgements

I would like to acknowledge the contribution of Dr. Andrew Goodhead in experimental design discussion and preparation of the concentration of aqueous samples via glass bead colonisation, his invaluable assistance with preparation of HT-BSTs and provision of training and assistance with performing bacterial community analysis.

Table 3-3 Summary of the practical considerations for implementing cell concentration methods for the preparation of inocula from aqueous samples for use in biodegradation screening tests

Practical Consideration	MF	TFF	C	CC
Availability of equipment within environmental laboratory	Standard lab equipment	TFF manifold not standard equipment	Standard lab equipment although existing centrifuge capacity may not be suitable	Standard lab equipment. Glass beads may need purchasing
Cost (assuming best and worst case scenario)	£1,000 - £5,000	£4,800 - £20,000	£4,000 - £20,000	£100 - £5,000
Operational and maintenance requirements	Vacuum pump; Filters	Peristaltic pump; Re-useable filters; Cleaning/Sanitising solutions; Periodic tubing replacement	Yearly service contracts (£1,000 - £2,000 p.a.); Replacement components; Electricity cost	Peristaltic pump; Syringes; Glass beads; Plastic tubing
Sample throughput (estimated in L h^{-1})	1.7	2.5-60	5	0.007-0.01

MF=Membrane Filtration; TFF=Tangential Flow Filtration; C=Centrifugation; CC=Glass bead (column) colonisation

Chapter 4

4. Investigation into the effect of increased cell concentrations and test volumes on the reliability of biodegradation screening tests

4.1. Introduction

The historical relevance of RBTs and their inadequacies as screening tests to characterise chemicals based on their persistence have been discussed previously (Sections 1.3.2.3; 1.3.3; 1.3.5). The poor definition of inocula characteristics, including bacterial cell concentration and inoculum source, are considered to be the predominant source of the high variability notoriously associated with standard ready biodegradability screening tests (Painter and King, 1985) affecting biodegradation kinetics (Simkins and Alexander, 1984; Battersby, 1990) and biodegradation outcome in screening tests (Thouand *et al.*, 1995).

Enhancements and modifications to existing biodegradation screening tests were proposed as an outcome from a European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) workshops on biodegradation and persistence (ECETOC, 2003; ECETOC, 2007; Comber and Holt, 2010; Snape, 2010). The purpose of these enhancements and modifications is to reduce the high variation typically observed in biodegradation screening tests and provide more reliable, reproducible degradation data from which assessments of persistency can be made. Enhanced and modified biodegradation screening tests were adopted into REACH legislation (EC, 2009), subject to a ring test with a set of reference chemicals of varying chemical persistence (Comber and Holt, 2010).

REACH was introduced in 2007 to replace existing legislation within the EU (Section1.3.4). It places an increased emphasis on intelligent approaches to Persistence, Bioaccumulation and Toxicity (PBT) testing. To this end, a number of enhancements and modifications to existing biodegradation screening studies have been identified to enable more effective prioritisation on persistence.

The enhancements proposed under REACH aim to develop a more environmentally relevant test, somewhere between a primary screening test and more expensive, higher tier testing (ECETOC, 2007; ECHA, 2008; Comber and Holt, 2010; ECHA, 2012), including:

i. **Extending the test duration beyond the minimum half-life threshold that defines persistence;**
ii. **Increasing the biomass density or concentration in the inoculum;**
iii. **Increasing the test vessel size;**
iv. Allowing pre-adaptation of inocula with the test substance;
v. Allowing semi-continuous test systems that include pre-adaptation and/or growth of potential degrader organisms to occur.

4.1.1. *Proposed enhancements to be investigated*

Extending test duration to better understand environmental fate and increasing inoculum concentration and test volume are discussed here.

The limitations regarding over-stringent testing durations in RBTs are discussed in Section 1.3.3. OECD RBTs have a test duration of 28 days (with the additional 10-day window to fulfil as previously described (OECD, 1992)). The predominant concern when testing with a relatively short time-frame is that the actions of slow growing organisms may be missed due to extensive lag phases. (Thouand and Block (1993); Thouand *et al.* (1996); Toräng and Nyholm (2005)) have all reported degradation of compounds following extended lag phases due to exposure of the test compound to the inoculum and the opportunity for slow growing degrader organisms to establish a community. There have been recommendations for tests assessing persistency to consider an extended testing period (ECHA, 2008; EC, 2009). The testing timeframe proposed within REACH (ECHA, 2008) is shown in Figure 4-1. It may be necessary to extend tests beyond 60 days in order to gain an accurate representation of chemical fate in the environment, particularly where degradation is occurring and has not yet reached zero order (i.e. three consecutive static observations with no increase in chemical removal), or where there is chemical or biological evidence to suggest the presence of a long lag phase.

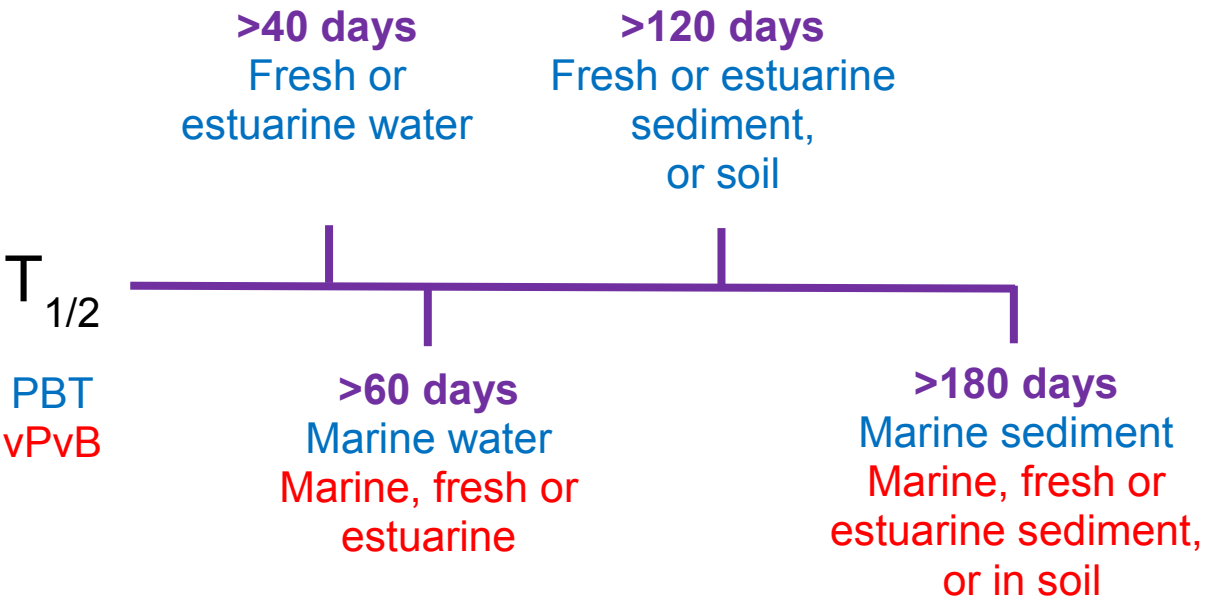


Figure 4-1 Proposed test durations for assessing persistency within REACH (ECHA, 2008)

The proposed enhancements of increasing inoculum concentration and cell concentration are focusing on increasing the total number of cells within a screening test. Nyholm *et al.* (1992) reported the association between low cell numbers and increased variation in screening tests. Goodhead *et* al. (2013) reported a correlation between increased diversity and the probability of a positive outcome in a high throughput biodegradation screening test. This is linked to the phenomenon termed the *'biodegradation lottery'* whereby the probability of a positive outcome in a biodegradation test is dependent upon the chance inclusion of specific degraders of the test compound within the test inoculum (Section 1.2.3). It seems a reasonable presumption that a greater total number of cells will increase the diversity of an environmental sample (Goodhead, 2009), in much the same way that using a larger sample size provides more reliable information about a population in human studies. Increasing the total cell number used within biodegradation screening tests is expected to provide a more environmentally representative inocula, with more consistency between replicates and tests, which will ultimately reduce the variation typically associated with screening tests and provide more reliable and reproducible degradation data.

4.1.2. *Existing research into the effect of cell concentration on biodegradation*

Davenport *et al.* (2010) showed a statistically significant link between an increase in inoculum diversity and the probability of a positive outcome in a high throughput RBT, allowing a more reliable prediction on biodegradation. Investigations by Goodhead (2009) built on work performed by Thouand *et al.* (1995), who characterised 11 inocula sources (activated sludge and rivers) based on suspended solids, total bacteria, cultivable bacteria and specific biodegraders and then used a descriptive model linking the probability of 4-nitrophenol degradation to the concentration of the characterised inocula. An inoculum concentration of 52 mg SS L^{-1} was determined as necessary for the model to return as close to a guaranteed probability of degradation as possible (99%), assuming the chemical being tested is biodegradable. Interestingly, the model was used to determine the probability of degradation using the inoculum characteristics defined within the OECD guidelines for the different types of RBT (OECD, 1992a). Probability of degradation was found to range from 0% to 99% based on the test selected and the range of concentrations recommended within each individual test (Thouand *et al.*, 1995). The largest intra-test variation arose within the Sturm test (5% to 65% probability of degradation dependent upon inoculum concentration). The variation in predicted biodegradation outcome observed in the model described by Thouand *et al.* (1995) is representative of the variation reported in laboratory experiments, showing disparity in biodegradation outcome dependent upon the test RBT applied (Gerike and Fischer, 1979; Gerike and Fischer, 1981), the inoculum source (Goodhead *et al*., 2013) and inoculum concentration (Chapter 3).

4.1.3. *Assessing study duration and biodegradation thresholds*

In addition to increasing the total cell numbers within screening tests, there has been a call for reassessing the duration of screening tests, which are currently set at 28 days with a ten day window for degradation (Section 1.3.2.3). The experiments presented in this Chapter assessed biodegradation success based on a 60 day test (with no degradation window) recommended for persistence assessments (Comber and Holt, 2010). This was extended beyond 60 days where degradation had commenced and not yet plateaued (i.e. 3 consecutive static observations with no increase in chemical removal), or where there was evidence to suggest that degradation would occur following a long lag phase.

A positive outcome in a biodegradation test is defined as a 60% reduction in theoretical oxygen demand (ThOD) or 60 % theoretical carbon dioxide evolution ($ThCO_2$), or a 70% reduction in DOC within ten days of 10% degradation being achieved in a 28 day test (OECD, 1992a).

Persistence is typically assessed based on half-life data, a measure that is currently not assessed in RBTs. It is also used as an input in modelling efforts to characterise chemical risk. The paucity of real half-life data leads to the use of read-across methods (OECD, 2013a) which can lead to erroneous values, for example the half-life calculated from an experiment where a readily biodegradable compound is exhibiting a long lag phase may lead to the compound being classed as persistent. The use of 50% endpoints would bring testing more in line with regulatory assessment of persistence and would facilitate modelling efforts.

4.2. Objectives

- *Investigate the effect inoculum concentration has on biodegradation outcome and inoculum characteristics in enhanced biodegradation screening tests*
- *Investigate the effect test volume has on biodegradation outcome and degradation characteristics in enhanced biodegradation screening tests*
- *Determine a recommended inoculum concentration and test volume to be used in future activated sludge and marine biodegradation screening tests*

4.3. Hypotheses

- *Increases in inoculum concentration and test volume will correlate with an increased probability of degradation in a biodegradation screening test*
- *Increases in inoculum concentration and test volume will result in decreases in variability between replicates and provide more reproducible data*
- *Increases in concentration will correlate to an increase in diversity within inocula used in biodegradation screening tests*

4.4. Materials and methods

An overview of the experimental process is provided for AS (Figure 4-2) and marine (Figure 4-3) inocula with more detail given where necessary.

4.4.1. *Sampling*

4.4.1.1. Activated sludge (AS)

AS was obtained from Buckland Sewage Treatment Works, Newton Abbot, Devon, UK. Buckland STW is a non-nitrifying plant with a population equivalent of approximately 80, 000, treating predominantly domestic waste.

AS was collected on two separate occasions: once for the cell concentration experiments and again for the test volume experiments. For each sampling event, 40 L AS were collected in sterile carboys. AS samples were collected using a sterile collection bucket secured with a length of rope. AS collected for concentration experiments and volume experiments was sampled in June and October respectively. Samples were aerated at room temperature until use on the same, or subsequent, day.

4.4.1.2. Marine inocula

Marine samples were collected from a 35,000 L on-site storage tank at Brixham Environmental Laboratory (AstraZeneca UK Ltd.), drawn from the sea adjacent to the laboratory. Typically 10-15,000 L per day is drawn through for husbandry purposes ensuring constant replenishing of the contents. Samples were collected after a 25 µm influent mesh screen but prior to any further on-site treatment. Samples were either collected as raw inocula or pre-screened through a filter with 10 µm nominal pore size. Marine samples for concentration experiments and volume experiments were sampled in March and November respectively.

4.4.2. *Inoculum preparation*

Preparation methods were selected based on the outcomes from Chapter 3. AS samples were concentrated using centrifugation due to the high initial biomass concentrations and relative density. Marine samples were concentrated using tangential flow filtration due to the high process throughput required.

4.4.2.1. Concentration of activated sludge samples

Following determination of TSS, 20 L of the AS was concentrated by transferring to 500 mL Nalgene bottles. AS was centrifuged at 30,000 x *g* for 10 minutes (Section 2.5.1), approximately half of the supernatant was discarded; the pellet was resuspended and returned to the aerated carboy. TSS was determined again. The remaining 20 L of AS was left as received for the low biomass concentration inocula. The inocula were amended with sterile OECD mineral media to give the concentrations shown in Figure 4-2 as detailed in Section 2.5.1.

4.4.2.2. Concentration of marine samples

Tangential flow filtration was used to concentrate marine inocula as previously described (Section 2.5.2; Figure 2-2). There were four levels of nominal concentration: (i) 100 × nominal concentration, (ii) 10 × nominal concentration, (iii) filtered and (iv) raw (Section 2.5.2.). Sterile mineral media (OECD, 1992b) was added to the seawater which was kept aerated at room temperature until use on the same day.

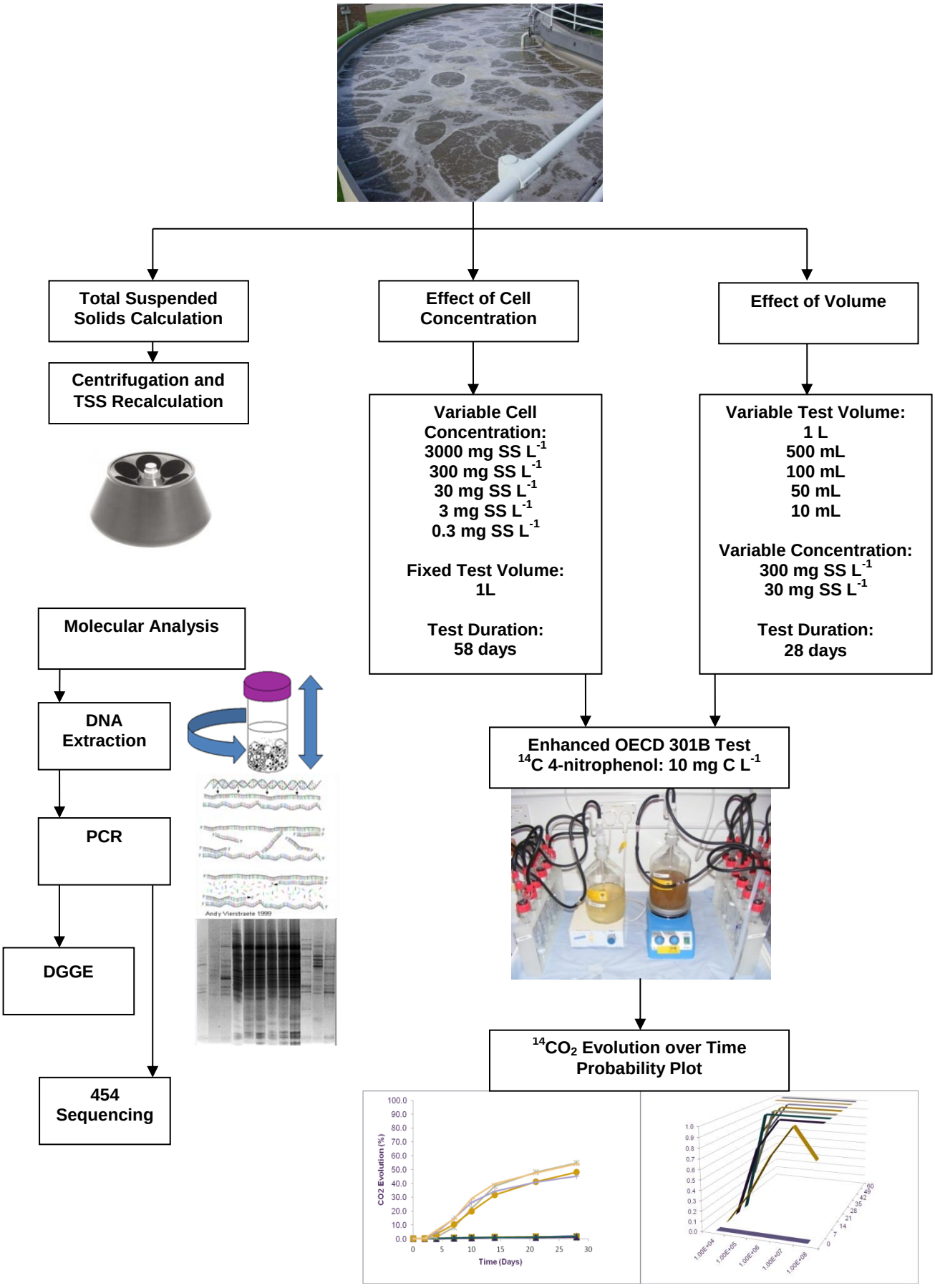
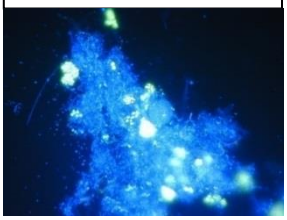


Figure 4-2 Summary of the methodological processes for investigating the effect of cell concentration, test volume and increased test duration in enhanced activated sludge biodegradation screening tests

Cell Concentration: TFF

Total Cell Counts



Effect of Cell Concentration

Variable Cell Concentration:
100X nominal conc.
10X nominal conc.
10µm filtered
Raw

Fixed Test Volume:
1L

Test Duration:
90 days

Effect of Volume and Concentration

Variable Concentration:
100X nominal conc.
10X nominal conc.
10µm filtered
Raw

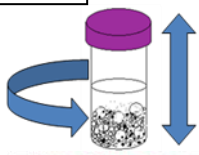
Varied Test Volume:
1L
500 mL
50 mL

Test Duration:
120 days

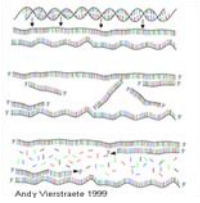
Enhanced OECD 301B Test
^{14}C 4-nitrophenol: 10 mg C L^{-1}
^{14}C Aniline: 10 mg C L^{-1}

Molecular Analysis

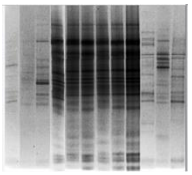
DNA Extraction



PCR

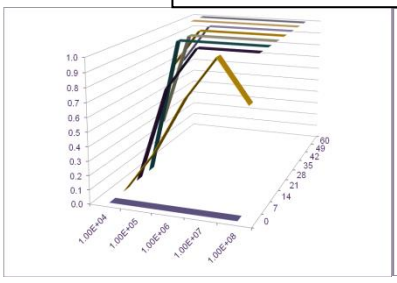


DGGE



454 Sequencing

$^{14}CO_2$ Evolution over Time
Residual ^{14}C Activity
Probability Plot



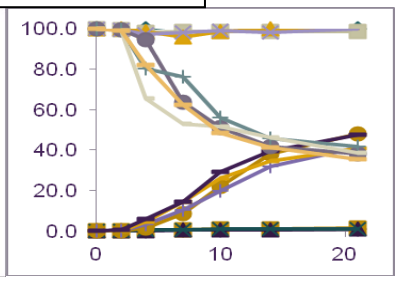


Figure 4-3 Summary of the methodological processes for investigating the effect of cell concentration, test volume and increased test duration in enhanced marine biodegradation screening tests

4.4.3. *Test chemical preparation*

Chemicals were prepared as previously described by first preparing concentrated stock solutions of unlabelled test compounds in sterile mineral media. The required amount of stock, based on the total volume of test inocula to be dosed, was calculated (i.e. 10 mL per L inoculum to be dosed based on a 1 g C L^{-1} stock solution and a test concentration of 10 mg C L^{-1}). Radiolabelled stock was added to the non-labelled stock; triplicate 0.1 mL subsamples were taken so as to calculate the applied radioactivity (Section 2.5.3).

4.4.4. *Enhanced biodegradation screening test preparation*

Test chemical solutions were prepared as in Section 2.5.3.

Triplicate biodegradation screening test systems were prepared, as shown in Figure 2-3, based on the variables defined in Figure 4-2 and Figure 4-3. A full summary of the systems used is given in Table 4-3. Test chemicals were applied to the relevant test vessels at a ratio of 10 mL dosing stock to 1 L test inocula to give final test concentrations of 10 mg C L^{-1}. The experiments were performed essentially as OECD 301 B biodegradation screening tests with enhanced inocula (OECD, 1992a). Systems were kept aerobic at 22ºC (±2ºC), in a temperature-controlled room with a red light to aid sampling.

The test systems which provided the fastest rates of degradation, shortest lag phases and smallest inter-replicate variation in the enhanced AS concentration study were selected to be involved in subsequent investigations into the effect of test volume on biodegradation outcome in enhanced AS screening tests.

4.4.5. *Determination of biodegradation*

Biodegradation outcome was assessed based on OECD pass thresholds (OECD, 1992) and the half-lives proposed for persistency assessments (ECHA, 2008). Probabilities of degradation were assessed against 60% removal of the test compound but were not confined by the 28-day test and 10-day window used in OECD RBTS (OECD, 1992a). A positive biodegradation outcome within OECD guidelines is classified as 60% degradation within a 10-day window of a 28-day test. Degradation was assessed in the present investigations based on 60% degradation and $t_{1/2}$ in tests that ranged in duration from 28 days up to 120 days. Where degradation had commenced and not yet reached zero order, or where there was

evidence to suggest the presence of a long lag phase, experiments were continued to a maximum of 120 days.

Biodegradation was determined by the capture of evolved $^{14}CO_2$ in 2M NaOH and LSC analysis (Section 2.5.5.1).

In marine experiments, the residual ^{14}C activity was also calculated as a measure of the chemical remaining in solution (Section 2.5.5.2). Residual activity was not calculated for 50 mL systems due to the requirement of a 5 mL subsample, which would have removed a significant proportion of the inoculum.

In all experiments, a mass balance was performed at the end of the experiment to account for the applied radioactivity (Section 2.5.5.3).

4.4.6. *Bacterial community analyses*

Extraction of total DNA, PCR, DGGE, preparation of amplicon libraries for 454 sequencing and subsequent analyses were performed as detailed in Section 2.6.

Samples used for DGGE analysis were collected at the start of the experiments and samples used for 454 sequencing were collected at the end of the respective experiments.

4.4.7. *Statistical analyses*

4.4.7.1. Analysis and interpretation of biodegradation data

The cumulative radioactivity at each sample point was used to calculate the percentage degradation of the test chemical, calculated as displayed in Equation 2-4 and Equation 2-5, whereby 1% $^{14}CO_2$ evolution was given to represent 1% degradation of the test chemical. The $^{14}CO_2$ evolution was plotted against time in SigmaPlot (Systat software Inc., London, UK) for both individual replicates of each system and average results.

Based on the current 60% degradation OECD RBT pass threshold, replicates were assigned a score of one, if the chemical had degraded, and zero if not. The average of the three replicates was used as the probability of a positive biodegradation outcome for a given system at a given time.

The degradation descriptors described in Table 1-1 were calculated for all systems, based on the $^{14}CO_2$ evolution curves. The 60% descriptors were based on the

current OECD pass threshold of 60% reduction in ThOD or $ThCO_2$. The 50% descriptors were based on the proposed 60 day half-life persistency assessments under REACH (ECHA, 2008) and the half-life requirements of the majority of predictive modelling tools. Several of these indices have been used previously (t_L and Δt_{50}) (Nyholm and Kristensen, 1992), with additional measures reflecting shifts in chemical assessment strategies ($t_{1/2}$, t_{60}, Δt_{60} and K_{max}).

4.4.7.2. Analysis of variance

In occurrences where the data was non-normal, the optimal transformation was determined using Box-Cox transformations and applied to the data, which was again tested for normality. In the case of non-normal transformed data, non-parametric analyses were performed including Moods-median and Kruskall-Wallis tests (Fry, 1993).

The coefficient of variation for degradation descriptors were calculated using Minitab (Minitab Ltd., Coventry, UK), which were then analysed using a one-way ANOVA to determine whether there was a significant difference in the inter-replicate variation between different systems.

In instances where biomass concentration and test volume were both investigated, a nested ANOVA and analysis of means was used to determine where variation occurred and where significant differences were present.

4.5. Results and Discussion

4.5.1. *Activated sludge inocula*

4.5.1.1. Effect of cell concentration in enhanced AS screening tests

There was a general trend for tests with higher biomass concentrations to exhibit more rapid rates of degradation and shorter lag phases (Figure 4-4). The highest biomass concentration of 3000 mg SS L^{-1}, however, showed longer lag phases, slower degradation rates and higher variability between replicates than the 300 mg SS L^{-1} system, with degradation descriptors akin to the 30 mg SS L^{-1} systems (Figure 4-4; Figure 4-5; Figure 4-6).

The most probable explanation is that the increased biomass concentration introduced a new source of energy such that the test chemical was no longer the sole carbon source and microbes preferentially metabolised the more readily available source. An alternative theory is that the competition for scarce resources triggered a self-mediated apoptosis within some cells in order to preserve the integrity of the community. This programmed cell death (PCD) is typically associated with phage-infected cells which present a danger to the population (Yarmolinsky, 1995), but has also been observed in starved populations where PCD has been initiated in bacteria to ensure the survival of a core population, with the nutrients relinquished by the perished cells used to sustain the residual population (Aertsen and Michiels, 2004). Future research into the effect of enhanced concentrations on concomitant DOC and biodegradation outcome may yield further information.

There was a trend of increasing inter-replicate variability with decreasing biomass concentration (Table 4-1; Figure 4-4): the rate of degradation and lag phase varied more at lower suspended solids concentrations than higher ones (Figure 4-5; Figure 4-6; Figure 4-7). Analogous variations have been associated with RBTs before, as a result of the low microbial inocula concentrations stipulated by OECD tests (OECD, 1992a). At lower biomass concentrations, the environmental sample would be expected to show less diversity and as a consequence have a reduced likelihood of specific degrader inclusion. Low microbial biomass densities stipulated in some OECD RBTs lead to greater variability between replicates with respect to the bacterial community composition, which is then reflected in the high degree of variability observed in the tests (Table 4-1; Figure 4-4) (Goodhead *et al*., 2013).

The coefficient of variation was calculated for each degradation descriptor at each concentration: $\boldsymbol{t_L}$, $\boldsymbol{t_{1/2}}$, $\boldsymbol{\Delta t_{50}}$, $\boldsymbol{t_{60}}$, $\boldsymbol{\Delta t_{60}}$ and $\boldsymbol{K_{max}}$ (Figure 4-5; Figure 4-6). The coefficients were analysed by ANOVA in Minitab and the degradation descriptors from the tests incorporating more concentrated inocula (3000 – 30 mg SS) were found to be significantly different from the tests incorporating a lower biomass concentration (3 mg SS: $p < 0.01$), but not significantly different to each other. The 0.3 mg SS biomass concentration showed lower variation between replicates than the 3 mg SS tests and was not significantly different to the higher biomass concentration coefficients. This result may be misleading as variation in $^{14}CO_2$ evolution was high in 0.3 mg SS tests (Figure 4-4). The lower variation was most

probably due to the degradation descriptors selected, particularly the low variation observed between K_{max} values which were considerably lower in 0.3 mg SS tests, in both magnitude and inter replicate variation, than in other biomass concentrations. An average of these measures is shown in Table 4-1. The general trend for increasing inter-replicate variability with decreasing biomass concentration is apparent with the greatest variability seen in the 3 mg SS L^{-1} system. The lowest variation was seen with the 300 mg SS L^{-1} inocula.

Table 4-1 Mean coefficient of variation for the following degradation descriptors: . t_L, $t_{1/2}$, Δt_{50}, t_{60}, Δt_{60} and K_{max} Values were significantly different ($P < 0.01$)

System (mg SS L^{-1})	Approximate cell concentration (cells mL^{-1})	Mean coefficient of variation (%)
3000	10^8	19.1
300	10^7	13.1
30	10^6	27.3
3	10^5	57.2
0.3	10^4	25.5

Greater probabilities of degradation were observed in shorter timeframes in higher biomass concentration tests (Figure 4-7). By the end of the study, all tests reported a 100% probability of degradation at both the 50% and 60% threshold. Although not all tests had achieved this within the ten-day window of a 28 day as stipulated in OECD RBTs (Figure 4-7).

OECD tests typically use biomass concentrations of 30 mg SS L^{-1} and 3 mg SS L^{-1} systems (OECD, 1992a); these biomass concentrations both degraded the test chemical, 4-NP, within 28 days and within a 10-day window i.e. a level sufficient to achieve a pass within OECD RBTs. Based on a half-life threshold of 60 day persistency assessment, all inocula concentrations would classify 4-NP as being non-persistent. This is in keeping with its current assessment as an inherently biodegradable compound (Comber and Holt, 2010).

4-NP is considered a difficult compound to assess due to its variable degradation in standard tests. Gerike and Fisher (1979) found ranges of 0-100% in 4-NP removal depending on the RBT selected. As previously discussed, Thouand *et al*. (1995) also

found variable degradation of 4-NP depending upon concentration of inoculum and the RBT selected. Goodhead (2009) found considerable variation in degradation outcome of 4-NP in AS, depending upon the source and the concentration of inoculum used in high throughput biodegradation screening assays. Enhanced screening tests incorporating more concentrated inocula appear to have a positive impact on reducing the variation typically associated with screening tests (Figure 4-4; Figure 4-5; Figure 4-6; Table 4-1).

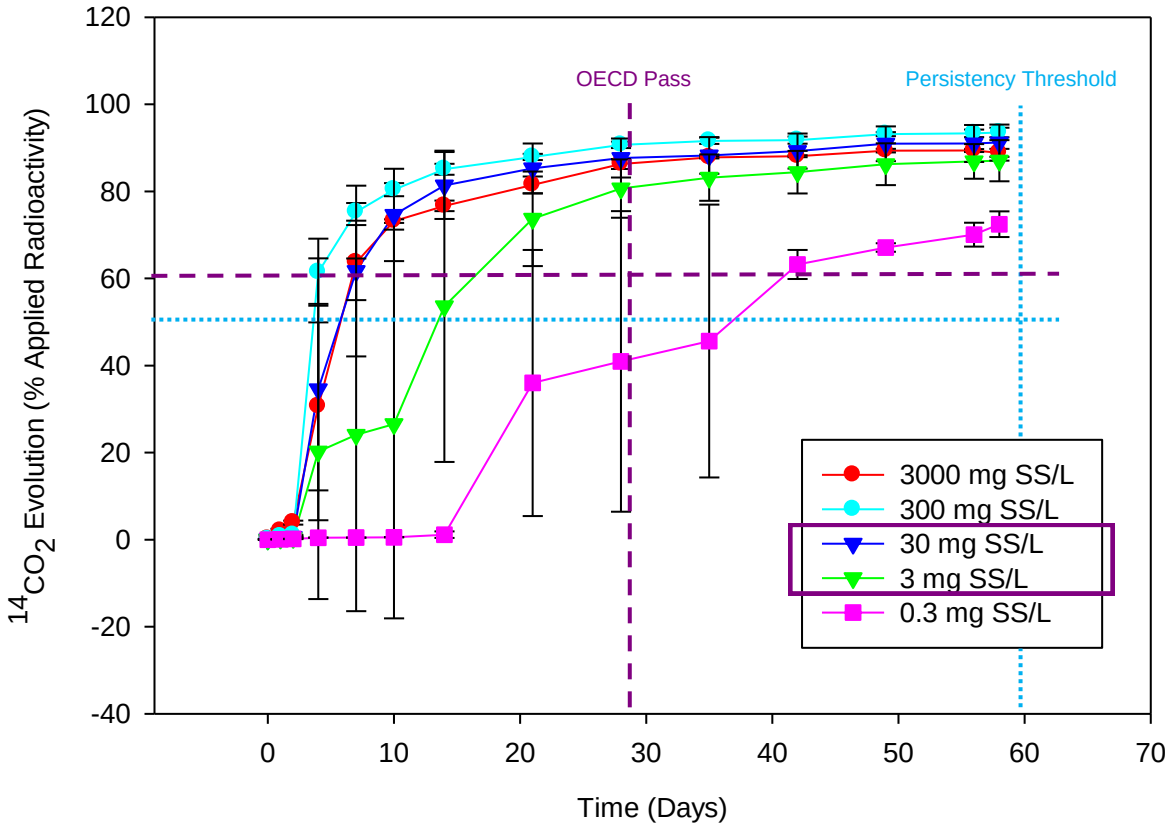


Figure 4-4 $^{14}CO_2$ evolution over time for activated sludge systems with different biomass concentrations. Error bars shown are +/- 1 s.d.

t_L:

Duration of the lag phase i.e. time taken to reach 10% degradation

$t_{1/2}$:

Time taken to reach 50% degradation from 0% degradation. Half-life. Used in predictive modelling and proposed as persistency assessment threshold

Δt_{50}:

Time taken to reach half-life from the end of the lag phase. This value indicates how quickly degradation might occur with sufficient density of degrader organisms, assuming that the lag phase allows the establishment of a competent degrader community whose catabolic activity has been activated

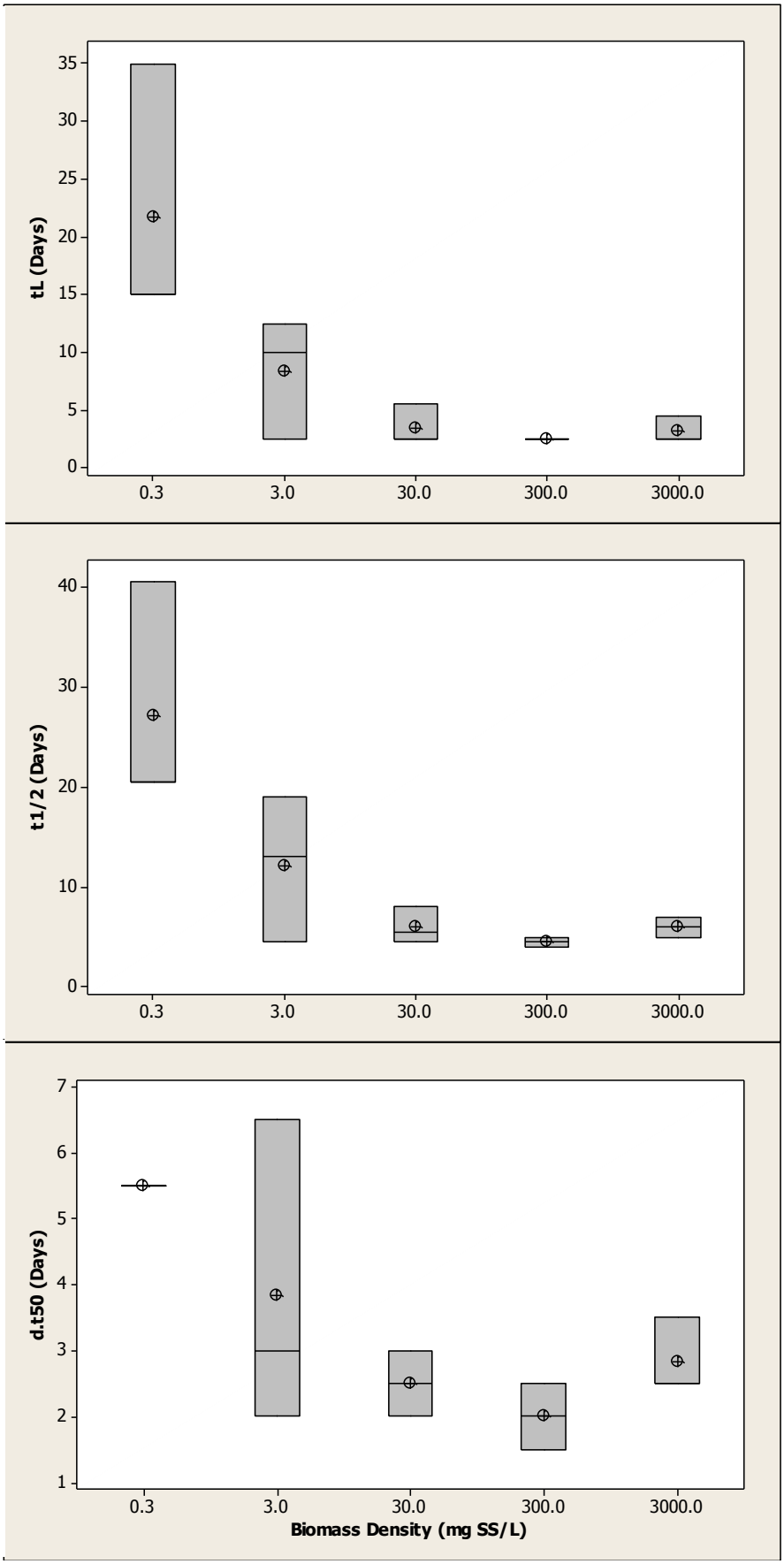


Figure 4-5 Boxplots of t_L, $t_{1/2}$ and Δt_{50} for different biomass concentrations from enhanced RBTs. Values were calculated from individual replicate data. Each replicate is represented by a horizontal line, with the mean of the three replicates represented by ⊕

t_{60}:

Time taken to reach the OECD RBT pass threshold of 60% reduction in $ThCO_2$

Δt_{60}:

Time duration from the end of the lag phase to the RBT pass threshold of 60% degradation. A value of less than 10 would be required to fulfill the ten day window aspect of the pass criteria

K_{max}:

Maximum rate of degradation observed. Determined by calculating the gradient at the steepest section of the $^{14}CO_2$ evolution curve. Values were calculated from the individual replicate curves as opposed to the average curve displayed in Figure 4-4.

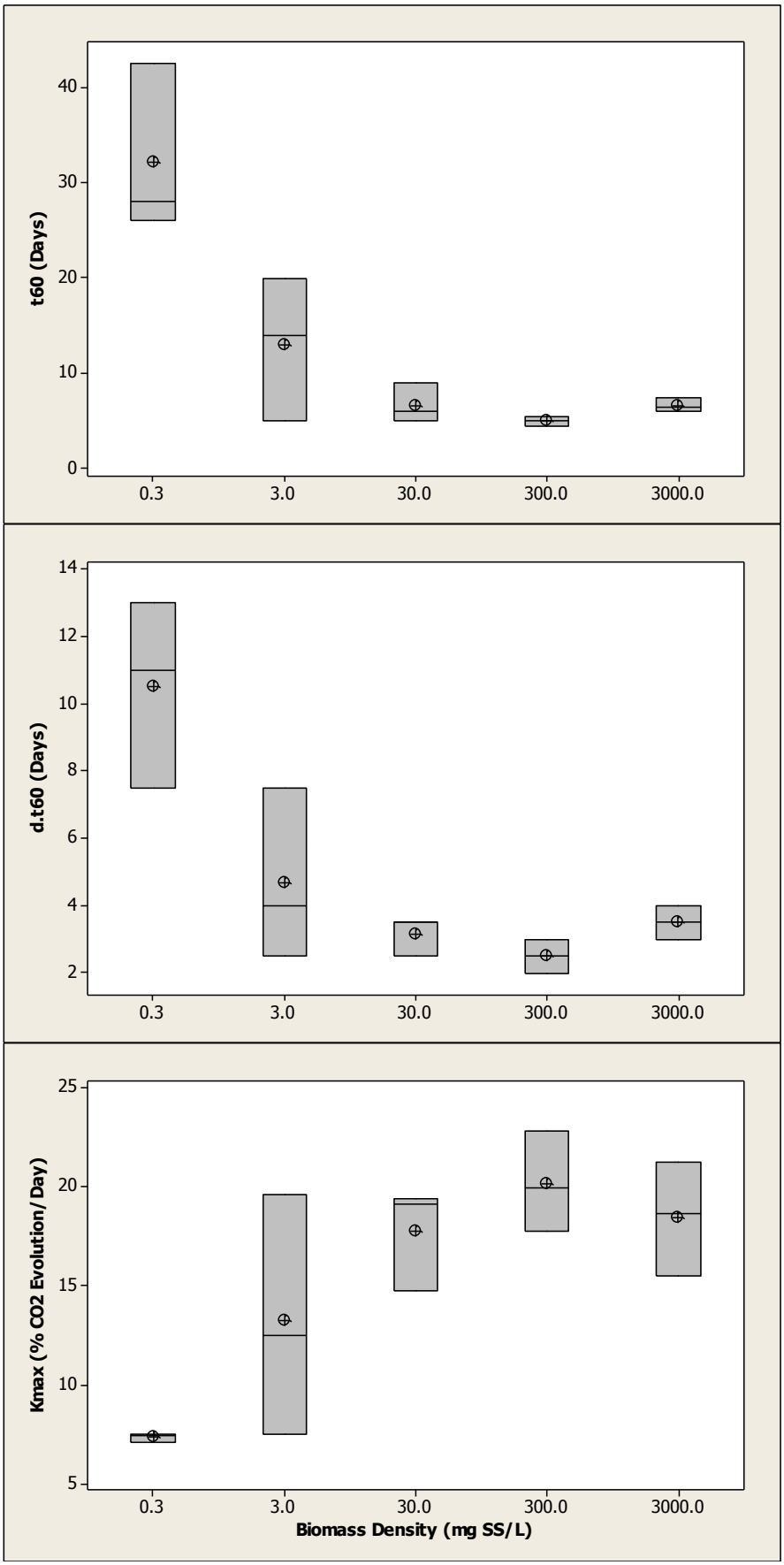


Figure 4-6 Boxplots of t_{60}, Δt_{60} and K_{max} different biomass concentrations from enhanced RBTs. Values were calculated from individual replicate data. Each replicate is represented by a horizontal line, with the mean of the three replicates represented by ⊕

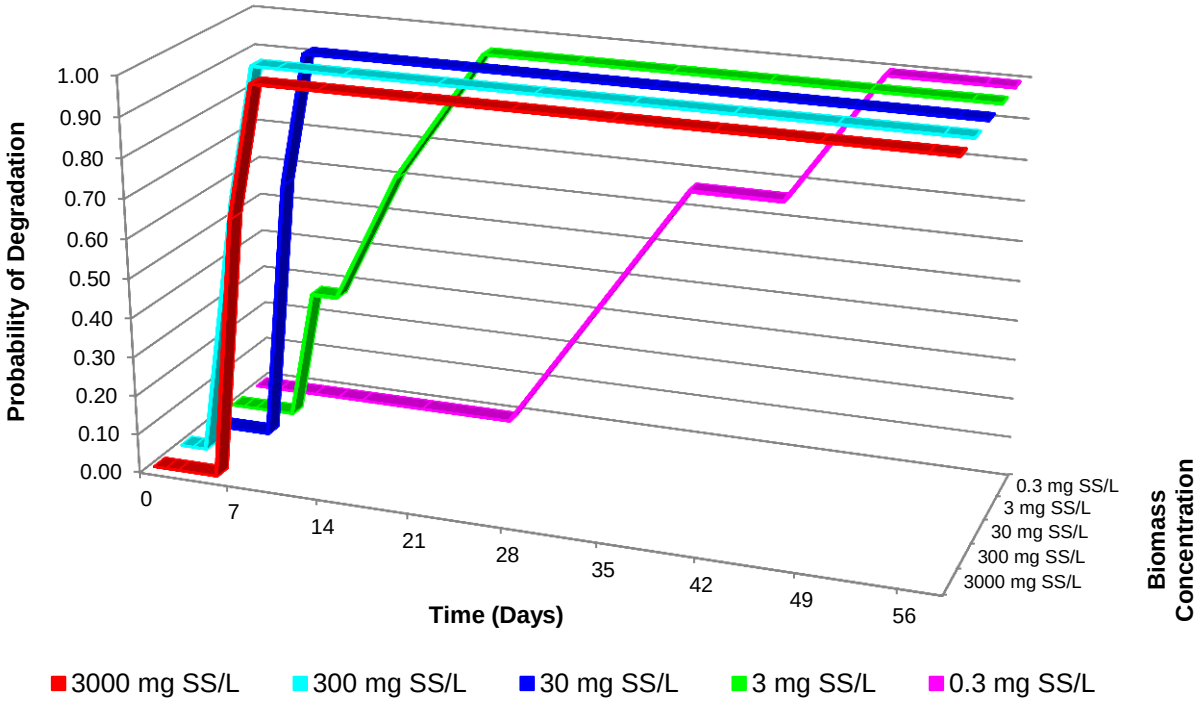


Figure 4-7 Probability of a positive outcome in a biodegradation screening test based on the OECD RBT pass threshold of 60% reduction in $ThCO_2$, for a given biomass concentration of activated sludge at a given time

4.5.1.2. Analysis of diversity

An estimate of operational taxonomic unit (OTU) richness of the most abundant taxa was made using DGGE images (Figure 4-8) by calculating the number of bands in each lane, where each band is given to represent an OTU. The position of the OTUs in the gel was also used to make comparisons of the community similarity between samples based on the presence and absence of bands. The data was grouped together using UPGMA clustering to produce a dendrogram indicating the percentage similarity of communities to each other. Primer facilitates NMDS analyses of these outputs to visualise community similarity, whereby the closer samples are to each other, the greater similarity they show (Figure 4-9).

The communities of enhanced tests with lower biomass concentrations were less similar and more variable than those with higher biomass concentration (60-75% similarity in highest biomass concentration in comparison with 25-50% similarity in lowest biomass concentration inocula, based on UPGMA clustering analysis in BioNumerics (Figure 4-8); this was highlighted by PRIMER analysis in Figure 4-9). In

addition, those with lower biomass concentrations typically showed lower band richness (mean OTU richness of 8 in lowest biomass concentration inocula and 26 in highest biomass concentration inocula (Figure 4-8)) with less similarity between replicates than those with high biomass concentrations. DGGE samples presented were collected at the beginning of the experiment (Figure 4-8 and Figure 4-9).

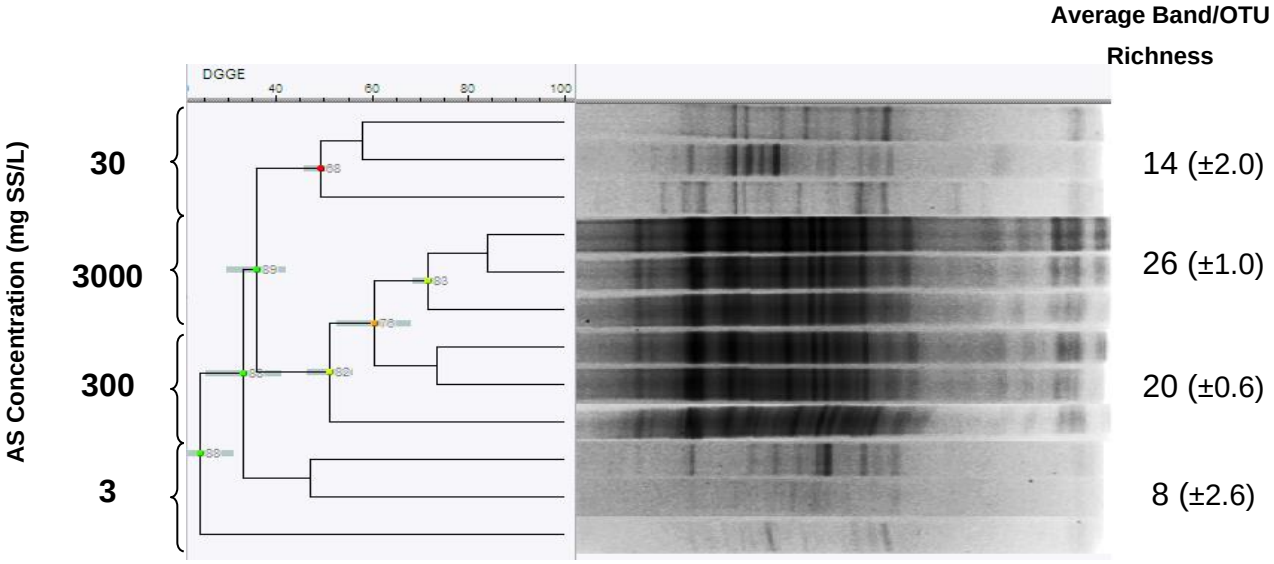


Figure 4-8 DGGE fingerprint gel (right) and dendrogram cluster analysis (left); the scale bar represents percentage similarity between samples based on the presence and absence of bands, as calculated using UPGMA clustering. On the far right of the figure is the average band richness for the different inocula concentrations. 0.3 mg SS L^{-1} systems were omitted due to PCR failure.

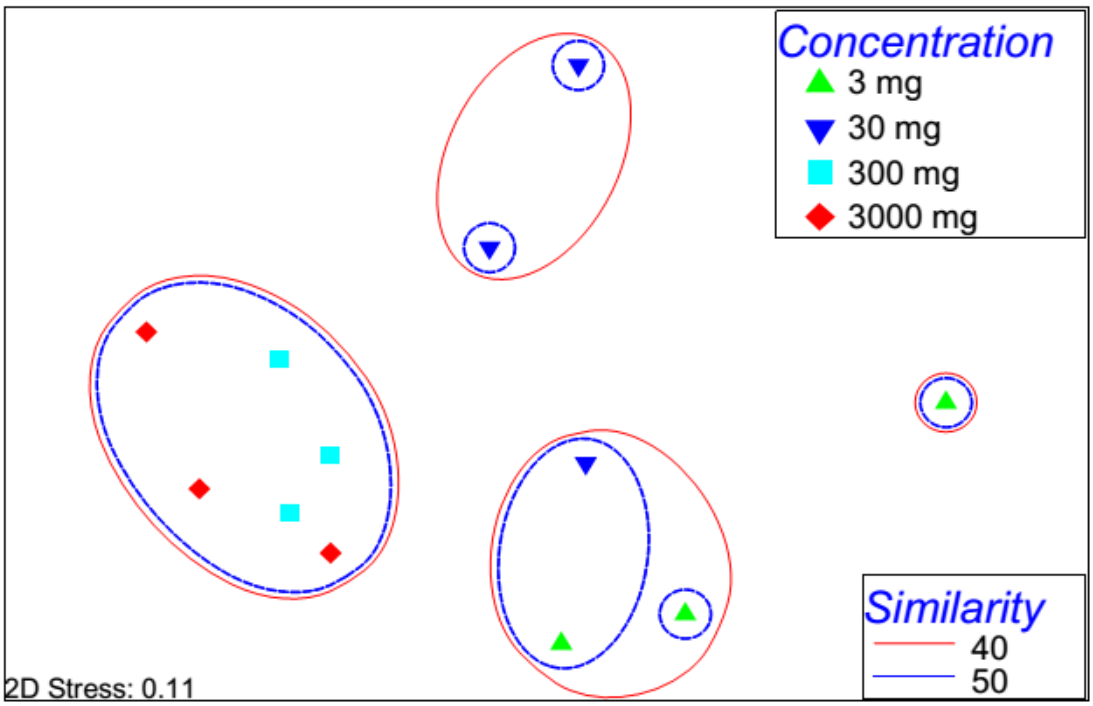


Figure 4-9 NMDS plots showing community similarity based on statistical analysis of the presence and absence of bands in the DGGE image shown in Figure 4-8. Samples cluster together based on their similarity. Greater similarity was seen with increasing inocula concentration. 0.3 mg SS L^{-1} systems were omitted due to PCR failure.

Diversity analysis demonstrated a trend of increasing inter-replicate variability with decreasing biomass concentration. The lower biomass concentration tests may have shown higher degrees of variation, simply because the systems themselves were so much more variable than high biomass concentration systems.

4.5.1.3. Sample coverage

Analysis of the 454 data allowed for calculation of α diversity indices within QIIME. The Chao1 diversity index can be used as a measure of how well an environment has been sampled, in other words how much of the true diversity contained within an environment has been captured (Hughes *et al.*, 2001). When chao1 is plotted against the number of reads per sample, data which plots a more pronounced asymptote is considered to be more completely sampled.

Figure 4-10 shows the chao1 data from samples collected for bacterial diversity analysis at the end of the respective studies. Higher biomass concentration samples typically exhibited a more pronounced asymptote than lower biomass concentration samples, which would suggest a more comprehensive assessment of the diversity within the environment. Higher biomass concentration inocula appeared to contain fewer OTUs than lower biomass concentration inocula, with several dominant species. This is probably an indication of enrichment of organisms which are capable of growth using the chemical as a substrate. Lower biomass concentration inocula appear to exhibit communities which are less enriched and therefore less dominated by a smaller number of OTUs. Lower biomass concentration inocula appeared to show communities with greater evenness as a result of lower levels of enrichment.

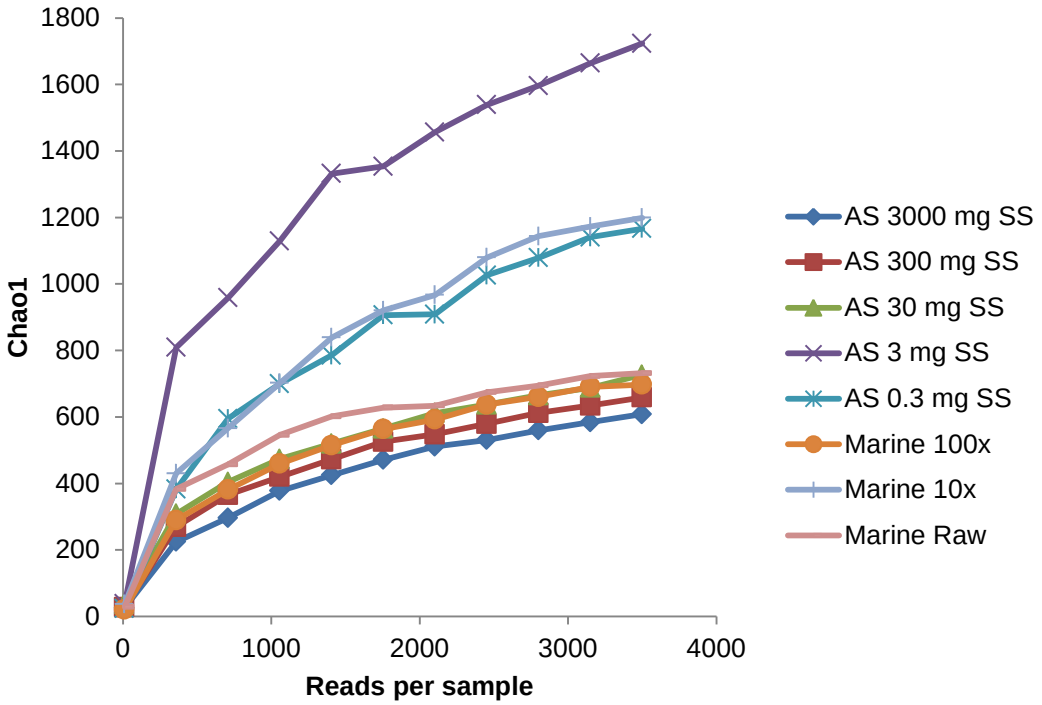


Figure 4-10 Average Chao1 values plotted against reads per sample for AS and Marine inocula sampled at the end of their relevant experiments. The closer to the downward concave of the curve the more representative the sampling procedure

4.5.1.4. Effect of test volume in enhanced biodegradation screening tests

Biodegradation studies using biomass concentrations of 300 and 30 mg SS L^{-1} were performed in 1L, 500 mL, 100 mL, 50 mL and 10 mL test systems with vessel geometry designed to maintain similar headspace in all systems (Figure 4-11).

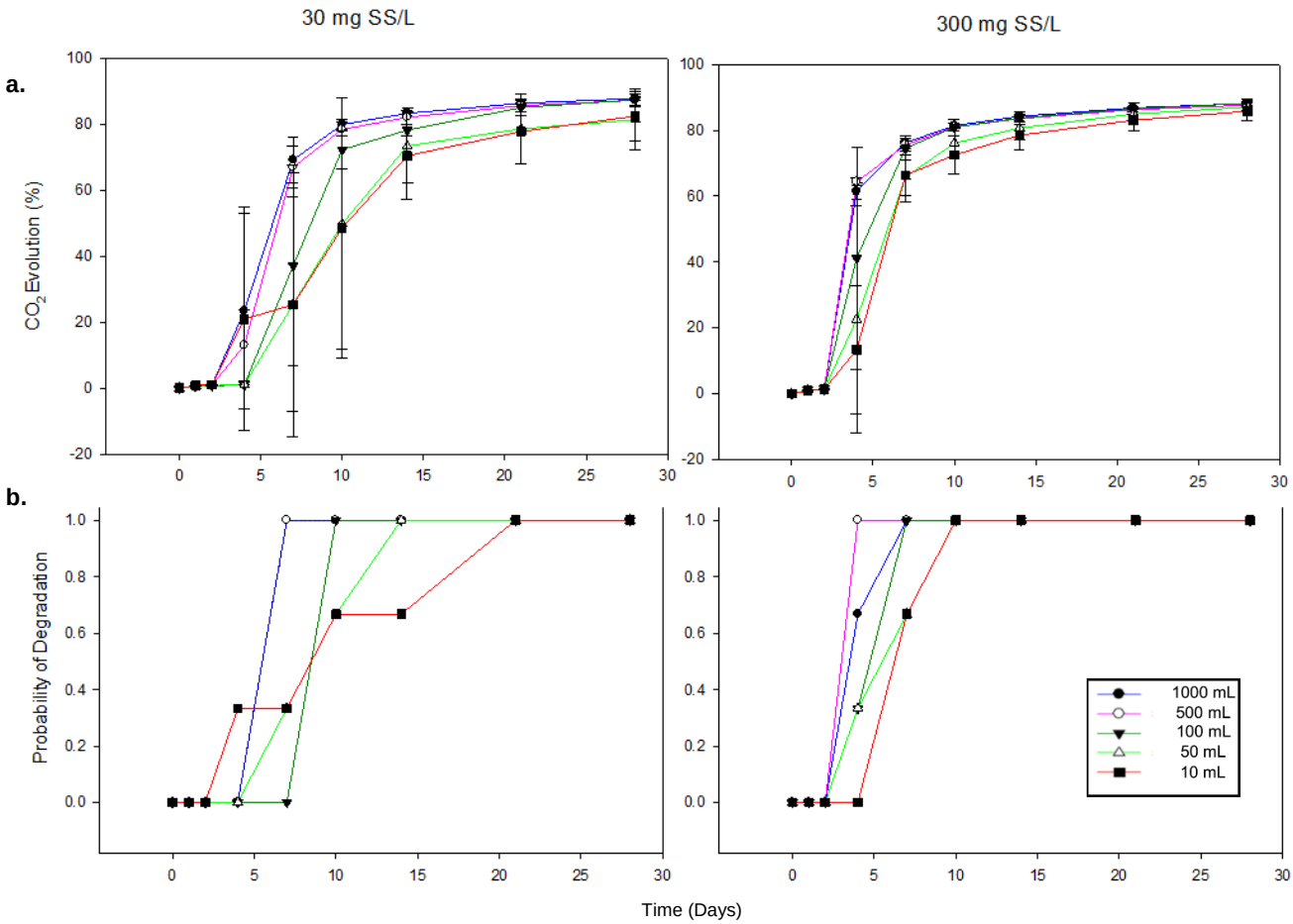


Figure 4-11 (a.) $^{14}CO_2$ evolution over time as a percentage of the originally applied radioactivity; **(b.)** probability of degradation for a given system at a given time. 30 mg SS L^{-1} systems are on the left with 300 mg SS L^{-1} on the right. Error bars are calculated as +/- 1 s.d.

All enhanced biodegradation tests sufficiently degraded 4-nitrophenol to 60% within 28 days and within a ten day window (Figure 4-11a). All systems ultimately reached a probability of 4-nitrophenol degradation of 100% (Figure 4-11b), although it was apparent that the smallest volume, lowest biomass concentration system of 10 mL at 30 mg SS L^{-1} took the longest time to reach this point, demonstrating the high inter-replicate variability within this system (Table 4-2).

Generally, larger volume systems exhibited shorter lag phases, shorter time to reach a pass threshold and smaller inter-replicate variability. However, the impact of biomass concentration appeared to be more pronounced than volume (Figure 4-11; Table 4-2). With respect to differences between the different inoculum concentrations, the 300 mg SS L^{-1} systems typically exhibited greater rates of degradation, shorter lag phases, shorter time to the pass threshold (both from the

beginning of the test and from the end of the lag phase), and less inter-replicate variability than observed with 30 mg SS L^{-1} biomass concentrations. (Table 4-2 and Figure 4-12; Figure 4-13).

The predominant impact of volume, rather than on biodegradation outcome, appears to be in reducing inter-replicate variation. At high volumes and high biomass concentrations, coefficients of variation are as low as 0.34%, indicating very powerful reliability at this volume and concentration (Table 4-2; Figure 4-13).

Table 4-2 Mean coefficient of variation for the following degradation descriptors: t_L, $t_{1/2}$, Δt_{50}, t_{60}, Δt_{60} and K_{max} in biodegradation screening tests for 4-NP incorporating different inoculum concentrations and volumes

Concentration (mg SS L^{-1})	**Volume (mL)**	**Mean coefficient variation (%)**
300	1000	0.68
300	500	0.34
300	100	30.21
300	50	33.11
300	10	19.58
30	1000	26.74
30	500	14.54
30	100	24.53
30	50	24.65
30	10	56.30

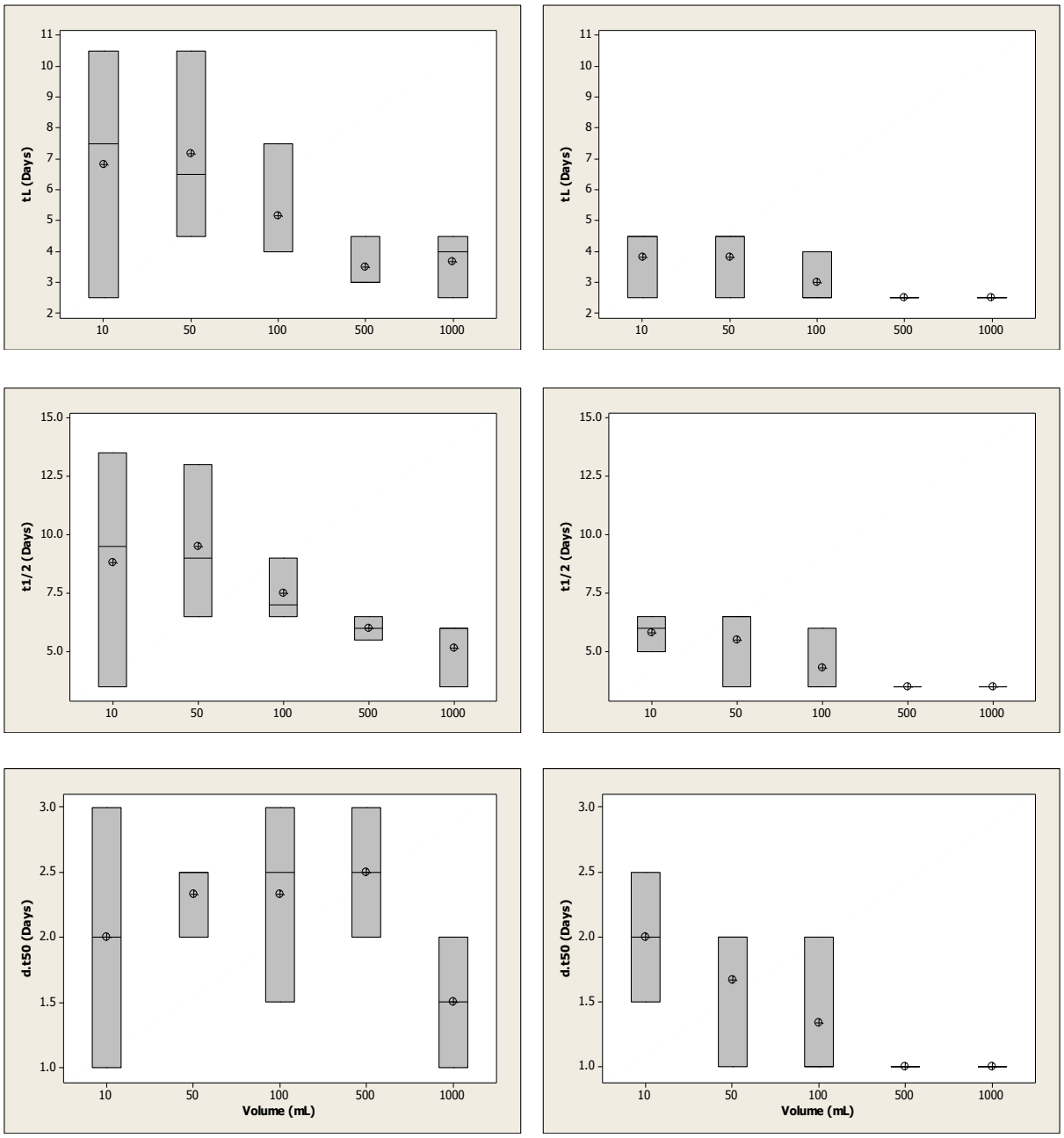


Figure 4-12 Boxplots of t_L, $t_{1/2}$ and Δt_{50} for different volumes from enhanced biodegradation screening tests. 30 mg SS L^{-1}degradation descriptors are shown on the left and 3000 mg SS L^{-1} on the right. Values were calculated from individual replicate data. Each replicate is represented by a horizontal line

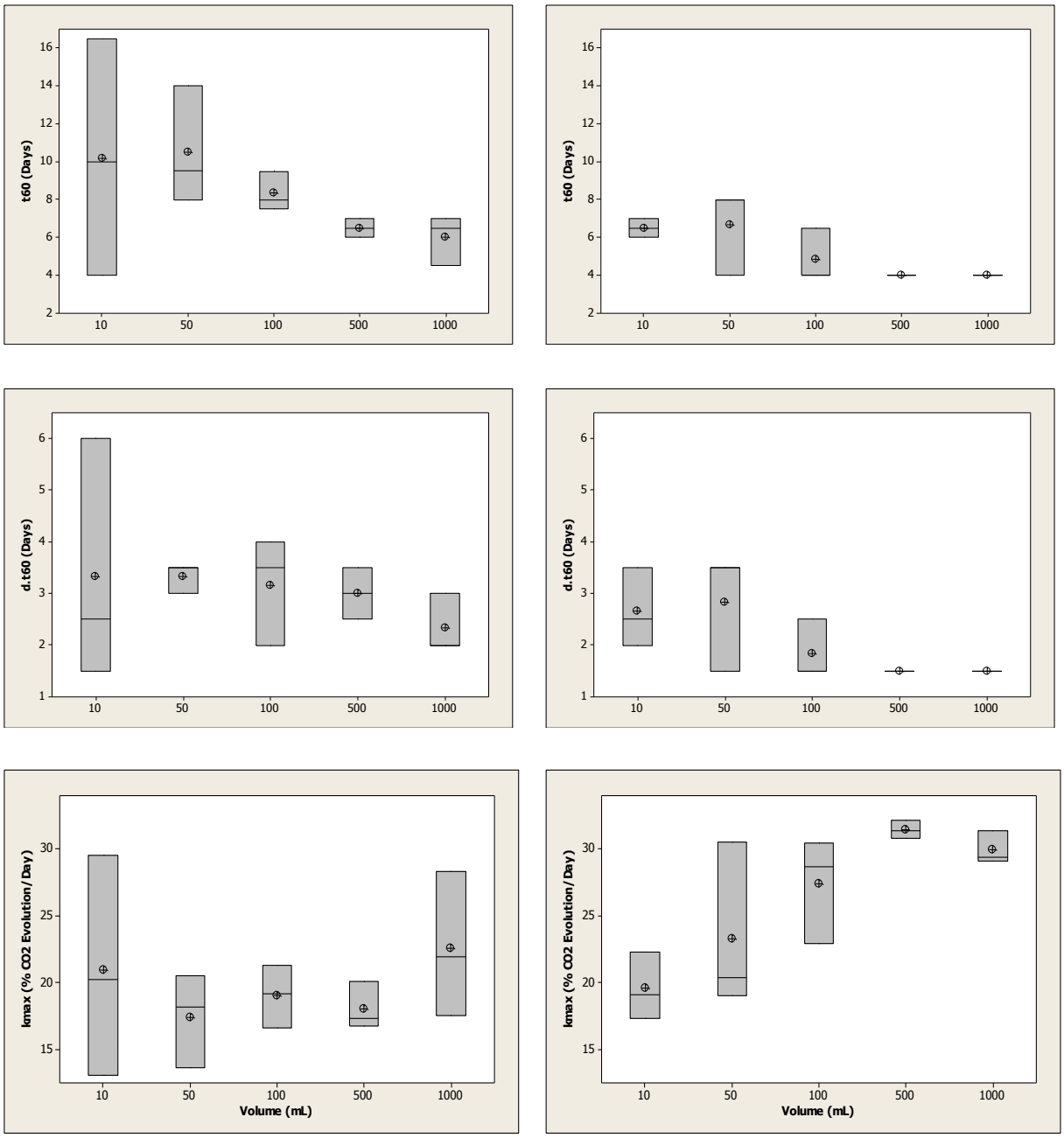


Figure 4-13 Boxplots of t_{60}, Δt_{60} and K_{max} for different volumes from enhanced biodegradation screening tests. Enhanced screening tests incorporating 30 mg SS L^{-1} sludge are shown on the left and 300 mg SS L^{-1} on the right. Values were calculated from individual replicate data. Each replicate is represented by a horizontal line

The most significant impact of volume appeared to be on inter-replicate variation (Table 4-2), rather than biodegradation outcome *per se*. All the systems resulted in degradation of 4-nitrophenol within the stringent OECD RBT requirements (Figure 4-11), but the coefficient of variation differed greatly between large (e.g. 1 L and 500 mL) and small (e.g. 50 mL and 10 mL) volumes, ranging from 0.34% in 500 mL volumes to 33.11% in 50 mL volumes (both at 300 mg SS L^{-1} biomass concentrations). However, the impact of concentration appeared to be more significant than test volume in reducing inter-replicate variability, providing faster degradation rates, shorter lag phases and shorter time taken to reach pass thresholds (Figure 4-12; Figure 4-13). A nested ANOVA performed in Minitab showed significant differences between concentrations but not volumes ($p<0.05$). An average of the variance components within the nested ANOVA for the degradation descriptors (t_L, $t_{1/2}$, Δt_{50}, t_{60}, Δt_{60} and K_{max}) show the majority of the variation was accounted for by inter-replicate variation (54%) followed by concentration (37%) and volume (9%).

A considerable concern in ready biodegradability testing is the degree of variation and subsequent failures inherently associated with the existing testing regimen. The observations and data gathered thus far suggest that, whilst inoculum concentration and test volume do appear to have an effect on overall extent of biodegradation, their most significant and important impact is on inter-replicate variation, which can be considerably reduced by utilising increased inoculum densities and test volumes.

The use of larger test volumes and greater inoculum concentrations in screening studies improves environmental realism by increasing the total number of cells and diversity to levels more similar to those observed in the natural environment. The OECD RBTs use inoculum concentrations ranging over four orders of magnitude, with maximum cell concentrations of 10^5 cells mL^{-1} (Table 1-2). Concentrations used in the enhanced AS screening tests discussed here are in the order of 10^8 cells mL^{-1}, a concentration which can feasibly be expected in a sewage treatment works (Goodhead, 2009). More realistic biomass concentrations provide a more robust basis for making extrapolations from laboratory-based studies to predict behaviour and effects at the local and regional scale.

4.5.2. *Marine inocula*

4.5.2.1. Concentration of marine samples for inoculum preparation

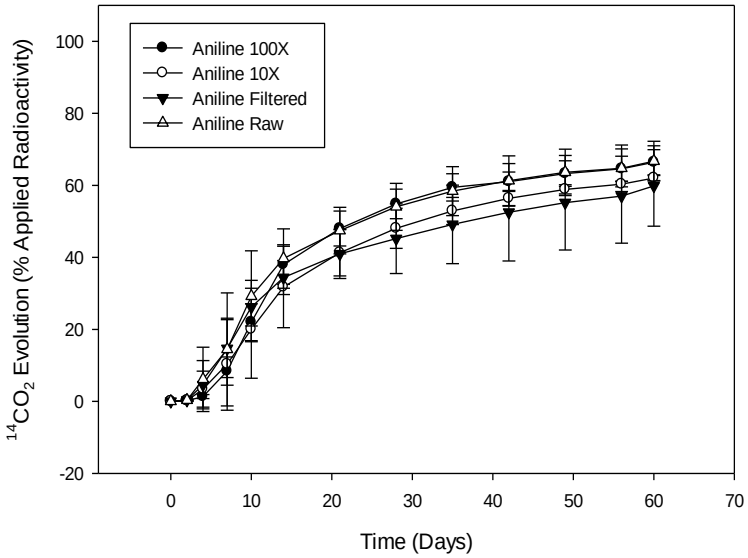
Initial cell concentrations were found to be in the 10^4-10^5 cells mL^{-1} range. This is at the lower end of what might be expected of marine samples (typically 10^5-10^6 cells mL^{-1}) but not atypically so for the region, based on cell numbers obtained during extensive periodic sampling of a neighbouring area (Gilbert *et al.*, 2009). Bacterial dip slides and total cell counts on untreated seawater and concentrated samples, indicated that the nominal concentrations ×100 and ×10 actually increased concentrations approximately ×60 and ×5 respectively. This is most likely due to some loss from the TFF manifold of the concentrated retentate under pressure during the final stages of processing. In addition, the backflow pressure may not have been great enough to fully resuspend all the material from the filter surface post-processing. The systems are referred to by their nominal concentrations throughout this Chapter (×100; ×10; Filtered and Raw).

4.5.2.2. Effect of cell concentration in enhanced marine biodegradation screening tests

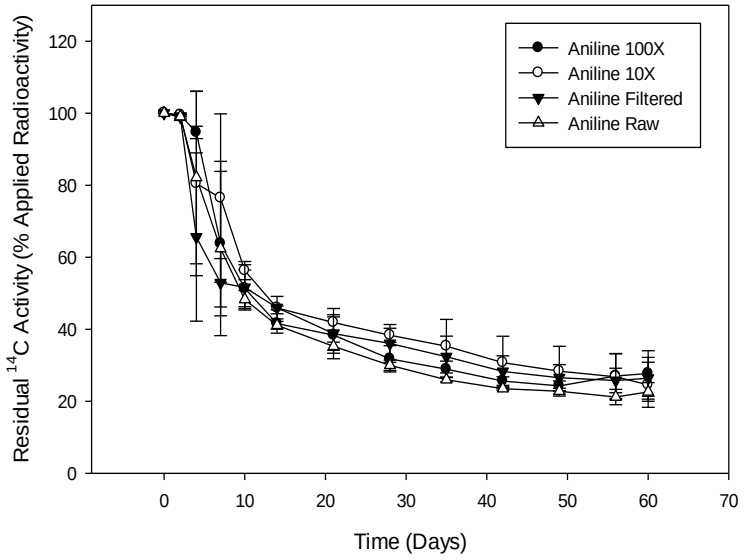
A pass in these biodegradation screening tests was considered to be 60% removal of the test compound within 28 days, and within a 10-day window as previously detailed (OECD, 1992a). Probabilities of degradation were assessed against a 60% removal with no time limit. For the purpose of persistency assessments, a compound with a half-life of less than 60 days in marine water was considered to be not persistent (ECHA, 2008).

Aniline was used as a reference compound throughout the marine studies. There was not a significant effect of cell concentration on biodegradation outcome for the reference compound ($P>0.05$). In all reference systems, a brief lag phase was followed by gradual removal until maximum degradation was reached at 60-70% evolution of $^{14}CO_2$ (Figure 4-14). Mass balances at the end of the study confirmed that the remainder of the radioactivity was either in solution (typically 20%) or bound to the biomass (typically 10%) (data not shown).

a.



b.



c.

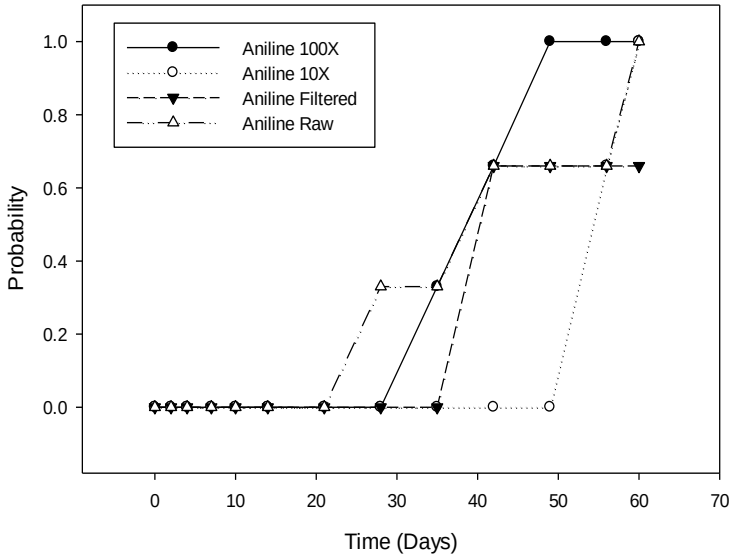


Figure 4-14 Effect of inoculum concentration on aniline biodegradation outcome in marine biodegradation screening tests: **a.** $^{14}CO_2$ evolution in 1L biodegradation tests with different inocula concentrations; **b.** residual ^{14}C activity; **c.** probability of a positive biodegradation outcome based on OECD 306 pass guidelines but with no study duration limit. Error bars represent ± 1 standard deviation.

The biodegradation screening test incorporating the highest cell concentration (×100) showed more rapid degradation of 4-NP than in other biodegradation tests with lower inoculum densities (Figure 4-15). This also resulted in higher probabilities of biodegradation within a shorter time frame for high, in comparison to low, inoculum concentration systems.

Much longer lag phases were observed in marine biodegradation tests (Figure 4-15) compared to those using activated sludge inocula (Figure 4-11). However, following these lag periods, 4-NP was relatively rapidly degraded with $\Delta\mathbf{t_{60}}$ values of less than 15 days for all ×100, ×10 and raw systems (filtered systems had a mean $\Delta\mathbf{t_{60}}$ of 19 days based on the two replicates where calculation was possible).

The ×100 screening test exhibited a shorter $\mathbf{t_L}$ and thus smaller $\mathbf{t_{1/2}}$ and $\mathbf{t_{60}}$ values, but similar rates of degradation to the ×10 and raw systems post lag phase. The more concentrated ×100 and ×10 systems showed marginally less variation between replicates. Generally, inter-replicate variation was considerably higher than in activated sludge systems (Figure 4-16).

The long lag phases observed with 4-NP have been reported previously by Nyholm and Kristensen (1992) who also encountered considerable variation between replicates and tests. The variation in degradation descriptors and biodegradation outcome is something that has typically been observed when conducting biodegradation screening tests with low biomass concentrations (Gerike and Fischer, 1979; Gerike and Fischer, 1981; Blok and Booy, 1984), which appear to lack degradative power of biodegradation screening tests which incorporate high biomass concentrations (Section 4.5.1; Nyholm *et al.*, 1984; Painter and King, 1985; Goodhead *et al*., 2013).

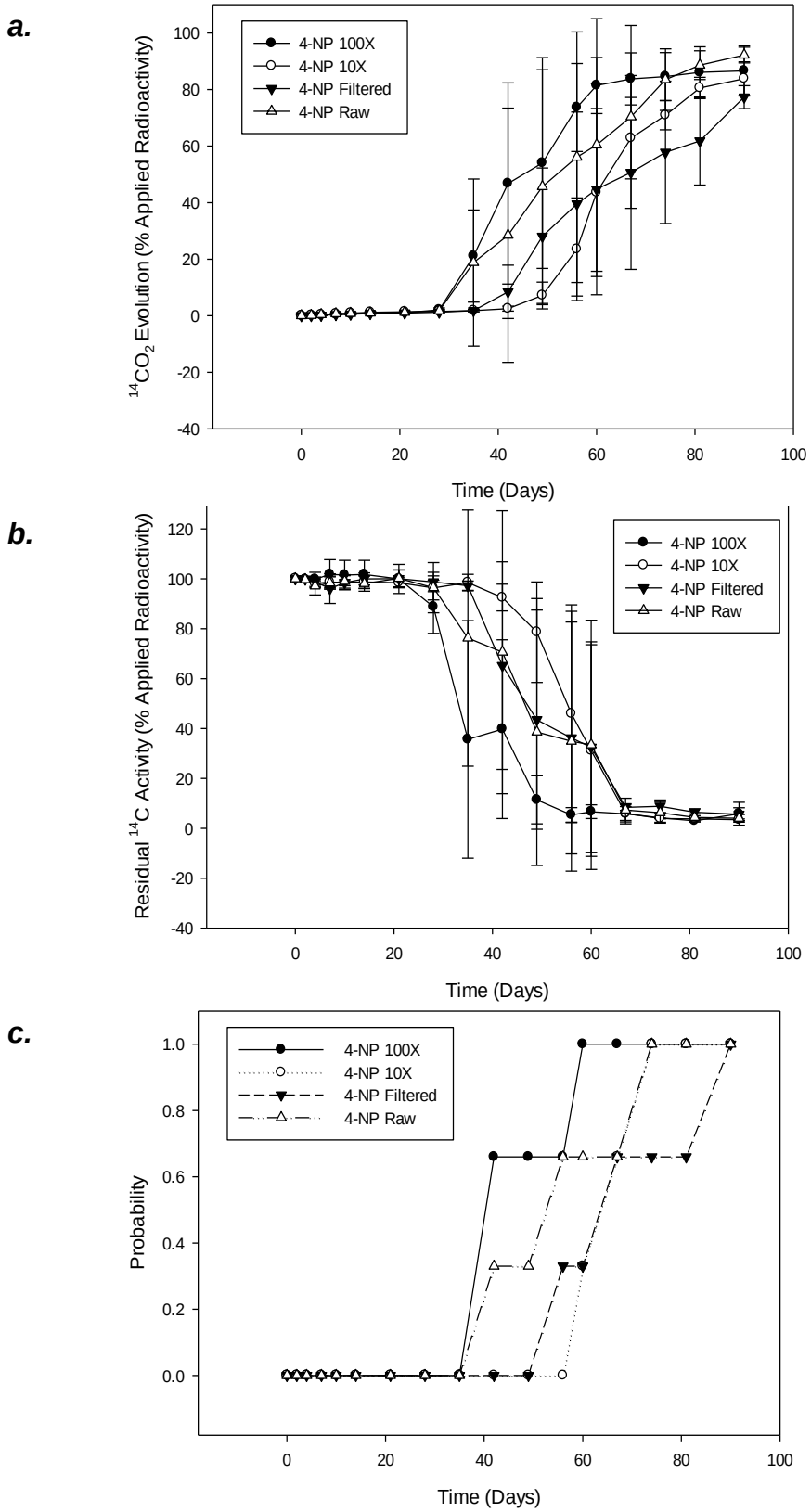


Figure 4-15 Effect of inoculum concentration on 4-NP biodegradation outcome in marine biodegradation screening tests: **a.** $^{14}CO_2$ evolution in 1L marine systems with varying inocula concentrations; **b.** residual ^{14}C activity; **c.** probability of a positive biodegradation outcome based on OECD 306 pass guidelines but with no study duration limit. Error bars represent ± 1 standard deviation.

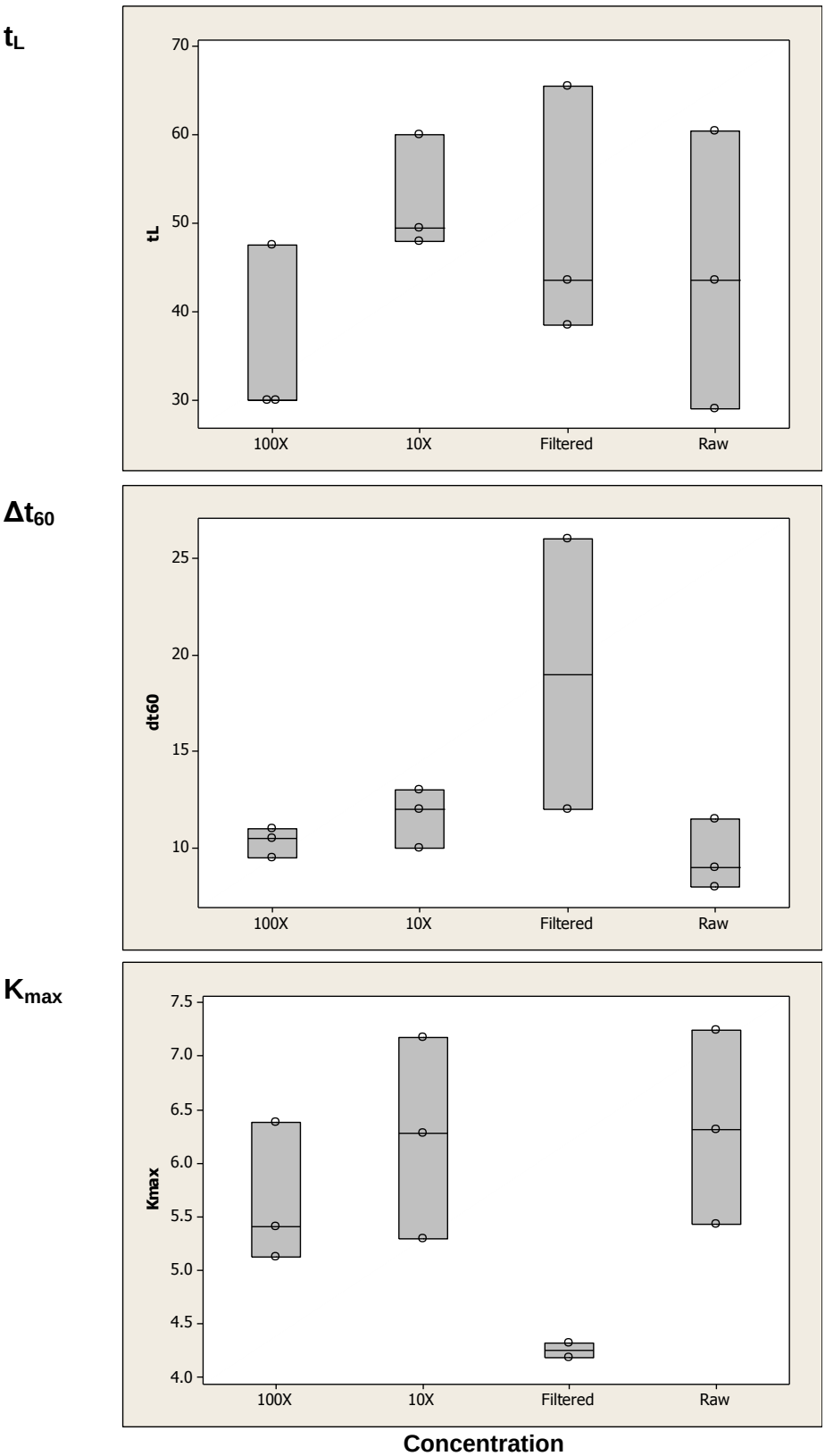


Figure 4-16 Comparison of t_L (lag time), Δt_{60} (interval from lag phase to RBT pass threshold) and k_{max} (fastest observed degradation rate) for 1L marine biodegradation screening tests with different biomass concentrations. (Degradation did not reach 50% in one of the filtered systems, therefore calculation of degradation descriptors were not possible, the values displayed represent the two replicates which achieved sufficient degradation to determine rate characteristics).

4.5.2.3. Effect of test volume in enhanced marine biodegradation screening tests

Degradation of the reference compound, aniline, occurred as previously observed, with a relatively short lag phase followed by gradual removal until maximum degradation of 60-70% was achieved (Section 4.5.2.2).

Degradation of the test compound, 4-NP, showed extensive lag phases of up to 90 days regardless of the volume used. Degradation failed to reach 10% in many biodegradation screening tests (Figure 4-17 a). The biodegradation screening test incorporating ×100 inoculum in 500 mL volume showed the highest $^{14}CO_2$ evolution and typically exhibited the shortest $\mathbf{t_L}$, $\mathbf{t_{1/2}}$, $\mathbf{\Delta t_{50}}$, $\mathbf{t_{60}}$, $\mathbf{\Delta t_{60}}$ values and fastest $\mathbf{K_{max}}$ for the replicates, which showed sufficient degradation to make degradation descriptor calculations possible. Additionally, this was the only system to return a 100% probability of degradation based on a 60% pass threshold within a 90 day test (Figure 4-17 a and Figure 4-17 c).

Biodegradation screening tests conducted in 1000 mL and 500 mL test volumes typically showed some level of biodegradation whereas tests conducted at lower volumes showed little degradation across all inocula concentrations. Whilst the extent of biodegradation in 4-NP was greatest in the biodegradation screening tests incorporating ×100 concentrated biomass in a 500 mL volume, the 1000 mL volume biodegradation screening tests showed potential for degradation in at least one of the replicates across all inocula concentrations, which was not seen for the remaining 500 mL and 50 mL volume biodegradation screening tests. The inter-replicate variation was also lower in larger volume biodegradation tests (where calculation of degradation descriptors was possible (Figure 4-18)). The increased lag phase and variation with lower volumes corroborates with prior research into the importance of volume in biodegradation screening tests (Ingerslev *et al.*, 2000).

Typically, biodegradation screening tests incorporating the ×100 and ×10 biomass concentration showed better extent and rates of degradation of the test compound across the different test volumes (Figure 4-17; Figure 4-18; Figure 4-19), suggesting that concentration, in addition to test volume, positively influenced the probability of degradation for the test compound 4-NP.

However, whilst the data suggests that inoculum concentration and test volume impact upon probability of degradation and inter-replicate variation, the initial concentration of microorganisms in marine samples was approximately five orders of magnitude lower than the highest biomass concentration used in AS biodegradation screening tests. This difference in cell concentration may explain why the extent of the effects observed in AS biodegradation screening tests was not observed in marine biodegradation screening tests.

From 12 marine biodegradation screening tests used to investigate the effect of volume on biodegradation outcome, 11 exhibited extended lag phases, which continued past the OECD RBT guidelines, and in some cases beyond the proposed persistence threshold for marine environments (ECHA, 2008). However, once degradation began, it progressed rapidly (Figure 4-15) and typically degraded the test compound beyond the 50% persistency threshold. These lag phases may be due to rare specific degraders which require time to establish a sufficient population density to degrade the chemical in question. Alternatively, the degraders may already be present and in sufficient abundance but require time to activate the requisite genes and metabolic pathways for degradation to occur.

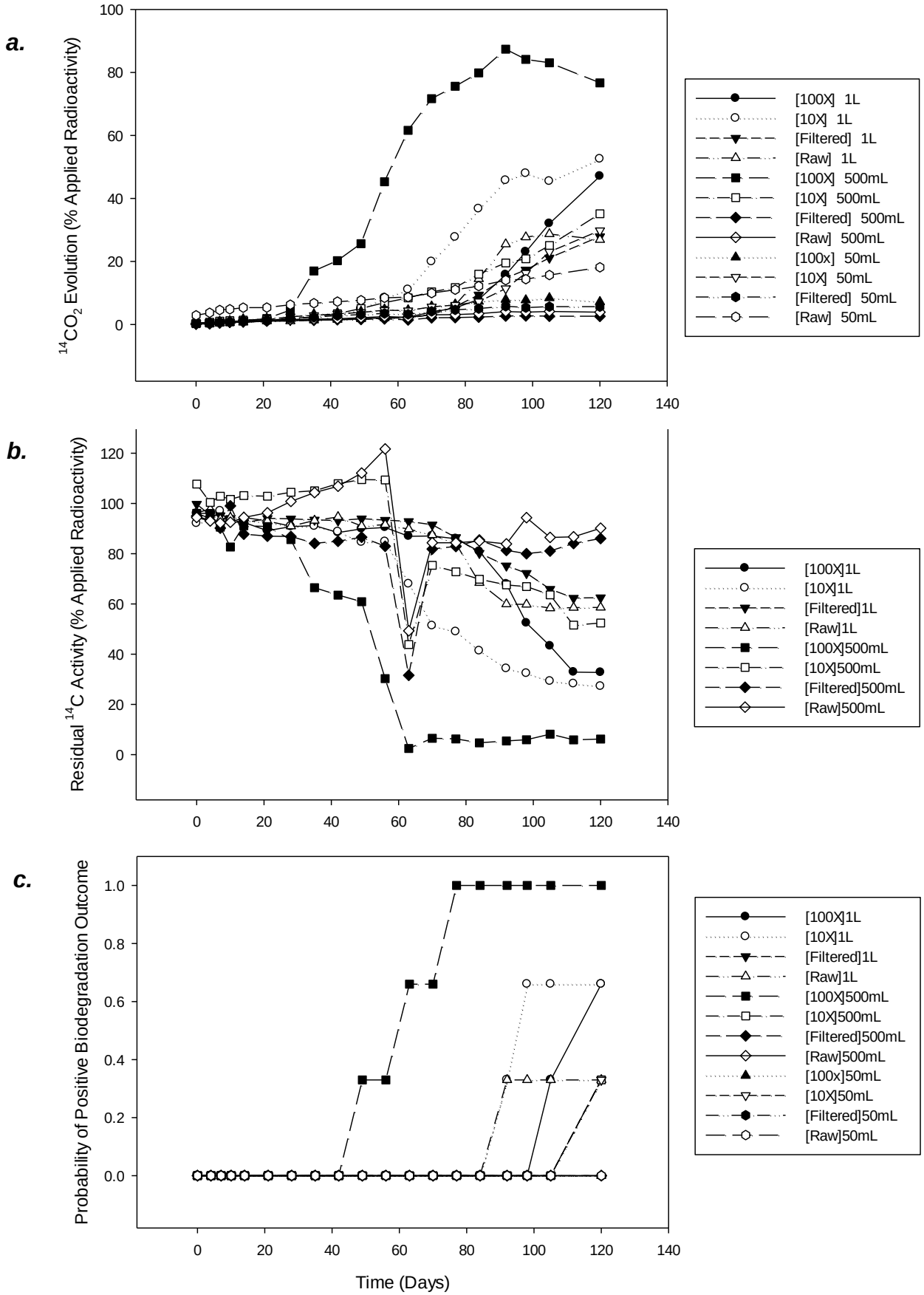


Figure 4-17 Plots showing the effect of test volume and inoculum concentration on 4-NP biodegradation outcome in marine biodegradation screening tests: **a.** $^{14}CO_2$ evolution over time in 1L, 500mL and 50mL marine biodegradation tests with varying inocula concentrations; **b.** radioactivity remaining in solution in 1000 mL and 500 mL systems (50 mL systems excluded due to low volume and the requirement to sample 5 mL at each sample point to determine residual activity) ; **c.** probability of a positive outcome in a biodegradation test for marine systems. No significant volume or concentration effects were observed (ANOVA; $P > 0.05$).

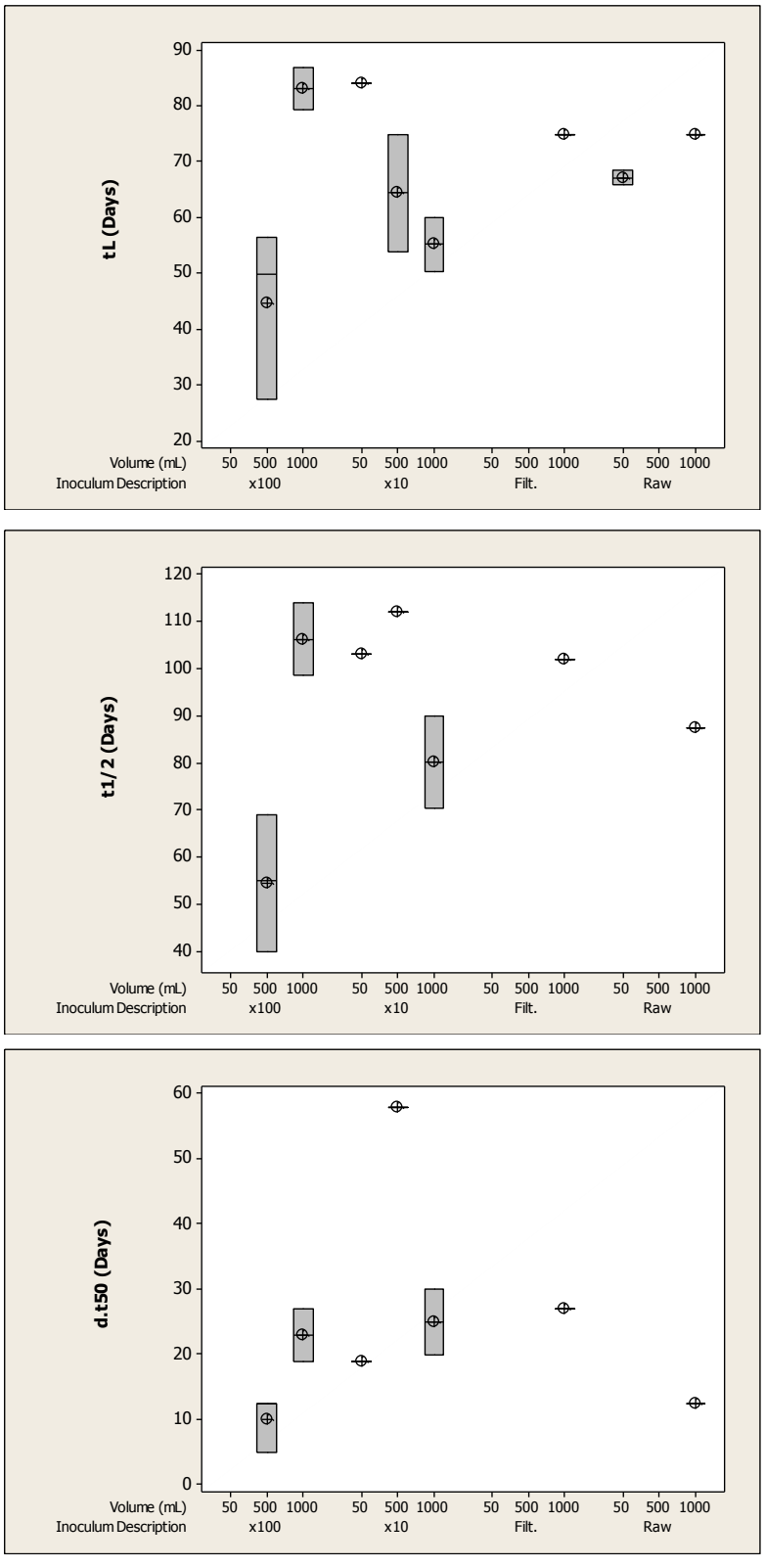


Figure 4-18 Boxplots of $t_{L,}$ $t_{1/2}$ and Δt_{50} for different volumes from marine biodegradation screening tests. Values were calculated from individual replicate data. The number of replicates used to calculate each descriptor is indicated in Table 4-4

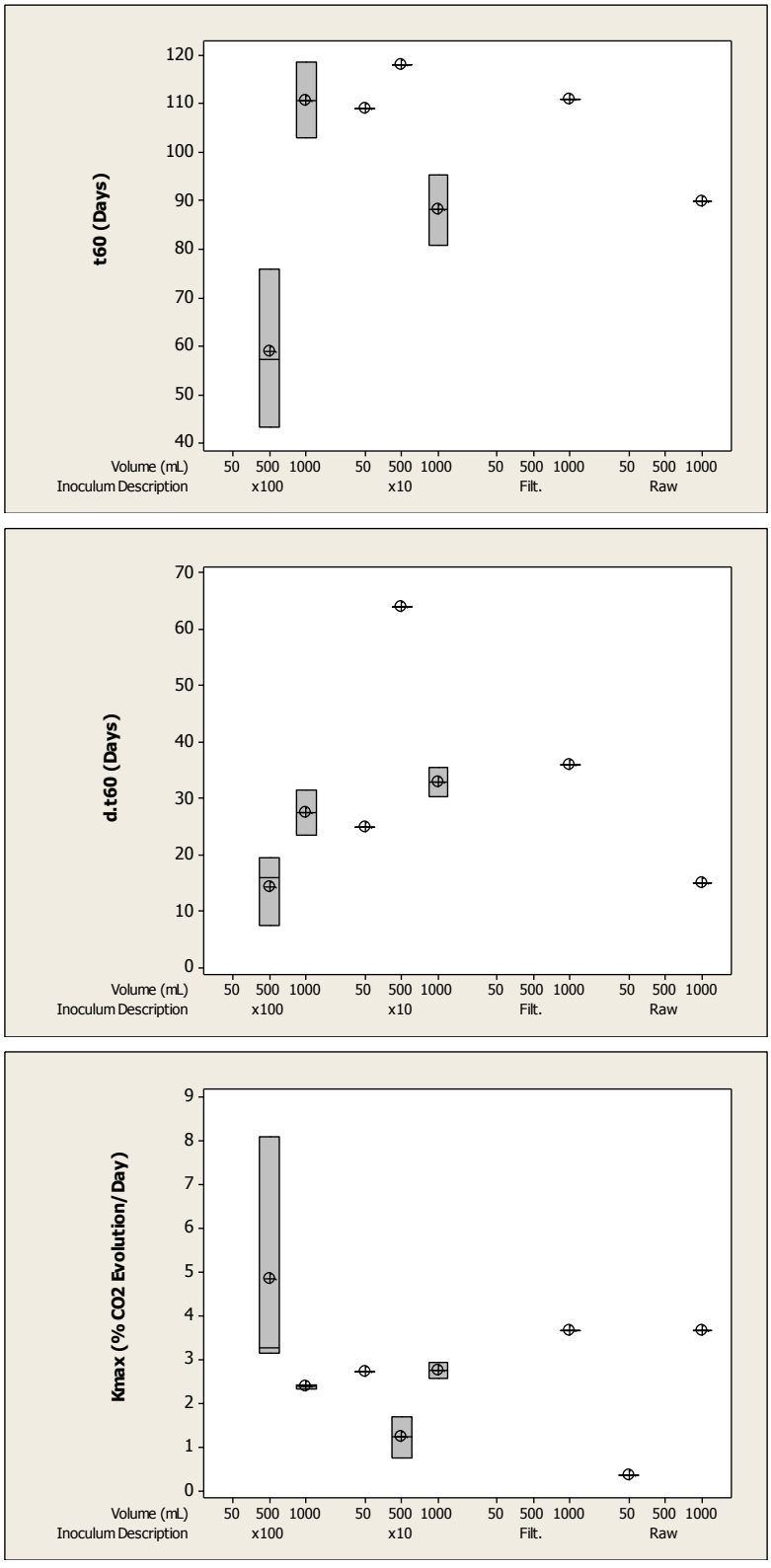


Figure 4-19 Boxplots of t_{60}, Δt_{60} and K_{max} for different volumes from marine biodegradation screening tests. Values were calculated from individual replicate data. The number of replicates used to calculate each descriptor is indicated in Table 4-4

4.5.3. *Discussion regarding F: M ratios in enhanced biodegradation screening studies*

A summary of the results for all enhanced biodegradation screening tests of 4-NP using activated sludge and marine inocula is given in Table 4-3 and Table 4-4 respectively.

The OECD RBTs stipulate the use of a low microbial biomass concentration and a relatively high chemical concentration. Typically, chemical concentrations may be expected in the ng L^{-1} range or lower, with µg L^{-1} concentrations being cause for concern (Kümmerer, 2010). Chemical concentrations in OECD RBTs vary (Table 1-2) but are typically in the mg L^{-1} range. Although some toxicity controls are incorporated to ensure that the chemical is not toxic to the inoculum at the test concentration, it is not clear how much of an impact the high F: M ratio seen in current screening tests may have upon degradation kinetics.

Microorganisms have shown the capacity to respond to changes in resources, for example by inducing apoptosis to preserve the core community when resources are scarce (Aertsen and Michiels, 2004) or evolving in response to resource variability (Lennon and Cottingham, 2008). They have also shown the capacity to degrade compounds over a range of chemical concentrations (Ingerslev and Nyholm, 2000; Toräng and Nyholm, 2005), with investigations into the effect of F: M ratios typically focused on altering the chemical concentration rather than the biomass concentration.

Ingerslev and Nyholm (2000) observed no significant variations in biodegradation outcome with chemical concentrations ranging from 1-100 µg L^{-1}. Toräng and Nyholm (2005) conversely reported significant increases in degradation rates when chemical concentrations were sufficiently high to induce adaptation. Table 4-3 highlights the F: M ratios used in this study for the enhanced biodegradation using activated sludge inocula to assess 4-NP degradation. There was a link between increasing F: M ratio and decreasing probability of degradation and reductions in degradation descriptors (F: M ≥ 3.33 resulted in an increased likelihood of failing an OECD RBT). This correlation did not appear to exist in marine biodegradation screening tests. The reduction in the probability of degradation with increasing F: M could be explained as a toxicity induced lag, which has previously been used to

explain the erratic behaviour of 4-NP in standard biodegradation assessments (Nyholm and Kristensen, 1992).

4.6. Conclusions and Recommendations

- Ultimately, all AS tests exhibited the same probability of degradation at the end of the respective tests. However, greater probabilities of degradation were observed in a shorter time frame with increasing biomass concentrations and test volumes. In marine tests, the most concentrated biomass typically resulted in a greater probability of degradation in a shorter timeframe.
- Generally, more concentrated inocula resulted in lower variation between replicates. Increasing AS concentration was found to be more significant in reducing inter-replicate variation than increasing the test volume. Marine systems did not show a significant concentration or volume effect.
- Increases in AS concentration appeared to correlate to an increase in OTU richness, based on DGGE outputs. Typically lower biomass concentrations produced communities with lower OTU richness and exhibiting less similarity to one another.

For biodegradation screening tests incorporating activated sludge inoculum, a concentration of 300 mg SS L^{-1} (10^7-10^8 cells mL^{-1}) and a volume of at least 500 mL are recommended to reduce the variation typically associated with standard biodegradation assessments and provide degradation data which is reproducible between tests.

For biodegradation screening tests incorporating marine inoculum, a best case system of ×100 nominally concentrated marine samples and a volume of at least 500 mL are recommended based on degradation observations, although statistically no significant volume or concentration effect was found (ANOVA; $P > 0.05$). The extent of improvements in variation between replicates and reproducibility of degradation data seen in activated sludge were not observed with marine inoculum. Further development of the marine biodegradation screening test is recommended to improve associated variation between replicates and tests.

Bacterial community analyses suggested that higher biomass concentration inocula exhibited greater similarity to each other and greater OTU richness than lower biomass concentration inocula. At the end of the study however, selective

enrichment within the typically faster degrading, higher biomass concentration inocula appeared to reduce the diversity of the samples, producing communities with several dominant OTUs. Investigations into understanding the mechanisms and pathways behind the enrichment may assist in designing future biodegradation screening tests.

Table 4-3 Summary of all activated sludge biodegradation screening tests used in assessing cell concentration and test volume impact upon 4-nitrophenol degradation. Biodegradation screening test which exhibited the same F: M ratios are shaded complementarily. OECD RBT outcomes were based on 60% reduction in $ThCO_2$ within the ten-day window of a 28 day test. Persistency assessments were based on $t_{1/2}$ of less than 40 days to be classed as not P. ***t_L, $t_{1/2}$, Δt_{60} and K_{max}*** degradation descriptors are described in Table 1-1. Values are an average of three replicates.

SS (mg SS/L)	Volume (L)	Total SS (mg SS)	Total chemical (mg C)	F:M ratio	OECD RBT Outcome	Persistency outcome (based on not P = t1/2 < 40 days)	t_L	$t_{1/2}$	Δt_{60}	K_{max}
3000	1	3000	10	0.003	PASS	Not P	3.2	6.0	3.5	18.4
300	1	300	10	0.033	PASS	Not P	2.5	4.5	2.5	20.1
30	1	30	10	0.333	PASS	Not P	3.5	6.0	3.2	17.7
3	1	3	10	3.333	FAIL	Not P	8.3	12.2	4.7	13.2
0.3	1	3	10	33.333	FAIL	Not P	21.7	27.2	10.5	7.4
300	1	300	10	0.033	PASS	Not P	2.5	3.5	1.5	30.0
30	1	30	10	0.333	PASS	Not P	3.7	5.2	2.3	22.6
300	0.5	150	5	0.033	PASS	Not P	2.5	3.5	1.5	31.4
30	0.5	15	5	0.333	PASS	Not P	3.5	6.0	3.0	18.0
300	0.1	30	1	0.033	PASS	Not P	3.0	4.3	1.8	27.4
30	0.1	3	1	0.333	PASS	Not P	5.2	7.5	3.2	19.1
300	0.05	15	0.5	0.033	PASS	Not P	3.8	5.5	2.8	23.3
30	0.05	1.5	0.5	0.333	PASS	Not P	7.2	9.5	3.3	17.4
300	0.01	3	0.1	0.033	PASS	Not P	3.8	5.8	2.7	19.6
30	0.01	0.3	0.1	0.333	PASS	Not P	6.8	8.8	3.3	21.0

Table 4-4 Summary of marine biodegradation screening tests used in investigating impact of cell concentration and test volume on 4-nitrophenol biodegradation in enhanced marine screening tests. Biodegradation test which exhibited approximately equal F: M ratios are shaded complementarily. OECD RBT outcomes are based on 60% reduction in $ThCO_2$ within 28 days. Persistency assessments are based on $t_{1/2}$ less than 60 days to be classed not P. t_L, $t_{1/2}$, Δt_{60} *and* K_{max} are degradation descriptors described in Table 1-1; D_{max} details the maximum extent of degradation achieved. Values are an average of three replicates, where data for three replicates was not available, the number of replicates from which the value was calculated is included in superscript next to the value. * indicates that calculation was not possible for any of the replicates.

Conc.	Approx. cell number (cells/mL)	Volume (L)	Approximate total cell number	Total chemical (mg C)	F:M ratio	OECD RBT Outcome	Persistency outcome (based on not P = t1/2 < 60 days)	t_L	$t_{1/2}$	Δt_{60}	K_{max}	D_{max}
100X	1.65E+07	1	1.65E+10	10	6.06E-10	FAIL	Not P	35.8	44.2	10.3	5.6	86.6
10X	2.75E+05	1	2.75E+08	10	3.64E-08	FAIL	P	52.5	61.8	11.7	6.3	83.9
FILT	5.50E+04	1	5.50E+07	10	1.82E-07	FAIL	Not P	49.2	51.5 [2]	19.0 [2]	3.9	77.3
RAW	5.50E+04	1	5.50E+07	10	1.82E-07	FAIL	Not P	44.3	51.5	9.5	6.3	92.2
100X	1.65E+07	1	1.65E+10	10	6.06E-10	FAIL	P	83.3 [2]	106.3 [2]	27.5 [2]	2.4 [2]	47.0
10X	2.75E+05	1	2.75E+08	10	3.64E-08	FAIL	P	55.3 [2]	80.3 [2]	33.0 [2]	2.7 [2]	54.1
FILT	5.50E+04	1	5.50E+07	10	1.82E-07	FAIL	P	75.0 [1]	102.0 [1]	36.0 [1]	3.7 [1]	27.9
RAW	5.50E+04	1	5.50E+07	10	1.82E-07	FAIL	P	75.0 [1]	87.5 [1]	15.0 [1]	3.7 [1]	29.3
100X	1.65E+07	0.5	8.25E+09	5	6.06E-10	FAIL	Not P	44.7	54.7	14.3	4.8	87.7
10X	2.75E+05	0.5	1.38E+08	5	3.64E-08	FAIL	P	64.5 [2]	112.0 [1]	64.0 [1]	1.2 [2]	35.1
FILT	5.50E+04	0.5	2.75E+07	5	1.82E-07	FAIL	P	*	*	*	*	2.6
RAW	5.50E+04	0.5	2.75E+07	5	1.82E-07	FAIL	P	*	*	*	*	3.9
100X	1.65E+07	0.05	8.25E+08	0.5	6.06E-10	FAIL	P	*	*	*	*	7.2
10X	2.75E+05	0.05	1.38E+07	0.5	3.64E-08	FAIL	P	84.0 [1]	103.0 [1]	25.0 [1]	2.7 [1]	29.8
FILT	5.50E+04	0.05	2.75E+06	0.5	1.82E-07	FAIL	P	*	*	*	*	5.7
RAW	5.50E+04	0.05	2.75E+06	0.5	1.82E-07	FAIL	P	67.3 [2]	*	*	0.4 [1]	18.1

Chapter 5

5. Developing an enhanced biodegradation screening test based on chemical persistence

5.1. Introduction

The problems with existing biodegradation screening tests and their inadequacies with respect to false negatives, high variability and their inability to identify persistence have been previously discussed (Section 1.3.3).

Several enhancements to existing screening studies (Section 1.3.4), including increased test duration, test volume and cell concentration, were proposed to enable a more effective prioritisation on persistence and reduce the high variability notoriously associated with RBTs (Figure 1-7). These enhancements were published in ECHA guidance (EC, 2009) with the recommendation that a ring-test be performed by international standards authorities to provide a weight of evidence for their adoption.

The need for a set of validation chemicals with characterised degradation behaviour was highlighted in an ECETOC workshop on persistence and biodegradation (ECETOC, 2007). A panel made up from academia, industry and the regulatory community described the need for a set of chemicals to ratify modifications and developments to biodegradation methods and to prevent screening tests becoming excessively protective or overly powerful (ECETOC, 2007).

Many years ago, the principle of microbial infallibility was discussed (Painter, 1974), whereby any and all organic chemicals were considered to be inherently degradable, given the right combination of microbes and enzymes. Subsequently, the inaccuracy of this concept has been realised and it is now known that organic compounds have a range of degradation capacities. The validation set imagined by the ECETOC workshop required inclusion of chemicals with a range of degradation characteristics, from those compounds which degrade rapidly to recalcitrant compounds (ECETOC, 2007).

Comber and Holt (2010) prepared a list of chemicals recommended for use as reference substances. Compounds were grouped together based on their degradation potential in collections referred to as bins, ranging from readily biodegradable through to persistent compounds. Their perceived contribution to, and

suitability for, biodegradation research was taken into consideration as well as their biodegradability (Comber and Holt, 2010).

The use of categories (or bins) for characterising compounds based on biodegradability has been discussed previously (SOMS, 2002; ECETOC, 2003), with Comber and Holt (2010) electing to use four persistency classes with chemicals selected predominantly based on performance in standard tests and known behaviour in the environment. The four bins are described in Table 5-1.

Table 5-1 Bin definitions for characterising chemicals based on biodegradability. Assignments are predominantly based on performance in standard tests and known behaviour in the environment. **$t_{1/2}$** categorisations are based on the median **$t_{1/2}$** from literature. Table adapted from information presented by Comber and Holt (2010)

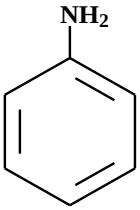
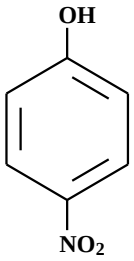
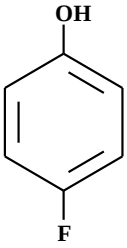
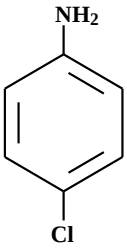
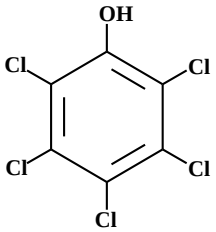
Bin	Standard Regulatory Test Outcome	Potential for Degradation	$t_{1/2}$ range from literature
1	Should pass OECD RBT and modified RBT	Very high; expected to degrade in all aerobic environmental compartments	≤ 15 days
2	Should pass enhanced screen but may fail OECD RBT	High potential for adaptation and growth-linked biodegradation	16-40 days
3	Should normally fail any screening test including modified RBTs and enhanced screens	Evidence for inherent biodegradability with no fulfilment of ready criteria	> 40 ≤ 60 days
4	Should never pass modified RBT or enhanced screen	No evidence for biodegradation	≥ 61 days

Comber and Holt (2010) used five distinct data sources to compile a preliminary list of validation chemicals: review of standard regulatory test reference chemicals; review of chemicals used in biodegradability test method development; review of results from standardised RBTs; review of regulatory priority lists and published literature containing measured $t_{1/2}$ data.

A group of chemicals were selected in the present study predominantly based on recommendations made in the ECETOC workshop report on biodegradation and persistence (ECETOC, 2007), recommendations for validation chemicals made by Comber and Holt (2010) and previous research performed by Goodhead (2009). The group contained chemicals from Bins 1, 2 and 3 to offer a range of persistence

characteristics. Multiple chemicals were selected from Bin 2 in acknowledgement of the fact that the enhancements to screening tests were principally being introduced to tackle the “difficult” chemicals i.e. those with erratic RBT behaviour. These chemicals were thought to be inherently, and under suitable conditions even readily, biodegradable but typically fail to show biodegradability under the stringent RBT criteria resulting in frequent misclassifications (Comber and Holt, 2010). The chemicals selected to validate the enhanced screening test used in the present study are listed in Table 5-2.

Table 5-2 Overview of chemicals used to validate the enhanced biodegradation screening test. Chemical data obtained from MSDS (Sigma-Aldrich, Gillingham, UK). Chemical structures drawn using ChemWindow (Bio-Rad, Hemel Hempstead, UK). Chemicals were universally ^{14}C labelled. *RBT refers to ready biodegradability test; IBT refers to inherently biodegradability test*

Chemical	Structure	Bin	Reasons for selection
Aniline C_6H_7N MWt: 93.13 g mol^{-1} CAS-No: 62-53-3 H_2O sol.: Soluble		**1**	Inclusion in Comber and Holt (2010) reference list; Used as reference compound in standardised RBTs (e.g. OECD 301); Vast body of literature (e.g. Gheewala and Annachhatre (1997))
4-Nitrophenol $C_6H_5NO_3$ MWt: 139.11 g mol^{-1} CAS-No: 100-02-7 H_2O sol.: 15 g L^{-1}		**2**	Considered for reference list but rejected due to insufficient confidence in biodegradation assessment; Build on previous research by Davenport *et al.* (2009) and Goodhead (2009); Vast body of literature (e.g. Nyholm *et al.* (1984) and Thouand *et al.* (1995))
4-Fluorophenol C_6H_5FO MWt: 112.10 g mol^{-1} CAS-No: 371-41-5 H_2O sol.: Soluble		**2**	Inclusion in Comber and Holt (2010) reference list; Build on previous research by Davenport *et al.* (2009) and Goodhead (2009); "Difficult" substance expected to occasion ally fail screening test
4-Chloroaniline C_6H_6ClN MWt: 127.57 g mol^{-1} CAS-No: 106-47-8 H_2O sol.: 2 g L^{-1}		**2**	Inclusion in Comber and Holt (2010) reference list; Potential for adaptation and growth-related biodegradation (Toräng and Nyholm, 2005); Typically fails RBTs but removal observed in IBTs
Pentachlorophenol C_6HCl_5O MWt: 266.34 g mol^{-1} CAS-No: 87-86-5 H2O sol.: Very low		**3**	Considered for reference list but rejected due to insufficient confidence in biodegradation assessment; Degradation ranging from none to rapid degradation following acclimation; Degradation linked to slow growing degraders

5.1.1. Reference chemicals for validation of enhanced biodegradation screening test

Additional information about the individual chemicals in Table 5-2 is provided below.

5.1.1.1. Aniline (Ani)

Aniline was named by Carl Fritzsche in 1841 following its preparation from indigo and potash, but is thought to have first been obtained in 1826 and subsequently found in coal tar in 1834. It acts as a precursor for many industrial chemicals; its origins lie in dye manufacturing but it is now predominantly used in polyurethane production (Budavari, 1989).

Aniline is recommended for use as a reference compound in regulatory tests, such as the OECD ready series (OECD, 1992a). There is a large body of research surrounding its degradation (Patil and Shinde, 1988; Nyholm *et al.*, 1996; O'Neill *et al.*, 2000; Vázquez-Rodríguez *et al.*, 2008) and its use as a positive control for comparing different standard tests (Gerike and Fischer, 1979), assessing adaptations to standardised tests (Ahtiainen *et al.*, 2003) and ring-testing new biodegradation assessment methods (Painter and King, 1985).

Gerike and Fischer (1979) found aniline removal of 92-100% depending upon the selected method (pre-OECD 301 ready tests (OECD, 1992a): coupled-units test; Zahn-Wellens test; MITI test; Sturm test; OECD screen test or closed bottle test). This research (Gerike and Fischer, 1979; Gerike and Fischer, 1981) provided a set of chemicals from which several were selected to ring-test a respirometric method (Painter and King, 1985). Painter and King (1985) also found aniline to be readily biodegradable, exhibiting lag phases of 2-4 days, greater than 60% degradation at all time points (lag phase (t_L) + 10 days; 14 and 28 days) and theoretical DOC removal of 96-99% after 28 days. Considerable variation was found between replicates but ultimately aniline was determined to be readily biodegradable in all tests (Painter and King, 1985).

Ahtiainen *et al.* (2003) compared the degradation of aniline in an ISO 14593 CO_2 headspace test (ISO, 1999) using standard chemical (20 mg C L^{-1}) and inoculum (4 mg SS L^{-1}) concentrations in addition to aniline biodegradation rates at low chemical concentrations (0.002 and 0.022 mg C L^{-1}) with different inoculum sources and concentrations (1.8×10^2 – 2.1×10^3 CFU mL^{-1} from surface water sources of varying

water quality). At standard chemical and inoculum concentrations aniline was degraded with a $t_{1/2}$ of five days (Ahtiainen *et al.*, 2003). In degradation tests with lower chemical concentrations and non-standard inoculum sources (ISO, 1999) aniline was found to degrade with $t_{1/2}$ ranging from 5 to 150 days.

Ingerslev and Nyholm (2000) and Nyholm *et al.* (1996) also looked at the effect of low, environmentally relevant (µg L^{-1} as opposed to mg L^{-1} used in standard tests (OECD, 1992a; ISO, 1999)) aniline concentrations on biodegradation tests using ^{14}C-labelled shake flask test and aerated SCAS reactors respectively. Aniline was found to degrade readily with short $t_{1/2}$ values of typically 5-20 days, although variation between concentrations and replicates was observed with a $t_{1/2}$ maximum of 116 days (Ingerslev and Nyholm, 2000). Aniline degradation was found to proceed relatively constantly, suggesting degradation by ubiquitous microbes, as opposed to degradation linked to the growth of competent degraders (Nyholm *et al.*, 1996).

5.1.1.2. 4-nitrophenol (4-NP)

4-nitrophenol (also referred to as *para*-nitrophenol or PNP; referred to forthwith as 4-NP) is used as a precursor for the synthesis of a number of industrial chemicals including the insecticide parathion (Budavari, 1989; Neilson and Allard, 2008). It is widely spread throughout the environment, present in many industrial wastewaters and of considerable interest to public and environmental health and protection authorities (Rezouga *et al.*, 2009). 4-NP solutions are bright yellow, with the intensity of the colour linked to the chemical concentration, a property which has been utilised by Thouand *et al.* (1996) and Goodhead (2009) to monitor 4-NP biodegradation in 96-well plate biodegradation tests. Changes in pH are also responsible for 4-NP solution colour changes (pH 5.6 colourless; pH 7 yellow) allowing for its use as a pH indicator (Budavari, 1989).

A review of mono-nitrophenols by the WHO details the standardised, and non-standardised, historical testing of 4-NP (WHO, 2000). It is a “difficult” substance with varied outcome in biodegradation tests which has led to its classification as inherently biodegradable. It has been used previously as a model compound for assessing developments in standard tests (Gerike and Fischer, 1979; Nyholm *et al.*, 1984; Ingerslev and Nyholm, 2000; Toräng and Nyholm, 2005) and is recommended as a reference compound for use in a standardised inherent biodegradability test

(OECD, 1981a). A review of the literature by Comber and Holt (2010) found a median $t_{1/2}$ in non-standard tests of 2.5 days, with a range of 1.3-77 days. This large variation is typical of the type of variation that enhanced screens are aiming to reduce; combined with the proven inherent biodegradability of 4-NP and a desire to build on previous research by Thouand *et al.* (1995) and Goodhead (2009), warranted its inclusion in this study, although it was rejected from the original reference list compiled by Comber and Holt (2010) due to the extent of the variation.

Gerike and Fischer (1979) showed 0-100% removal by biodegradation based on the screening tests (pre-OECD 301 screening tests (OECD, 1992a): MITI and closed bottle tests showed low removal (0-60%); coupled units, Zahn-Wellens, AFNOR, Sturm and OECD screening test showed high removal (90-100%)).

Marvin-Sikkema and Bont (1994) reviewed specific microorganisms capable of degrading nitroaromatics and postulated that whilst rapid, complete degradation of 4-NP occurs in freshwater environments, a dense population of competent degraders is required and best obtained by an acclimation or adaptation period (Marvin-Sikkema and Bont, 1994). Gérald Thouand investigated 4-NP biodegradation in the context of reducing the variation observed within screening tests by:

- Determining what inoculum concentration would be required to give a guaranteed probability of degradation (52 mg SS L^{-1}) (Thouand *et al.*, 1995);
- Pre-conditioning or acclimating inocula to reduce inter-replicate variability (coefficient of variation range reduction from 101-257% to 27-74% with acclimation to test compounds) and increase test outcome reproducibility (Thouand and Block, 1993);
- Investigating pre-adaptation and increasing inoculum concentration to show a guaranteed (99.9%) probability of degradation using pre-adapted inocula at lower cell concentrations (Thouand *et al.*, 1996).

Toräng and Nyholm (2005) also investigated the impact of acclimation on biodegradation outcome and false negative rate via adaptation with a ‘semi-continuous pre-exposure procedure’ (SCEP). 4-NP was found to be completely degraded within pre-adapted systems, which exhibited reduced and more reproducible lag phases after 5 weeks adaptation than observed in initial, non-exposed tests (Toräng and Nyholm, 2005).

The findings regarding acclimation within 4-NP research suggest the degree of additional information which may be obtained by extending enhanced biodegradation screening tests beyond the 28 days used within OECD RBTs (OECD, 1992a) in order to allow mechanisms of adaptation to occur, whether the growth of rare or slow growing specific degraders or the expression of the genes required to switch on the required metabolic pathways.

Goodhead *et al.* (2013) criticised the inocula preparation techniques within OECD RBTs (OECD, 1992a), using a high throughput 96-well plate colorimetric assay to show increased variability in 4-NP biodegradation outcome when using pre-conditioning. Molecular analyses suggested a reduced total cell number accompanied by reduced detectable diversity when pre-conditioning. This reduction in diversity was proposed as being responsible for the lack of 4-NP degradation observed when using pre-conditioned inocula even when testing at cell concentrations 1000× larger than those recommended in OECD RBTs (Goodhead *et al.*, 2013)

5.1.1.3. 4-fluorophenol (4-FP)

The potential environmental impacts of fluorinated organics have gained less precedence globally than other halogenated compounds, due in part to a perceived biological inertness (Zhang *et al.*, 2007). More recently, however, increasing occurrence of these compounds in the environment (Key *et al.*, 1997) has awakened environmental interest in them (Neilson and Allard, 2008). The stability of the C-F bond lending chemicals towards persistence and the electronegativity of fluorine impacting upon the bond and encouraging biological interactions with fluorine substituents (Ferreira *et al.*, 2008). Organofluorine compounds have a broad range of uses which includes the manufacture of water repellents, pharmaceuticals and agrochemicals.

4-fluorophenol (4-FP) has been proposed by Comber and Holt (2010) as a reference compound for assessing enhanced screening methods. Davenport *et al.* (2009) used 4-FP as a reference compound in investigating the importance of cell concentration and microbial diversity in standard biodegradability test inocula. Goodhead (2009) used 4-FP as a reference compound in the development of a novel colorimetric assay based on an azo-coupling reaction which was subsequently used in 96-well

plate high throughout biodegradation assays for the rapid detection of phenolics (Goodhead, 2009).

Davenport *et al.* (2010) reported a varied biodegradation outcome in a 96-well plate high throughput biodegradation assay. Four different environmental compartments (AS, river, estuarine and seawater) were each represented by six different sites at inoculum concentrations ranging from 10^3-10^8 cells mL^{-1}. Biodegradation outcome was typically varied with larger cell concentrations generally providing a higher probability of 4-FP degradation: 67% of biodegradation tests using inocula concentrations greater than 10^6 cells mL^{-1} provided a guaranteed probability (100%) of degradation (based on 70% reduction in chemical concentration) (Davenport *et al.*, 2010).

The varied outcome in standard biodegradation tests suggest 4-FP is likely to fail occasionally even in an enhanced screening test (Comber and Holt, 2010).

5.1.1.4. 4-chloroaniline (4-CA)

4-chloroaniline is an important precursor in the chemical industry, used in the manufacture of pesticides, pharmaceuticals and dyes. It is also a predominant intermediate of other prolific chemicals, leading to its virtually ubiquitous presence in the environment (Vangnai and Petchkroh, 2007). Historically, 4-CA has shown varied biodegradation potential but currently is expected to fail a standard OECD RBT (Comber and Holt, 2010).

Ingerslev and Nyholm (2000) reported a failure to degrade in some biodegradation tests when assessing the biodegradability of 4-CA with different inoculum sources and concentrations and varied 4-CA concentrations. Where degradation did occur it proceeded gradually with no distinct lag period; $t_{1/2}$ values ranging from 21-173 days were recorded (Ingerslev and Nyholm, 2000). Ahtiainen *et al.* (2003) also observed slow, gradual removal of 4-CA, making a lag-phase in the traditional sense (i.e. a period of little degradation, followed by rapid removal once competent degraders have sufficiently established themselves in a community to degrade a compound) imperceptible. Ahtiainen *et al.* (2003) report $t_{1/2}$ values which ranged from 25 days to more than 500 days, where they could be calculated.

In contrast to these results, which would not suggest any increase in biodegradation rates from acclimation for 4-CA, Nyholm *et al.* (1996) proposed that pre-exposed sludge was responsible for faster 4-CA degradation rates than non-adapted sludge with higher 4-CA concentrations. Toräng and Nyholm (2005) also observed reduced lag phases (from 88 to 9 days) with considerably shorter half-lives (average 3.3 days ranging from 1.2-39 days)) in adapted Semi-Continuous preExposure Procedure (SCEP) tests than in non-adapted systems.

Comber and Holt (2010) report $t_{1/2}$ values in non-standard tests ranging from 93-150 days with large variations in lag phase duration and extent of biodegradation observed.

5.1.1.5. Pentachlorophenol (PCP)

Pentachlorophenol (PCP) is an organochlorine compound which has several industrial uses including the manufacture of pesticides and wood preservation (Budavari, 1989). PCP is the most recalcitrant compound included in the set of chemicals (Table 5-2) used to validate the enhanced screening method described in Chapter 4. It was considered for inclusion in the reference list of chemicals prepared by Comber and Holt (2010) for assessing enhanced and modified screening methods and to be used in future biodegradation and persistence studies (ECETOC, 2007; Comber and Holt, 2010). During the course of a literature review, Comber and Holt (2010) reported $t_{1/2}$ values in non-standard tests ranging from 50-150 days with a median of 128 days. The large variability in $t_{1/2}$ is the most likely reason for PCP ultimately not being selected in Comber and Holt's reference list.

Lapertot and Pulgarin (2006) reported no degradation of PCP in a ready biodegradability test. Nyholm *et al.* (1996) observed little degradation of PCP but did note an increase in degradation rate when using adapted rather than non-adapted sludge, suggesting a link between PCP degradation and the occurrence of a sufficient number of slow-growing organisms.

Ingerslev *et al.* (1998) observed a varied degradation outcome of PCP in ready biodegradability tests when using a range of inoculum sources (water and sediment from a polluted river and an unpolluted stream), test chemical concentrations (1-74,000 µg L^{-1}) and adaptation periods. Adaptation periods of 10-20 days allowed for rapid degradation of PCP (where concentrations of PCP were not toxic). Ingerslev

and Nyholm (2000) encountered $t_{1/2}$ values which ranged from 6-69 days with inoculum adapted over a period of five weeks.

Although degradation of PCP has been observed in biodegradation tests, and appears to benefit from an acclimation or adaptation period, it is not expected to routinely pass an enhanced screening test and would normally be expected to fail any RBT or screening test, including modified and enhanced tests.

5.1.2. The importance of reference chemicals

In order for a novel screening method to be accepted there are two main concerns which have to be addressed: the ECETOC workshop on biodegradation and persistence encouraged the modification of existing biodegradation studies and development of new, enhanced methods, but also urged caution that any new methods show not be overly protective or too powerful (ECETOC, 2007; Snape, 2010). This statement effectively relates to false negatives and false positives whereby a chemical is mistakenly classified as persistent or not persistent respectively.

The occurrence of false negatives questions the reliability of the selected biodegradation test method, typically arising as a result of the high variation currently observed in OECD RBTs. As previously discussed (Section 1.3.3), this variation results in the need for more intensive, higher tier testing. The proposed enhancements aim to reduce this variation by addressing the discrepancy in total cell number and microbial diversity which is currently encountered in standard tests, both between tests and within tests between replicates. It is postulated that providing a more environmentally relevant, representative inoculum will result in more reliable and reproducible chemical assessments.

Ensuring no occurrence of false positives may be considered more important than reducing false negatives. It is critical that these tests do not falsely identify persistent chemicals as being not persistent. A small number of false negatives would be acceptable but occurrence of false positives would strongly undermine the concerted effort to modernise regulatory testing and lend support to simply retaining the existing testing regimen.

5.2. Objectives

- *Design an enhanced biodegradation screening test capable of characterising chemicals based on their persistence*
- *Validate the enhanced biodegradation screening test against a reference set of chemicals with a range of known persistence*

5.3. Hypotheses

- *The enhanced screening test will deliver an accurate characterisation of chemical persistence with no false positive results*
- *Microbial community composition will change in response to chemical addition*

5.4. Methods

A summary of the methods used in validating the enhanced screen with both activated sludge and marine inocula is provided in Figure 5-1.

Activated sludge experiments were performed at separate times for the following groups of chemicals: 4-NP; Aniline and 4-CA; 4-FP and PCP (Aniline and 4-CA data were provided by Miss. Abigail Bartram and the Brixham Environmental Laboratory (BEL) environmental fate unit as part of a CEFIC-LRi project (Davenport *et al.*, 2009); experimental parameters including the inoculum source, inoculum preparation and biodegradation determinations were maintained between experiments performed within the same environmental compartment but at different time).

Marine experiments were performed at separate times for the following groups of chemicals: 4-NP and Aniline; 4-FP, 4-CA and PCP.

5.4.1. Sampling and inoculum preparation

5.4.1.1. Activated sludge (AS)

AS was obtained from Buckland Sewage Treatment Works, Newton Abbot, Devon, UK and prepared as previously detailed (Sections 4.4.1.1 and 4.4.2.1 respectively) on three separate occasions. At each sampling event 40L were collected in sterile carboys and kept aerated at room temperature until use on the same day. AS was concentrated or diluted to give concentrations of 3, 30, 300 and 3000 mg SS L^{-1}.

5.4.1.2. Marine samples

Marine samples were collected on two separate occasions from an on-site storage tank at Brixham Environmental Laboratory and prepared using tangential flow filtration as previously detailed (Sections 4.2.1.2. and 4.2.2.2. respectively). Four levels of processing were performed: 100× nominal concentration; 10× nominal concentration; filtered and raw (Section 4.2.2.2.).

5.4.2. Test chemicals

Test chemicals were selected as previously discussed in 5.1.1

Test chemicals were prepared as concentrated stock solutions to be dosed to vessels as previously detailed (Section 2.5.3) with the exception of PCP. PCP was prepared as a stock solution in ethanol rather than water due to its low water solubility.

5.4.3. Enhanced biodegradation screening test preparation

Triplicate 1 L batch biodegradation test systems were constructed based on the variables defined in Figure 5-1. The concentrated dosing stocks were dosed to the biodegradation tests at a ratio of 10 mL dosing stock to 1 L test inoculum to give final test concentrations of 10 mg C L^{-1}.

Test apparatus was constructed as shown in Figure 2-2. Experiments were performed as enhanced OECD 301B biodegradation screening tests (OECD, 1992a). Systems were kept aerobic in the dark at 22ºC (± 2ºC), in a temperature controlled room with a red light to fulfil the dark or diffuse light requirement during sampling.

5.4.4. Determination of biodegradation

Biodegradation was determined by the capture of evolved $^{14}CO_2$ in 2M NaOH and subsequent analysis via liquid scintillation counting (Section 2.5.5.1).

Residual ^{14}C activity was calculated as a measure of the chemical remaining in solution, as described previously in Section 2.5.5.2

5.4.4.1. Analysis and interpretation of biodegradation

The $^{14}CO_2$ evolution data was processed as detailed in Section 4.4.7.1 to give a measure of degradation over time.

5.4.5. Bacterial community analysis

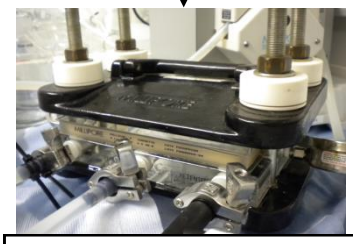
Extraction of total DNA, preparation of amplicon libraries for 454 sequencing and subsequent analysis were performed as in Section 2.6

Enhanced persistency screen

Activated Sludge
Buckland STW

Marine
Brixham Environmental Laboratory

Concentration by centrifugation



Concentration by TFF

Test Chemicals:

[10 mg C L^{-1}]

Aniline

4-Nitrophenol

4-Fluorophenol

4-Chloroaniline

Pentachlorophenol

SS Concentrations:

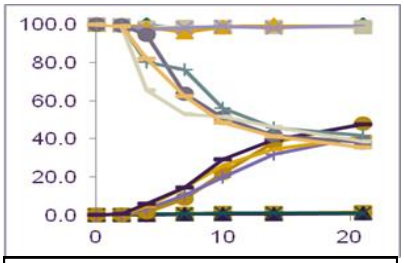
3; 30; 300; 3000 mg SS L^{-1}

Test volume: 1L

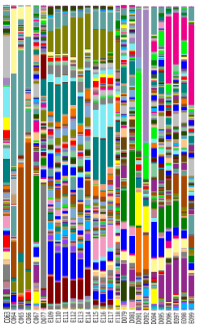
Test duration:

42 days *(Ani + 4-CA)*

60 days *(4-NP + 4-FP + PCP)*



$^{14}CO_2$ Evolution over time



454 Sequencing

Test Chemicals:

[10 mg C L^{-1}]

Aniline

4-Nitrophenol

4-Fluorophenol

4-Chloroaniline

Pentachlorophenol

Test Inoculum:

100×; 10×; Filt.; Raw

Test volume: 1L

Test duration:

60 days (*Ani*)

90 days (*4-NP + PCP*)

110 days (*4-FP + 4-CA*)

Figure 5-1 Summary of methodology for validating an enhanced regulatory screen for assessing the persistence of chemicals using activated sludge and marine inocula

5.5. Results and Discussion

5.5.1. Activated sludge enhanced biodegradation screening test validation

The Bin 1 chemical, aniline, was degraded in all activated sludge enhanced screening tests. The extent of aniline degradation and biodegradation descriptors were greatest with 300 mg SS L^{-1} inoculum concentrations. Lag phases of 2-4 days, a $t_{1/2}$ range of 5-23 days and removal of 57-90% were observed depending upon the inoculum concentration (Figure 5-2). Similar lag phases were found by Painter and King (1985) (2-4 days) when assessing a new method for determining biodegradation with aniline as a reference compound. Ingerslev and Nyholm (2000) reported a similar $t_{1/2}$ value range (5-20 days) in pre-exposed systems, although considerable variation was exhibited between different inoculum concentrations and between replicates.

The Bin 2 chemicals showed varied enhanced biodegradation screening test outcomes as expected (Comber and Holt, 2010). 4-NP was rapidly degraded at all inoculum concentrations, with a lag phase range of 2-3 days, $t_{1/2}$ range of 3.5-14 days and removal of 87-94% depending upon the inoculum concentration (Figure 5-3). The 300 mg SS L^{-1} concentration was again responsible for the shortest $t_{1/2}$ and greatest extent of degradation recorded (Figure 5-3). 4-NP has been used in several investigations into pre-exposure (Thouand and Block, 1993; Thouand *et al.*, 1996) due to variable lag phases and reported variation in degradation outcome (Comber and Holt, 2010). The supposition exists that the establishment of a relatively dense population of specific degrading organisms is required in order for 4-NP to be degraded (Marvin-Sikkema and Bont, 1994). The rapid degradation observed in the biodegradation screening tests presented here suggests an established, and sufficiently dense, community of 4-NP degraders present in the Buckland STW inoculum (Figure 5-2).

Degradation of 50-63% was observed for 4-FP across the range of inoculum concentrations employed (Figure 5-2). Lag phases of 1-2 days were followed by relatively slow but gradual removal of 4-FP with $t_{1/2}$ values which range from 16-60 days. The 3000 mg SS L^{-1} and 300 mg SS L^{-1} inoculum concentrations showed very similar degradation profiles, exhibiting the greatest rate and extent of 4-FP removal across the four inoculum concentrations used in validating the enhanced screen. 4-

FP was sufficiently degraded by all four concentrations used in this biodegradation screening test ($t_{1/2}$ ≤ 60 days), but barely reached this pass threshold when testing with low inoculum concentrations (Figure 5-2). It is expected that 4-FP may occasionally fail enhanced biodegradability screening tests (Comber and Holt, 2010) which could feasibly be expected based on the data presented here, if low inoculum concentrations, or inocula with low abundances of competent degrading organisms, were used. This is in keeping with research conducted by Davenport *et al.* (2010) suggesting 4-FP degradation is dependent upon source and concentration.

The longest lag phases amongst the Bin 2 chemicals tested were encountered when assessing 4-CA using the enhanced biodegradability screen (8-15 days). A $t_{1/2}$ range of 14-38.5 days was observed, with the enhanced screens using the 3000 mg SS L^{-1} inoculum concentration not showing sufficient degradation to calculate a $t_{1/2}$ value, although the degradation profile suggests that given time 50% removal would have been achieved (Figure 5-2). Similar degradation profiles were observed when applying the 300 and 30 mg SS L^{-1} inoculum concentration to the enhanced biodegradability test, with optimum degradation rates and a maximum extent of degradation at 74% attributed to these assessments (Figure 5-2). 4-CA has historically shown varied outcomes in standard assessments and has shown $t_{1/2}$ ranges in non-standard tests of 93-150 days (Comber and Holt, 2010). Values exceeding this have also been found when using 4-CA as a reference compound (173 days (Ingerslev and Nyholm, 2000); > 500 days (Ahtiainen *et al.*, 2003)).

The chemicals included in Bin 3 should normally fail any assessment, whether a modified RBT or an enhanced screening test. This is the area where the occurrence of false positives is most likely. PCP is inherently biodegradable, in the sense that, given favourable conditions, removal of PCP may be observed in standard type tests (Ingerslev *et al.*, 1998; Ingerslev and Nyholm, 2000) but it would not be expected to pass a standardised test or an enhanced test. The recalcitrance of PCP means that it poses a persistent threat in the environment, despite its recorded biodegradation potential. As such it needs to be identified by an enhanced screen characterising persistence. No removal of PCP was observed in the enhanced screen at any of the inoculum concentrations tested (Figure 5-2).

There was a significantly greater probability (ANOVA, $P < 0.05$) of a correct assessment of chemical persistency being made using the enhanced test (AS concentration of 300 mg SS L^{-1} with a pass threshold of a half-life of 60 days or less) compared to an RBT assessment (AS concentration of 30 mg SS L^{-1} with a pass threshold of 60% degradation within the ten-day window of a 28 day test).

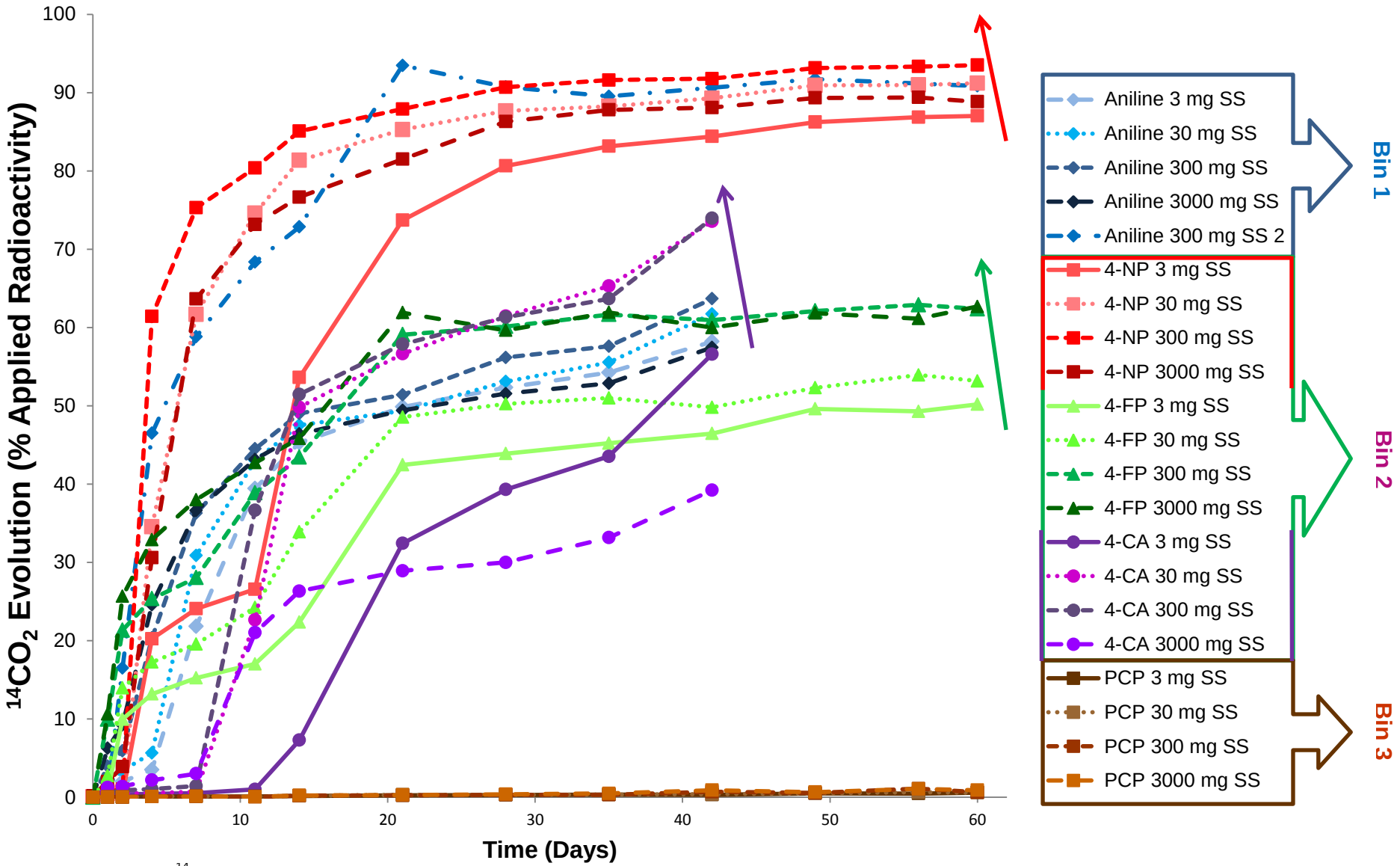


Figure 5-2 Activated sludge $^{14}CO_2$ evolution data for the reference set of chemicals selected to validate the enhanced biodegradation screening test. Arrows indicate a general increase in rate and extent of biodegradation with increasing inoculum concentration. Arrow colours are representative of the chemical they refer to. Increased inoculum concentrations also typically resulted in reduced lag phase and reduced variation between replicates. Data presented are the average of triplicate biodegradation tests. Variation between replicates is presented as coefficients of variation (%) in Table A-3 contained within Appendix B.

5.5.2. Marine enhanced biodegradation screening test validation

Aniline was degraded (60-67%) in the enhanced biodegradability screening test at all levels of marine inoculum processing (100×; 10×; Filtered; Raw), with lag phase ranging from 5-7 days and $t_{1/2}$ values of 23-36 days (Figure 5-3). There did not appear to be a concentration effect as the enhanced screen showed similar degradation profiles for aniline when using raw, unprocessed seawater and 100× nominally concentrated seawater (Figure 5-3). The extent of degradation is lower than might be expected based on previous research, for example Gerike and Fischer (1979) observed 92-100% removal of aniline (dependent upon test method) and Painter and King (1985) reported 96-99% removal within 28 days. Longer lag phases, however, have been known to cause false negative outcomes in aniline biodegradability assessments (Comber and Holt, 2010); whilst the rate and extent of degradation are not overwhelming, the enhanced screen would correctly identify aniline as not persistent.

The Bin 2 compounds showed more variable degradation outcomes in enhanced marine assessments than observed in enhanced AS assessments. Long lag phases were witnessed in 4-NP screening tests for all marine inocula, ranging from 31-50 days. Following the end of the lag phase, degradation occurred rapidly with $t_{1/2}$ values which ranged from 45-66 days (Δt_{50} range of 12-23.5 days). Ultimately 4-NP was sufficiently removed in all of the enhanced screening tests (77-92% dependent upon inocula), for it to be classified as not persistent (Figure 5-3). Enhanced biodegradation tests performed with 100× nominally concentrated inocula typically exhibited the shortest lag phases and $t_{1/2}$ values, but there was no significant difference in biodegradation screening test outcome between different inoculum concentrations. There was also no evidence of a reduction in inter-replicate variation with increasing inoculum concentration, with variation between replicates being relatively high at all concentrations (Appendix B). The long lag phase observed here was also seen in further investigations into the impact of volume on 4-NP degradation outcome (Chapter 4) and has been previously reported by Nyholm and Kristensen (1992) amongst others (Comber and Holt, 2010). It highlights the importance of extending biodegradation screening tests beyond the currently accepted 28 days used in OECD RBTs (OECD, 1992a) in order to gain a more comprehensive understanding of chemical fate in the environment. Based on a 28

day test both aniline and 4-NP would have failed and required higher tier testing; using an extended persistency screening test the compounds are both correctly identified as not persistent and resources can be redirected towards those chemicals which truly require further testing to protect human and environmental health. Despite 4-NP degradation data in Figure 5-3 showing rapid removal after a long lag phase, degradation outcome and degradation descriptors were not reproduced in subsequent 4-NP marine studies (Section 4.5.2) suggesting that the enhancements adopted for this biodegradation screening test have not sufficiently reduced the variation between replicates and experiments to repeatedly provide reliable degradation data for 4-NP.

4-FP was only degraded when the biodegradation screening test incorporated 100× nominally concentrated marine inocula, exhibiting a $t_{1/2}$ of 70 days (Figure 5-3). Degradation occurred gradually at all inoculum concentrations, with no perceptible lag phase, reaching 38-55% (Raw<Filtered<10×<100×) removal of 4-FP after 110 days (Figure 5-3). Slow but constant degradation was observed in all enhanced screening tests at all concentrations; extrapolation of the degradation curve would suggest that 4-FP would have been removed by at least 50% in all biodegradation screening tests, given sufficient time and presuming no change in degradation rate (Figure 5-3). As previously mentioned, it is expected that 4-FP may occasionally fail enhanced screening tests (Comber and Holt, 2010). Based on previous research by Davenport *et al.* (2010) and the data presented in Figure 5-3, the probability of a positive outcome in a 4-FP screening test is greater when the screen incorporates more concentrated inocula. There was no discernible reduction in variation between replicates in response to increased inoculum concentrations.

There was no degradation of 4-CA observed in any of the enhanced screening tests at any of the inoculum concentrations employed (Figure 5-3). Although long lag phases have been observed with 4-CA (Comber and Holt, 2010) there was no evidence to suggest that degradation had begun in any of the screening test individual replicates after 110 days (Figure 5-3). 4-CA is known to behave unpredictably in standard tests: Ingerslev and Nyholm (2000) observed varied biodegradation outcome when using 4-CA to assess the impact of inoculum sources and concentrations on biodegradation outcome, as did Ahtiainen *et al.* (2003) when using 4-CA as a reference compound in assessing the impact of chemical

concentration and inoculum source on biodegradation. However, it is known to degrade given the opportunity (Nyholm *et al.*, 1996; Toräng and Nyholm, 2005) and would be expected to pass an enhanced screening test, although fails may occasionally be experienced. Additionally, at least 50% removal was recorded in 83% of the activated sludge enhanced screen individual replicates (Figure 5-2).

No degradation of PCP was expected and none was observed (Figure 5-3). The toxic, persistent threat posed by PCP should mean that it is prioritised by an enhanced screening test for further environmental fate testing. No false positive results were observed for any of the environmental screens assessing PCP degradation using any of the activated sludge or marine inocula (Figure 5-2 and Figure 5-3).

There was no significant difference between the enhanced test and an RBT assessment in accurately characterising chemical persistency (ANOVA, $P > 0.05$).

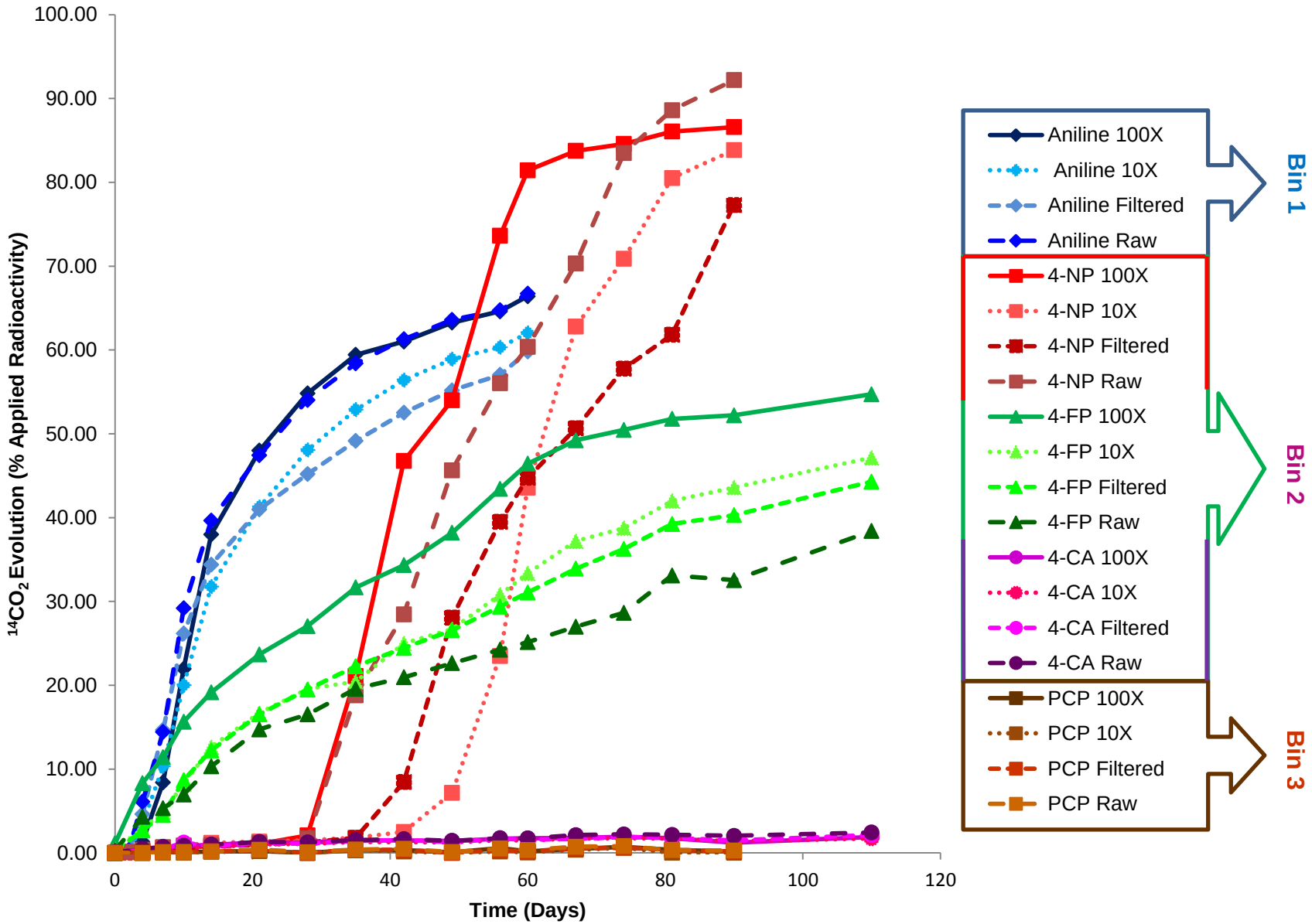


Figure 5-3 $^{14}CO_2$ evolution data for the reference chemicals selected to validate the biodegradation screening test. Generally, chemicals degraded as expected. No false positive outcomes were observed. There was a general trend for increased extent and rate of degradation and reduced lag phase with increased biomass; although the extent of the impact observed in activated sludge biodegradation screening tests were not seen in marine biodegradation screening tests. Variation between replicates is presented as coefficients of variation (%) in Table A-4 contained within Appendix B

5.5.3. Analysis of 454 sequencing data

5.5.3.1. Activated sludge

Differences in community composition were calculated using weighted UniFrac methods (Lozupone and Knight, 2005) which allow comparisons between communities to be made based on both the occurrence of OTUs and the relative abundance of those OTUs (Figure 5-4). The outcome is a distance metric, with a value of 0.0 only applied to identical samples. Figure 5-4 compares samples taken from the same location but at different times and subjected to different chemical applications. Group (1) is an assemblage of the AS (at all the different concentrations: 3000-0.3 mg SS L^{-1}) prior to any chemical addition; Group (2) is an assemblage of the AS (all concentrations) inocula following addition of 4-NP; Group (3) is an assemblage of the AS (all concentrations) used in assessing the impact of volume on 4-NP degradation; Group (4) is an assemblage of the AS (all concentrations) inocula following addition of 4-FP and PCP (AS samples were taken for molecular analysis as part of the CEFIC-LRi study which provided the aniline and 4-CA data). There is a greater degree of difference within AS samples following chemical addition (Groups 2 and 4) than within fresh samples (Group 1) (Figure 5-4); this suggests a varied reaction from individual systems in response to the addition of a test chemical, a supposition supported by the individual taxa plots showing an increased dissimilarity between samples following chemical addition (Figure 5-5).

The individual taxa plots represent the occurrence and relative abundance of OTUs present within individual samples where each colour is a distinct OTU and the area of the colour is representative of that OTU abundance within the sample (Figure 5-5). The individual taxa plots allow for areas of enrichment to be observed. There is significant enrichment of members of the *Peptostreptococcaeceae*.

The enhanced screening test outcomes when using 300 mg SS L^{-1} inoculum showed the greatest degree of reliability and reproducibility between tests, they also showed significantly less dominance of any one OTU than was observed in the 3000 mg SS L^{-1} inocula (i.e. appeared to show greater evenness). Similar observed OTU numbers were estimated for all inocula concentrations bar the 3 mg SS L^{-1} (Table 5-3). The lowest concentration inocula appeared to deliver the highest number of observed OTUs in 454 sequencing (Table 5-3), which may be an indication that less

enrichment occurred and therefore the communities were less dominated by a small, select number of abundant OTUs. The lack of a small number of abundant OTUs indicated a more even distribution of individual organisms between OTUs in lower biomass concentration inocula, which may also have contributed to more, different OTUs being observed. The apparent similarity in diversity in higher biomass concentration inocula may be indicative of a proportional increase in similar OTUs as a result of enrichment.

It is important to note that OTUs have been selected against a database of known 16S rRNA sequences; therefore only sequences that matched those contained within the database were recorded as observed species. This may have resulted in lower numbers of OTUs than might otherwise be expected and may have led to bias in taxa estimates

Table 5-3 Observed OTU calculated from next generation sequencing performed on a 454 GS-FLX platform. Chao1 values indicated representative sampling for all inocula except at 3 mg SS L^{-1}. No sequencing results were obtained for Filtered marine inocula

Inoculum source	Description	454 Observed OTU
Activated Sludge	3000 mg SS L^{-1}	608
	300 mg SS L^{-1}	659
	30 mg SS L^{-1}	727
	3 mg SS L^{-1}	1723
	Original	612
Marine	× 100	800
	× 10	873
	Original (RAW)	732

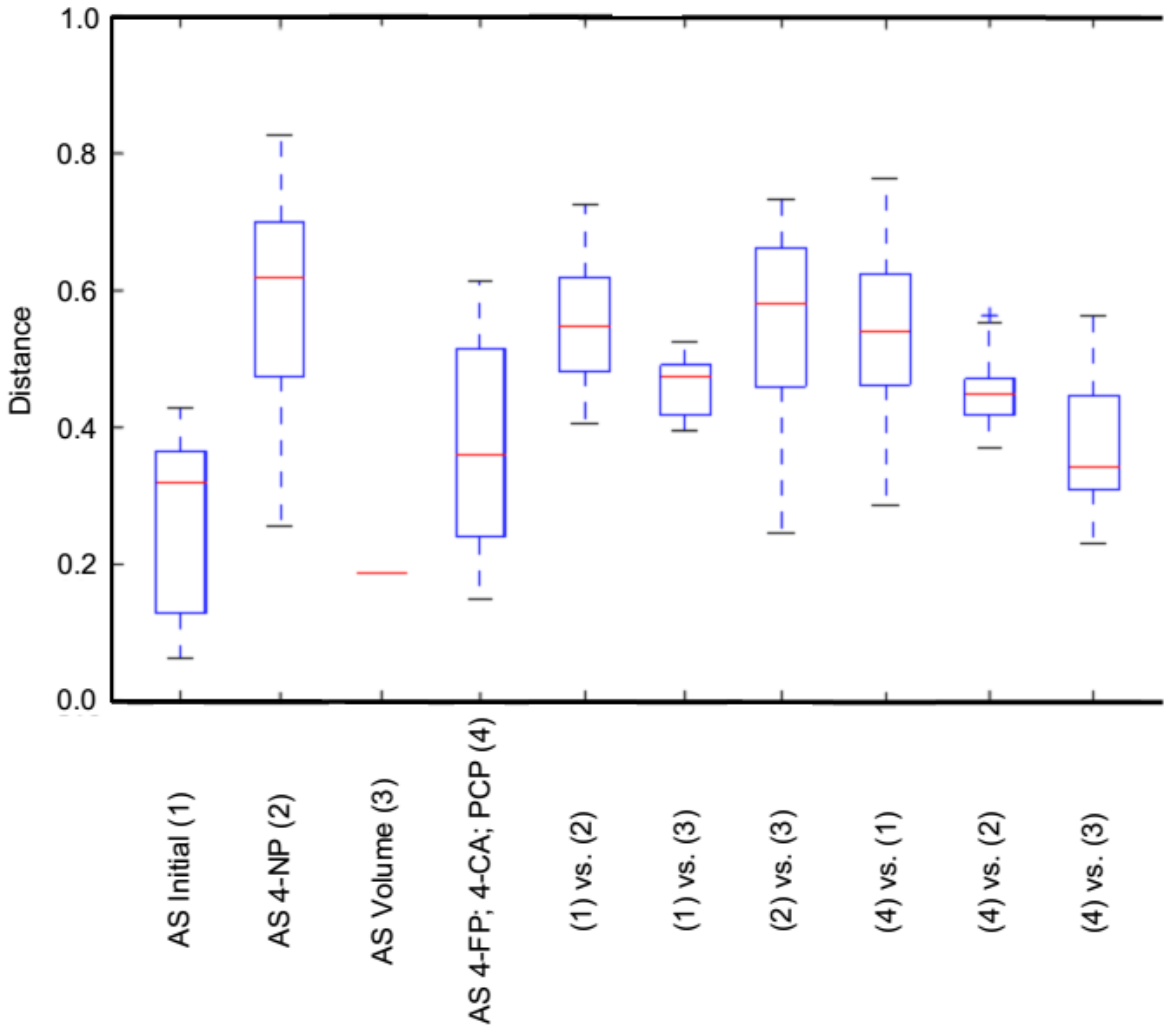


Figure 5-4 Weighted UniFrac boxplots (accounting for occurrence and abundance of OTUs) showing the community differences within and between the activated sludge biodegradation tests. Comparisons are made between the unprocessed AS, the processed AS used in assessing 4-NP biodegradation ; the processed AS used in assessing the impact of volume on 4-NP biodegradation (Chapter 4) and the processed AS used in assessing biodegradation of 4-FP, 4-CA and PCP. Box plot whiskers indicate 1.5 times the inter-quartile range (IQR).

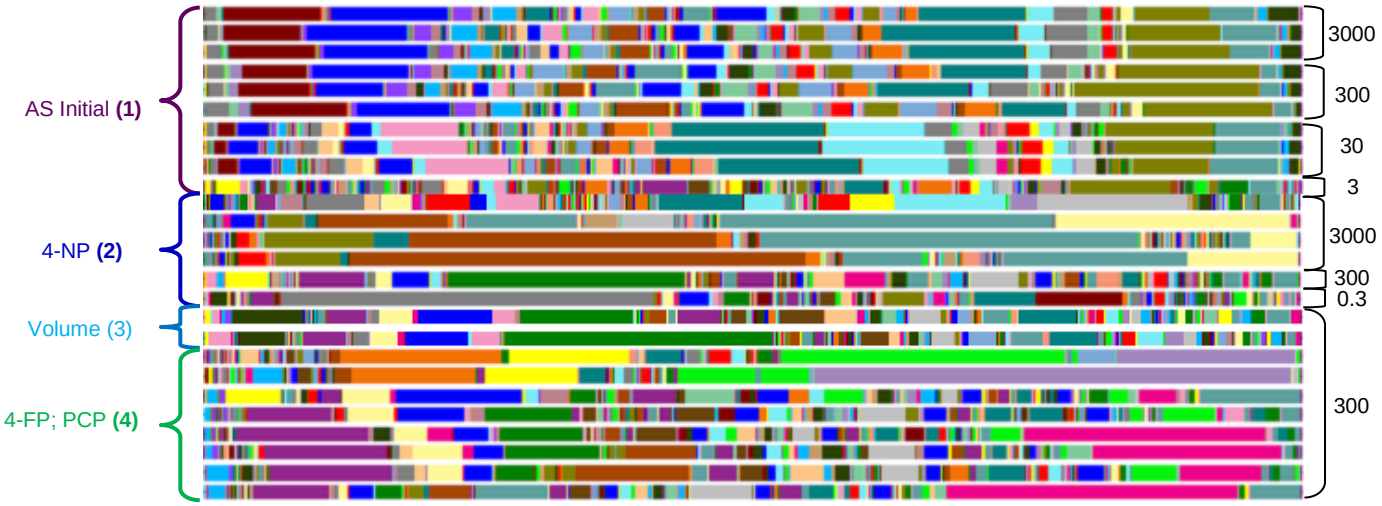


Figure 5-5 Individual taxa plots showing the composition of AS samples sequenced using the 454 platform. Each colour is representative of a distinct OTU, the abundance of which is proportional to the area covered by that colour. The distinct OTU colour is maintained across all samples allowing the visual comparison of composition and abundance. Numbers in parentheses refer to the weighted UniFrac boxplots in Figure 5-4. Values on the right represent the inoculum concentration in mg SS L^{-1}

5.5.3.2. Marine

The concentration method generally resulted in an inoculum which was a reliable representation of its environment. The strength of sampling increased with increasing cell concentration (Figure 4-10).

All inoculum concentrations appeared to produce communities with relatively similar diversity, based on observed species calculated from 454 sequencing data (Table 5-3).

Marine inocula appeared to be more similar to one another (Figure 5-6) than AS inocula (Figure 5-4) based on weighted UniFrac analyses. The addition of a chemical appeared to result in a decrease in the UniFrac distance, suggesting enrichment of certain bacteria and more similar communities produced in response to the chemical (Figure 5-4).

The individual taxa plots (Figure 5-7) showed significant enrichment of members of the SAR 11 clade or *Pelagibacteraceae*, a group of free living marine organisms commonly found in the ocean, following concentration of marine inocula (Rodriguez-Ezpeleta and Embley, 2012).

Raw marine samples contained a significant number of *Stramenopiles*, a diverse collection of marine microbes. Their absence from the concentrated samples is most likely due to their being screened out in the pre-filtering stage rather than as a response to chemical addition; they range in size from 2-20 µm (Lin *et al.*, 2012) and a pre-screen of 10 µm was applied.

As with the AS inocula (Figure 5-5) the marine inocula which powered the most reliable and reproducible marine enhanced screens did not have a single dominant bacteria; more often they exhibited numerous bacteria at relative abundances of 1-10% and a considerable number at lower populations constituting 0-1% of the overall community sampled.

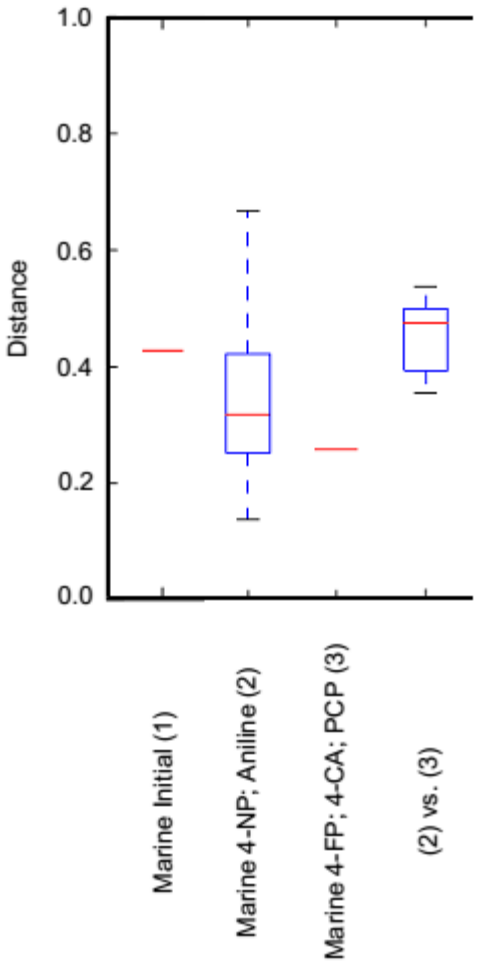


Figure 5-6 Weighted UniFrac boxplots (accounting for occurrence and abundance of OTUs) showing the community composition differences within and between the marine biodegradation tests. Comparisons are made between: the raw marine inocula; the processed marine inocula used in assessing the biodegradation of 4-NP and aniline; the processed marine inocula used in assessing the biodegradation of 4-FP, 4-CA and PCP. Box plot whiskers indicate 1.5 times the inter-quartile range (IQR).

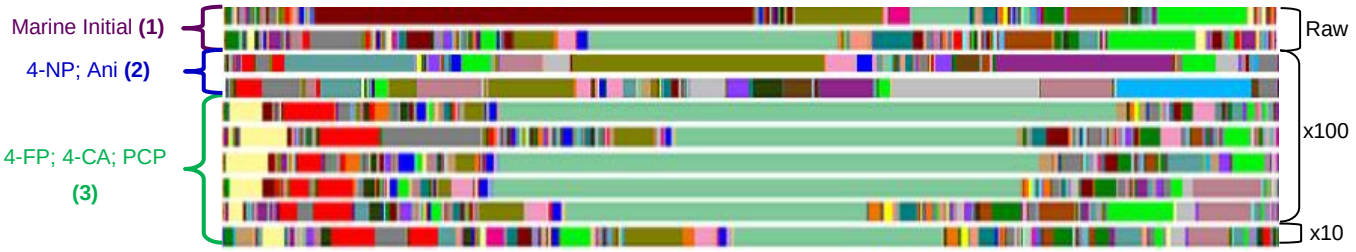


Figure 5-7 Individual taxa plots showing the composition of marine samples sequenced using the 454 platform. Each colour is representative of a distinct OTU, the abundance of which is proportional to the area covered by that colour. The distinct OTU colour is maintained across all samples allowing the visual comparison of composition and abundance. Numbers in parentheses refer to the weighted UniFrac boxplots in Figure 5-6. Values on the right of the figure represent the nominal concentration of the respective marine sample

5.6. Conclusions and Recommendations

- The enhanced biodegradation screening test delivered an accurate characterisation of the persistence of the reference chemicals tested. No false positive results were observed.
- Preliminary 454 analysis suggested enrichment of a small number of OTUs following chemical exposure. Further analysis is required to support preliminary findings.

It is recommended that the activated sludge biodegradation screening test described here should be used as an enhanced method described within REACH (EC, 2009) to identify chemicals based on their persistence. The biodegradation screening test has been shown to provide reliable, reproducible degradation data, significantly reducing the high levels of variation which are typically associated with standard biodegradation screening tests. The method described presently has been validated against a reference set of chemicals reporting no false positive or false negatives with respect the anticipated persistency assessments of the reference chemicals.

It is recommended that the marine biodegradation screening test described here requires more development to deliver reliable, reproducible degradation data and reduce the variation associated with these tests. Future method developments should focus on attempts to better understand mechanisms for adaptation and how these might be incorporated into a standard biodegradation assessment without making the test overly powerful.

5.7. Acknowledgements

I would like to acknowledge the contribution of Miss. Abigail Bartram, of the Brixham Environmental Laboratory (BEL) Environmental Fate Unit, for the provision of 4-chloroaniline and aniline degradation data in activated sludge enhanced biodegradation screening tests as previously remarked.

Chapter 6

6. General discussion

The chemical demand of the burgeoning global population is increasing year on year. It is important to provide accurate risk assessments for these chemicals to minimise the impact on human and environmental health. The present study answers the call to provide enhanced biodegradation screening tests which allow reliable and reproducible identification of those chemicals which are liable to persist in the environment (ECETOC, 2003; 2007). The current biodegradation screening tests (OECD, 1992a) are not effective at characterising persistence; they are notoriously highly variable and produce a large number of false negatives, estimated to account for 20-80% of RBT fails (ECETOC, 2007). The Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) adopted into legislation the proposed recommendations from prior ECETOC workshops in 2009, awaiting a comprehensive ring test with a set of reference chemicals (EC, 2009). These recommendations for enhanced tests typically focused on increasing the total number of cells within biodegradation screening tests and increasing the opportunity for adaptation of the inoculum to the test chemical:

- Increasing test duration beyond 28 days;
- Use of semi-continuous systems;
- Increasing inoculum concentration and test volume;
- Use of two ready tests in series

The present study provides the first comprehensive review of bacterial cell concentration methods in aqueous samples for the purpose of providing an enhanced inoculum to be used in biodegradation screening methods. It is recommended that the cell method to be used should be selected on a case-by-case basis, principally dependent upon the source and volume of the inoculum to be concentrated.

Additionally, a framework of scientific and practical criteria has been developed which can be used to assess future method developments in this area. The criteria within the framework were all given equal weighting; however, it may be decided, following feedback from industry and the regulatory community, that further

assessments should give preferential weighting to certain criteria. The importance of providing a concentrated inoculum which is representative of the sampled environment and has not been subject to selective enrichment and diversity bias may lead to community similarity being more heavily weighted, for example.

The general increased probability of degradation with increased inoculum concentrations in biodegradation assessments has been reported previously when assessing chemical degradation using different inoculum sources which naturally have different cell concentrations (Painter and King, 1985; Thouand *et al.*, 1995; Ahtiainen *et al.*, 2003). Davenport *et al.* (2010) reported a correlation between increased diversity and increased probability of degradation. Increases in inoculum concentration in the present study appeared to produce more similar bacterial communities than less concentrated inocula. DGGE data suggested this was also associated with an increase in diversity, although this was not necessarily supported by 454 sequencing data, which suggested approximately proportional increases in OTU abundance from original samples without necessarily introducing new diversity.

The present study carried out the most comprehensive investigation to date into enhanced biodegradation screening tests with particular attention on the variation between replicates and tests. Probability of biodegradation was highest when using concentrated activated sludge inocula. There was evidence that there is a concentration threshold in reducing variation and improving the reliability (probability) of biodegradation at 10^7-10^8 cells mL^{-1}, beyond which variation increases and reliability is reduced. There was limited evidence to suggest that increased cell concentrations led to these same effects when using concentrated marine inocula. Larger test vessel volumes typically generated faster degradation rates (or descriptors of them), including shorter lag phases and half-lives, but did not prove to be as significant as inoculum concentration with respect to these effects. Volume effects have been seen previously in larger scale tests: Ingerslev *et al*. (2000) reported reduced lag phases at larger volumes but found no link between lag phase and test volume in the 10-1000 mL volume range as used in the present study.

Reduced variation between replicates and tests was observed with increased inoculum concentration and test volume. Coefficient of variation between replicates was as low as 0.34% in biodegradation screening tests incorporating concentrated

inoculum in larger test volume (activated sludge: 10^7 cells mL^{-1} in 500 mL). The reduced variation is arguably the most important effect seen in these studies. Standard biodegradation assessments are notoriously associated with high variability within and between tests (Gerike and Fisher, 1979) and its reduction is a principal driver in changing biodegradation screening tests. The use of degradation descriptors allows for a comprehensive overview of variation between replicates, with reference to measureable endpoints used within OECD standard tests (OECD, 1992a) and proposed persistency endpoints (ECHA, 2008).

The aim of using concentrated inocula and other methods to increase total cell number should never be to increase the probability of biodegradation. Rather, the aim is to provide more realistic, representative samples of the environment, which result in more reliable, reproducible biodegradation data when applied to biodegradation screening tests. Where improvements in biodegradation potential are repeatedly observed, they should be considered as an indication that the environment has been representatively sampled and the inoculum has been given sufficient opportunity to give an accurate degradation assessment of the chemical within the defined parameters of the test. As mentioned previously, there are other factors relating to environmental relevance which have not been investigated during the course of this research (e.g. pH, temperature and light), and would need to be considered in the course of making truly environmentally relevant assessments.

The concentration of samples does not just increase the total number of cells within biodegradation screening tests but goes someway to normalising the number of cells and to some extent the diversity within inocula, which explains the reductions in variation between replicates and tests observed. It is a combination of the range of inocula concentrations and the increased variation in diversity between low inocula concentration replicates that is thought to lead to the high variation observed between replicates in OECD biodegradation tests (Comber and Holt, 2010).

It is important to note that, although the inoculum is referred to as being of increased concentration and the tests are referred to as enhanced tests, this is relative to the inoculum characteristics defined within OECD RBTs (OECD, 1992a). The approximate “concentrated” inocula concentrations of 10^8 cells mL^{-1} in activated sludge and 16^6 cells mL^{-1} in marine inocula are more akin to the concentrations you

might expect to find in the environment. This is particularly true for activated sludge inocula but perhaps does not extend to marine environments where concentrations ranging from 10^3-10^5 cells mL^{-1} are frequently encountered. However, a similar argument may still be applied: the inoculum which has been nominally concentrated 100 times, in theory, contains the bacteria from 100 litres of seawater. This equates to a cubic volume of approximately 0.1 m^3. I hypothesise that a chemical released into the marine environment would typically disperse beyond a 0.1 m^3 area and have the opportunity to interact with a much greater percentage of the estimated 10^{29} bacterial cells contained in the ocean than encountered in the present study (Whitman *et al.*, 1998). Indeed, data from the literature suggest that flow, transport and dispersal in seawater are high. Hydrological models of Norwegian coastal current flow have been estimated as 1.3-1.9 × 10^6 m^3 s^{-1}, with flushing times for the whole of the East England coastline of 10-50 days (OSPAR, 2000). Furthermore, validated 2D hydrological modelling of tidal date on the Hudson estuary suggest wide variation in eddy diffusivity and vertical turbulent salt flux of 10^{-5}-10^0 m^2 s^{-1} and 10^{-5}-10^{-1} kg m^{-2} s^{-1} respectively (Peters, 1997), which suggests that mixing can be high. However, determining contaminant transport is extremely complex and highly dependent on location, environmental conditions and climate.

The present study is the first to provide reliable and reproducible data assessing the impact of bacterial concentration and test volume in an activated sludge enhanced biodegradation screening test.

The variation seen in degradation data between the marine concentration experiments and the marine volume experiments indicated that the proposed biodegradation screening test does not currently have the capacity to provide reliable, reproducible degradation data regarding chemical behaviour in the marine environment. The variation observed was probably due to relatively low inoculum concentration levels (compared to AS) and the inherent problems associated with assuming that a 0.1 m^3 (the cubic volume when sampling 100 L of water) sample of seawater will provide an accurate representation of this environment. There are practical aspects to consider when contemplating the future development of a marine biodegradation screening test. Principally, it is infeasible to increase concentration and volume further than was attempted in this study. However, adaptation is an area of previous research (Thouand and Block, 2003; Thouand *et al*., 1995; Thouand *et*

al., 1996; Toräng and Nyholm, 2005) that has yet to be incorporated into a standard biodegradation screening tests but which may yield reliable, reproducible degradation data if applied appropriately. Methods that provide the opportunity for adaptation to occur, e.g. extended test duration and use of semi continuous test systems, are suggested within REACH guidelines (EC, 2009) but do not currently enjoy the confidence of regulators (Davenport, 2013). Efforts should be made to better understand the mechanisms that govern adaptation and how the mechanism might be accurately reproduced in biodegradation screening tests without making the tests overly powerful.

The study is the first to validate any enhanced biodegradation screening test for the assessment of chemical persistence. The enhanced biodegradation screening test was validated for use with activated sludge inocula against a reference set of chemicals with different, known, biodegradation behaviour in tests using environmental inocula. The test was neither overly protective nor overly powerful with the expected assignation of the correct Bins for the chemicals selected (Comber and Holt, 2010). No false positive or false negative results were reported with increased inocula concentrations. It is recommended that future enhanced biodegradation screening tests with activated sludge should incorporate inocula concentrations of 300 mg SS L^{-1} (10^7-10^8 cells mL^{-1}) in test volumes of 500 mL or greater.

The enhanced biodegradation screening test for marine inocula requires further investigation as previously described. The test did not prove to be overly powerful with respect to the reference set of validation chemicals. The failure to degrade 4-CA in any biodegradation test and the failure of 4-FP to reach at least 50% degradation in all but the biodegradation screening tests with ×100 inocula, may be an indication that the test is overly protective. It is certainly an indication that the erratic behaviour and variation in biodegradation outcome previously observed in other marine tests for these compounds has not been removed (Ahtiainen *et al.*, 2003; Davenport *et al.*, 2009; Goodhead, 2009; Davenport *et al.*, 2010).

Investigations to better understand the mechanisms and pathways by which enrichment appeared to occur within some higher biomass concentration inocula may provide additional information for the design of biodegradation screening tests.

Chapter 7

7. Conclusions, Recommendations and Future Research

7.1. Conclusions

- Selected methods were successful in concentrating bacterial cell concentrations in aqueous samples;
- No single selection method was suitable for all aqueous samples. Instead, the cell concentration method should be selected on a case-by-case basis;
- Increasing inoculum concentrations appear to lead to proportional increases in OTUs from the original samples. DGGE data suggests increases in diversity are linked to increases in inoculum concentration;
- Increased inoculum concentration typically resulted in decreased variation between replicates in activated sludge biodegradation screening tests;
- Increased inoculum concentrations typically produced more reproducible degradation data and degradation descriptors;
- Increased inoculum concentrations and test volumes were generally associated with an increased probability of degradation in enhanced biodegradation screening tests;
- There appears to be an upper threshold for increasing inoculum concentration in biodegradation screening tests using activated sludge. Beyond this threshold, the observed increases in extent of degradation and degradation descriptors begin to decrease, although there is no evidence to suggest a significant decrease in reproducibility between replicates and tests;
- Methods used to increase bacterial cell concentrations produced inocula that provided a robust representation of the environment sample;
- The enhanced biodegradation screening test delivered an accurate characterisation of chemical persistence in activated sludge, reporting no false positive results;
- Enhanced marine biodegradation screening tests using increased inoculum concentrations and test volumes did not significantly reduce the variation and erratic biodegradation behaviour that has previously been observed in previous biodegradation assessments.

7.2. Recommendations

- The appropriate cell concentration method should be selected on a case by case basis. Where there are no significant differences in scientific criteria, the inoculum source and required throughput will be the predominant factors. The gold standard method is membrane filtration, where sample volumes and initial sample bacterial concentrations allow its use; samples with high initial bacterial cell concentrations and relative densities are most suited to centrifugation; scenarios which require a large volume of low initial bacterial cell concentration to be concentrated would benefit most from tangential flow filtration;
- The enhanced biodegradation screening test described here is recommended for screening chemicals that are liable to persist when using activated sludge inocula. An inoculum concentration of 300 mg SS L^{-1} (10^{7}-10^{8} cells mL^{-1}) in a test volume of 500 mL or greater should be applied in the biodegradation screening test;
- The marine enhanced biodegradation screening tests described in the present study require further development to improve their reliability and reproducibility. It is recommended that future method developments should focus on incorporating adaptation into a regulatory test or the use of mixed sediment-seawater systems;
- Greater emphasis should be placed on the importance of the inocula within biodegradation screening tests. Molecular methods should be used more commonly to draw correlations between degradation of chemicals and the microbial community. In time, a database of degradation descriptors and community composition can be compiled. Using this information, it may be possible to make predictions of chemical fate based on the microbial community of a receiving environment prior to the release of a given chemical. Considering that the cost of sequencing is reducing at a faster rate than Moore's law, it is conceivable to think biological analysis may become cheaper than chemical analysis and facilitate shifts towards assessing the capacity of native communities to degrade compounds. Dependent upon the strength of the database, this could prove to be a powerful tool in risk assessment, particularly in tackling pollution at a local level.

7.3. Future work

- A considerable amount of data was generated during 454 sequencing, which I have at present only been able to analyse on a relatively superficial level, looking at measures of species richness and diversity. I would like to spend further time analysing the substantial volume of data returned from sequencing efforts and deliver a more comprehensive assessment of any correlations between sequencing observations and degradation descriptors.
- Continue the development of an enhanced marine biodegradation screening test to decrease variation between replicates and improve reproducibility of degradation data. Comprehensive analysis of the 454 sequencing data may yield suggestions as to providing marine inocula that are capable of driving a successful and reproducible biodegradation screening test.
- There is a need for a universal high throughput biodegradation screening test to allow a rapid prioritisation of chemicals based on their persistence. Good head (2009) has developed a 96 well plate high throughput biodegradation assay, but it is only suitable for phenolics. Johnsen *et al.* (2009) have developed a radiorespirometric method for measuring the evolved radiolabelled $^{14}CO_2$ in a 96 well plate format but it has not yet been applied to deliver a biodegradation screening test. It would be useful to develop a radiolabelled 96 well plate high throughput biodegradation assay, which accounts for evolved $^{14}CO_2$, residual ^{14}C activity in solution and residual ^{14}C bound to biomass, allowing a mass balance to be completed. The cost of radiolabelled chemicals can be prohibitive, so additionally methods should be developed that can be used universally e.g. by taking into consideration CO_2 production, the formation of carbonate and subtle changes in pH with compound degradation that could be measured.

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Appendices

APPENDIX A

Table A-1: Sequence for the 24 forward primers used for the amplification and downstream detection of sampled selected to be analysed via 454 sequencing

Primer Description	Bar Code	Adaptor	Forward Primer
A_BC1_f515	TATATGCG	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC2_f515	TATAGCTC	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC3_f515	TATCTCGT	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC4_f515	TATCAGAC	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC5_f515	TATCGTCA	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC6_f515	TATGCTAG	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC7_f515	TACTATCG	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC8_f515	TACTCAGT	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC9_f515	TACACGTA	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC10_f515	TACGTATG	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC11_f515	TACGACAT	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC12_f515	TAGTACGA	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC13_f515	TAGTGTAC	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC14_f515	TAGACTCT	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC15_f515	TCTACTGA	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC16_f515	TCTCTATC	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC17_f515	TCTCGCAG	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC18_f515	TCTGATCT	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC19_f515	TCATACTC	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC20_f515	TCATCACA	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC21_f515	TCATGTGT	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC22_f515	TCACTGCT	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC23_f515	TCAGTCGA	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA
A_BC24_f515	TCAGAGAG	CGTATCGCCTCCCTCGCGCCATCAG	GTGNCAGCMGCCGCGGTAA

Table A-2: Sequence for the 24 reverse primers used for the amplification and downstream detection of sampled selected to be analysed via 454 sequencing

Primer Description	Bar Code	Adaptor	Reverse Primer
B_BC1_rB926	TATAGCTC	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC2_rB926	TATCTCGT	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC3_rB926	TATCAGAC	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC4_rB926	TATCGTCA	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC5_rB926	TATGCGTA	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC6_rB926	TACTACGA	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC7_rB926	TACTCTAC	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC8_rB926	TACATGCT	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC9_rB926	TACGTATC	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC10_rB926	TAGTCACA	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC11_rB926	TAGTGTGT	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC12_rB926	TCTACTCT	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC13_rB926	TCTAGAGA	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC14_rB926	TCTCACTA	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC15_rB926	TCTGTCAC	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC16_rB926	TCATAGTC	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC17_rB926	TCATCTGA	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC18_rB926	TCATGCAT	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC19_rB926	TCACTACA	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC20_rB926	TCGATGTA	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC21_rB926	TCGCATAT	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC22_rB926	TGTGATGT	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC23_rB926	TGATCGCT	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT
B_BC24_rB926	TGACTCTC	CTATGCGCCTTGCCAGCCCGCTCAG	CCGYCAATTYMTTTRAGTTT

APPENDIX B

Table A-3: COV (%) values showing the variation seen between $^{14}CO_2$ evolution data for triplicate AS biodegradation screening tests at sampling points (indicated as days after dosing) when using a validation set of reference chemicals (Figure 5-2).

Coefficient of variation (%)

Test System	0	0.5	1	2	3	4	5	7	9	10	11	14	21	28	35	42	49	56	58	60
Aniline																				
3000	0.0		49.6	5.0		14.4		4.8			4.4	3.7	4.0	4.1	3.5	3.1				
300	0.0		56.8	52.0		16.3		11.6			7.7	11.8	14.3	10.1	9.8	10.4				
30	0.0		87.2	31.2		1.5		4.8			5.9	3.8	5.5	3.0	1.1	2.1				
3	0.0		53.4	30.6		2.8		63.2			16.2	9.7	5.1	4.4	3.4	7.1				
Aniline 2																				
300	0.0	34.1			49.1		12.7	5.4		2.3		1.6	2.6	1.7	1.6	2.6	2.3	1.3		1.7
4-NP																				
3000	38.7		26.5	11.3			63.0	13.5	0.5			1.6	2.3	1.3	0.3	0.2	0.5	0.4	1.0	
300	58.8		11.4	7.3			12.5	2.7	1.9			1.5	0.8	0.8	0.8	0.9	0.5	0.9	1.2	
30	126.9		11.3	4.6			87.1	31.8	14.2			9.5	6.7	5.1	4.8	4.5	4.4	4.7	4.5	
3	92.8		49.7	21.8			167.5	168.3	168.0			66.7	14.7	8.3	6.4	5.8	5.6	4.6	5.4	
4-FP																				
3000	0.0	63.8			10.8		4.0	4.6		6.8		5.1	1.4	4.4	3.0	4.4	2.5	3.4		2.8
300	0.0	12.3			1.4		2.1	4.4		6.6		3.2	2.6	1.6	2.5	3.7	2.4	3.3		1.1
30	0.0	69.3			0.5		3.3	1.1		19.8		8.1	4.3	3.1	2.3	2.9	4.5	4.6		6.4
3	0.0	57.5			5.9		2.9	1.7		1.2		23.9	5.5	7.8	5.6	5.0	1.1	5.9		7.0
4-CA																				
3000	0.0		53.7	95.7		76.6		66.2			85.4	82.8	82.6	82.1	76.0	67.7				
300	0.0		2.0	6.1		3.4		3.1			7.9	4.4	4.4	3.3	5.2	2.9				
30	0.0		5.6	13.1		8.4		12.6			94.6	5.9	4.6	4.7	4.1	4.7				
3	0.0		32.7	10.5		5.2		1.2			64.9	155.9	87.1	83.5	76.3	48.0				
PCP																				
3000	0.0	173.2			173.2		21.2	24.5		162.3		26.4	66.2	35.5	34.3	52.1	10.0	34.1		9.6
300	0.0	173.2			164.0		22.4	7.3		76.5		26.5	5.8	14.3	20.5	53.8	27.8	83.4		36.3
30	0.0	86.6			79.1		11.2	18.0		82.4		16.7	22.9	13.8	32.6	29.1	24.9	32.9		24.0
3	0.0	0.0			0.0		1.9	1.7		25.5		1.4	1.1	18.6	13.6	2.5	34.8	30.9		42.3

Table A-4: COV (%) values showing the variation seen between $^{14}CO_2$ evolution data for triplicate marine biodegradation screening tests when using a validation set of reference chemicals (Figure 5-3).

System	0	2	4	7	10	14	21	28	35	42	49	56	60	67	74	81	90	110
Aniline																		
100X	0.0	84.8	42.6	46.6	23.3	14.5	10.1	7.5	6.4	4.4	5.6	5.4	5.3					
10X	0.0	41.3	147.1	123.6	68.0	35.6	15.6	11.6	7.1	3.9	2.2	1.3	1.2					
Filtered	0.0	56.4	145.0	54.8	20.0	13.7	16.7	21.4	22.2	25.7	23.8	22.9	18.6					
Raw	0.0	107.3	146.1	108.6	43.3	20.8	13.6	12.1	11.6	11.3	10.1	10.0	8.3					
4-NP																		
100X	0.0	25.6	33.2	33.1	34.9	22.9	15.4	32.5	77.1	76.0	69.0	21.1	12.2	11.0	10.0	10.5	10.2	
10X	0.0	54.8	76.8	49.3	40.5	29.2	28.6	29.4	24.7	34.3	66.1	77.2	68.2	22.9	7.2	4.7	7.1	
Filtered	0.0	33.6	86.1	42.9	43.8	36.1	22.7	11.2	22.8	111.4	86.2	82.4	83.4	67.7	43.5	25.2	5.2	
Raw	0.0	18.0	48.2	36.2	31.9	29.1	18.0	25.9	157.1	158.0	90.5	79.1	74.0	46.0	13.0	5.7	3.0	
4-FP																		
100X	33.2		26.7	34.9	32.2	25.2	21.4	8.7	5.1	5.6	8.7	5.6	3.4	1.8	0.5	0.8	1.3	1.3
10X	46.1		38.9	22.4	33.8	19.1	14.0	9.7	12.1	15.1	20.5	21.8	20.1	16.7	13.9	9.3	6.9	4.5
Filtered	17.8		11.6	13.7	46.5	30.2	25.6	24.5	20.7	21.1	18.5	15.7	13.2	14.5	14.8	17.9	16.9	15.1
Raw	71.5		28.1	35.4	34.6	38.9	29.6	25.4	20.5	19.7	18.6	18.0	18.4	18.3	19.0	16.0	8.7	7.4
4-CA																		
100X	51.9		57.2	53.1	39.8	28.2	18.0	17.2	16.5	11.9	17.4	7.3	20.9	10.3	10.7	7.2	7.6	22.2
10X	16.1		24.9	22.2	12.7	18.2	11.6	12.7	4.7	8.6	6.9	5.8	6.1	7.1	1.2	4.0	1.0	10.2
Filtered	50.4		20.8	13.4	85.7	3.0	2.3	0.3	5.2	7.1	7.3	6.2	10.3	12.5	7.1	7.6	22.5	7.0
Raw	65.4		18.0	16.8	8.9	15.3	9.2	7.4	5.9	8.7	5.9	12.8	9.4	2.0	3.3	6.4	6.6	14.7
PCP																		
100X	173.2		98.7	50.0	91.7	49.1	4.8	39.6	3.2	22.3	136.5	33.6	158.8	31.1	22.0	134.7	125.7	
10X	86.6		86.6	99.8	165.5	65.3	1.7	17.5	1.1	42.4	16.0	1.5	148.0	1.0	1.1	16.2	16.2	
Filtered	173.2		165.1	1.8	19.8	1.3	42.1	129.2	18.1	1.7	8.5	45.8	91.1	2.1	2.6	111.1	111.5	
Raw	0.0		173.2	48.5	89.6	48.5	33.7	94.5	55.9	25.0	151.4	74.1	97.1	35.4	34.8	130.2	127.3	

Coefficient of variation (%)