

**BIODEGRADATION OF NONYLPHENOL
ETHOXYLATES (NPEOs) IN A MEMBRANE
AERATED BIOFILM REACTOR (MABR)**

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DECLARATION OF ORIGINALITY

I hereby declare that this thesis and the work reported herein was composed by and originated entirely from me. Information derived from the published and unpublished work of others has been acknowledged in the text and the relevant references are included in this thesis.

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Imperial College London, May 2013

❧ ABSTRACT ❧

The degradation intermediates of NPEOs surfactants (NP and short chain NPEOs) are of growing concern in environmental studies. These intermediates, recognised as endocrine disrupting chemicals (EDCs), are more toxic and refractory than their parent compounds. Their formation is assisted by anaerobic process, while their further breakdown to less harmful compounds is more easily achieved in aerobic environments. In this study, an hybrid MABR was exploited to completely degrade NPEOs, based on the concept of a multi-layered biofilm in the MABR that permits a simultaneous anaerobic-aerobic process to occur in a single reactor. This is the first study conducted on NPEOs biodegradation in an MABR. Batch microcosm experiments were conducted primarily to simulate NPEOs biodegradation behaviour in the MABR. The results showed that NPEOs removal was improved in a simultaneous anaerobic-aerobic system, as compared to a fully anaerobic system. A microporous polypropylene membrane with a non-woven polypropylene scrim heat-sealed to the surface was then used as an aeration device and biofilm support in a flat sheet MABR. Under steady state conditions (NPEO_{ave9} surface loading of 0.49 g/m².d; at 48 hr HRT), the reactor achieved an excellent removal of NPEOs (up to >99%) and organics in terms of COD (up to 93%). The disruption of MABR performance was less pronounced under hydraulic shock loads (reduced HRT) compared to organic shock loads (increased NPEO_{ave9} concentration), and this was postulated to be due to improved NPEO_{ave9} mass transfer into the biofilm. Despite the slow MABR recovery from shock loads, a stable NPEOs removal of more than 95% was achievable after the recovery periods. Based on HPLC-UV and GC-MS analyses, the EO units of NPEO_{ave9} were sequentially shortened (commonly via a non-oxidative pathway) over 500 days of operation to the major intermediate of NPEO₁. Nevertheless, complete removal of NPEO₁ was unsatisfactory, and more work needs to be done to optimise and investigate the role of the aerobic layer in degrading the compound. Nevertheless, this study has shown that the MABR is very reliable for the removal of both COD and NPEOs under long term operation, and the presence of toxic intermediates did not appear to inhibit overall reactor performance.

œ To my beloved mother and father œ

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NOTATION

AFM	Atomic Force Microscopy
ANAMMOX	anaerobic ammonium oxidation
AOB	ammonia-oxidising bacteria
AOPs	advanced oxidation processes
APEOs	alkylphenol ethoxylates
APHA	American Public Health Association
ATA	anaerobic toxicity assay
BMP	biochemical methane potential
BSA	N,O-bis(trimethylsilyl)acetamide
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
CABR	conventionally aerated biofilm reactor
CAS	conventional activated-sludge
CAPECs	carboxyalkylphenoxy ethoxy carboxylates
C:N	carbon to nitrogen ratio
COD	chemical oxygen demand
CSLM	confocal laser scanning microscope
CSTR	Continuous Stirred Tank reactor (CSTR)
DE	dead-end lumen
DEA	diethylamino
DGGE	denaturing gradient gel electrophoresis (DGGE)
DO	dissolved oxygen
EAWAG	Swiss Federal Institute of Aquatic Science and Technology
EDCs	endocrine disrupting compounds
EI	electron ionisation

EMB	extractive membrane bioreactor
EO	ethylene oxide/ethoxylate
EPS	extracellular polymeric substances
ES	electrospray
FISH	fluorescent in situ hybridisation
GC	gas chromatography
HF	hollow fibre
HILIC	hydrophilic interaction liquid chromatography
HPLC	high performance liquid chromatography
HRT	hydraulic retention time
KHP	potassium hydrogen phthalate
LOD	limit of detection
LOQ	limit of quantification
LLE	liquid–liquid extraction
MABR	membrane aerated biofilm reactor
MAD	microwave assisted derivatisation (MAD).
MAE	microwave-assisted extraction
MBfR	membrane biofilm reactor
MBR	membrane bioreactor
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
MW	molecular weight
NOB	nitrite oxidising bacteria
NP	nonylphenol

p-NP	para-Nonylphenol
NPEOs	nonylphenol ethoxylates
NPEO _{ave9}	nonylphenol ethoxylates (n = 9(average))
NPEO _{ave5}	nonylphenol ethoxylates (n = 5(average))
NPEO _{ave1.5}	nonylphenol ethoxylates (n = 1.5(average))
NPEO ₁	nonylphenol monoethoxylates (n = 1)
NPEO ₂	nonylphenol diethoxylates (n = 2)
NPEO ₃	nonylphenol triethoxylates (n = 3)
NPECs	nonylphenol ethoxy carboxylates
NPEC ₁	nonylphenol carboxylic acid (n =1)
NPEC ₂	nonylphenol diethoxy carboxylates (n = 2)
OE	open ended lumen
OECD	Organization for Economic Cooperation and Development
OPEOs	octylphenol ethoxylates
OUR	oxygen uptake rate
PBBR	packed-bed biofilm reactor
PCE	perchloroethylene
PCR	polymerase chain reaction
PVPDVB	poly (N-vinylpyrrolidone-divinylbenzene)
PLE	pressurised liquid extraction
RSD	relative standard deviation
SAMBR	submerged anaerobic membrane bioreactor
SEM	scanning electron microscopy
SMP	soluble microbial products
SPE	solid phase extraction

SRT	solids retention time
STPs	sewage treatment plants
TCD	thermal conductivity detector
TIC	total ion current
TMS	trimethylsilyl
TSS	total suspended solid
UASB	up-flow anaerobic sludge blanket
USE	ultrasonic assisted extraction
VFAs	volatile fatty acids
VSS	volatile suspended solids

❧ CHAPTER 1 ❧

INTRODUCTION

In the past few decades, a significant amount of evidence has emerged on the disturbance of hormonal function in wildlife caused by chemicals released into the environment. These chemicals, known collectively as endocrine disrupting compounds (EDCs) have become a prevalent subject of scientific and public debate. The EDCs have been defined as exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behaviour (USEPA, 1997). The disturbance of human and wildlife reproductive systems, in inducing an estrogen-like response, is believed to be the most profound effect of EDCs being addressed (Colborn *et al.*, 1993; McLachlan, 2001). Furthermore, measurable amounts of EDCs in aquatic environments, soil and food products have been recorded in different parts of the world. Thus, stringent legislative measures have been implemented in some countries to protect the environment and public health from the deleterious effects of EDCs.

Numerous chemicals have been identified as endocrine disrupters, or potential endocrine disrupters, by various environmental organisations worldwide. These include the specific groups of steroid compounds, pesticides, polyaromatic compounds and surfactants (Birkett, 2003). The key to an assessment of the fate of these organic chemicals in the environment is a realistic evaluation of their susceptibility to mineralisation (Grady, 1985). In other words, vast arrays of microbial species should be able to synergistically transform the organic chemicals completely to halide (e.g., chlorine, bromine), CO₂ and/or methane

(Rochkind-Dubinsky *et al.*, 1987). However, this is not always the case as the progress of biodegradation is commonly impeded by various environmental factors, and the nature of the compound itself. These often result in the formation of problematic metabolites, which are more toxic, lipophilic, recalcitrant and/or estrogenic compared to those of the parent compound.

A group of non-ionic surfactants, referred to as nonylphenol ethoxylates (NPEOs), have received considerable attention since they were introduced in the 1940s, due to their issues with respect to biodegradation pathways. It is estimated that about 60-65% of these compounds that enter the sewage treatment plants (STPs) are discharged into the environment (Ahel *et al.*, 1994a). In general, the primary anaerobic biodegradation of NPEOs involves a step-wise shortening of the ethoxylate (EO) chain length, generating refractory estrogenic shorter chain NPEOs as intermediates such as nonylphenol (NP) and NPEO mono to triethoxylates (NPEO₁₋₃) (Giger *et al.*, 1984). For instance, NP is reported to induce approximately 10 times more toxicity than its ethoxylate precursors (Renner, 1997). Moreover, a large body of literature exists on animal bioassays developed to elucidate the toxic effect and estrogenicity of nonylphenolic species in the environment. These findings clearly demonstrate that the intermediates induce damaging effects in fish, avian and mammalian cells, and that they mimic the effects of a natural hormone (17 β -estradiol) by binding to the estrogen receptor (White *et al.*, 1994).

The fate and biodegradation of NPEOs are largely influenced by their physical-chemical properties, such as solubility and partitioning onto organic material. The hydrophilicity of NPEOs is greatly reduced as the ethoxylate chains shorten. For example, the NP exhibits a high octanol-water partition coefficient value (Log K_{ow}) of 4.48, thus

conforming to the lipophilic nature of these intermediates which have a strong affinity for sediment (John *et al.*, 2000) and organic matter (Stasinakis *et al.*, 2010), as well as bioaccumulating in the lipids of organisms. This, in turn, prolongs its existence in the environment. In addition to anaerobic conditions, the NPEOs can also be degraded aerobically, producing hydrophilic intermediates of carboxylated NPEOs (NPECs) and dicarboxylated NPEOs (CAPECs) (DiCorcia *et al.* 1998). Despite being less estrogenic than anaerobic intermediates, their high concentration and low biodegradability suggests the possibility that they might also contribute to the endocrine disrupting effect in water, either individually or as a mixture (Chiu *et al.*, 2010). Therefore, there have been on-going efforts to identify and quantify these intermediates. On top of this, the fate of NP in STPs and aquatic environments is the most commonly and extensively reviewed intermediate due to its low biodegradability in most anaerobic environments. However, aerobic conditions have been proven to facilitate further biotransformation of NPEOs intermediates more easily than anaerobic conditions (Ying *et al.*, 2002). Therefore, as suggested by Luppi *et al.* (2007), further development of sequential anoxic/anaerobic – aerobic biodegradation strategies for the treatment of sewage containing NPEOs is essential to achieve its complete mineralisation.

The applied treatment strategy for NPEOs removal focuses primarily on conventional activated-sludge (CAS) technology, however, slight improvements have been made by the use of a membrane bioreactor (MBR) with a nanofiltration membrane (Wintgens *et al.*, 2002). Nevertheless, no study has been conducted on NPEOs mineralisation under simultaneous anaerobic and aerobic degradation processes. With respect to combining both aerobic to anaerobic biological environments, the membrane aerated biofilm reactor (MABR) has shown enormous potential for the biotreatment of wastewater containing xenobiotic pollutants. In the MABR, a biofilm with a high concentration of active microbial

communities is immobilised on a gas permeable membrane. Apart from providing a high rate of oxygen transfer through the membrane, counter diffusion of oxygen and substrates through the biofilm creates a unique concentration profile of both elements in the biofilm, thus enabling the formation of layered aerobic and anaerobic microenvironments. This type of biofilm is promising for the degradation of compounds with refractory intermediates which require both environments for complete mineralisation. Considerable effort has been devoted to studying the potential of MABRs from both a fundamental and applied aspect over the last 20 years. Apart from organic carbon and nitrogen removal, interest in xenobiotic pollutant removal in MABRs has increased due to its ability to promote diverse microbial communities responsible for degrading an array of pollutants in different microenvironments, while retaining slow growing microorganisms in the biofilm (Syron and Casey, 2008). For example, the exploitation of both processes in degrading xenobiotic compounds with toxic intermediates was successfully conducted for perchloroethylene (PCE) with complete mineralisation in a flat sheet MABR (Ohandja and Stuckey, 2007).

Therefore, it is hypothesised that the complete mineralisation of NPEOs under both aerobic and anaerobic conditions can be accomplished using the MABR. The use of a scrim-covered membrane appears to be ideal for immobilising and stabilising a biofilm on the membrane surface. As the biofilm grows, different layers of biofilm will be formed based on oxygen diffusion, thus providing a range of varying aerobic to anaerobic microenvironments in the same reactor. The anaerobic metabolites of NPEOs will diffuse into the aerobic layer driven by a concentration gradient, and vice versa. In addition, bubbleless aeration enhances oxygen transfer into the biofilm, thus overcoming oxygen limitations for efficient metabolites degradation. Hence, the biodegradation of NPEOs in this reactor could be achieved via oxidative and non-oxidative pathways.

1.1 PHD OBJECTIVE

Hence, the overall aim of this study was to investigate the biodegradation of NPEOs in batch microcosms, as well as designing and operating the MABR for NPEOs treatment, and monitoring the formation and mineralisation of intermediates in the reactor. In addition, the study also aimed to develop comprehensive analytical techniques to detect and quantify the degradation intermediates in the aforementioned experiments.

1.2 THESIS STRUCTURE AND PRESENTATION

The thesis contains eight chapters, and brief descriptions of each chapter are as follows:

- ❧ **Chapter 2: The literature review** represents a comprehensive review of the occurrence, fate and toxicity of NPEOs and their possible intermediates in natural environments and STPs. These include an overview of various interrelated factors that influence the compound's mineralisation, such as physical-chemical properties and the present of electron acceptors. The review also provides a brief description of the MABR technology, the mechanisms influencing its process performance, and its application in wastewater treatment.
- ❧ **Chapter 3: Materials and methods** lists all the analytical methods, experimental set ups and working procedures implemented in this study.

- ❧ **Chapter 4: Analytical method development for nonylphenol ethoxylates (NPEOs) and intermediates** represents optimisation of analytical techniques, including sample preparation steps and compounds separation, detection and quantification by instrumental analyses.
- ❧ **Chapter 5: Batch microcosm studies of nonylphenol ethoxylates (NPEOs)** evaluates the extent of the mineralisation of compounds using serum bottle techniques under different process conditions, and monitors the formation and disappearance of their intermediates using chromatographic techniques. The new approach of using a hybrid anaerobic-aerobic batch microcosm is tested on NPEOs.
- ❧ **Chapter 6: The treatment of nonylphenol ethoxylates (NPEOs) in a membrane aerated biofilm reactor (MABR) – a preliminary study;** in which the reactor is acclimatised to NPEOs, and the evolution of intermediates is monitored under steady state conditions. The work will provide a preliminary understanding on the prospect of NPEOs mineralisation in an MABR under long term operation.
- ❧ **Chapter 7: The treatment of nonylphenol ethoxylates (NPEOs) in a membrane aerated biofilm reactor (MABR) – the effect of organic and hydraulic shock loads;** is a second part of the MABR study, in which the MABR stability for NPEOs removal under different hydraulic and organic loads is investigated. At the end of the MABR operation, the physical structure of the biofilm is analysed microscopically.

œ **Chapter 8: MABR concluding discussion** summarises highlighted outcomes of the reactor studies over their long term operation. General remarks about reactor performance are also discussed.

œ **Chapter 9: Final conclusions and recommendations** represent a summary of the thesis, and the limitations of the study, and recommendations for future work.

❧ CHAPTER 2 ❧

LITERATURE REVIEW

2.1 ENDOCRINE DISRUPTING COMPOUNDS (EDCs)

A vast quantity of synthetic chemicals have been manufactured and discharged into the environment since World War II. Unfortunately, since then there has been a considerable amount of evidence gathered on the environmental and human health impacts linked to the exposure to synthetic chemicals. An alarming decrease in bird populations and reproducibility due to their exposure to pesticides was mentioned in the classic publication of Rachel Carson's "Silent Spring" (Carson, 1962). In addition to this evidence, the intersexuality of male fish was first discovered in an English River around the 1970s (Sumpter and Johnson, 2008). On top of this, a notable publication in the British Medical Journal in 1992 revealed a substantial decrease in the quality of semen amongst men from 1938 to 1990, and this was based on 61 reports published worldwide (Carlsen *et al.*, 1992).

Although profound cases of health problems related to these 'hormonal triggering' chemicals have been widely reported before the 1990s, a larger picture of the problems had not yet appeared. It was not until a meeting in July 1991 in Wingspread, Wisconsin, where key researchers gathered to address the issue associated with hormone-like effects of chemicals in fish, wildlife and humans did this issue becomes clear (Colborn and Clement, 1992). These chemicals were later lumped together as "endocrine disrupting compounds (EDCs)".

According to the USEPA (1997), EDCs can be defined as exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behaviour. Their responses are understood to be due to their ability to: (1) mimic the action of endogenous hormones such as estrogens and androgens; (2) antagonise the effect of endogenous estrogen; (3) disrupt and alter the pattern and alter the synthesis and metabolism of natural hormones; and, (4) disrupt and modify the hormone receptor levels (Sonnenschein and Soto, 1998).

To date, most of the EDCs antagonising effects have been found to be associated with causing reproductive disturbances in wildlife and aquatic organisms (Colborn *et al.*, 1993), as well as birth defects in the reproductive system in humans (McLachlan, 2001). Of these, aquatic organisms or organisms closely associated with the aquatic environment are profoundly affected by the EDCs as these chemicals have their major sink in rivers, groundwater and lakes (Trudeau and Tyler, 2007). The EDCs enter water bodies primarily from sewage treatment plants (STPs). A broad range of EDCs can be classified into: (1) natural steroidal estrogens such as estrone and estradiol; (2) xenobiotic or synthetic chemicals such as alkylphenol ethoxylates (APEOs), bisphenol A, pesticides and heavy metals; (3) synthetic estrogens such as 17 α -ethinylestradiol; and (4) phytoestrogens.

Current research has shown staggering numbers of suspected or identified synthetic chemicals in the environment that may induce endocrine disrupting responses, even at very low levels of exposure. According to the European Water Framework Directive (Directive 2000/60/EC, 2000), nonylphenol and its ethoxylates were identified as priority hazardous substances, and their use are subsequently regulated; this restriction applies to the products

and product formulations that equal or exceed 0.1% of NP and NPEOs by mass (Directive 2003/53/EC, 2003). These compounds have been replaced in household applications in most European countries (i.e., Scandinavian countries, United Kingdom, and Germany), Canada and Japan - either by the legal or voluntary ban, or by less toxic alcohol ethoxylates (Soares *et al.*, 2008). Nevertheless, the NP and NPEOs are still widely used in many parts of the world, such as the United States, China and other Asian countries. The US Environmental Protection Agency (EPA), however, has recognised the risk of NP and prepared guidelines that recommend the NP concentrations to be below 6.6 µg/L and 1.7 µg/L in freshwater and saltwater, respectively (Brooke and Thursby, 2005; Soares *et al.*, 2008). In addition, the proposed Environmental Quality Standards (EQS) for nonylphenols based on Directive 2008/105/EC are 0.3 µg/L and 2.0 µg/L for annual average (AA) and maximum allowable concentrations (MAC), respectively. The estrogen-like properties of alkylphenol were accidentally discovered by Soto *et al.* (1991), when an unknown contaminant leached out from the plastic centrifuge tubes and subsequently induced both cell proliferation and progesterone receptors in human estrogen-sensitive MCF₇ breast tumor cells. The contaminant was then identified as the alkylphenolic species of para-Nonylphenol (p-NP). To date, a large body of literature exists on animal bioassays envisioned to elucidate the toxic effect and estrogenicity of alkylphenolic species in the environment. In addition, these compounds have a strong tendency to persist and bioaccumulate in waterborne organisms and birds. For instance, the detected concentration of alkylphenolic derivatives is 10-1000 times higher than that of surrounding environments (Sonnenschein and Soto, 1998). Alkylphenols found in nature mainly originate from the primary breakdown of APEOs surfactants, and this surfactant is one of the most widely used industrial surfactants.

2.2 ALKYLPHENOL ETHOXYLATES (APEOs)

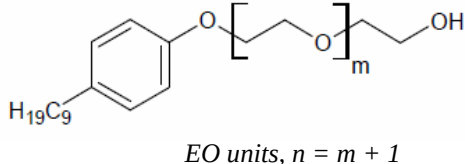
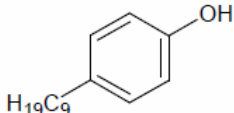
Introduced in the 1940s, the non-ionic surfactants of alkylphenol ethoxylates (APEOs) are the second largest group of surfactants used as detergents, wetting agents, dispersants, emulsifiers, solubilisers and foaming agents in various industrial and commercial applications. Examples of these include the production of pulp and paper, textiles, pesticides, household and industrial cleaning products, and other miscellaneous consumer products (Ying *et al.*, 2002; Staples *et al.*, 2008). It has been estimated that more than 60% of APEOs produced ends up in the aquatic environment, most entering via sewage treatment plants (White *et al.*, 1994). Based on a survey of 95 organic water contaminants in 139 United States streams by Kolpin *et al.* (2002), detergent metabolites of APEOs are the most frequently detected at higher concentrations than most of the other contaminants. Nonylphenol ethoxylates (NPEOs) and octylphenol ethoxylates (OPEOs) are the two most commercially important APEOs on the market. NPEOs are the most highly distributed APEO compounds in nature, and constitute about 80% of the total APEOs used commercially.

2.3 NONYLPHENOL ETHOXYLATES (NPEOs)

NPEOs are commercially produced by ethoxylation of nonylphenol isomers with ethylene oxide, forming a complex mixture of NPEO oligomers with different ethoxylate (EO) chain lengths; as a result, NPEOs are typically sold as a mixture of different oligomers and isomers. NPEOs with an average EO chain length of 9 units (having an EO chain length varying between 1 and 14), are simply denoted as NPEO_{ave9}, and are the most widely used NPEO mixture for commercial application. The surfactant-like structure of NPEO_{ave9} is shown in Table 2.1; the compound consists of both an hydrophobic alkyl phenolic portion,

and a long hydrophilic ethylene oxide (EO) chain part. In general, the primary biodegradation of NPEOs in sewage treatment plants and in the environment involves a step-wise shortening of EO chain length, generating lower molecular weight NPEO metabolites such as nonylphenol (NP) (refer Table 2.1) and NPEO mono to triethoxylates (NPEO₁₋₃) (Giger *et al.*, 1984). These metabolites are more persistence and hydrophobic than those of the parent compounds, and the physical properties of NPEOs and NP are shown in Table 2.1.

Table 2.1: Structure and physical properties of nonylphenol ethoxylates (NPEOs) and nonylphenol (NP).

Compound	Properties	Value	Reference
NPEOs  EO units, $n = m + 1$	Water solubility (mg/L) Molecular weight (g/mol)	Miscible 616	Huntsman, 1999a,b -
NP 	Water solubility (mg/L) Molecular weight (g/mol) Boiling Point (°C) Octanol-water partition coefficient (Log K_{ow})	6 (pH 7) 220 293 to 297 4.48	Weston, 1990 - Merck, 2006 Ahel and Giger, 1993a

One apparent reason for increasing global concern over the detrimental effect of NPEOs on the environment is the characteristics of their degradation products or metabolites. Thus, extensive reviews of nonylphenolic compounds have been published over the last two decades regarding their occurrence, fate, toxicity and extent of degradation in sewage treatment plants (STPs) and the natural environment (Bennie, 1999; Ying *et al.*, 2002; Staples *et al.*, 2008; Soares *et al.*, 2008; Chiu *et al.*, 2010). The intermediates of NPEOs are known to

be more toxic than their parent compounds, and possess a higher ability to mimic natural hormones by interacting with the estrogen receptors (Ying *et al.*, 2002). According to a study by White *et al.* (1994), short chain NPEOs such as nonylphenol diethoxylates (NPEO₂) and NP are estrogenic in fish, and avian and mammalian cells, by mimicking the effects of 17 β -estradiol in binding to the estrogen receptor. In addition, the hydrophobic alkylphenolic metabolites are prone to adsorb onto sediment and soil as well as tending to bioaccumulate in the lipids of organisms in the environment (John *et al.*, 2000; Stasinakis *et al.*, 2010). This, in turn, extending their persistence in the environment.

Among the early comprehensive studies on the occurrence and fate of nonylphenolic compounds in real environmental conditions were those conducted by EAWAG, Switzerland since the 1980s; these encompassed wastewater treatment plant effluents and rivers in the Glatt valley water catchment area (Giger *et al.*, 2009). The researchers found metabolites of short chain NPEO₁, NPEO₂ and NP in treated municipal wastewater, while exceptionally high levels of NP (mean 1 g/kg dry weight) were measured in digested anaerobic sewage sludge (Giger *et al.*, 1984). Alongside short chain NPEOs, carboxylated metabolites of NPEOs, e.g. nonylphenol diethoxy carboxylates (NPEC₂) and nonylphenol carboxylic acid (NPEC₁), were also detected in primary and secondary effluents of STPs along the Glatt River, as well as downstream of the river (Ahel *et al.*, 1994a,b). From these findings the authors postulated that the biotransformation of NPEOs proceeds under aerobic and anaerobic conditions, via oxidation of the EO chain (producing NPECs) and shortening of the EO chain (producing lower NPEOs oligomers and NP), respectively. The concentration of NPEOs and their intermediates found in surface water, wastewater effluent, sediment, and groundwater have been reported worldwide. Some of the selected data in different environmental media are shown in Table 2.2. The data shows a wide variation in

Table 2.2: Reported concentrations of NPEOs and their metabolites in environmental and wastewater samples (adapted from Campbell *et al.* (2006) and Petrovic *et al.* (2004)).

Compounds	Surface water (ng/l)	Sediments (µg/g)	Groundwater (ng/l)	Drinking water (ng/l)	Wastewater effluent (ng/l)	Sewage sludge (µg/g)
NP	<100–15000 ^{[1],a}	0.022–0.645 ^{[1],a}	200–760 ^{[8],k}	2.50–16 ^[3]	25–770 ^[3]	5.4–887 ^[7]
	<10–920 ^{[1],c}	<0.05–0.26 ^{[1],b}		10–2700 ^{[9],n}	18–185 ^[4]	
	<110–640 ^{[1],d}	<0.003–2.96 ^{[1],d}				
	<20–1200 ^{[1],e}	2.35–4.61 ^[1]				
	<77–420 ^{[1],f}	<0.01–1.05 ^{[1],g}				
	6.7–134 ^[3]	0.03–9.05 ^{[1],h}				
	<33–225 ^[4]	6.4–154 ^{[5],m}				
	100–7300 ^{[9],n}					
NPEO ₁ – NPEO _x	<220–1050 ^[2]	0.05–30 ^[2]	<10–8400 ^{[8],k}	100–300 ^{[9],n}	320–1570 ^{[6],b}	<0.5–254 ^[7]
	<100–31000 ^{[1],a}	<0.015–38 ^{[1],c} (NPEO ₁)	14000–38000 ^{[13],o} (NPEO ₂)			
	<20–10000 ^{[1],c} (NPEO ₂)	<0.003–0.17 ^{[1],d} (NPEO ₁)				
	<60–600 ^{[1],d} (NPEO ₁)	0.16–3.97 ^{[1],h} (NPEO ₁)	2900–22400 ^{[8],k}			
	<40–520 ^{[1],j} (NPEO ₂)	0.04–0.25 ^{[1],i}				
	<20–11000 ^{[1],g}	0.05–30 ^{[1],f}				
	1000–97600 ^{[9],n}					
Acidic metabolites	8400–20000 ^{[8],k} (NPEC ₁)				2000–2900 ^{[11],j} (NPEC ₁₋₂)	
	ND–2000 ^[10] (NPEC ₁)				14700–67200 ^{[12],l} (CAPEC ₁₋₂)	
					1000–15000 ^{[12],l} (NPEC ₃₋₂₀)	

Abbreviations: ND: Not detected; NPEC_x: nonylphenol ethoxy carboxylates; CAPEC_x: dicarboxylated nonylphenol.

Continue: Table 2.2

Country/location: a) Spain; b) Germany; c) USA and Canada (Great Lakes); d) USA streams; e) Krka estuary (Croatia); f) USA (Jamaica Bay); g) Spanish coastal area; h) English estuaries; i) Netherlands (Scheldt Estuary); j) Japan; k) Switzerland; l) Italy; m) Czech Republic; n) China; and o) Massachusetts, USA.

References: [1] Petrovic *et al.* (2004); [2] Ferguson *et al.* (2001); [3] Kuch and Ballschmiter (2001); [4] Heisterkamp *et al.* (2004); [5] Hilscherova *et al.* (2002); [6] Korner *et al.* (2000); [7] La Guardia *et al.* (2001); [8] Ahel *et al.* (1996); [9] Shao *et al.* (2005); [10] Field and Reed, 1996; [11] Isobe and Takada, 2004; [12] DiCorcia *et al.*, 2000; and [13] Rudel *et al.*, 1998.

nonylphenolic metabolite concentrations in different locations. The anthropogenic activities and the performance of local STPs strongly influenced the level of nonylphenolic metabolites in the environment.

2.4 MICROBIAL DEGRADATION OF NONYLPHENOL ETHOXYLATES (NPEOs)

The key to the assessment of the fate of organic chemicals in the environment is a realistic evaluation of their susceptibility to mineralisation (Grady, 1985). Mineralisation refers to the complete (ultimate) degradation of an organic chemical to carbon dioxide, water, and various inorganic forms. It can be achieved by means of chemical reaction such as hydrolysis and photodegradation (abiotic degradation), or biological reaction by microorganisms (biodegradation). In the environment, biodegradation is usually the only way in which an organic compound can be completely mineralised (Pavan and Worth, 2008). Depending on the ambient conditions, different modes and rates of biodegradation may predominate. In aerobic environments, microorganisms use oxygen (O₂) as a terminal

electron acceptor to convert the organics to carbon dioxide (CO_2), water and new cells. In the absence of O_2 , or in other words, in anaerobic environments, organic carbon is converted by subsequent oxidations and reductions in the presence of alternate electron acceptors (such as nitrate, sulfate and carbonate) to produce CO_2 and methane (CH_4). Unlike the large amount of biological sludge produced by aerobic process, in anaerobic processes only a very small portion of the degradable waste is converted to new cells, while the major portion of it is converted to CH_4 gas, which in turn represents better waste stabilisation (McCarty, 1964).

The ultimate biodegradation or mineralisation of NPEOs is of greater environmental significance than its primary biodegradation; however, mechanistic studies on the degradation pathways of NPEOs are still scarce. It is, however, well accepted that the NPEOs biodegradation can be achieved via oxidative (aerobic) and non-oxidative (anaerobic) pathways, and these biodegradation pathways are shown schematically in Figure 2.1. The most favorable bacterial attack on NPEO molecules occurs at the two end-points of the molecule, namely: (a) attack at the far end of the hydrophilic ethoxylate chain, progressing inward, (b) attack on the hydrophobic alkyl chain (Staples *et al.*, 2008). A hydroxyl shift mechanism for the anaerobic degradation of NPEOs has been proposed by John and White (1998). Biodegradation by the proposed mechanism preceded via successive excisions of the ethoxylate chains, and involved the cleavage of the terminal ethoxyl unit releasing acetaldehyde to give the corresponding shortened NPEOs until NPEO_2 is formed. In other words, the anaerobic biodegradation of NPEO starts with a shortening of the ethoxylate (EO) chain, leading to short-chain NPEOs and complete de-ethoxylation with the formation of NP. Carboxylated intermediates such as short-chain nonylphenol ethoxy carboxylates (NPECs) and alkylphenoxy acetic acid (NPEC_1) were only detected under aerobic conditions (Zhang *et al.*, 2008b).

Figure 2.1: Metabolic pathway of NPEOs; a: John and White (1998); b: Jonkers *et al.* (2001); c: DiCorcia *et al.* (1998); d: Zhang *et al.* (2008b); e: Telscher *et al.* (2005); f: Zhang *et al.* (2009); g: Corvini *et al.* (2006).

According to Jonkers *et al.* (2001), aerobic oxidation of the terminal alcohol group of NPEOs in a laboratory scale biofilm reactor occurs rapidly; nearly 99% reduction of the parent compound after 100hr. A slow conversion of long chain NPECs to their corresponding short chain NPECs (mainly NPEC₂) is then observed afterwards. Then, the oxidation of the alkyl chain starts with the formation of CAPECs (e.g. CA₅₋₉PEC₂), and disappeared completely after 500hr. Lower biodegradation performance was observed under the batch OECD modified Sturm test by DiCorcia *et al.* (1998). They found that the alcohol metabolites of NPEOs disappear by day 30, while the NPECs formed were almost eliminated by day 175. A gradual increase in CAPECs formation was observed during the test with an increasing abundance of CAPEC₁. However, these metabolites resisted further transformation, and remained in the system even after 5 months of their formation.

To date, only small numbers of studies have been carried out to detect further transformations of NP, the most potent EDC among nonylphenolic compounds. Based on current understanding, NP can only be degraded under aerobic condition. The metabolite of NP with a nitro-group at the ortho-position of the aromatic ring was detected in batch experiments with soil and sewage sludge (Telscher *et al.*, 2005), and in agricultural soils (Zhang *et al.*, 2009). In addition, a pathway via ipso-hydroxylation of NP was also elucidated in a study using the Spingomonads strain (Corvini *et al.*, 2006; Gabriel *et al.*, 2005). Under this pathway, the benzylic carbon will cleave and the alcohols (ROHs) will subsequently detach. This provides evidence that the aromatic ring needs to be cleaved from the alkyl side chain in order to be degraded and utilised. Nevertheless, an attack on the alkyl side is difficult due to the highly branched alkyl chain (>22 isomers), and its degradability is strongly dependent on the structure of the isomers (Montgomery-Brown *et al.*, 2003; Gabriel *et al.*, 2005). The pathway shown in Figure 2.1 is simplified in Table 2.3.

Table 2.3: NPEOs biodegradation mechanisms.

Conditions	Attack point	Action	Products
Anaerobic	End of EO chain	Ether hydrolysis; EO chain shortening	Short chain NPEOs and NP
Aerobic	End of EO chain	Terminal alcohol oxidation	Carboxylic acids (NPECs, NPEC ₁)
	Alkyl chain	Terminal methyl group oxidation; Alkyl chain shortening	Di-carboxylic acids (CAPECs)
		Cleavage of benzylic carbon	Metabolites of NP and alcohol

2.4.1 FACTORS AFFECTING NPEOs BIODEGRADATION: A REVIEW FROM BATCH BIODEGRADATION STUDIES

The potential of a chemical to be degraded under anaerobic conditions, and its toxicity towards the anaerobic process, can be effectively monitored by the “serum bottle method” that uses total gas production (pressure derived from the end-products CH₄ and CO₂) as the measure of ultimate degradation. The method was first described by Owen *et al.* (1979), namely the anaerobic toxicity assay (ATA) and biochemical methane potential (BMP). The method was subsequently evaluated and optimised by Shelton and Tiedje (1984). After further modification by Birch *et al.* (1989), it was later drafted as the Organisation for Economic Cooperation and Development (OECD) Guideline 311, which was finalised on March 23, 2006 (Meylan *et al.*, 2007). Although different terms have been used by authors to describe the anaerobic bioassay, generally, these serum bottle methods provide a relatively simple and easy way of evaluating biodegradation.

Numbers of batch microcosm studies on NPEOs and their derivatives have been carried out under aerobic and anaerobic conditions. Table 2.4 lists the biodegradation studies of the compounds conducted under batch conditions, as described above. The sources of inoculum or biomass were commonly obtained from STPs (i.e. digester sludge and activated sludge). Some studies used pure bacterial cultures, mainly isolated from activated sludge of a STP, and a wide range of degradation rates have been reported depending on the kind of test system and conditions employed. The biodegradation of a highly reduced organic molecule is thermodynamically favorable when sufficient terminal electron acceptors with relatively high redox potential, such as nitrate and sulphate, are supplied (Heider *et al.*, 1999). Since NPEOs are highly reduced organic molecules, the anaerobic biodegradation rate of the NPEOs can be enhanced by supplying sufficient terminal electron acceptors, such as nitrate (Luppi *et al.*, 2007), Iron (III) (Lu *et al.*, 2007a) and sulphate (Lu *et al.*, 2007c). NPEOs can serve as a sole carbon source for microorganisms, and some studies show that addition of organic matter such as glucose and acetate greatly reduced the degradation rate of NPEOs (Lu *et al.*, 2007c; Lu *et al.*, 2007b). One possible explanation for this is that the organic matter competes with NPEOs for electron acceptors. Nevertheless, addition of yeast extract only resulted in a slight inhibition of NPEO degradation (Lu *et al.*, 2007b), and enhanced the aerobic degradation of NP (Chang *et al.*, 2005). Another study by Hayashi *et al.* (2005) found that the presence or absence of organic matter (methanol, glucose and yeast extract) could influence the biodegradation pathway, in which the parent compound of NPEO₁₀ was only oxidised to the corresponding carboxylated metabolite of NPEC₁₀ without shortening of the EO chain. The reported data also shows that the biodegradation rates were higher for the long chain NPEO oligomers than for the shorter ones (Hayashi *et al.*, 2005). As a result, short chain NPEOs and NP accumulated in the system.

Table 2.4: Studies on the biodegradation of NPEOs in batch microcosms.

No	Compounds	Inoculum	Process	Electron acceptor	Substrate	References
1	NPEOs	<i>Pseudomonas</i> sp. strain TR01	Aerobic	Sulphate, Nitrate		Maki <i>et al.</i> , 1994
2	NPEOs	<i>Pseudomonas putida</i>	Anaerobic			John and White, 1998
3	NPEOs	Filtered fresh effluent of a STP	Aerobic			Di Corcia <i>et al.</i> , 1998
4	NPEOs	Municipal digester solids	Anaerobic			Salanitro and Diaz (1998).
5	NPEO ₁ , NPEO ₂	Anaerobic digester from a pulp plant; Dewatered digester sludge from landfill; Municipal solid waste	Anaerobic			Ejlertsson <i>et al.</i> , 1999
6	NPEOs	Filtrate of STP secondary effluent	Aerobic		Methanol, glucose, yeast	Hayashi <i>et al.</i> , 2005.
7	NP Isomers	<i>Sphingomonas xenophaga</i> Bayram				Gabriel <i>et al.</i> , 2005
8	NP, NPEO ₁	Sludge from STP	Aerobic	Hydrogen peroxide, aluminium sulphate	Yeast	Chang <i>et al.</i> , 2005
9	NP, NPEO ₁	Soil	Aerobic	aluminium sulphate	Yeast	Chang <i>et al.</i> , 2007
10	NPEOs	Activated sludge	Aerobic			Zhao <i>et al.</i> , 2006
11	NPEOs	Freshwater sediment	Anaerobic	Fe(III)		Lu <i>et al.</i> , 2007a
12	NPEOs	Denitrifying activated sludge from STP	Anaerobic	Nitrate	Methanol, acetate, glucose, and yeast extract	Lu <i>et al.</i> , 2007b
13	NPEOs	Anaerobic digester sludge	Anaerobic	Nitrate, sulphate	Acetate, lactate, glucose	Lu <i>et al.</i> , 2007c
14	NP	Nitrifying activated sludge from STP	Aerobic	Nitrate, Ammonium, Nitrite	Glucose	Kim <i>et al.</i> , 2007
15	NPECs	Activated sludge from STP	Aerobic			Zhang <i>et al.</i> , 2007
16	NP, NPEOs	Natural water				Zhang <i>et al.</i> , 2008a
17	NPEOs	Activated sludge from mixed industrial and domestic waste	Anaerobic	Nitrate		Luppi <i>et al.</i> , 2007

STP: Sewage treatment plant
NPEOs: Nonylphenol ethoxylates (long chain)
NPEO₁: Nonylphenol monoethoxylate
NPEO₂: Nonylphenol diethoxylate
NPECs: Nonylphenol ethoxy carboxylates
NP: Nonylphenol

Ejlertsson *et al.* (1999) investigated the anaerobic degradation of NPEO₂, NPEO₁ and NP in anaerobic digester sludge, landfill sludge, and landfill municipal solid waste (MSW) inocula. All inoculates used degraded NPEO₁ and NPEO₂ at 2 mg/L, with NP forming the ultimate degradation product; however, the NP was not further degraded. They also observed that for 2 mg/L of NPEOs, CH₄ production was similar to the controls; creating the possibility that the NPEO₁₋₂ and NP could hinder CH₄ production by adversely affected the acetate-utilising methanogens. In addition, the amount of EO released during the transformation of NPEO₁₋₂ and NP would have been too low to generate enough methane. Kim *et al.* (2007) used a nitrifier-enriched activated sludge in the batch degradation of NP and discovered that ammonium oxidising activity is more influential on NP removal compared to nitrite oxidising activity. It is believed that the co-metabolic activity of ammonium oxidising cells could play a role in the degradation of NP.

2.5 NPEOs TREATMENT TECHNOLOGIES

The treatment of wastewater containing NPEOs can be conducted using physical, chemical and biological treatment. In recent days, studies using laboratory-scale reactors have been widely devoted to chemical treatment, including advanced oxidation processes (AOPs)(i.e. photolysis with UV, photocatalytic oxidation and ozonation), and biological treatment including conventional activated sludge (CAS) and biofilm reactors. For instance, NPEC₁ and NP were effectively removed by ozonation, but NPEO₁ was poorly eliminated in the same system, within the same reaction time (Ike *et al.*, 2002). Moreover, the AOPs often produce unsuspected, persistent and potentially toxic by-products, such as benzoquinones in a UV photolysis reaction (Neamtu and Frimmel, 2006; Di Gioia *et al.*, 2009). However, their high demand for energy and cost largely impeded the practical and commercial exploitation

of chemical oxidation. On the other hand, biological treatment usually offers the most cost effective treatment option. Moreover, the majority of EDCs in wastewater are expected to be removed by biodegradation, but for nonylphenolic compounds (i.e NPEOs and NP) removal efficiencies vary between 12-95%, depending on the type and condition of the treatment (Liu *et al.*, 2009; Mcadam *et al.*, 2011). The CAS, for instance, is the most popular treatment option for large scale NPEOs-containing wastewater. Nevertheless, the extensive used of bubble aeration in CAS is costly and in some cases, mass transfer is insufficient to sustain the aerobic treatment of low ethoxylate compounds. Thus, substantial amounts of refractory hydrophobic intermediates are formed and associated with sludge, and the sludge then need to be treated by anaerobic digestion (Johnson and Sumpter, 2001). Improvements on removal efficiency have also been made by using membrane bioreactors (MBR) with nanofiltration membranes (Wintgens *et al.*, 2002). Although there are only a limited number of studies on MBR application, it appears that MBRs result in slightly higher EDCs elimination efficiencies compared to CAS processes (Zhou *et al.*, 2011).

A study by Clara *et al.* (2005) confirmed that the removals of micropollutants, especially EDCs, are strongly related to solids retention time (SRT). Higher SRT (>10days) enhanced the elimination of estrogenic micropollutants in most full scale treatment plants studied (e.g. CAS and MBR). Hence, biofilm systems are especially suitable for the treatment of estrogenic micropollutants because of their excellent retention of microbial biomass. Biofilms are a complex mixture of highly distinguished microorganisms embedded in extracellular polymeric substances that are attached to a material surface (Watnik and Kolter, 2000). In previous lab scale studies, a packed-bed biofilm reactor has proven to be effective in the removal of wastewater containing nonylphenol (Fujii *et al.* 2003; Soares *et al.* 2006), and NPEO_{ave5} (Di Gioia *et al.*, 2009). Despite its potential for effectively treating xenobiotic

contaminants, biofilm-based systems are rarely exploited for the treatment of NPEOs. Since oxygen transfer is a critical factor in facilitating further biotransformation of NPEOs intermediates such as NP, frequent occurrences of mass transfer limitations in conventional biofilm systems need to be overcome. Using a membrane as a biofilm support and an aeration device has tremendously improved the oxidation capacity of a biofilm system. The concept, operation and application of an emerging advanced biofilm technology, called a Membrane Aerated Biofilm Reactor (MABR), will be discussed in the next section.

2.6 MEMBRANE BIOFILM REACTOR

Watnik and Kolter (2000) describe a biofilm as “a city of microbes”- an assemblage of complex, highly distinguished microorganisms embedded in extracellular polymeric substances (EPS) that are attached to a material surface; much like our own city that provides a convenient way of life and safety for its inhabitants. A biofilm, as a whole structure, is a complex matrix. Not only can a diverse group of bacteria, fungi, algae and protozoa species thrive in it, but the biofilm also contains secreted polymers, cell lysis products, absorbed nutrients and metabolites and noncellular materials such as mineral crystal and particulates, depending on the environment in which the biofilm has developed (Singh *et al.*, 2006; Donlan, 2002). A biofilm can develop on various surfaces of living and non-living materials, such as living cells, sediments, and a piping system. The excreted polymers in a biofilm play an important role as a shelter for microorganisms by protecting them from harsh and changing environmental conditions. Furthermore, according to a modern theory, biofilms predominate in 95-99% of microorganisms' existence in nature, whereas only a small fraction of them exists in the form of isolated, drifting cells in suspension (planktonic) (Nikolaev and Plakunov, 2007). Hence, there is no doubt that biofilm formation is a prevalent way of

existence for microorganisms in nature. These attached biofilm systems have long been exploited for biological wastewater treatment applications, e.g. static biofilm systems (e.g. trickling filters) and particulate biofilm systems (e.g. biofilm fluidised bed reactors, upflow anaerobic sludge blanket reactors and biofilm airlift suspension reactors) (Nicolella *et al.*, 2000). Widespread use of biofilms in wastewater treatment is driven by several factors such as: (1) natural and mixed microbial populations are readily formed in the biofilm; (2) excellent biomass retention and accumulation (higher SRT), eliminating the need for solids-liquid separation devices; and (3) high biomass concentrations promote better volumetric reaction rates.

In spite of these advantages, the biotreatment of wastewaters using biofilms is often subjected to oxygen transfer limitations at higher biofilm thicknesses. In a biofilm system, substrates and electron acceptors diffuse into the depth of the biofilm, where subsequent microbial reactions take place. When aerobic conditions are highly desirable, dissolved oxygen should be able to fully penetrate the biofilm. However, this is challenging as the biofilms formed are frequently thicker than the oxygen penetration depth, which is typically 50-150 μm (Van Loosdrecht and Heijnen, 1993). This common phenomenon inhibits the penetration of oxygen to a certain depth of the biofilm, and substrate removal performance may also change radically as the biofilm grows. The oxygen transfer constraints, however, can be overcome by using a gas-permeable membrane for gas transfer that has the potential to improve oxygen transfer to the active biomass by a bubbleless mode of aeration, and subsequently reduces the energy cost associated with gas transfer (Sommers, 2008). As the biofilm starts to develop on the membrane, oxygen is diffused to the microorganisms inside the biofilm, which in turn reduces the overall transfer rate of oxygen to the bulk liquid (Wilderer, 1995).

The technology of a membrane biofilm reactor (MBfR) fully exploits the concept of using membranes for gas delivery and/or support for biofilm growth. This system is different from that of a membrane bioreactor (MBR), in which the membrane in the MBR only acts as a filtration device to separate the biomass from the effluent. The MBR is, however, susceptible to an accumulation of inorganic deposits, organic deposits, and microorganisms on the membrane surface, or in other words membrane biofouling, causing flux decline and an increase in pressure drop. According to Lewandowski and Beyenal (2005), this biofouling is unavoidable and cannot be eliminated entirely by manipulating operating conditions. In contrast, the phenomenon of a biofilm growing on the membrane surface is advantageous in the MBfR. The MBfR consists of several types of reactor configuration and operational principles. According to Casey *et al.* (1999a), the MBfR can be classified into three smaller groups, mainly differentiated by their principle of operation:

1. The membrane biofilter, in which contaminated gases pass through the membrane and biodegradation takes place in the biofilm immobilised on the outside surface of the membrane (Parvatiyar *et al.*, 1996).
2. The extractive membrane bioreactor (EMB), in which only selected contaminants in the wastewater stream are transported through the membranes into biofilms where biodegradation takes place (Brookes and Livingstone, 1994).
3. The membrane aerated biofilm reactor (MABR), where oxygen/air diffuses through the membrane and oxidation of substrate takes place in the biofilm.

A dual-functioning membrane of the MABR acts as a biofilm support and for supplying bubbleless aeration to the biofilm at the same time. The spatial distribution of

oxygen in the biofilm is important in the MABR, and the following discussion will focus on MABR operation, design and performance.

2.7 MEMBRANE AERATED BIOFILM REACTOR (MABR)

Although the first patent for biological wastewater treatment using microorganisms supported on microporous hollow fiber membranes was filed in 1980 (Onishi *et al.*, 1980), a substantial increase in MABR research was only seen after the first review of MABRs by Casey *et al.* (1999a). Since then, a consistent number of peer-reviewed journal articles in this subject have been produced focusing on fundamental aspects including modeling, and MABR applications for organic carbon and nitrogen removal (Hwang *et al.*, 2009a). Studies have shown that the MABR represents a promising alternative to the conventional biofilm reactor for removing contaminants from wastewater (Satoh *et al.*, 2004). In contrast to the conventional use of membranes for solid-liquid separation, the MABR exploits the membrane uniquely for aeration and biofilm support.

The operational principles of an MABR are shown schematically in Figure 2.2. As can be seen in the figure, the system consists of a biofilm immobilised on the membrane surface, a substrate supplied to the biofilm-liquid interface and air/oxygen supplied from the membrane side. This counter-diffusion of oxygen and substrate through the biofilm creates unique concentration profiles of both elements in the biofilm. Above a critical biofilm thickness, depending on the loading rate, reaction kinetics and mass transfer resistances, an oxygen depleted zone can exist (Syron and Casey, 2008). This leads to strata of micro-niches across the biofilm depth (Gong *et al.*, 2008). The region nearest to the membrane is oxygen-rich with aerobic microbes predominating, whereas in the oxygen depleted region next to the

biofilm-liquid interface anaerobic microbes predominate. In addition, an anoxic region can occur in-between these two layers that consists of facultative microorganisms.

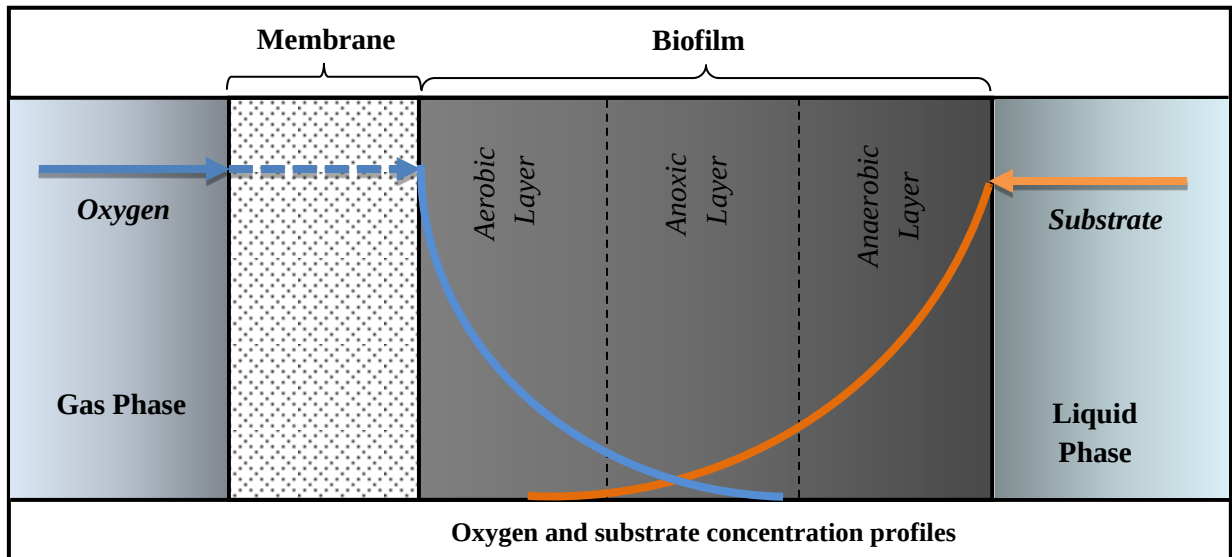


Figure 2.2: Schematic diagram of the operational principle of the MABR.

The unique operational principle of an MABR confers some advantages on the MABR compared to other biofilm reactors in terms of the role of the membrane and the nature of the biofilm. The use of a membrane for aeration enhances oxygen transfer to the biofilm by providing bubbleless aeration, in which the molecular oxygen diffuses through the membrane lumen directly into the biofilm. In contrast with conventional bubble aeration, the oxygen dissolves into the surrounding water and then this dissolved oxygen is transported by convection and diffusion to the active biomass within the reactor (Semmens, 2008). It has been hypothesised that under favorable design and operational features, close to 100% oxygen utilisation efficiency is achievable (Pankhania *et al.*, 1994). By overcoming oxygen limitations, a substantial amount of operational cost related to aeration could be saved. In addition, bubbleless aeration also prevents stripping of volatile organic compounds during the biotreatment. The membrane can also segregate the methane and oxygen gas phases to overcome explosion risks (Rishell *et al.*, 2004). The existence of spatial stratification of

microbial activity in the biofilm is an important feature to achieve different treatment objectives, such as: (1) nitrogenous pollutant removal by simultaneous nitrification and denitrification (Terada *et al.*, 2003), and by anaerobic ammonium oxidation (ANAMMOX) (Gong *et al.*, 2008); and (2) mineralisation of compounds with problematic intermediates by simultaneous aerobic and anaerobic reactions (Ohandja and Stuckey, 2006). The mechanism involved will be elaborated upon later. The MABR has been the subject of several reviews; a brief, introductory review of MABR technology and its prospects was written by Casey *et al.* in 1999, while in 2008 Syron and Casey listed recent progress in the technology, its technical aspects, and future development requirements for high-rate biotreatment. Recently, Hwang *et al.* (2009a) published a review on the chronological and regional distribution of MBfR studies published worldwide. For the purpose of this thesis, this review is limited to MABRs in which oxygen is supplied through the membrane for oxidation of substrates. This technology is, however, still in its developmental stage and only limited pilot trials have been conducted to scale up the technology for future commercial exploitation.

2.7.1 DESIGN AND FUNDAMENTAL ASPECT OF MABR

A series of bench scale studies on the MABR have provided a basis for fundamental understanding of the technology and its prospect for commercialisation. A representative sample of various MABR system configurations and performance data is summarised in Table 2.5. The process performance, usually judged by the rate and removal efficiency of a targeted pollutant, is influenced by various interrelated factors, as illustrated in Figure 2.3. Of these, the membrane characteristics and configuration, oxygen transfer rates to the biofilm, and biofilm characteristics are some of the most significant factors influencing process performance (Syron and Casey, 2008).

Table 2.5: Membrane aerated biofilm reactors: Configuration, applications and performances.

Pollutant	Membrane material	Membrane module configuration	Aeration mode	Reactor volume (l)	Specific surface area (m ² /m ³)	Removal efficiency (%)	Reference
Organic carbon and inorganic nitrogen	Gore-tex fabric (polytetrafluoroethylene + nylon woven fabric)	Single tube	Air and Pure O ₂	3.15	19	TOC: 50% T-N: 75%	Timberlake <i>et al.</i> , 1988
Organic carbon	Polypropylene	HF, DE	Pure O ₂	1.345	511	COD: 86%	Pankhania <i>et al.</i> , 1994
Inorganic nitrogen	Polyethylene	HF, DE	Pure O ₂	0.43	185	NH ₄ ⁺ : 99%	Brindle <i>et al.</i> , 1998
Organic carbon	Polypropylene	HF, DE	Pure O ₂	1.35	511	COD: 89%	Pankhania <i>et al.</i> , 1999
Organic carbon and inorganic nitrogen	Polyethylene	HF, OE	Air	7	422	COD: 95% T-N: 90%	Semmens <i>et al.</i> , 2003
Organic carbon and inorganic nitrogen	DEA-grafted polyethylene	HF, DE	Air	0.15	50	TOC: 96% T-N: 83%	Terada <i>et al.</i> , 2003
Inorganic nitrogen	DEA-grafted polyethylene	HF, DE	Air	0.1	57	TOC: 90% T-N: 90%	Hibiya <i>et al.</i> , 2003
Organic carbon and inorganic nitrogen	Polyurethane	HF, OE	Pure O ₂	4.5	56	COD: 90% NH ₄ ⁺ : 95%	Satoh <i>et al.</i> , 2004
Inorganic nitrogen	polyacrylonitrile	HF, DE	Air	0.86 0.76 0.63	290 660 1190	NH ₄ ⁺ : 100% NH ₄ ⁺ : 100% NH ₄ ⁺ : 97.7%	Terada <i>et al.</i> , 2006
Organic carbon and inorganic nitrogen	Carbon	HF, OE	Air	4	45	COD: 90% NH ₄ ⁺ : 95%	Liu <i>et al.</i> , 2007
Inorganic nitrogen	Carbon (covered with non-woven polyester fabrics)	Tubular	Air	4	14	NH ₄ ⁺ : 92%	Gong <i>et al.</i> , 2007
Organic carbon and inorganic nitrogen	Carbon	Tubular	Air	2.4	75	COD: 86% NH ₄ ⁺ : 94% T-N: 84%	Hu <i>et al.</i> , 2008
Inorganic nitrogen	Silicone	Flat Sheet	Air	0.5	16		Lackner <i>et al.</i> , 2010
Xenobiotic pollutants							
Xylene	Silicone	Tubular, OE	Pure O ₂	111	0.18	98%	Debus <i>et al.</i> , 1994
Chlorophenol	Silicone	Tubular, DE	Pure O ₂	200	19	95%	Wobus <i>et al.</i> , 1995
1,2-dichloroethane	-	Flat sheet, OE	Air	0.008	500	79%	Hage <i>et al.</i> , 2004
Perchloroethylene (PCE)	Silicone	Tubular, OE	Air	0.43	0.715	95%	Ohandja and Stuckey, 2006
Perchloroethylene (PCE)	Polypropylene	Flat sheet, DE	Air and Pure O ₂	0.19	4.048	99%	Ohandja and Stuckey, 2007; 2009
Acetonitrile	Polypropylene	HF, DE	Pure O ₂	1.42	84.5	96%	Li <i>et al.</i> , 2008a
Fluoroacetate	Silicone	Tubular, OE	Pure O ₂	0.2	27.8	91.5%	Heffernan <i>et al.</i> , 2009

Abbreviations: DE, dead-end lumen; OE, open ended lumen; HF, hollow fiber DEA: diethylamino

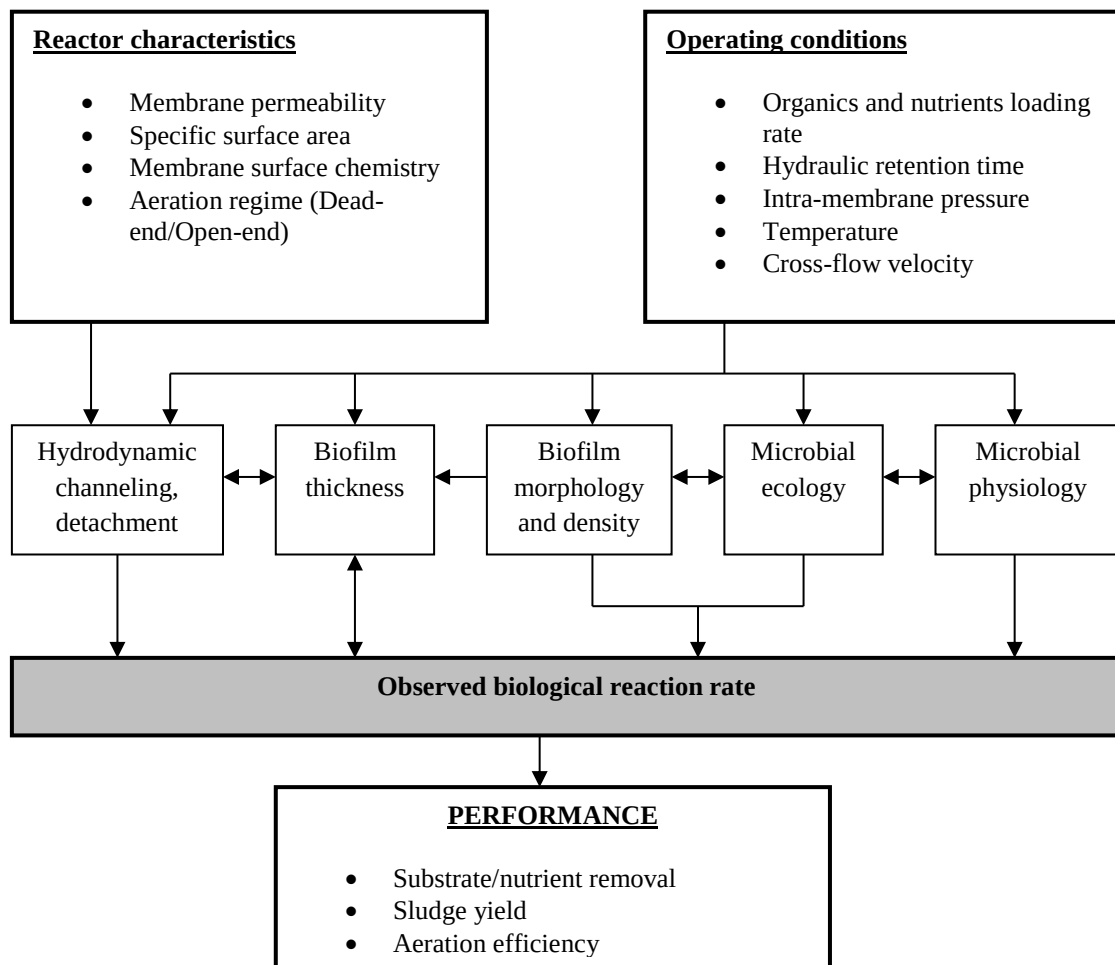


Figure 2.3: Interrelationships between various factors influencing process performance (Syron and Casey, 2008).

2.7.1.1 Selection of membrane material and membrane module configuration

One of the most important factors in meeting the needs of the membrane to efficiently transport the air/oxygen to the biofilm, and at the same time providing a firm support for biofilm growth, is the selection of an appropriate membrane material. The material chosen should be able to provide long lasting resistance to water penetration (wetting), and mechanical and chemical stress (Stricker *et al.*, 2011). To date, a wide range of membrane materials have been used in the MABR, and can be classified as microporous, dense and

composite membranes. Microporous membranes, manufactured from hydrophobic materials such as polypropylene or polyethylene can provide very high oxygen permeability. The oxygen transfers through the dry pores by gaseous diffusion, and the oxygen transfer rate is directly proportional to membrane porosity. During operation, the gas pressure is maintained below the bubble point to avoid detachment of the biofilm by bubbles. With a dense polymer membrane, typically silicone rubber tubing, oxygen is absorbed in the polymer on the gas side and is transported by diffusion to the biofilm. However, liquid penetration inside the porous membrane significantly increases gas transfer resistance (Yasuda and Lamaze, 1972). There was also evidence of colonisation of bacteria inside the pores (Rothmund *et al.*, 1994). In contrast, nonporous or dense silicone membranes can be operated at high pressure without bubble formation. However, silicone membranes are usually much thicker than microporous membranes; their thickness and their nonporous nature present a higher resistance to mass transfer than microporous membranes (Ahmed and Semmens, 1992).

In addition, improvements have been made to conventional microporous membranes to reduce membrane clogging by coating or embedding a thin layer of dense material onto a microporous membrane; this composite membrane is, however, not fully exploited in MABRs. In MABR, surface modification of a membrane can be done by grafting specific functional groups onto the membrane surface to enhance electrostatic interactions between bacterial cells and the membrane surface (Terada *et al.*, 2003), as well as by the attachment of a non-woven scrim to the membrane surface to avoid complete biofilm sloughing while protecting and securing the thin biofilm (Alvarez *et al.*, 2008). Hu *et al.* (2008) applied a carbon membrane covered with a nonwoven fiber in the MABR and observed the enhancement of biofilm attachment, which could accelerate the startup of the MABR. The membrane module configurations reportedly used in a MABR are hollow fiber, flat sheet and

tubular. High specific surface areas can be achieved in a hollow fiber configuration, but when tightly packed they are susceptible to biofilm clogging (Semmens *et al.*, 2003). The use of flat sheet membranes in MABR studies is less common, however, due to the lower specific surface area (Timberlake *et al.*, 1988; Semmens, 2005). Nevertheless, this type of configuration is suitable for microelectrode studies on an MABR (Syron and Casey, 2004).

2.7.1.2 Oxygen transfer rate to the biofilm

In MABRs the intra-membrane pressure has a marked effect on the oxygen uptake rate (OUR) as a consequence of the higher oxygen concentrations at the biofilm-membrane interface resulting in deeper penetration into the biofilm. Therefore, oxygen pressure must be balanced carefully in order to maintain the anoxic/anaerobic zone at the biofilm-liquid interface if both aerobic and anoxic/anaerobic degradation is required. For the treatment of high-strength wastewaters the intra-membrane oxygen pressure is likely to be the rate controlling factor (Syron and Casey, 2008).

Due to the low solubility of oxygen in water, the maximum oxygen diffusion rate in conventionally aerated biofilms is typically ca. 10 g/m²/day/bar, which is low enough to cause oxygen limitations in many wastewater biotreatment processes (Tijhuis *et al.*, 1994). In contrast, Casey *et al.* (1999b) reported that OUR up to 20 g/m²/day/bar could be achieved experimentally, and that a mathematical modeling study predicts that a 30 g/m²/day/bar OUR is possible for an optimised biofilm thickness. In addition, Pankhania *et al.* (1994) demonstrated 100% oxygen conversion efficiencies by the use of dead ended membranes to achieve high organic carbon removal rates in MABR.

2.7.1.3 Biofilm characteristics

Biofilm thickness control is one of the most significant parameters influencing process performance in an MABR. Depending on the treatment objective, a thin biofilm would promote better oxygen transfer through the biofilm, whereas a thick biofilm would increase the resistance to oxygen and nutrient diffusion, resulting in the stratification of active regions in the MABR. Biofilms are highly stratified and characterised by a heterogeneous structure, not only in the composition and the distribution of EPS, but also by defined aerobic/anoxic zones within the biofilm depth (Bishop *et al.*, 1995). The distribution of EPS in the biofilm matrix is important in biofilm cohesiveness and biofilm adhesion to the surface. Ahimou *et al.* (2007) used Atomic Force Microscopy (AFM) to measure the level of biofilm cohesive energy per unit volume in the MABR and found that the biofilm cohesiveness was strongly correlated with biofilm polysaccharide concentration, which increased with depth, but not with the age of the biofilm.

According to the most recent study on EPS distribution in a MABR by Li *et al.* (2008b), up to 81 to 92% of the total EPS in the biofilm was associated with bound EPS (i.e., bound polysaccharide and bound protein), and hence the soluble EPS was less significant. Bound EPS is more hydrophobic and non-readily biodegradable, thus improving biomass adhesion and biofilm stability. Oxygen-sensing microelectrodes were used in some studies to determine the oxygen penetration depth in the biofilm, computed based on the oxygen distribution (LaPara *et al.*, 2006). Based on measurements by LaPara *et al.*, (2006), mass transfer was strongly controlled by diffusion rather than bacterial uptake. Moreover, the oxygen profile in the biofilm largely depends on the differences in experimental conditions.

2.7.2 APPLICATIONS OF THE MABR

Table 2.5 shows the application of the MABR in the treatment of a wide range of contaminants. Of these, the removal of organic carbon and inorganic nitrogen were the most comprehensively studied; either via aerobic heterotrophic oxidation, autotrophic nitrification, heterotrophic denitrification, or a combination of heterotrophic organic carbon oxidation with nitrification-denitrification. In addition, the MABR has also been used successfully for xenobiotic pollutant removal (Xenobiotic refers to man-made pollutants that are foreign to nature).

2.7.2.1 Organic carbon and inorganic nitrogen removals

The feasibility of using an MABR for organic carbon, inorganic nitrogen and simultaneous organic carbon and inorganic nitrogen removal has been thoroughly investigated (Table 2.5). Highly stratified MABRs provide favourable environments for total inorganic nitrogen removal by means of simultaneous nitrification and denitrification. Nitrification is a microbial process in which reduced nitrogen compounds (primarily ammonia) are sequentially oxidised to nitrite and nitrate. The nitrification process is primarily accomplished by two groups of autotrophic nitrifying bacteria, ammonia-oxidising bacteria (AOB) and nitrite-oxidising bacteria (NOB) that can build organic molecules using energy obtained from inorganic nitrogen compounds. In the first step of nitrification, AOB oxidise ammonia to nitrite according to equation [2.1] using O_2 as an electron acceptor. In the second step of the process, NOB oxidises nitrite to nitrate according to equation [2.2].



In the process of denitrification, nitrate serves as an electron acceptor and is reduced to nitrogen gas through a sequence of intermediates that include nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O), as shown in equation [2.3]. Proceeding under anoxic conditions, the denitrifiers are typically facultative heterotrophic bacteria and require organic carbon for maintenance and growth.



Timberlake *et al.* (1988) suggested that the occurrence of a vertical stratification of nitrification, aerobic heterotrophic oxidation, denitrification and anaerobic fermentation zones from the bottom to the surface of the biofilm promoted organic carbon removal, and nitrification and denitrification in the single MABR. Satoh *et al.* (2004) verified that nitrification occurred in the zones near the membrane surface and denitrification mainly occurred just above the nitrification zones using microelectrode measurements of O_2 , NH_4^+ , NO_3^- and NO_2^- profiles in the biofilm. Studies using the fluorescence in situ hybridisation method (FISH) also confirmed that AOB were mainly distributed near to the membrane surface (Hibiya *et al.*, 2003), and denitrifying bacteria were mainly distributed in the outer layer of the biofilm (Hu *et al.*, 2008). High O_2 and low carbon concentrations facilitate nitrification at the bottom of biofilm, while the oxygen-depleted conditions and high carbon concentrations near the bulk liquid are favourable for heterotrophic denitrification. In order to maintain good nitrification and denitrification, the bulk dissolved oxygen (DO) should be zero, or at very low levels, an adequate carbon/nitrogen (C/N) ratio should be provided; and

the biofilm should be thick enough (>1mm) to develop aerobic and anaerobic regions (Semmens *et al.*, 2003). Several modeling studies on the effect of organic carbon on nitrogen removal have been carried out using AQUASIM software (Shanahan and Semmens, 2004; Matsumoto *et al.*, 2007; Lackner *et al.*, 2008). Matsumoto *et al.* (2007) for instance, predicts that the efficient simultaneous COD and total nitrogen removal (of more than 80% and 70%, respectively) is attained in the range of a C/N ratio from 3.0 to 5.25. When too high organic load is present, heterotrophic bacteria will outcompete slow growing nitrifying bacteria for oxygen, thereby inhibiting nitrification. In contrast, low organic carbon and a thin biofilm with excellent oxygen penetration encourages more nitrification activity and inhibits denitrification (Terada *et al.*, 2004). Gong *et al.* (2007) developed a single-stage autotrophic nitrogen removal process based on partial nitrification and anaerobic ammonium oxidation (ANAMMOX) in an MABR. Under optimised conditions, about 83.8% removal of the total nitrogen present can be achieved. The AOB found at the membrane-biofilm interface oxidises ammonia to nitrite, and the nitrites produced are then utilised by ANAMMOX bacteria to convert the remaining ammonia to nitrogen gas in an anoxic region of the biofilm-liquid interface where the oxygen is limited. The existence of these two bacterially active layers in an MABR has been verified by FISH analysis (Gong *et al.*, 2008). It has been postulated that a thick biofilm provides protection for the sensitive ANAMMOX bacteria from exposure to oxygen.

2.7.2.2 Degradation of xenobiotic pollutants

The MABRs have been tested to biodegrade a number of xenobiotic pollutants, namely xylene (Debus *et al.*, 1994), phenol (Woolard and Irvine, 1994), chlorophenol (Wobus *et al.*, 1995), perchloroethylene (PCE) (Ohandja and Stuckey, 2006; Ohandja and

Stuckey, 2007), acetonitrile (Li *et al.*, 2008), and flouroacetate (Heffernan *et al.*, 2009). Table 2.5 shows that more than 90% contaminant removal could be achieved in most systems under optimised conditions. Promising features of an MABR regarding its capacity to remove xenobiotics are:

1. A diverse microbial community in the biofilm which can synergistically degrade a wide range of contaminants. The biofilm also provides a protective environment for slow growing microorganisms against shock load.
2. Bubbleless membrane aeration can result in a substantial reduction in the gas stripping of volatile organic compounds, such as xylene, as well as overcoming oxygen transfer limitations in thick biofilms.
3. Simultaneous removal of xenobiotics and nutrients in a single biofilm (Li *et al.*, 2008).
4. The creation of both aerobic and anaerobic microenvironment in a single biofilm is advantageous for the degradation of compounds with problematic intermediates, such as perchlorethylene (PCE) (Ohandja and Stuckey, 2006; Ohandja and Stuckey, 2007).

Ohandja and Stuckey (2007) reported the highest value for volumetric PCE mineralisation rates in a flat sheet MABR so far, which was 246.58 mM of PCE h⁻¹.m⁻³. Degradation of PCE under anaerobic conditions often results in the accumulation of less chlorinated biodegradation intermediates, such as trichloroethylene (TCE), dichloroethylene isomers (DCEs), and vinyl chloride (VC). These intermediates, known to be more toxic than the parent compound, are readily degradable under aerobic conditions. As a result, anaerobic degradation of PCE can be allowed to occur in the anaerobic layer of the biofilm, whereas aerobic degradation of the intermediate products occurs in the aerobic layer of the biofilm. Besides, the presence of ethylene and methane could facilitate the growth of aerobic

chloroethene degraders and the synthesis of oxygenase enzymes such as methane and alkene monooxygenases that are responsible for aerobic chloroethene cometabolism. Cometabolism is defined as “the concomitant transformation of a non-growth substrate by growing cells in the presence of a growth substrate, or by resting cells grown on a substrate capable of supporting microbial growth” (Horvath, 1972).

Methane monooxygenase, an enzyme produced by methanotrophic bacteria, is known for its ability to cometabolise refractory compounds, such as chlorinated aliphatic hydrocarbons (CAH), including TCE (Clapp *et al.*, 1999). A methanotrophic MABR, developed by Casey *et al.* (2004) for methane oxidation, reached a maximum methane utilisation rate of 13.9 g/m².d compared to 1.6 g/m².d measured in a conventional methanotrophic rotating drum biofilm reactor (Arcangeli *et al.*, 1994). They postulated that the potential of an MABR to exploit methanotrophic cometabolism is due to its potential to control microbial stratification, which in turn minimises the competitive inhibition under CAH cometabolic conditions. In addition, the metabolites of methanotrophic oxidation can act as electron donors for coexisting denitrifiers for denitrification – a new concept which has been applied in a membrane biofilm reactor supplied with oxygen and methane (Modin *et al.*, 2008). Biodegradation of certain xenobiotics can also produce high concentrations of nutrients, for instance, the generation of NH₄⁺ and/or NO₃⁻ from the biodegradation of organonitrile compounds. Li *et al.* (2008) exploited the concept of an aerobic/anoxic/anaerobic zone in an MABR for simultaneous oxidation of acetonitrile (an organonitrile compound) coupled with nitrification and denitrification for total nitrogen removal in a single biofilm. The acetonitrile removal was approximately 98.6% and 83.3%, in terms of total organic carbon and total nitrogen, respectively. Despite promising volatile organic carbon (VOCs) removal in an MABR, the VOCs tend to diffuse through the biofilm

and membrane into the gas phase (Ohandja and Stuckey, 2007). This may be due to physical properties of the membrane, and/or biofilm structure. In addition, suitable conditions for their biodegradation may not have been established so that little or no biodegradation of VOCs occur (Debus, 1995). However, such undesirable situations can be prevented by controlling the intra-membrane oxygen pressures so that appropriate conditions for biodegradation are established in particular layers of the biofilm, as required (Casey *et al.*, 1999).

2.7.3 MABR SCALE-UP

To date, scale-up and commercialisation of the MABR has not occurred due to several obstacles in terms of its technical aspects and cost effectiveness, and an overview of process scale up has been carried out by Syron and Casey (2008). These authors highlighted the principal issue experienced in many bench scale trials, which is the control of excess biomass. Up to 4mm of biofilm was seen in a pilot scale test, thus reducing overall performance (Semmens, 2005). This can be overcome by improving membrane materials and modules (membrane robustness and adequate membrane spacing), and by selecting appropriate scouring methods. Furthermore, the economic feasibility of an MABR with respect to conventional biofilm processes should be assessed before it can be commercialised.

A recent pilot scale trial has been successfully conducted by Stricker *et al.* (2011) to treat high-strength industrial wastewater (4700 mgCOD/L; 145 mgTKN/L) for 16 months. The improved system, named ZeeLungTM, consists of extremely fine diameter hollow fiber membranes (50-70 μm) with a specific surface area of 810 m^2/m^3 at only 1 % packing density. An overall mass balance on the 3 reactors in series (total volume of 12.2 L) indicated only 1 % and 11 % of the total COD and TKN constituents in the effluent, respectively.

However, the control of excess biomass was uncertain, either by using intermittent mixing or air sparging. It was suggested that aggressive air sparging could be effective if applied frequently. Most importantly, a thin biofilm (200-350 μm) observed in this system reduced mass transfer limitations and so it could be fed efficiently with low pressure air.

2.8 SUMMARY OF THE LITERATURE

Previous researchers have highlighted the fact that the study of NPEO biodegradation is complicated, and considerably more work is needed to understand the degradation pathways. The reasons for these are:

1. The complex physico-chemical properties of NPEOs. NPEOs contain a mixture of oligomers of varying ethoxylate chain lengths. In addition, each oligomer consists of a mixture of isomers (i.e. >22 isomers) having highly branched alkyl chains which are attached to either the ortho- or para- position on the benzene ring.
2. Both side chains (ethoxylates and alkyl) can be degraded simultaneously, anaerobically or/and aerobically. This will result in different biodegradation pathways and a considerable number of intermediates will also be formed.
3. Some of the intermediates identified are novel, and hence their toxicity and fate in the environment are not fully understood.

One apparent reason for increasing concern over the detrimental effect of NPEOs on the environment is the characteristics of their degradation intermediates. Despite the occurrence of novel intermediates, NP and short chain NPEOs are still some of most scrutinised intermediates in biodegradation studies conducted over the past 30 years. These

intermediates, are well recognised as being more toxic, persistent and estrogenic (induce endocrine disrupting effects) than their parent compounds. The formation of these intermediates involves a successive excision of the ethoxylates chain, based on a non-oxidative hydroxyl shift mechanism. Although the process is rapid, further biotransformation of NP and short chain NPEO (i.e. NPEO₁) is uncommon under anaerobic conditions.

In contrast, NP has been widely reported to be degraded under aerobic conditions; hence most efforts have been devoted towards aerobically degrading the NPEOs. The use of aerobic treatment on NPEOs, however, will result in the production of acidic NPECs due to carboxylation of the ethoxylates. This carboxylate chain will be shortened until NPEC₁ is formed, but its exact mechanism is unverified. Nevertheless, a study has also found that the chain was not shortened in the presence of additional substrates. Other than NPECs, the shorter chain NPEOs were also evident in some of the aerobic systems, thus making the system more complex (in terms of intermediates formation). The advantages and disadvantages of anaerobic and aerobic treatment of NPEOs are summarised in Table 2.6. The studies on NPEOs biodegradation are largely devoted to investigating the role of inoculum, external electron acceptors (other than O₂), and additional substrates on the NPEOs biodegradation behaviour. The studies were either carried out under anaerobic or aerobic conditions. Based on our current knowledge, no study has been done to provide both anaerobic and aerobic conditions for the complete degradation of NPEOs in wastewaters. Based on Table 2.6, we propose the following treatment scheme:

1. First stage: Primary degradation of the parent compounds (NPEOs) under anaerobic condition provides a rapid removal of long chain NPEOs and a limited number of nonylphenolic species are formed.

2. Second stage: The intermediates (i.e. NP, NPEO₁ and NPEO₂) are subjected to aerobic treatment to be mineralised by mixed microbial culture.

Table 2.6: Advantages and disadvantages of anaerobic and aerobic treatment of NPEOs.

Condition	Stages			
	1. Primary degradation		2. Intermediates degradation	
	Advantage(s)	Disadvantage(s)	Advantage(s)	Disadvantage(s)
Anaerobic	Rapid process; produces less intermediate species	Produces refractory, toxic and estrogenic intermediates	-	Slowly/not degraded
Aerobic	Rapid process	Produces more complex mixtures of intermediates; mostly unknown intermediates	Efficiently degraded	Produces possible refractory intermediates

In the MABR, a layered biofilm which contains both aerobic and anaerobic conditions is grown on a gas permeable membrane. This property has been exploited primarily for nitrification/denitrification coupled to organic removal. In fact, only a single researcher has manipulated the design of the MABR for simultaneous anaerobic/aerobic treatment of a xenobiotic pollutant with problematic intermediates, which is PCE. Other xenobiotic studies in an MABR have only focused on sole aerobic treatment. On top of that there is no report on the use of an MABR for NPEOs biodegradation, as well as EDCs in general. In the present study, it is hypothesised that an MABR could promote a suitable environment for simultaneous anaerobic/aerobic biodegradation of NPEOs and their intermediates, in a single reactor.

2.9 OBJECTIVES

Based on the literature review above, the specific objectives of this study are as follows:

1. To develop comprehensive analytical methods for the determination of NPEOs and their intermediates in solid and aqueous samples.
2. To investigate NPEO_{ave9} biodegradability in a batch microcosm, based on the biochemical methane potential (BMP) and anaerobic toxicity assay (ATA) methods, under anaerobic and simultaneous anaerobic/aerobic conditions.
3. To design and operate an MABR using a gas permeable membrane to achieve complete mineralisation of NPEO_{ave9}, and to monitor the biodegradation intermediates under long term operation.
4. To study MABR stability for NPEO_{ave9} removal under different organic and hydraulic shock loads.

❧ CHAPTER 3 ❧

MATERIALS AND METHODS

3.1 INTRODUCTION

This chapter contains a description of the materials and methods used in this study, including the experimental set-up, MABR system design, and analytical techniques. A summary of the methods is shown in Figure 3.1.

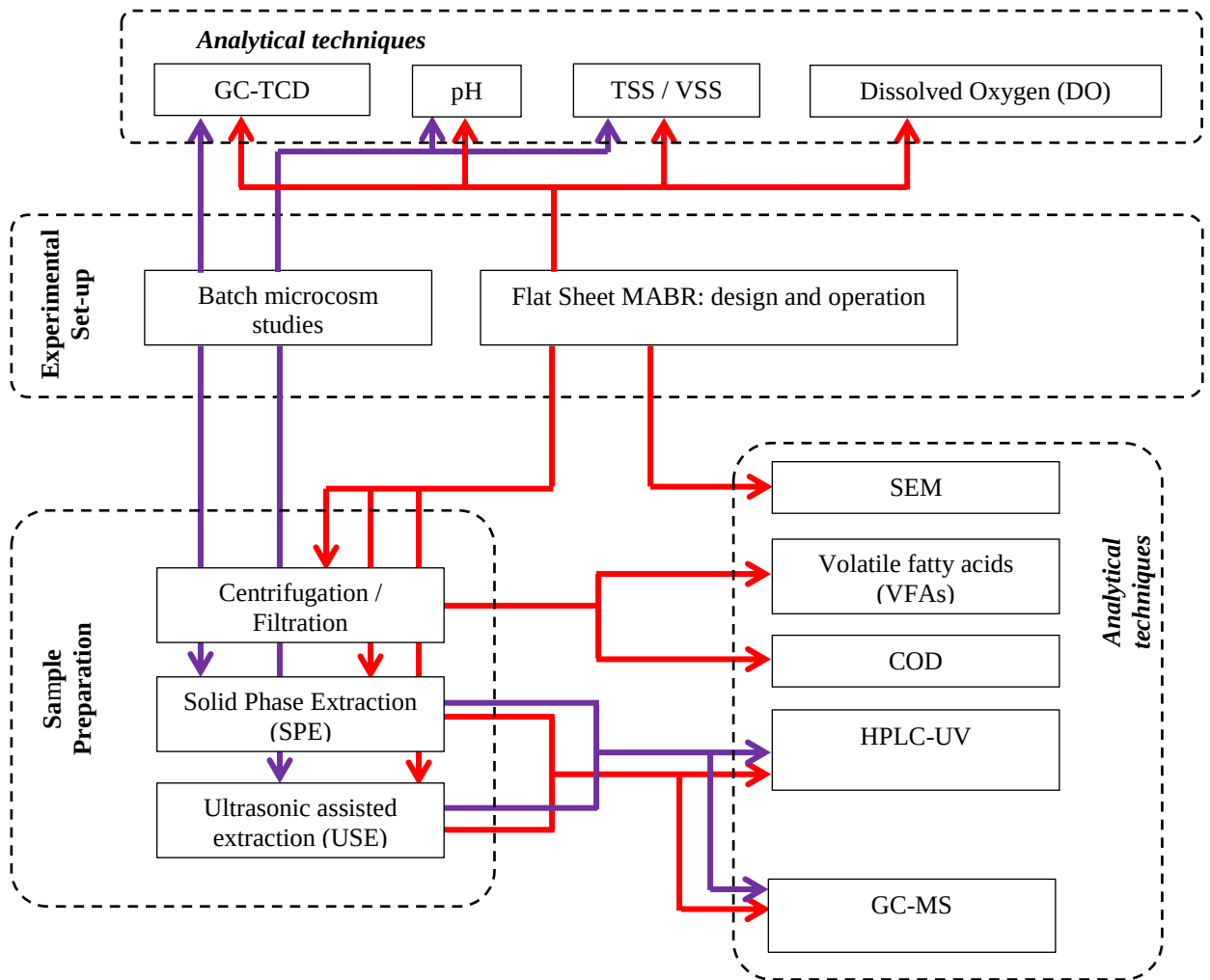


Figure 3.1: The method structure.

Some of the experimental procedures and modifications will be clarified in subsequent chapters.

3.2 MATERIALS

The mixed-isomer technical grade nonylphenol (NP) and NPEOs surfactant mixtures of IGEPAL CO-630 and IGEPAL CO-210 contained branched-chain nonylphenol attached to ethylene oxide (EO) chains averaging 9 and 1.5 EO units, and collectively referred to as NPEO_{ave9} and NPEO_{ave1.5}, respectively, were obtained from Sigma Aldrich (Dorset, UK). Standards of nonylphenoxy diacetic acid (NPEC₂) were purchased from QMX Laboratories (Essex, UK). The derivatisation agents of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), the internal standard of d-chrysene, and other solvents such as dichloromethane, pyridine and acetonitrile were obtained from Sigma Aldrich (Dorset, UK).

All other reagents including HPLC grade n-hexane, isopropanol, acetone and methanol were purchased from VWR (Leicestershire, UK). All other reagents used were of analytical grade. Stock solutions of standards were prepared by dissolving a known amount of each standard in methanol (1 mg/mL), and storing it at -18°C. Working standard solutions were obtained by further diluting the prepared stocks with analytical grade deionised water.

3.3 SAMPLE PREPARATION FOR INSTRUMENTAL ANALYSIS

Sample preparation or pretreatment was designed to eliminate matrix interference while improving enrichment and sensitivity of the targeted compounds. Extraction of targeted

compounds from an aqueous suspension was achieved by Solid Phase Extraction (SPE), whereas analyte extraction from a solid matrix (i.e. suspended particulate matter and sludge) was achieved by simultaneous ultrasonic assisted extraction (USE) and SPE. For SPE, an adsorbent used was an Oasis HLB (30mg) cartridge, obtained from Waters (Hertfordshire, UK). The extraction was performed according to a modification of the Oasis generic methodology (Waters, 2003). For the isolation of analytes from a solid matrix, the sample was ultrasonicated twice in 5 mL solvent, and then subjected to SPE for clean-up and analysed chromatographically. Detailed procedures of SPE and its use will be given in Chapter 4.

3.4 SOURCE OF INOCULUM

The biological inoculum used in this study was collected from a sewage treatment plant (Mogden, West London, UK). The digested sludge was screened and inoculated into two Continuous Stirred Tank Reactors (CSTR) under anaerobic and aerobic conditions. Each 2L reactor was operated in batch mode and supplemented with biomedica based on the formulation by Owen *et al.* (1979) (see Table 3.1). For the anaerobic reactor, fresh feed of biomedica and glucose (1.0 gCOD/gVSS) were supplied every week and the methanogenic activity of the biomass was monitored by means of methane production. Compressed air was supplied to the aerobic reactor to facilitate the growth of aerobic bacteria. Fresh feed of biomedica and glucose (0.1 gCOD/gVSS) were then introduced every 4 days; while COD profiles were monitored to see whether the bacteria were acclimatising. The acclimation phase for each reactor was maintained for at least 3 months prior to the biodegradation study and MABR start-up.

3.5 BATCH MICROCOSM STUDIES

The preparation of defined media and the serum bottle technique was conducted using the anaerobic biodegradation assay developed by Owen *et al.* (1979). The bottles were flushed with a gas mixture containing 70% N₂ and 30% CO₂ at a flow rate of approximately 0.5 L/min to remove traces of oxygen. Serum bottles of 160 mL and 75 mL were used in the study. The inoculated biomass from the batch reactor (2 gVSS/L), a source of substrate, and defined media containing nutrients and vitamins for microbial cultures (Table 3.1) were transferred into the serum bottles under strict anaerobic conditions by continuously flushing the bottles with a mixture of N₂ and CO₂ before capping the bottles with a leak proof Teflon seal. The serum bottles were placed in a mechanical shaker at 30°C. The volumetric gas productions were measured by the syringe displacement method (Owen *et al.*, 1979) and the gas compositions were analysed by gas chromatography (GC).

3.6 THE MEMBRANE AERATED BIOFILM REACTOR (MABR): DESIGN AND OPERATION

A laboratory-scale flat sheet MABR was fabricated from stainless steel. The different parts of the flat sheet MABR are depicted in Figure 3.2. The reactor consisted of the upper compartment (liquid compartment), lower compartment (gas compartment), a flat sheet membrane with a stainless steel support screen (Millipore) and the o-rings for insulation. The membrane used to separate the two compartments was a microporous polypropylene membrane with a non-woven polypropylene scrim heat-sealed to the surface, and was made by the 3M Corporation, St Paul, Minnesota, USA.

Table 3.1: Chemical composition of biomedica used in BMP bottles (Owen *et al.*, 1979).

Compounds	Final Concentration in Medium (mg/L)
Resazurin	1.0
(NH ₄) ₂ HPO ₄	80.1
<u>Trace Elements solution:</u>	
CaCl ₂ ·2H ₂ O	250.5
NH ₄ Cl	399
MgCl ₂ ·6H ₂ O	1800
KCl	1300.5
MnCl ₂ ·4H ₂ O	19.95
CoCl ₂ ·6H ₂ O	30
H ₃ BO ₃	5.7
CuCl ₂ ·2H ₂ O	2.7
Na ₂ MoO ₄	2.55
ZnCl ₂	2.1
FeCl ₂ ·4H ₂ O	370
Na ₂ S·9H ₂ O	500
<u>Vitamin Solution:</u>	
Biotin	0.02
Folic acid	0.02
Pyridoxine hydrochloride	0.1
Riboflavin	0.05
Thiamin	0.05
Nicotinic acid	0.05
Pantothenic acid	0.05
B ₁₂	0.001
p-aminobenzoic acid	0.05
Thioctic acid	0.05
NaHCO ₃	4667

The scrim-covered membrane appears to be ideal for immobilising and stabilising a biofilm on the membrane surface. The average scrim height was $470 \pm 40 \mu\text{m}$ (Alvarez *et al.*, 2008). Upper and lower compartments had a volume of 190 mL and 192 mL, respectively, and yielded a specific membrane surface area of $20.25 \text{ m}^2/\text{m}^3$. The reactor was designed as dead-ended in order to maximise the oxygen transfer. The design was a modified version of a flat sheet MABR used by Ohandja (2008). The glass cover was replaced with a flat stainless steel cover in order to minimise the void volume of gas in the upper compartment. In addition, the modification should also reduce stripping and accumulation of volatile compounds in the upper compartment.

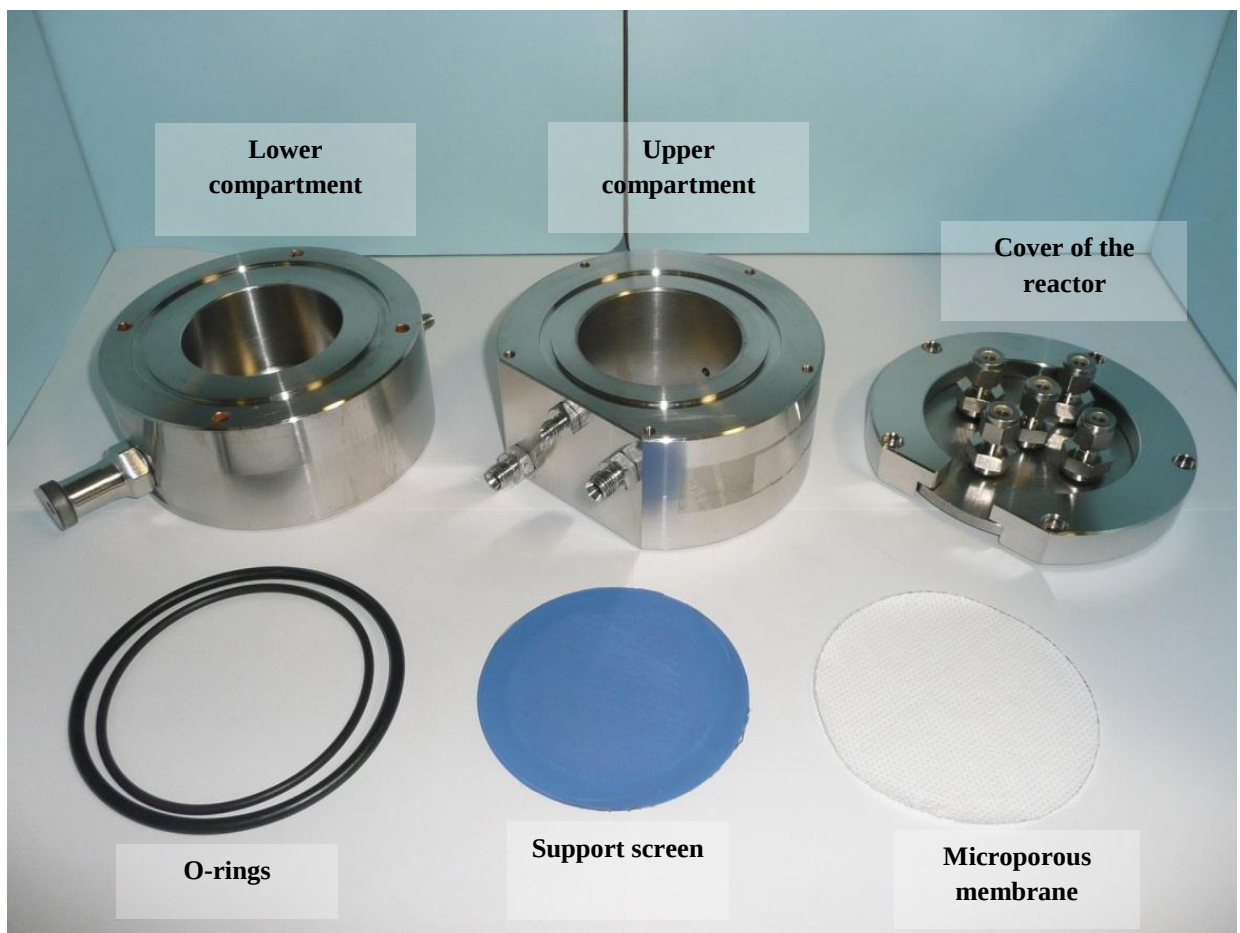


Figure 3.2: Different parts of the flat sheet MABR.

Figures 3.3 and 3.4 show the schematic experimental set-up of the flat sheet membrane MABR, and a photograph of the whole reactor system, respectively. Compressed air was supplied to the lower compartment at a pressure of 15mbar by means of a pressure-regulating valve so as to avoid gas bubbling on the membrane surface. The MABR was inoculated with 2 g VSS/L of mixed aerobic and anaerobic biomass (1:1) from the aforementioned seed CSTRs. During the 3 months of the start-up period, the reactor was supplemented with readily biodegradable organic substrates (glucose and yeast extract) and biomedica in both batch and continuous modes of operation. The biomedica feed into the reactor was modified from its initial composition by Owen *et al.* (1979), as shown in Table 3.2. Influent was introduced into the upper compartment at a specified hydraulic retention time (HRT) that was set by peristaltic pumps. Continuous mixing was provided by a recirculation pump at a flow rate of 70 mL/min, and dissolved oxygen (DO) levels were measured by a DO meter connected to the recirculation line. The reactor was manufactured by Imperial College Chemical Engineering Workshop.

3.7 ANALYTICAL TECHNIQUES FOR NPEOs AND METABOLITES

This section will provide a brief description of the apparatus and methods used for analytical determination of a complex mixture of NPEOs and their metabolites. A detailed protocol will be given in the next chapter.

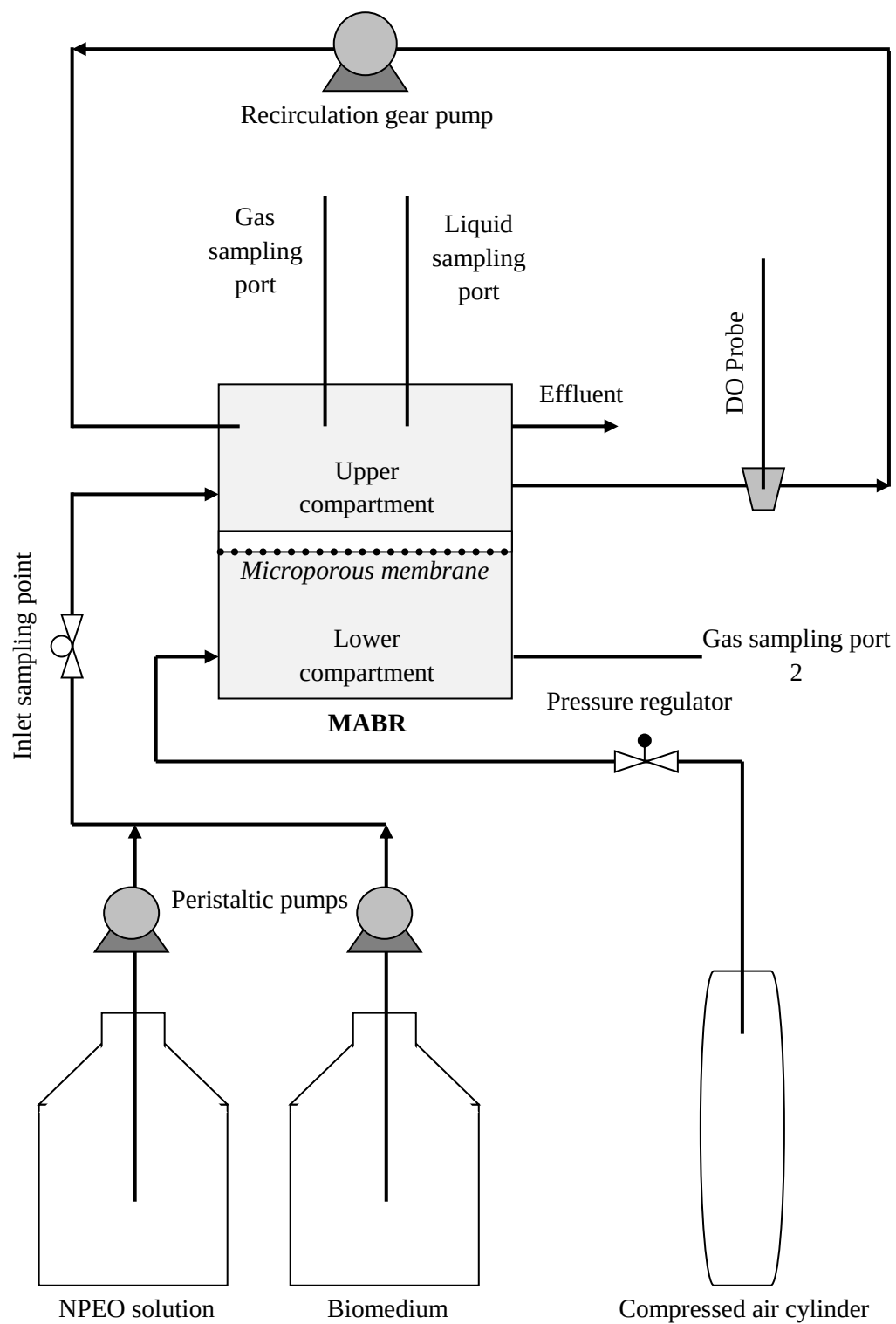


Figure 3.3: The schematic experimental set-up of the flat sheet MABR.



Figure 3.4: A photograph of the whole reactor system.

Table 3.2: Chemical composition of biomedica used in the MABR.

Compounds	Concentration (mg/L)	Compounds	Concentration (mg/L)
$(\text{NH}_4)_2\text{HPO}_4$	80.1	NaHCO_3	2000
<u>Trace Elements solution:</u>		<u>Vitamin Solution:</u>	
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	250.5	Biotin	0.02
NH_4Cl	399	Folic acid	0.02
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	1800	Pyridoxine hydrochloride	0.1
KCl	1300.5	Riboflavin	0.05
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	19.95	Thiamin	0.05
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	30	Nicotinic acid	0.05
H_3BO_3	5.7	Pantothenic acid	0.05
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	2.7	B12	0.001
Na_2MoO_4	2.55	p-aminobenzoic acid	0.05
ZnCl_2	2.1	Thioctic acid	0.05

3.7.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The chromatographic work on the analytical determination of nonylphenolic compounds was carried out using a LC-10Avp HPLC system (Shimadzu Co., Kyoto, Japan) consisting of two LC-10AS pumps, a SCL-10A system controller, a SIL-10AXL auto injector and a SPD-10A UV-Vis absorption detector. The separation was performed under isocratic and gradient elution using a 250 x 4 mm I.D., 5 μ m, Nucleosil C18 column (Macharey-Nagel, Duren, Germany) and 250 x 4.6 mm I.D., 5 μ m, TSK GEL Amide-80 column (Tosoh Bioscience, Yamaguchi, Japan). Detailed analytical procedures and their corresponding relative standard deviation will be elaborated on in Chapter 4.

3.7.2 GAS CHROMATOGRAPHY – MASS SPECTROMETRY (GC-MS)

Samples were derivatised in a glass vial by the addition of selected silylation agents, namely BSTFA and MSTFA, prior to GC-MS analysis. These were done to increase the target compounds volatility and sensitivity. The GC-MS analyses were performed using a gas chromatograph model G1800A (Hewlett-Packard, CA, USA) coupled with an electron ionization detector (EID) (Hewlett-Packard, CA, USA). Analytes were separated in a crosslinked 5% diphenyl–95% dimethylsiloxane (Elite 5-MS, Perkin Elmer, Massachusetts, USA) capillary column (30m $\times$ 0.25mm i.d.; 0.25 μ m film thickness). Helium was used as the carrier gas at a constant flow of 1.0 mL/min. Injection of a 1 μ L sample was performed by an HP autosampler in a splitless mode, at a temperature of 285°C. The GC column temperature was programmed as follows: 1 min equilibrium time at 80°C; 8°C/min to 220°C; 10°C/min to 300°C; and maintained at 300°C for 4 min. The total analysis time was approximately 31

min. The mass spectrometer was operated in a full scan mode. Detailed analytical procedures and their corresponding relative standard deviations will be elaborated on in Chapter 4.

3.8 ANALYTICAL TECHNIQUES: OTHERS

3.8.1 VOLATILE FATTY ACIDS (VFAs)

Seven volatile fatty acids in the bulk liquid and effluent samples (formic, acetic, propionic, isobutyric, n-butyric, isovaleric and n-valeric acids) were separated on an Aminex HPX-87H ion exclusion column (300 mm x 7.8 mm) (Bio-Rad, USA), and analysed by HPLC-UV (Shimadzu 10-AS). Operating conditions were as follow: (1) Mobile phase: 0.01M H₂SO₄; (2) Flow rate: 0.68 ml/min; (3) Column temperature: 55°C; (4) Injection Volume: 50 µL; and (5) UV detector wavelength: 210 nm. The limit of detection for VFAs was 5 mg/L, and the relative standard deviation (RSD) was ±10% for five identical samples. Raw samples were prepared by filtration through a 0.45 µm filter prior to analysis.

3.8.2 BIOGAS COMPOSITION

The biogas composition (CH₄ and CO₂) was monitored using a Shimadzu Gas Chromatograph (GC) equipped with a TCD detector and fitted with a Porapak N column (1500mm x 6.35mm). The carrier gas was helium, at a flow rate of 50 mL/min. The temperature of column, detector and injector were 28°C, 38°C and 128°C, respectively. One mL of gas sample was manually injected into the GC injection port using 1 mL plastic syringes (Terumo). The results of peak area were calculated and printed out on a Shimadzu Chromatopac C-R6A integrator. The RSD for 5 identical samples was within ±2%.

3.8.3 CHEMICAL OXYGEN DEMAND (COD)

The measurement of COD was based on the Standard Closed Reflux Colorimetric Method described in section 5220-D of Standard Methods (APHA, 1999). Digestion solution was first prepared by adding 10.216 g of $K_2Cr_2O_7$ (Merck, UK) (previously dried for 2 hours at 103°C), 167 mL of concentrated H_2SO_4 (Merck, UK) and 33.3 g of $HgSO_4$ (Merck, UK) into 500 mL of distilled water. The mixture was then left to cool at room temperature before diluting to 1000 mL. The sulphuric acid reagent was prepared by adding Ag_2SO_4 technical grade powder (5.5 g Ag_2SO_4 /kg H_2SO_4) to concentrated H_2SO_4 . Standard solutions in the range of 20-1000 mg/L COD were prepared using potassium hydrogen phthalate (KHP) (Merck, UK), in which the theoretical COD was 1.176 mg COD/mg KHP. As far as soluble COD measurement was concerned, raw liquid samples were first filtered through a 0.45 μ m syringe filter to remove fine suspended material and any residual biomass. The tubes were tightly sealed and inverted three times to ensure complete mixing. One mL of liquid sample was then added to a Hach reflux tube, followed by 0.6 mL of digestion solution and 1.4 mL of sulphuric acid reagent. The mixture was then refluxed in a HACH COD reflux reactor (Model 45600) at 150°C for two hours. After cooling, the samples were analysed on a Shimadzu UV-Vis scanning spectrophotometer (Model UV-2101/3101 PC) at a wavelength of 600 nm. The relative standard deviation for 10 identical samples was within $\pm 5\%$.

3.8.4 pH

The pH was measured using a calibrated pH meter (Jenway, Model 3020). Values obtained were accurate to within ± 0.02 units.

3.8.5 TOTAL SUSPENDED SOLIDS (TSS) AND VOLATILE SUSPENDED SOLIDS (VSS)

The measurement of TSS and VSS were conducted according to Sections 2540-B and 2540-E of Standard Methods (APHA, 1999). After washing with deionised water, a 1.2 μm glass microfiber filter (Whatman) was placed on a foil tray and dried in a muffle furnace (Carbolite) at 550°C for 30 min. Initial weight of the filter was recorded after cooling in a desiccator. For TSS measurement, a known volume of sample was filtered under vacuum, and dried in an oven at 103-105°C for 1.5 hr. The filter and residue was then burn in the furnace at 550°C for 20 min for VSS measurement. Both methods involved cooling in a desiccator before final weight was recorded. TSS and VSS calculations were performed according to Standard Methods (APHA, 1999). The RSD was $\pm 5\%$ for 5 identical samples.

3.9 BIOFILM CHARACTERISATION: SCANNING ELECTRON MICROSCOPY (SEM)

Membrane and biofilm structure were analysed by Scanning Electron Microscopy (SEM). Samples were fixed overnight at 4°C in 3.0% glutaraldehyde in 0.1M phosphate buffer (pH 7.2), and then dehydrated in a graded ethanol/water series (10-30-50-70-90-100%) for 20 minutes at each concentration. Samples were then dried overnight at 30°C. Gold splutter coating (30mA for 2.5 minutes, vacuum 0.2 Torr) were applied to the samples prior to SEM analysis. Specimens were later examined and photographed under a scanning electron microscopy (JEOL JSM-5610LV). The above procedure (dehydration and fixation of the biological sample) may change some of the characteristics of the initial sample and are known as artifacts.

❧ CHAPTER 4 ❧

ANALYTICAL METHOD DEVELOPMENT FOR NONYLPHENOL ETHOXYLATES (NPEOs) AND INTERMEDIATES

Outline:

- 4.1 Introduction
- 4.2 Analytical strategies for the determination of NPEOs and intermediates
- 4.3 Results and discussion
 - 4.3.1 Detection and Quantification of NPEO_{ave9} by HPLC
 - 4.3.1.1 Reversed phase HPLC
 - 4.3.1.2 Hydrophilic interaction liquid chromatography (HILIC) of NPEOs
 - 4.3.1.3 Normal phase chromatography of NPEOs
 - 4.3.2 Detection and Quantification of NPEO_{ave1.5} and NP by GC-MS
 - 4.3.2.1 Derivatisation
 - 4.3.2.2 GC-MS analysis
 - 4.3.3 Sample preparation of NPEOs
- 4.4 Summary

4.1 INTRODUCTION

In the last few decades, modern instrumental analysis has contributed much to our knowledge on the fate of trace organic contaminants in the environment. Progress in this area is not only aided by technological advances in instrumentation, but also by the improvements in its methodological schemes. According to Ahel and Giger (1985b), the procedures used for the qualitative and quantitative determination of synthetic surfactants must cope with the compositional complexity of these chemicals. NPEOs are commercially produced by ethoxylation of a mixture of nonylphenol isomers with ethylene oxide monomers. Depending on the reaction conditions and the molar ratio of the reaction compounds, the end products typically consist of a polydispersed mixture of NPEO oligomers having different numbers of repeating hydrophilic ethoxylate (EO) units (typically ranging from 4 to 50) with a wide arrangement of isomeric, branched-chain hydrophobic nonyl groups. NPEOs with an average number of EO units of 9 (having an EO chain length from 1 to 15), simply denoted as NPEO_{ave9}, are the most widely used NPEO mixture for commercial application. The difficulty of chromatographic separation, detection and quantification of NPEOs is not only due to their complex physicochemical properties among the parent compounds, but also their degradation products. Based on previous studies, bacteria attack at different points along the molecules, leading to various biodegradation mechanisms and numbers of possible intermediates (Staples *et al.*, 2008). Structural configurations of the intermediates have been previously shown in Figure 2.1, in Chapter 2. Figure 4.1 summarises several discrete steps, generally applied for the determination of polar pollutants, including nonylphenolic compounds, in both water and particulate samples (sludge, sediment and soil) (Reemtsma and Quintana, 2002).

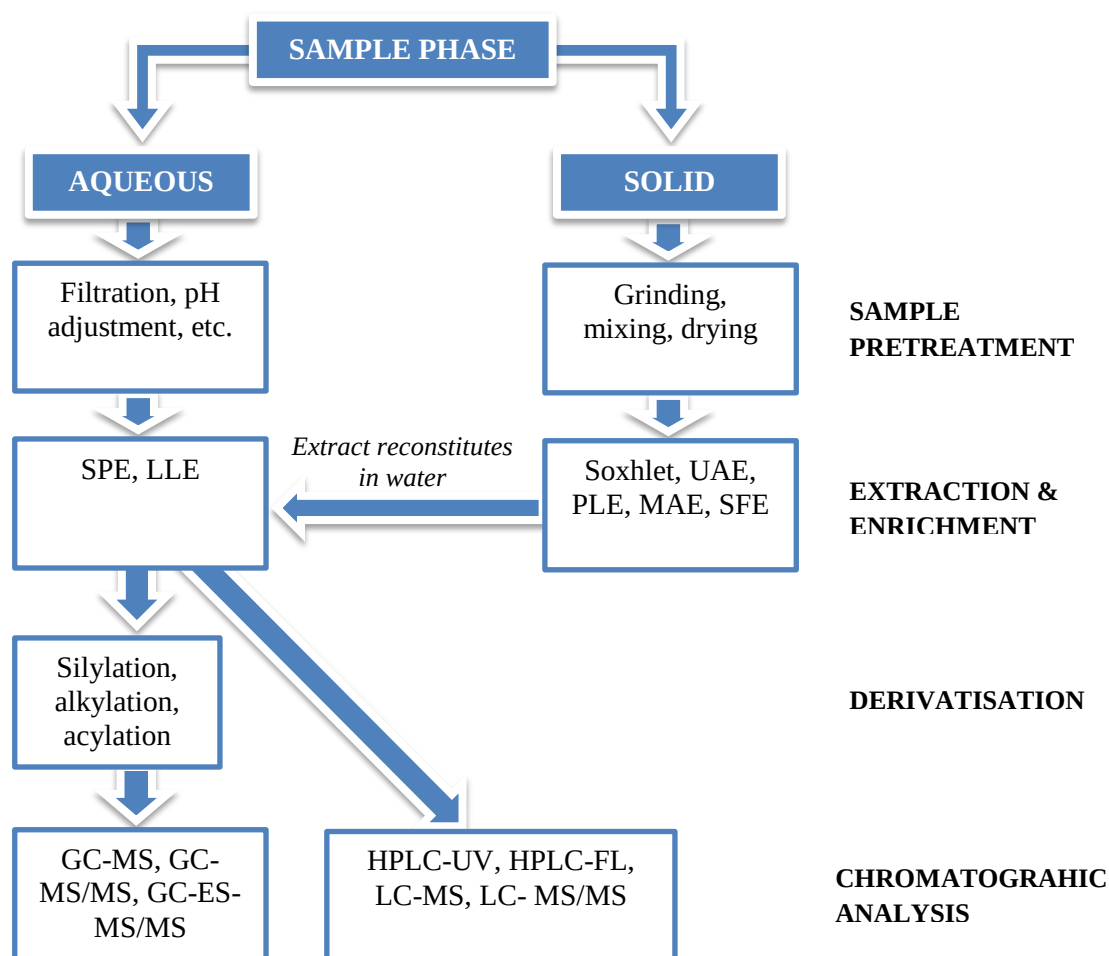


Figure 4.1: Overview of the analytical scheme. SPE: Solid Phase Extraction; LLE: Liquid-Liquid Extraction; UAE: Ultrasonic assisted Extraction; PLE: Pressurised Liquid Extraction; MAE: Microwave Assisted Extraction; SFE: Supercritical Fluid Extraction; GC-MS: Gas Chromatography – Mass Spectrometry; MS/MS: Tandem Mass Spectrometry; ES: Electrospray; HPLC: High Performance Liquid Chromatography; FL: Fluorescence. Adapted from Reemtsma and Quintana (2002).

A sample is often subjected to a series of preparative steps, including sample pretreatment, extraction and enrichment, prior to its chromatographic analysis. This labour intensive procedure aims to remove interfering species from the sample matrix, to adapt the physical or chemical state of the sample to the requirements of the instrument, and to

concentrate the compounds of interest, thereby enhancing the sensitivity of analysis (Domini *et al.*, 2006). The sophistication of the applied techniques depends on the nature of the sample on which the analytes are associated with, and the objective of the analysis.

Although the methods for analytical determination of NPEOs and their intermediates have been extensively reviewed (Petrovic and Barcelo, 2002; Scrimshaw *et al.*, 2004; Morales *et al.*, 2009); the involvement of many key parameters produces different yet contradictory results, and thus it is difficult to establish a standard method for these compounds. In some cases, the methods need further modification and optimisation in order to compensate for equipment differences in other facilities. The aim of this work, therefore, was to develop comprehensive analytical methods for the determination of NPEOs and their intermediates in solid and aqueous samples. A mechanistic study is not of priority in the present report, but rather to search for appropriate analytical techniques that are safe, reliable and fast, and which will be used throughout this study - most importantly for routine identification and quantification of biodegradation intermediates.

4.2 ANALYTICAL STRATEGIES FOR THE DETERMINATION OF NPEOs AND INTERMEDIATES

A brief summary of proposed analytical strategies for the determination of NPEOs and intermediates is given in Table 4.1. The work involved an evaluation of chromatographic separation and detection of pure standards; and the samples were then subjected to a series of extraction procedures to purify the analytes of interest from an interfering matrix. Some of the methods were modified from previous researchers, while others are proposed and further

evaluated for their efficiency. The materials and apparatuses used for method development were mentioned briefly in Chapter 3, and the details will be discussed later in this chapter.

Table 4.1: Summary of analytical strategies.

Analytical strategies	Methods		Objectives
Detection and quantification of NPEOs and intermediates	HPLC-UV	Reversed phase	Separation of low and high MW NPEO ₁₋₁₅ and NP
		HILIC and Normal phase	Separation of low and high MW NPEO ₁₋₁₅ and NP
	GC-MS	Derivatisation	Increased volatility of NPEOs and NP
		High temperature column	Separation of low MW NPEO ₁₋₂ and NP
Sample preparation	Solid phase extraction (SPE) by polymeric sorbent Ultrasonic assisted extraction (USE) with SPE		Extraction of all nonylphenolic compounds from aqueous phase Extraction of all nonylphenolic compounds bound to solid biomass

4.3 RESULTS AND DISCUSSION

4.3.1 DETECTION AND QUANTIFICATION OF NPEO_{AVE9} BY HPLC

According to Kiewiet and de Voogt (1996), the analysis of non-ionic surfactants is one of the most difficult in the environmental analytical field, where the separation of all the different oligomers and isomers of the non-ionic surfactants in one analysis seems almost unachievable. High Performance Liquid Chromatography (HPLC) is the prevalent method for

NPEOs separation as it covers a broad range of polarity. Moreover, the presence of a phenol ring chromophore enables their direct UV absorbance (at 277-280 nm) (Kibbey *et al.*, 1996; Kiewiet and de Voogt, 1996). Numbers of HPLC methods have been developed by researchers for NPEOs determination in environmental samples using different stationary phases (columns), mobile phases, detectors and operating conditions (Morales *et al.*, 2009). In this study, HPLC-UV methods were evaluated under three elution modes, namely reversed phase, normal phase and hydrophilic interaction liquid chromatography (HILIC). This was done to select the method that could determine the ethoxy (EO) chain length distribution of NPEO_{ave9} most effectively.

4.3.1.1 Reversed phase HPLC

In reversed phase elution, the mobile phase is significantly more polar than the stationary phase (e.g. microporous silica-based material with chemically bonded alkyl chains – C₁₈, C₈) (McNaught and Wilkinson, 1997). The reversed phase elution was performed on a Nucleosil C18 column using acetonitrile and water as the mobile phase. The reversed phase chromatogram of the NPEO_{ave9} commercial standard, IGEPAL CO-630, is shown in Figure 4.2; a single peak is observed at a short retention time. According to its manufacturer, IGEPAL CO-630 contains a mixture of NPEO_{ave9} oligomers Gaussian distributed with an average chain length of 9 ethoxy groups. Thus, the occurrence of a single peak confirms that the retention of IGEPAL CO-630 on this column was not influenced by the ethoxy chain length. In fact, the stationary phase (column) exhibited a strong interaction with the hydrophobic moiety, thus eluting all the individual oligomers as a single peak (Thiele *et al.*, 1997). According to Núñez *et al.* (2007), it is almost impossible to separate peaks

corresponding to NPEOs with more than 3 EO units on a reversed phase C18 column, due to the increase in polarity as the number of EO units increases.

This phenomenon has been commonly observed in various reversed phase systems (Ahel and Giger, 1985a,b; de Voogt *et al.*, 1997). However, the advantage claimed for this method is that it increases the peak intensity and detection sensitivity (Morales *et al.*, 2009). If the separation of individual oligomers is desired, the use of a stationary phase with stronger hydrophilic interactions should be considered.

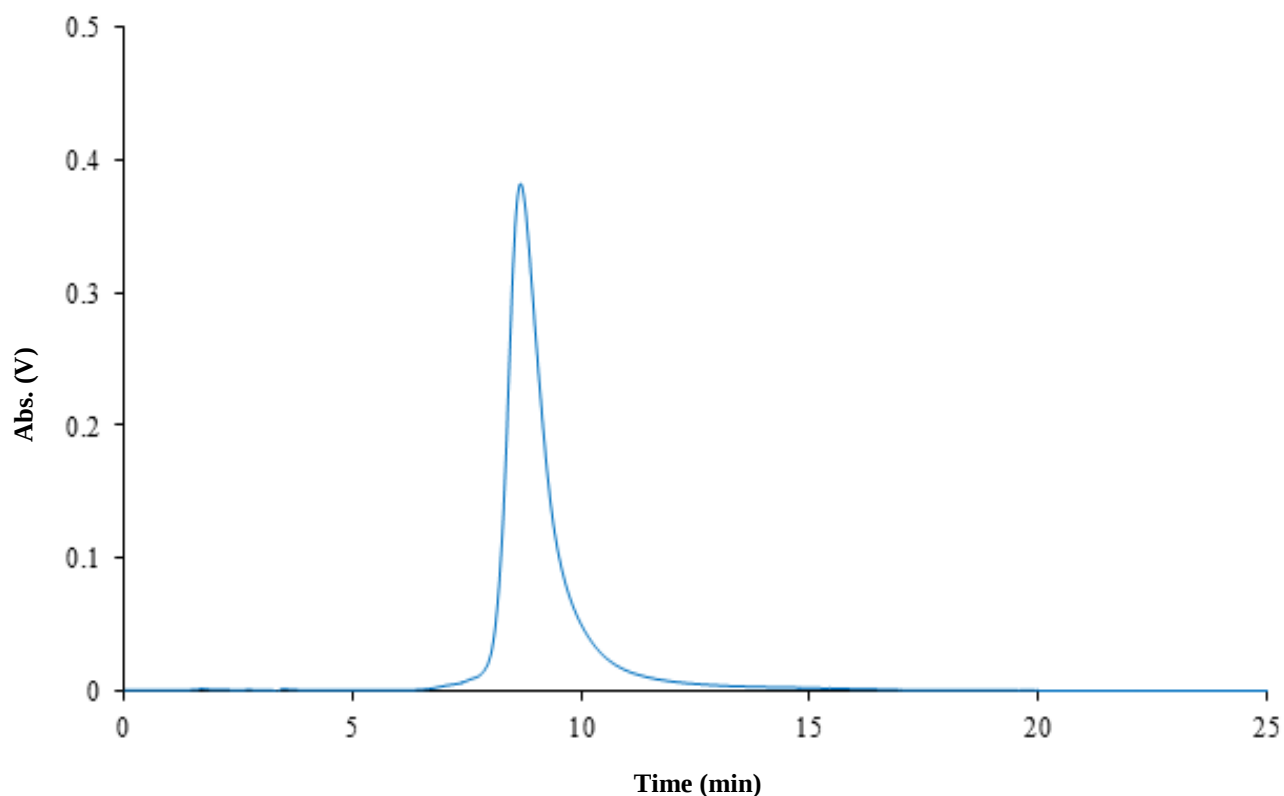


Figure 4.2: Reversed phase chromatogram of an IGEPAL CO-630 commercial standard solution. Chromatographic conditions: Nucleosil reversed phase C18 column; flow-rate, 1 ml /min; mobile phase, acetonitrile–water (80:20); detection, UV at 277 nm; concentration, 1000 mg/L; injection volume, 50 μ L.

4.3.1.2 Hydrophilic interaction liquid chromatography (HILIC) of NPEOs

HILIC is a relatively new type of chromatography introduced by Alpert (1990). It is commonly used to separate polar compounds, or compounds that are not sufficiently retained under reversed phase elution, such as peptides, amino acids, carbohydrates, oligonucleotides, small drug molecules and metabolites, and surfactants (Zhang *et al.*, 2008c; Liu and Pohl, 2008). In HILIC, polar molecules can be retained on polar stationary phases (e.g. silica gels modified with polar functionalities such as amide, diol and cyano) by using high concentrations of organic mobile phases (e.g. acetonitrile) containing aqueous buffer (Liu and Pohl, 2008; Ikegami *et al.*, 2008). Several reviews on HILIC have been published (Hemström and Irgum, 2006; Jandera, 2008; Ikegami *et al.*, 2008) in which the fundamental aspects and applications of HILIC have been discussed. Nevertheless, only a few applications of HILIC for surfactant analysis have been reported so far, including the NPEOs.

For the purpose of this study, an HILIC column of TSK GEL Amide-80 (Tosoh) was used to separate IGEPAL CO-630 (NPEO_{ave9}) according to their respective EO units. Amide-80 is one the most widely used HILIC columns for the separation of monosaccharides, peptides, and amino acids (Ikegami *et al.*, 2008). The amide phase consists of carbamoyl groups attached to the silica surface through an aliphatic carbon chain (Tosoh, 2003); this conveys good chemical and pH stability due to the low reactivity of the amide moiety. The application of this column for HILIC elution of NPEOs has not been reported previously in the literature. The advantage of this elution mode is it provides simpler isocratic elution with an aqueous mobile phase, instead of a complex gradient of a non-polar mobile phase under conventional normal phase elution.

The retention behaviour of NPEO_{ave9} under HILIC elution is shown in Figure 4.3. The analyses were performed isocratically, and the acetonitrile to 0.1M ammonium acetate buffer contents were varied from 60/40, 80/20, 90/10 to 99/1. At higher aqueous contents (less than 80% acetonitrile), the retention behaved similarly to that of reversed phase elution. The oligomers collapse into a single peak, and the sensitivity increased substantially. However, by increasing the content of the non-aqueous solvent (acetonitrile), the retention time increased and the oligomers were slightly separated, showing typical HILIC behaviour. In HILIC, retention is proportional to the polarity of the compounds, and inversely proportional to the polarity of the mobile phase.

A similar trend was also reported by Liu and Pohl (2008) in which more than 90% acetonitrile was required in the mobile phase to retain individual oligomers of octylphenol ethoxylates (OPEOs) on HILIC columns, and behaved as a reversed-phase column when the mobile phase contained less than 75% acetonitrile. Nevertheless, the retention behaviour of NPEOs shown in the above figure is inadequate to separate the oligomers effectively, compared with the previous author. The authors used a laboratory fabricated stationary phase of undecyl-1,2-diol ligands bonded to a silica surface through an alkyl chain, which differs in retention properties compared to Amide-80. In addition, OPEOs exhibits lower hydrophobicity than NPEOs, thus improving the OPEOs retention on this type of column. The underlying mechanism of HILIC is not fully understood, nevertheless, these findings suggest that the Amide-80 column could be used for mixed-mode chromatography for NPEOs using a simple isocratic elution.

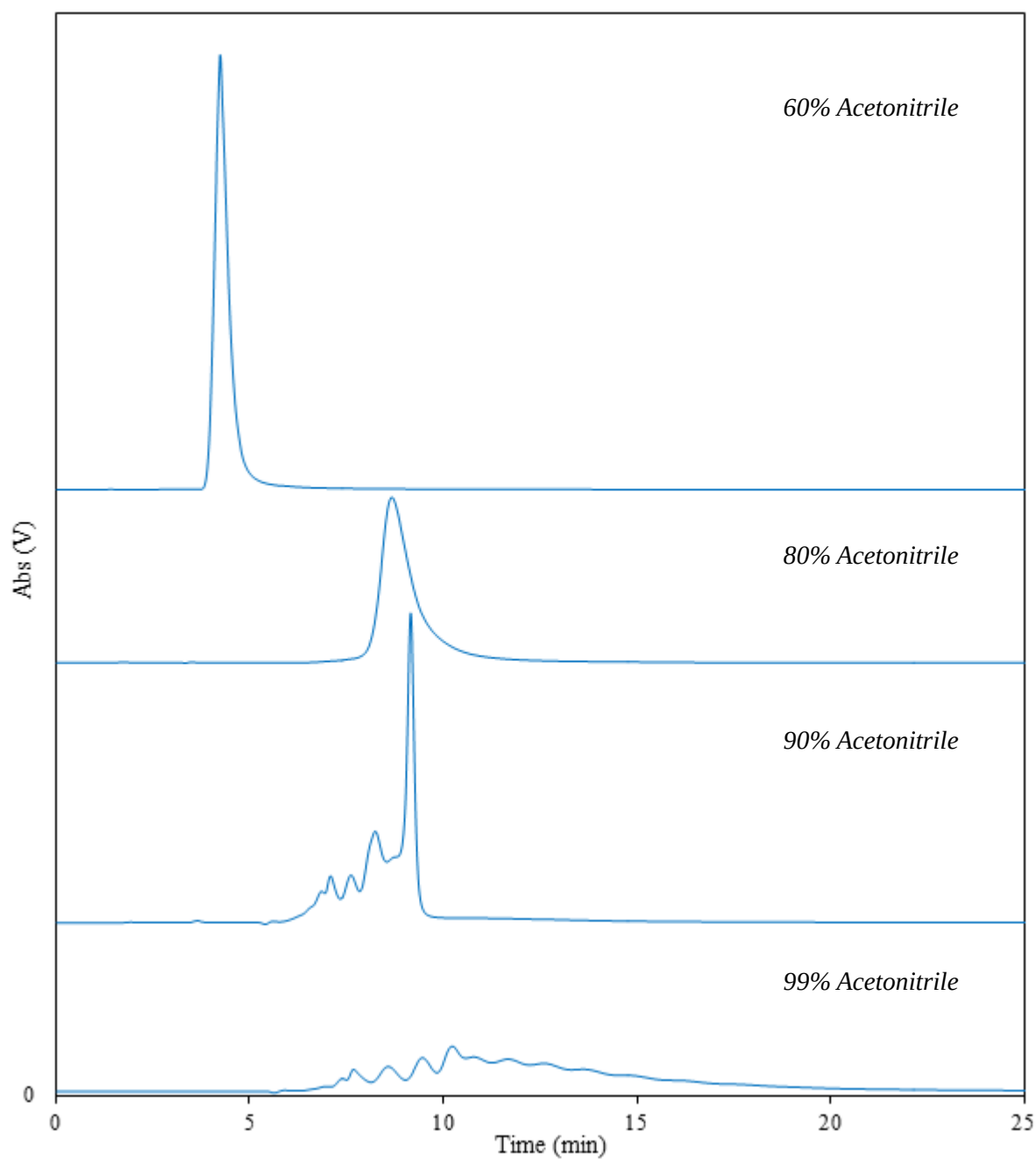


Figure 4.3: HILIC chromatograms of IGE PAL CO-630 commercial standard solutions, at different acetonitrile compositions. Chromatographic conditions: TSK GEL Amide-80 column; flow-rate, 0.7 mL/min; mobile phase, acetonitrile–0.1M ammonium acetate; detection, UV at 277 nm; concentration, 1000 mg/L; injection volume, 50 μ L.

Therefore, changes in operating conditions, such as the introduction of a less polar mobile phase, are essential to improve the chromatographic separation of NPEOs. The application of a non-polar mobile phase (i.e. hexane) is similar to conventional normal phase elution in which the stationary phase (e.g. microporous silica-based material with chemically bonded polar phase – amino, nitrile, diol) is more polar than the mobile phase (McNaught and Wilkinson, 1997). Subsequent trials will elucidate whether we can apply normal phase elution for IGEPAL CO-630 on a TSK GEL Amide-80 column.

4.3.1.3 Normal phase chromatography of NPEOs

The distribution of NPEOs oligomers is normally determined by a procedure based on normal phase elution. In the present study, a normal-phase HPLC method was modified from Maki *et al.*, (1996) by using the aforementioned column under gradient elution. The elution program is presented in Table 4.2, and the normal phase chromatogram of IGEPAL CO-630 is illustrated in Figure 4.4(a).

Table 4.2: Composition of mobile phase and gradient elution program in normal phase HPLC analysis.

Time	Eluent A (%)	Eluent B (%)
0	100	0
9	76	24
20	2	98
30	2	98

Eluent A composition: Hexane/2-Propanol/Methanol – 95/3.5/1.5

Eluent B composition: 2-Propanol/Methanol – 70/30

Flow rate: 0.7 mL/min; Injection volume: 50 µL

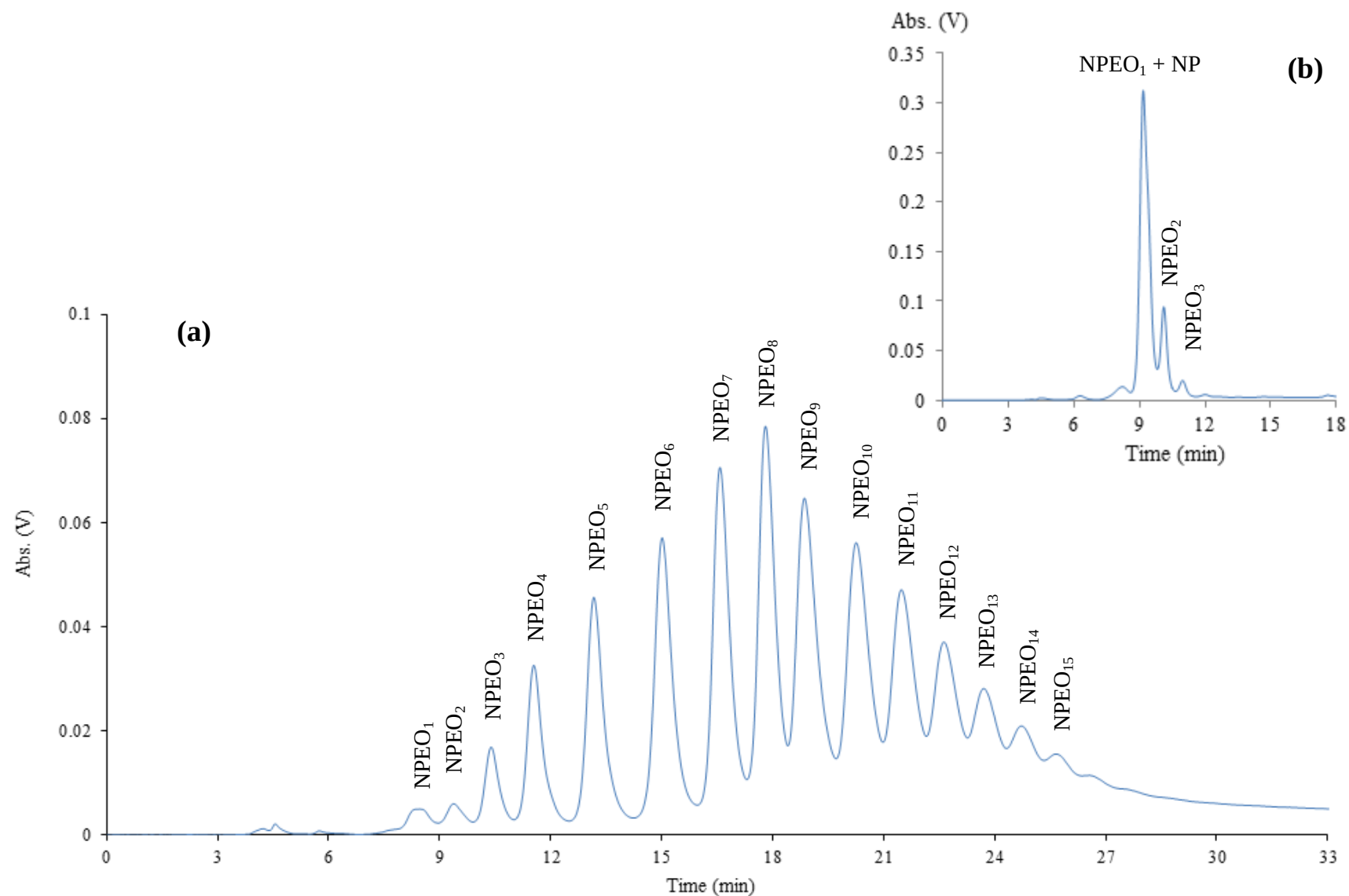


Figure 4.4: (a) Chromatogram of IGEPAL CO-630, and (b) chromatogram of IGEPAL CO-210 and NP commercial standard solutions, under normal phase elution; Chromatographic conditions: TSK GEL Amide-80 column; flow-rate, 0.7 ml/min; mobile phase, Hexane/2-Propanol/Methanol (gradient elution); detection, UV at 277 nm; concentration, 1000mg/L; injection volume, 50 μ L.

The chromatogram reveals 15 well resolved oligomers, in less than 27 min of retention time. The EO units (n) are assigned to each individual oligomeric peak. The gradient starts with 95% of a non-polar solvent, hexane. Long chain oligomers are more strongly retained on the stationary phase than the short ones due to the strong interactions of hydroxyl groups with the stationary phase. By increasing the solvent polarity over time, the surface occupied by oligomers will be successively replaced by a higher polarity solvent, i.e. propanol/methanol; hence, surfactant oligomers with fewer EO groups are eluted first, while those with greater numbers of EO groups are eluted later (Kibbey *et al.*, 1996). Thus, the retention of compounds is inversely proportional to the polarity of the solvent. Complete separations of a wide range of NPEOs oligomers have also been reported using other normal phase columns, such as Lichrosorb-NH₂ and Hypersil APS (Ahel and Giger, 1985b).

The validity and reproducibility of the method were evaluated. Table 4.3 lists the instrumental reproducibility as relative standard deviation (RSD) of three replicates for 15 NPEOs oligomers, as well as their corresponding limit of detection (LOD) and limit of quantification (LOQ). LOD is defined as the concentration that corresponds to 3 times the signal of the background noise, while LOQ were calculated as 10 times the background noise. As can be seen in Table 4, LOD was varied from 42 to 214 µg/L, and LOQ was from 140 to 716 µg/L. The RSD obtained from the replicates were less than 15%, most of which less than 2%. This demonstrates a good precision of the method. Moreover, a linear calibration fit with a high correlation coefficient ($r^2 = > 0.99$) was obtained for the compounds studied.

Table 4.3: HPLC-UV reproducibility and performance.

Compounds	Retention time, % RSD*	Peak area, % RSD*	LOD (µg/L)	LOQ (µg/L)
NPEO ₁	0.81	13.49	42	140
NPEO ₂	1.34	1.83	49	163
NPEO ₃	0.96	1.53	56	187
NPEO ₄	0.72	1.43	63	210
NPEO ₅	0.63	1.65	70	233
NPEO ₆	0.23	0.62	75	250
NPEO ₇	0.21	1.42	83	275
NPEO ₈	0.25	1.13	90	301
NPEO ₉	0.22	1.63	105	350
NPEO ₁₀	0.13	1.81	131	436
NPEO ₁₁	0.12	1.66	156	519
NPEO ₁₂	0.08	1.73	186	621
NPEO ₁₃	0.11	1.83	193	644
NPEO ₁₄	0.10	1.91	204	680
NPEO ₁₅	0.07	3.55	215	716

* Standard of 20 mg/L of IGEPAL CO-630 (n=3)

It is known that the molar absorptivities are identical for all the oligomers at the selected UV detection wavelength due to the presence of only one phenyl ring (chromophore) in all NPEO molecules (Ahel and Giger 1985b; Wang and Fingas, 1993). The molar amounts of each NPEOs oligomer were therefore calculated from the HPLC peak area derived from standard solutions. This can serve as a basis to determine the EO distribution and average moles of EO. The mole fraction of each oligomer, and average EO numbers, were calculated according to Wang and Fingas (1993), using the following equations:

$$F_i = \frac{A_i}{\sum_{i=0}^n A_i} \quad (1)$$

where F_i and A_i represent the mole fraction of each oligomer, and the peak area of individual oligomer, respectively. Then, the average EO number can be determined as:

$$\tilde{n} = \sum n_i F_i \quad (2)$$

where $\tilde{n}$ and n_i are the average EO number of the sample, and the number of EO units in each individual oligomer, respectively. Then, the average molecular mass of the total NPEO sample can be calculated as:

$$M_r = (M_r)_R + \tilde{n} (M_r)_{EO} \quad (3)$$

in which $(M_r)_R$ is the molecular mass of the nonylphenol group ($C_9H_{19}C_6H_5-$); and $(M_r)_{EO}$ is the molecular mass of an ethylene oxide unit $(CH_2CH_2O-)_n$. The average EO number for IGEPAL CO-630 from three measurements ($n = 3$) was calculated to be 8.73 (0.14% RSD), and the average molecular mass was 604 g/mol (0.09% RSD). The values obtained were close to the values stated by the manufacturer, and hence the above method was sufficient to separate and determine the EO chain length distribution of NPEO_{ave9}, especially in monitoring the long chain NPEOs. However, as can be seen in Figure 4.4(b), the separation of low MW NPEOs intermediates is unachievable; for instance, the peak of NPEO₁ co-elutes with NP. This is due to the weak hydrophobic interactions between the polar stationary phase and hydrophobic analytes. In a study by Shao *et al.* (2002), NPEOs with short EO chain lengths of less than 5 could not be separated on a normal phase bare silica column without the hydrophobic C18 pre-column. The use of an additional pre-column is, however impractical,

while improving the gradient elution profiles in the early stage of the chromatograms can only partly solved the separation. Hence, a GC-MS technique was chosen for the determination of low molecular weight intermediates because of its high sensitivity and selectivity.

4.3.2 DETECTION AND QUANTIFICATION OF NPEO_{AVE1.5} AND NP BY GC-MS

Long ethoxy chain NPEOs (i.e. >4 EO units) are not analysable with GC separation due to their high polarity. In the present study, a comprehensive analytical method using GC-MS was developed for technical grade nonylphenol (NP) and IGEPAL CO-210 (NPEOs with an average 1.5 EO units). Based on previous studies, analysing short ethoxy chain NPEOs requires their derivatisation prior to analysis in order to improve the appearance of the chromatographic peaks, and to achieve the highest possible detection sensitivity (Gatidou *et al.*, 2007).

4.3.2.1 Derivatisation

Derivatisation aims to increase volatility and the thermal stability of polar compounds. Silylation is the most prevalent derivatisation reaction for nonylphenolic compounds. During silylation, active hydrogen on hydroxyl groups are replaced with trimethylsilyl (TMS) groups. The TMS groups are supplied by selected derivatisation agents such as N,O-bis-(trimethylsilyl)-trifluoro acetamide (BSTFA), N-methyl-N-trimethylsilyl trifluoro acetamide (MSTFA) and N,O-bis(trimethylsilyl)acetamide (BSA). The derivatised compounds are more amenable to standard GC/MS analysis, and this will also enhance their chromatographic separation and detection sensitivity. It is believed that the enhanced reaction

kinetic is attributed to the thermal-kinetic effect caused by the rapid transfer of energy into the bulk of the reaction (Fiamigos *et al.*, 2010). The derivatisation reaction is normally operated at elevated temperature (~60°C) in block heaters or water baths, and often require from 30 min up to several hours for the bulk solution to reach conditions capable of completing the reaction (Bowden *et al.*, 2009). Recent improvements have been made to replace the conventional heating method with microwave irradiation technology, known as microwave assisted derivatisation (MAD). The main objective of MAD is to overcome heat transfer limitations while reducing the reaction time (i.e. <5 min), but since its early introduction in the 1990s, limited trials have been reported. The technique has been successfully implemented for sugars, amino acids, fatty acids, aliphatic alcohols, steroids, and EDCs; mostly using domestic microwave ovens (Bowden *et al.*, 2009). A review on this subject has also been published recently by Söderholm *et al.* (2010). To our knowledge, MAD has not been tested on any nonylphenolic compounds.

Figure 4.5 shows the flow diagram of a derivatisation procedure; a conventional derivatisation was conducted according to Gatidou *et al.* (2007), while MAD was modified from Bowden *et al.* (2009) using a domestic microwave oven with a maximum power output of 700W. It was noted that the operating conditions of MAD were systematically optimised for steroidal compounds (Bowden *et al.*, 2009). For the purpose of this study, it was assumed that the nonylphenolic compounds will behave almost similarly to steroids. According to Bowden *et al.* (2009), MSTFA (as a reagent) and acetonitrile (as a solvent) are the most effective for microwave heating due to their ability to absorb microwave energy efficiently. In this study, the response factors of the GC spectra acquired from microwave assisted derivatised compounds were very similar to the response factors achieved from the derivatives using the conventional method. Indeed, microwave heating only takes 1 min to

complete the reaction, compared with 20 min for conventional heating to reach the same yield. Hence, the time and energy efficient MAD was chosen for future derivatisation work prior to GC-MS analysis.

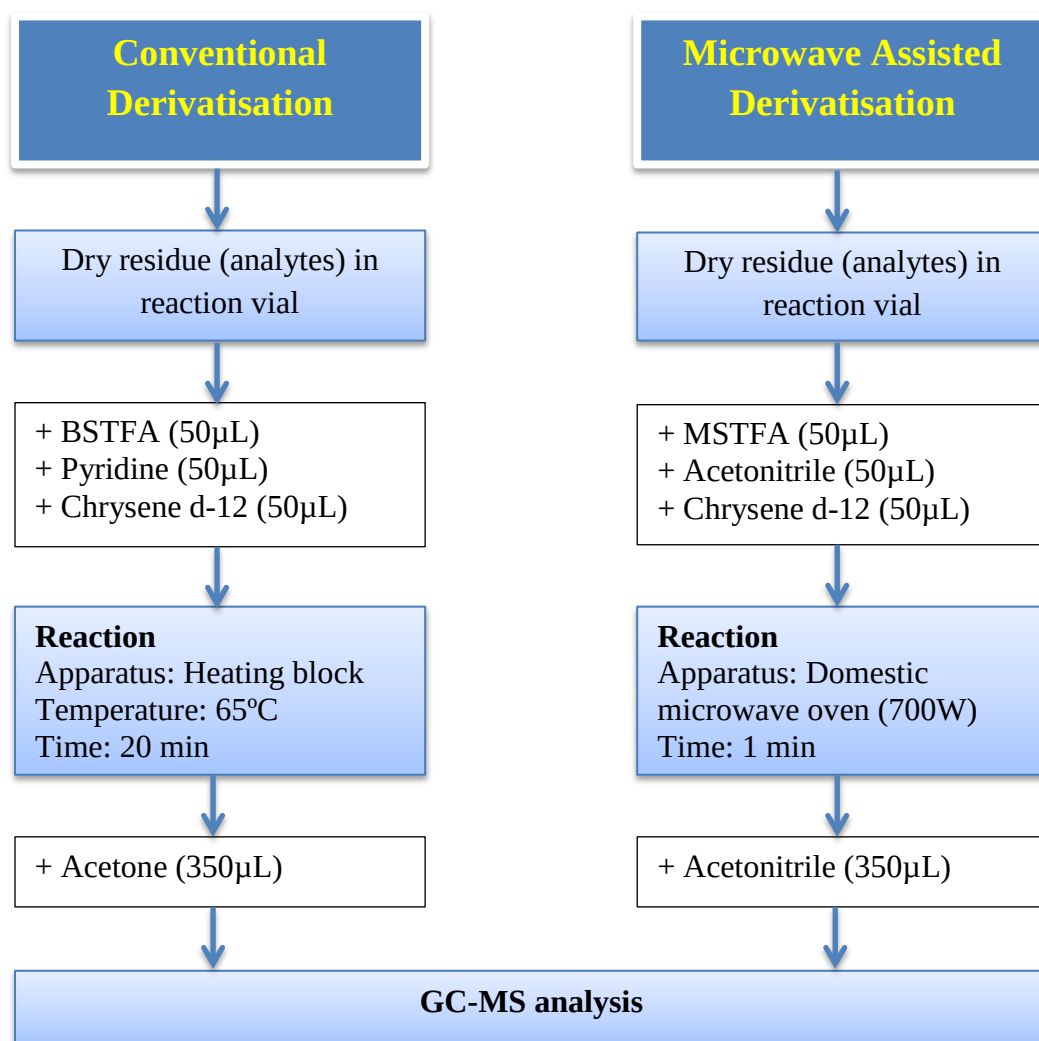


Figure 4.5: Flow diagram of the derivatisation procedure for NP and NPEOs; a comparison between conventional and microwave assisted derivatisation. (The procedures were conducted according to Gatidou *et al.* (2007) and Bowden *et al.* (2009)).

4.3.2.2 GC-MS analysis

Figure 4.6(a) illustrates the full-scan electron ionisation (EI) chromatogram of trimethylsilylated NP, NPEO₁ and NPEO₂, and Figure 4.6(b), (c) and (d) represents respectively, their corresponding mass spectra detected in a standard solution. The characteristic ions were chosen based on the most abundant ions in each spectrum. The ions are 179, 193 and 221 for NP; 251 and 265 for NPEO₁; 295 and 309 for NPEO₂; and 242 for the internal standard (Chrysene d-12). As can be seen from the figure, each compound consists of a mixture of isomers separated by the GC. Hence, the quantification of each compound will be based on the sum of the peak areas of all isomeric clusters. This finding is in line with previously reported trimethylsilylated NP, NPEO₁ and NPEO₂, in terms of qualitative comparison of the chromatograms and the selected characteristic ions (Gatidou *et al.*, 2007; Liu *et al.*, 2004; Hernando *et al.*, 2004). For a spiking level of 10 mg/L, the RSD obtained of the peak area (n=3) were 11.04%, 4.99% and 6.38% for NP, NPEO₁ and NPEO₂, respectively, which indicates good reproducibility of the analysis. In addition, the instrument LOD and LOQ for NP, NPEO₁ and NPEO₂ were 3.1 µg/L, 0.9 µg/L and 0.8 µg/L; and 10.4 µg/L, 3.1 µg/L and 2.7 µg/L, respectively.

4.3.3 SAMPLE PREPARATION OF NPEOs

A good chemical analysis not only relies on the sensitivity of the equipment, but also on how the sample is prepared. Liquid–liquid extraction (LLE) and solid phase extraction (SPE) techniques have been described for the enrichment of nonylphenolic compounds from water, sediment and sludge samples. To date, SPE is the most popular sample extraction technique due to its low solvent requirement, speed and flexibility.

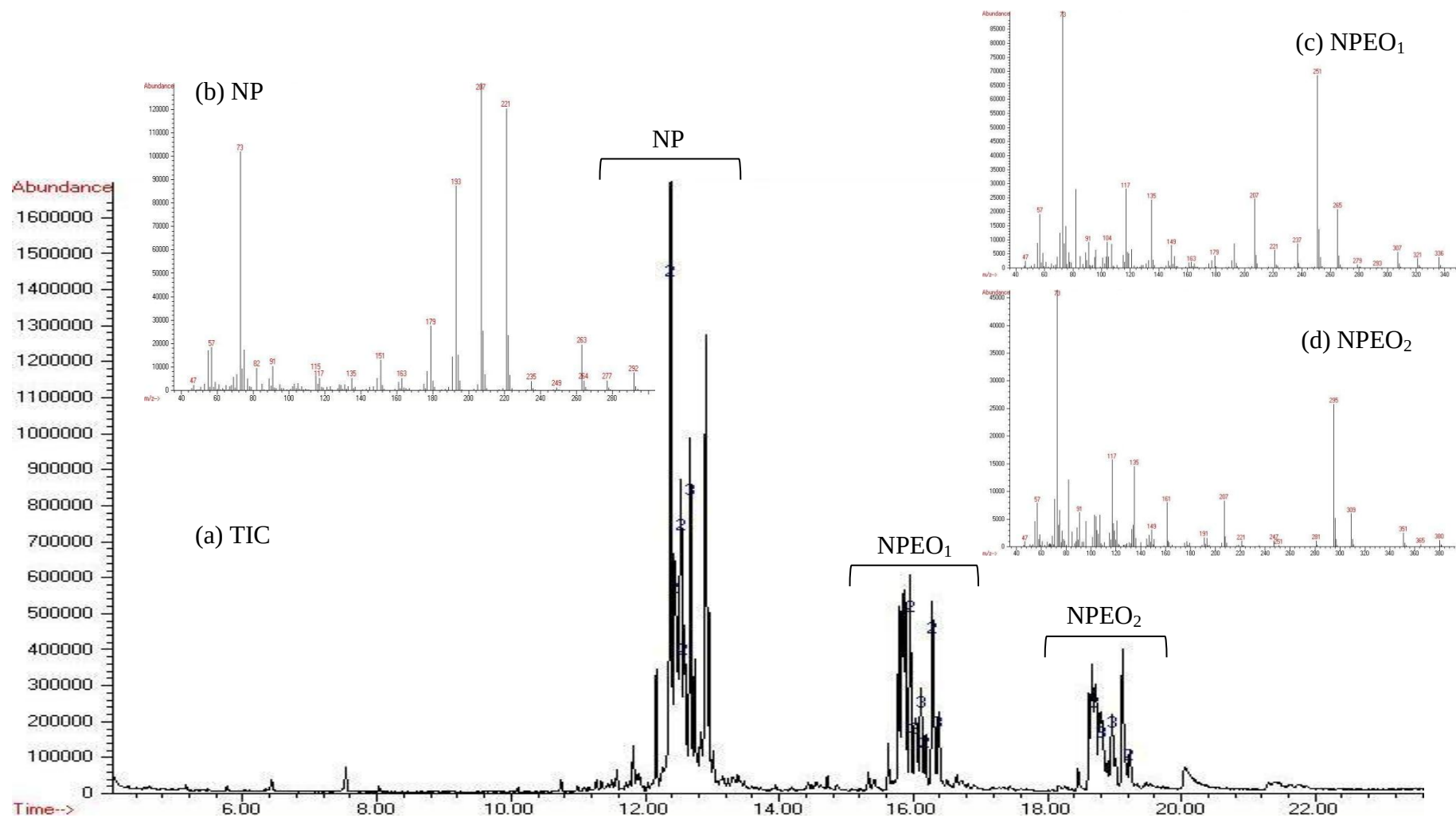


Figure 4.6: (a) GC-MS total ion current (TIC) profile of the trimethylsilyl derivatives of NP, NPEO₁ and NPEO₂; (b), (c) and (d) Electron ionisation (EI) mass spectra for selected isomers of trimethylsilylated NP, NPEO₁ and NPEO₂, respectively.

Varieties of SPE packing materials are introduced to the market to fit a particular extraction objective. For instance, one of the most widely used polymeric SPE sorbents for NPEOs, the Oasis HLB cartridge (Waters, Milford, MA, USA), has shown a superior extraction performance to other silica-based sorbents (e.g. C18), due to its better wetting characteristics and extra possibilities for interactions with functional groups, thus retaining a wide spectrum of polar and nonpolar compounds (Boitsov *et al.*, 2004). The Oasis HLB is a macroporous poly (N-vinylpyrrolidone-divinylbenzene) (PVPDVB) copolymer and has a specific surface area of 800 m²/g (Fontanals *et al.*, 2007). In this study, the extraction of analytes from an aqueous phase was performed according to a modification of the Oasis generic methodology (Waters, UK). The cartridge was first placed on a vacuum pump and conditioned sequentially with 1mL ethyl acetate, methanol and analytical grade deionised water. Then, 2 mL of sample was percolated through the cartridge and dried under a gentle nitrogen stream. The cartridge was washed with 1 mL of 5% methanol to remove impurities. The targeted compound was then eluted with 1.5 mL of ethyl acetate. The extract was completely evaporated to dryness under a nitrogen stream and submitted to the derivatisation procedure. The SPE gave good recoveries for NP and NPEO₁₋₁₅ ranging from 71 to 108%. The method detection limit (MDL) and method quantification limit (MQL) of NP and NPEO₁₋₁₅ via SPE-HPLC and SPE-GC-MS are shown in Table 4.4.

The fate and partitioning of organic chemicals into water/solid is largely influenced by their physico-chemical properties. When the hydrophilic parent compounds of NPEOs degrade, intermediates of short chain NPEOs and NP will be predominantly formed. These predominant intermediates are hydrophobic in nature (Ahel and Giger, 1993b), and have a strong affinity for sediment (John *et al.*, 2000) and organic matter (Stasinakis *et al.*, 2010). The extraction of these compounds from a solid matrix has been accomplished by Soxhlet

extraction, microwave-assisted extraction (MAE), ultrasonic-assisted extraction (USE), and pressurised liquid extraction (PLE) (Arditsoglou and Voutsas, 2008). The USE method will be evaluated in this study due to its low solvent requirement and less time required compared to that of the conventional method of Soxhlet extraction (Petrovic and Marcello, 2002).

Table 4.4: Method detection limit (MDL) and method quantification limit (MQL) of NP and NPEO₁₋₁₅.

Instrument	Compounds	MDL (ug/L)	MQL (ug/L)
GC-MS	NP	1.6	5.2
	NPEO ₁	0.3	1.1
	NPEO ₂	0.3	1.0
HPLC-UV	NPEO ₃	17	56
	NPEO ₄	18	61
	NPEO ₅	20	65
	NPEO ₆	20	67
	NPEO ₇	23	75
	NPEO ₈	24	79
	NPEO ₉	26	88
	NPEO ₁₀	35	118
	NPEO ₁₁	40	134
	NPEO ₁₂	45	151
	NPEO ₁₃	47	157
	NPEO ₁₄	46	153
	NPEO ₁₅	49	163

The test was conducted using a solution of 2.0 gVSS/L of sterilised anaerobic sludge, spiked with 20 mg/L of NP and IGEPAL CO-210 (NPEO₁ and NPEO₂) each. The compounds were allowed to be homogenised overnight before the extraction procedure. The procedure is given in Figure 4.7. Briefly, the suspension was first subjected to centrifugation

and then the solid was re-suspended in 5 mL methanol/acetone (1:1). In order to ensure sufficient contact with the solvent, the suspension was vortex stirred and placed in a mechanical shaker for 30 min. It was later immersed into the ultrasonic water bath and ultrasonically extracted for 10 min in 2 sequences. The extracted solvent was then evaporated and re-suspended in H₂O/MeOH: 9:1 (v/v) before subsequent extraction according to the aforementioned SPE method using an Oasis HLB (30mg) cartridge. The extract was then subjected to GC-MS analysis.

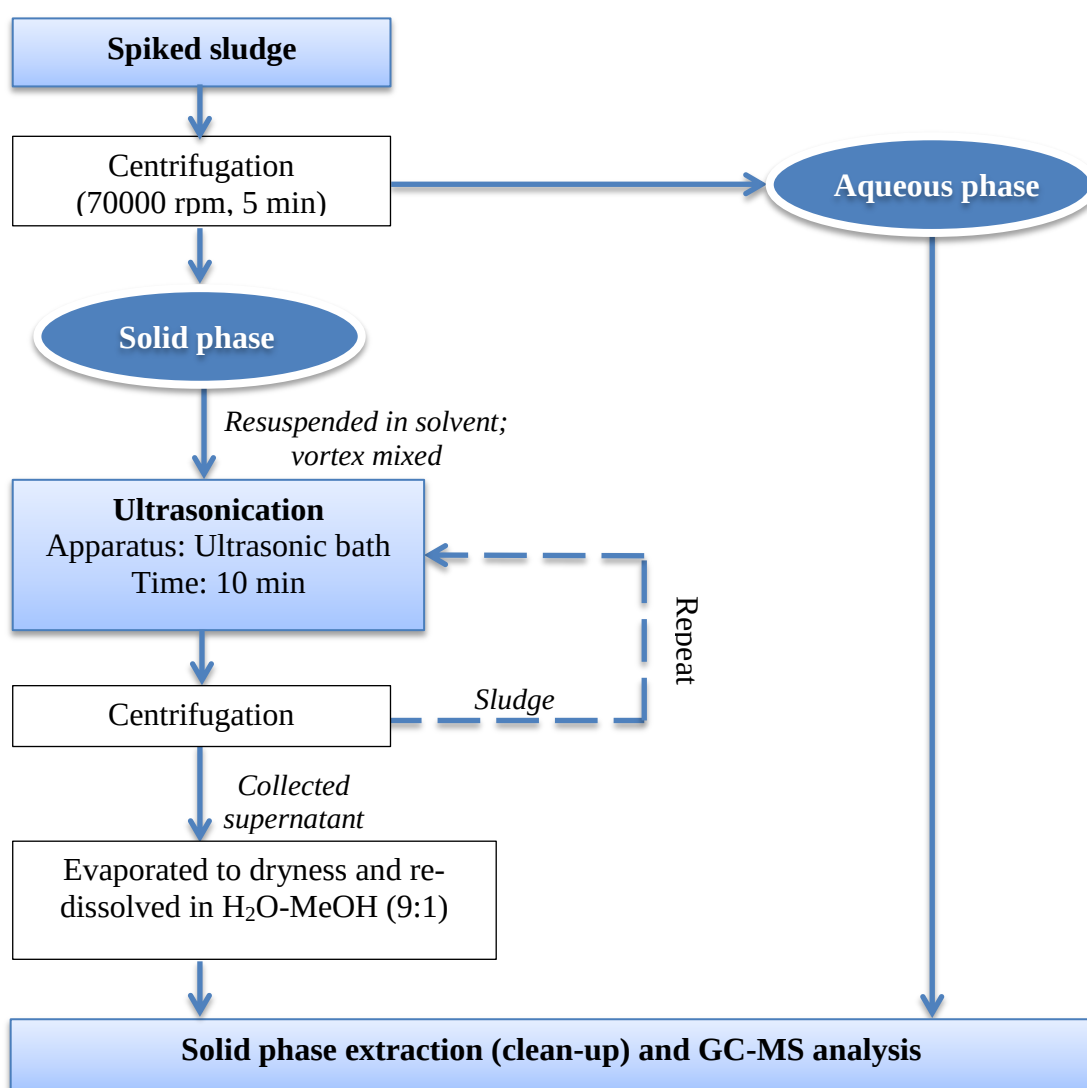


Figure 4.7: The ultrasonic assisted extraction (USE) procedure for NP and NPEOs from a sludge sample.

The recovery efficiencies of five different solvents with a wide range of polarity (acetone, methanol, acetone-methanol (50:50, v/v), ethyl acetate and *n*-hexane) were tested. The results obtained are shown in Figure 4.8.

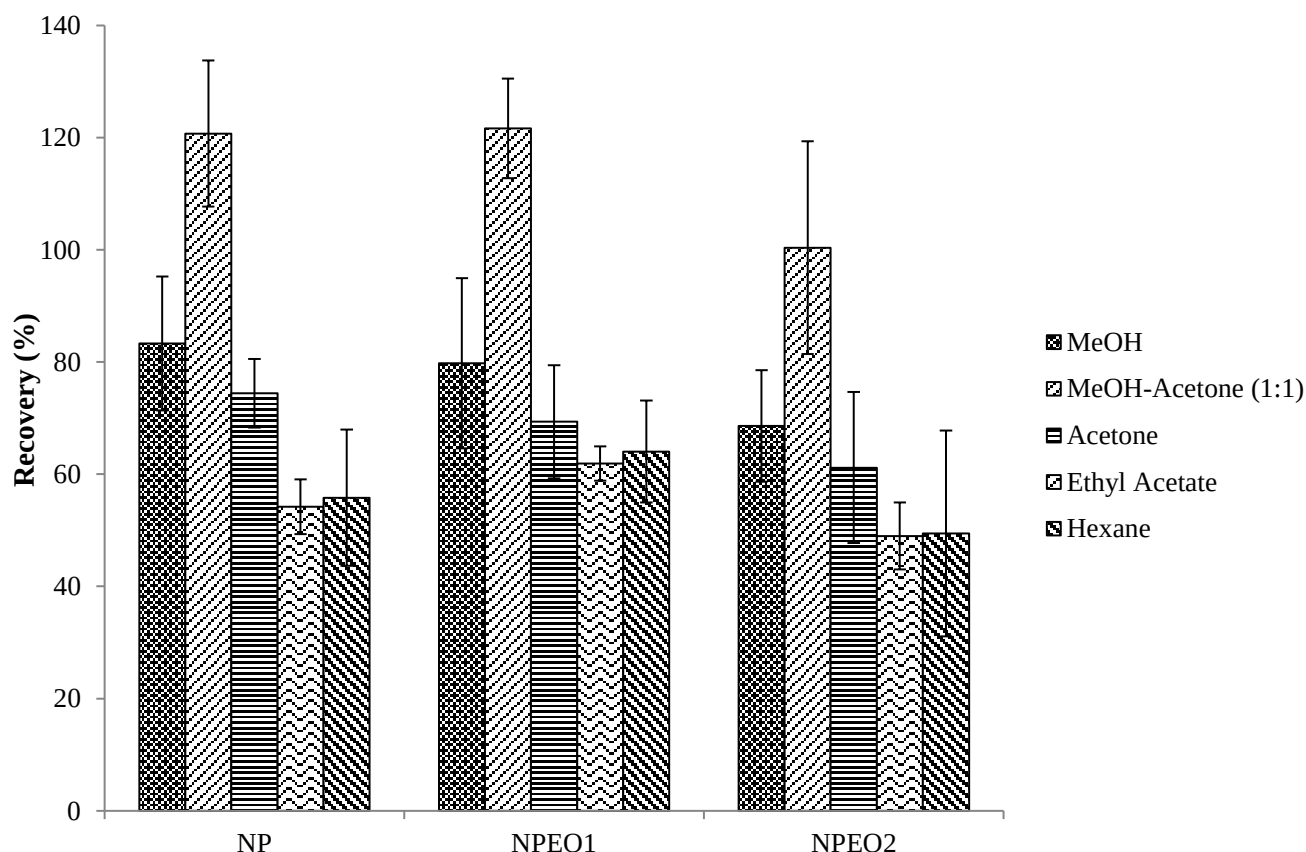


Figure 4.8: Overall recoveries of NP, NPEO₁ and NPEO₂ from sludge employing USE, clean up through SPE cartridge and elution with different solvents (spike levels 20 mg/L of NP and IGEPAL CO-210, n=3).

The calculated recovery percentages were relative to the blank (pure standard solution subjected to SPE-GC-MS). From the figure, the acetone-methanol (50:50, v/v) system achieved the best extraction recoveries, ranging from 100 to 121% for all compounds, whereas *n*-hexane exhibited the lowest recoveries ranging from 49 to 64%. These results are in agreement with Núñez *et al.* (2007), in which NP, NPEO₁ and NPEO₂ were poorly

extracted by n-hexane (~20%), while more than 100% recoveries were obtained when methanol was used. This shows that polar solvents might favour the extraction of analytes with hydrophilic-hydrophobic mixed character, especially for NPEOs having one or more EO units in its structure. In addition, Arditoglou and Voutsas (2008) found that a mixture of acetone-methanol (50:50, v/v) exhibited the best extraction recovery of up to 145% for NPEO₂ from sediment. The exceeded 100% recovery might be due to evaporation of solvent. The other satisfactory solvent systems that have been used in ultrasonication of NPEOs are methanol-dichloromethane (70:30, v/v) (Petrovic and Barcelo, 2000; Shang *et al.*, 1999) and hexane-acetone (60:40, v/v) (Petrovic *et al.*, 2001). In the present study, acetone-methanol (50:50, v/v) was found to be the best solvent system to extract nonylphenolic compounds by USE from sludge/biomass.

4.4. SUMMARY

An integrated method for the simultaneous determination of NP and NPEO with 1 to 15 EO units in an aqueous or solid phase was developed. For solid samples, adequate isolation of hydrophobic analytes (i.e NP, NPEO₁₋₂) was obtained using an ultrasonic-assisted extraction (USE) procedure followed by a clean-up step prior to their determination by instrumental analysis. For aqueous samples, a solid phase extraction (SPE) using a polymeric Oasis HLB cartridge was employed for the isolation of a broad range of NPEOs and intermediates. The extracted eluent was then analysed using HPLC-UV (effectively for NPEO₃₋₁₅) and GC-MS analysis (for low molecular weight intermediates, i.e. NP and NPEO₁₋₂). A successful separation and determination of individual oligomers was obtained using a TSK gel amide-80 column, under normal phase elution. GC-MS analysis was found to be relatively more sensitive and selective than HPLC, in which each individual alkyl chain

isomer was almost separated. A microwave assisted derivatisation (MAD) was performed to increase the volatility of NPEOs and make them more amenable to GC analysis in a very short period of time. The method developed therefore provided a viable alternative to the previous literature methods, especially when dealing with fast and routine analysis for a wide range of nonylphenolic compounds.

❧ CHAPTER 5 ❧

BATCH MICROCOSM STUDIES OF NONYLPHENOL ETHOXYLATES (NPEOs)

Outline:

- 5.1 Introduction
- 5.2 Methods
- 5.3 Results and discussion
 - 5.3.1 Anaerobic biodegradability screening of NPEO_{ave9}
 - 5.3.2 Anaerobic biodegradation behavior of NPEO_{ave9} with and without co-substrate
 - 5.3.3 The effect of supplying oxygen on NPEO_{ave9} biodegradation
- 5.4 Summary

5.1 INTRODUCTION

The biodegradation of nonylphenol ethoxylates (NPEOs) has been the subject of many studies, in most of which the NPEOs are seen to undergo partial degradation to less complex, but often more harmful intermediates, depending on the reaction conditions. In aerobic environments, NPEOs are oxidised to a number of acidic compounds, including nonylphenoxy acetic acid (NPEC₁) and carboxylated alkylphenoxy acetic acids (CAPECs) (Di Corcia *et al.*, 1998). Despite the persistency of these intermediates, their toxicity and estrogenicity are not fully understood. In contrast, the intermediates of anaerobic breakdown, such as nonylphenol monoethoxylates (NPEO₁) and nonylphenol (NP), are of greater

environmental significance due to their adverse effects on endocrine functions in living organisms (Giger *et al.*, 1984). These compounds could be strongly adsorbed onto sludge and sediments, and this prolongs their persistence under restricted aeration (Thiele *et al.*, 1997).

Thus, on-going efforts have been made by researchers to find ways to degrade or mineralise these compounds completely into their inorganic constituents, i.e. CO₂, CH₄ and H₂O, or at least into their less harmful states. Aerobic conditions were found to be adequate for NP removal, while NP was hardly degraded under anaerobic conditions (Staples *et al.*, 1999; Ejlerstsson *et al.*, 1999; Zhang *et al.*, 2008b). Nevertheless, there are good reasons for evaluating the anaerobic biodegradability of these compounds since: (1) there is increasing demand for anaerobic treatment options in current sewage treatment plants due to biogas recovery; (2) anaerobic treatment is effective for high-strength industrial wastewater, such as pulp and paper mill wastewater, in which significant amounts of NPEOs surfactants are commonly found (Strevett *et al.*, 2002; Hawrelak *et al.*, 1999; Zhang *et al.*, 2008b).

The ultimate anaerobic biodegradation of a specific solute can be ascertained by measuring its disappearance or decrease, and the production of biogas using standard biodegradation assays (Staples *et al.*, 2001). In this study, the anaerobic biodegradability and toxicity of NPEOs was first screened using biochemical methane potential (BMP) and anaerobic toxicity assays (ATA), respectively; developed by Owen *et al.* (1979). This preliminary assessment is based on the amount of biogas produced in a batch microcosm. In addition, various parameters are known to affect NPEOs biodegradation including the nature of the electron donor, and the presence of oxygen. The feasibility of simultaneous anaerobic and aerobic treatment on NPEOs biodegradation will also be assessed by monitoring the disappearance of the compound(s), and the formation of degradation intermediates.

5.2 METHODS

The biodegradation assay followed in this chapter was based on the techniques described by Owen *et al.* (1979). Known amounts of substrate and anaerobic sludge (2 gVSS/L) were added to serum bottles and incubated at 37°C. The first part involved the anaerobic biodegradability screening of NPEO_{ave9} using standard BMP and ATA assays. In the second and third part, the technique was modified by supplying air and oxygen, and sampling the test liquor. The assay was conducted according to the experimental scheme shown in Table 5.1. Biogas (i.e. CH₄) and nonylphenolic compounds were analysed according to the analytical techniques described earlier in Chapter 4. The biodegradation intermediates were extracted from aqueous and solid phases using the ultrasonication (USE) and SPE methods described earlier. The naming of compounds were denoted as NPEO_n, in which “n” is substituted for ethylene oxide (EO) units. For instance, the parent compound of IGEPAL CO-630 was represented as NPEO_{ave9}, (an average number of EO units of 9). All tests were carried out in duplicate.

Table 5.1: Experimental flow scheme.

Day	Without Co-substrate			With Co-substrate		
	P _{An}	P _{Air}	P _{Ox}	S _{An}	S _{Air}	S _{Ox}
1	Anaerobic	Anaerobic	Anaerobic	Anaerobic	Anaerobic	Anaerobic
3	Anaerobic	Anaerobic	Anaerobic	Anaerobic	Anaerobic	Anaerobic
5	Anaerobic	Anaerobic	Anaerobic	Anaerobic	Anaerobic	Anaerobic
7	Anaerobic	Air injected	Pure O ₂ injected	Anaerobic	Air injected	Pure O ₂ injected
15	Anaerobic	Air injected	Pure O ₂ injected	Anaerobic	Air injected	Pure O ₂ injected
22	Anaerobic	Air injected	Pure O ₂ injected	Anaerobic	Air injected	Pure O ₂ injected
36	Anaerobic	Air injected	Pure O ₂ injected	Anaerobic	Air injected	Pure O ₂ injected

P_{An}: Anaerobic without co-substrate

P_{Air}: Anaerobic-Aerobic (air supplied) without co-substrate

P_{Ox}: Anaerobic-Aerobic (pure O₂ supplied) without co-substrate

S_{An}: Anaerobic with co-substrate

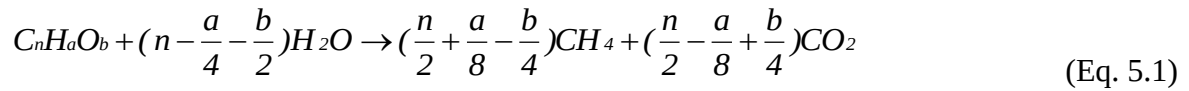
S_{Air}: Anaerobic-Aerobic (air supplied) with co-substrate

S_{Ox}: Anaerobic-Aerobic (pure O₂ supplied) with co-substrate

5.3 RESULTS AND DISCUSSION

5.3.1 ANAEROBIC BIODEGRADABILITY SCREENING OF NPEO_{AVE9}

In BMP tests, NPEO_{ave9} was used as a sole carbon source, and no other easily biodegradable organic matter was added. Hence, methane production was interpreted as a direct measure of the potential for the test compound to degrade via methanogenesis (Benjamin *et al.*, 1984). With gas measurement, the extent of anaerobic biodegradation is commonly expressed by comparing the observed CH₄ production to the theoretical CH₄ production calculated using Buswell's equation (Symons & Buswell, 1933; Shelton and Tiedje, 1984):



The equation is based on the stoichiometry of the reaction, assuming that all the substrates are used exclusively for metabolic energy, i.e. cellular synthesis is neglected (Labatut *et al.*, 2011). Figure 5.1 provides a representative set of the cumulative methane production data obtained with the BMP test for the blank (biomass only) and the serum bottle with added 20 mg/L NPEO_{ave9}. The size of the serum bottle used was 160 mL. The figure shows a slight difference in gas production between the sample and the blank. A paired sample student's t-test was used to test the significance of difference between two sampling means. The results shows a significant difference in the methane production of the blank (M = 1.86, SD = 2.09) and the test sample (M = 2.09, SD = 2.31); $p = 0.0027 < 0.05$. Thus, a slightly higher CH₄ production of the sample to the blank indicates the CH₄ generated was from the degradation

of NPEO_{ave9}. The small concentration of NPEO₉ used generated very low amounts of CH₄ production (Ejlertsson *et al.*, 1999).

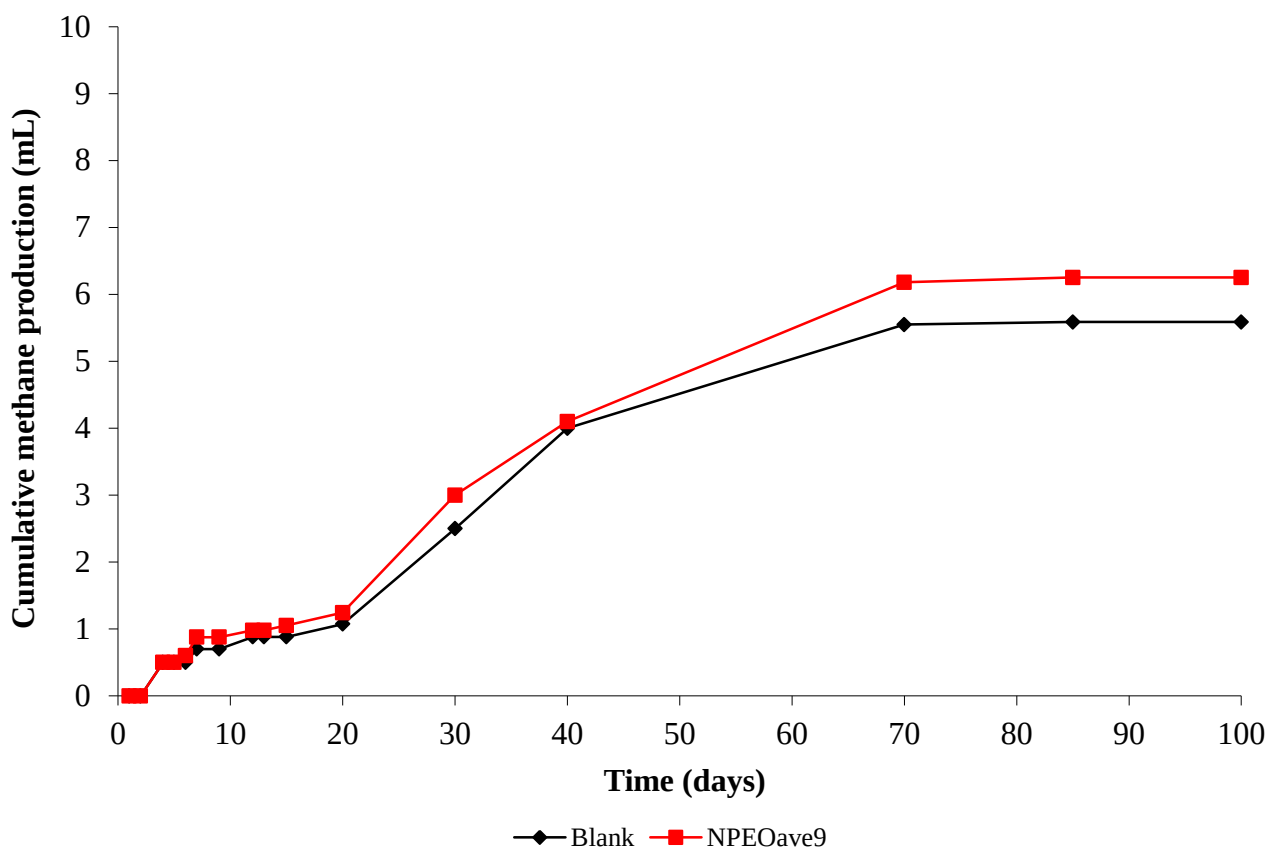


Figure 5.1: Methane production from anaerobic NPEO_{ave9} biodegradation.

From the experimental results, calculating the difference in cumulative gas production from the blank, and compared to Equation 5.1, the NPEO_{ave9} was found to be partially mineralised (approximately 37.4% of the theoretical CH₄ production) under the given conditions. NPEO_{ave9} is known to be partially mineralised under anaerobic conditions, in which toxic nonylphenol (NP) is potentially released and accumulates in the system. Ejlertsson *et al.* (1999) suggested that the phenol ring structure remained intact during this period of incubation, while the EO units liberated as ethylene glycol during the degradation of long chain NPEOs could have been further degraded to CH₄ and CO₂. In addition, Salanitro and Diaz (1995) reported that 4 mg C/L of NPEO_{ave9} was only mineralised by 32-

43% of the theoretical CH₄, but this increased to 70% when the concentration was reduced to 0.8 mg C/L. Hence, the high concentration of NPEO_{ave9} used in this test (equivalent to 12.9 mg C/L) may have dampened anaerobic activity due to the release of inhibitory NP, and hence slowed the catabolic reaction rate. As depicted in Figure 5.1, the reaction proceeded at a very slow rate, and only reached the ultimate CH₄ yield on day 70.

Inhibition indicates a detrimental effect that a test substance causes on the activity of a microbial population. In a system containing easily degradable compounds, the presence of a toxicant may decrease overall CH₄ production. It is also found that surfactants are relatively toxic to CH₄ production compared to other organic chemicals (Shcherbakova *et al.*, 1999). It has also been proven that high concentrations of the most potent estrogenic intermediates of NPEOs, such as NP, are inhibitory to the anaerobic biodegradation of NPEOs (Lu *et al.*, 2008b). The next batch assay, the anaerobic toxicity assay (ATA), aims to elucidate the effect of different concentrations of NP on the rate of CH₄ production from an easily degradable methanogenic substrate, i.e. acetate and propionate, as described by Owen *et al.* (1979). The cumulative gas production over a 28-day period is presented in Figure 5.2. The NP, dissolved in methanol, was added to 75 mL serum bottles to give final concentrations of 10, 20 and 30 mg/L, while the controls were supplied with methanol corresponding to the amount used for the NP additions. From the figure, maximum substrate utilisation of the control occurred from day 3 to 8; during this period the gas production rate was at its peak. The gas production rate of each sample corresponding to a slope value during the stipulated period was used for computing MRR (Maximum Rate Ratio) in which each respective rate was normalised with the rate of a control. According to Owen *et al.* (1979), an MRR of less than 0.95 suggests possible inhibition, and a MRR of less than 0.9 suggests significant inhibition. The MRR data are shown in Table 5.2.

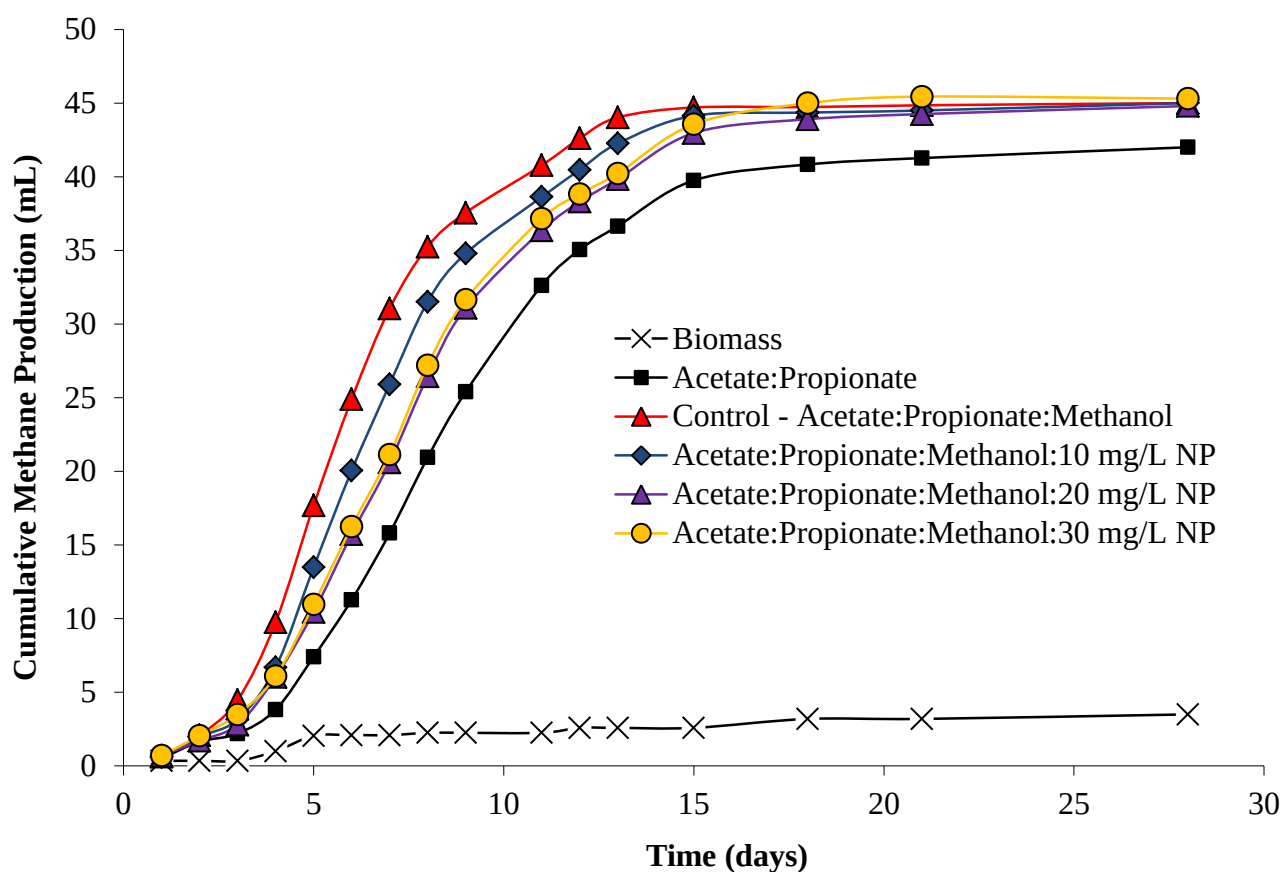


Figure 5.2: The effect of different concentrations of NP on methane production.

Table 5.2: MRR for anaerobic toxicity assays (ATA) of NP.

Sample	k*	r ²	MRR ⁺ *	Remarks
Control (Ace:Pro:MeOH)	6.43	0.9926	-	-
Sample 1 (Ace:Pro:MeOH: 10 mg/L NP)	5.89	0.9949	0.92	Possible inhibition
Sample 2 (Ace:Pro:MeOH: 20 mg/L NP)	4.78	0.9927	0.74	Significant inhibition
Sample 3 (Ace:Pro:MeOH: 30 mg/L NP)	4.83	0.9894	0.75	Significant inhibition

Ace: Acetate; Pro: Propionate; MeOH: Methanol; k: CH₄ production rate (mL/day)

* Period used between day 3 and 8

⁺ MRR = Maximum Rate Ratio = $\frac{k \text{ value of sample}}{k \text{ value of control}}$

These findings suggest that at 10 mg/L, NP causes possible inhibition, whereas at 20 and 30 mg/L, NP significantly inhibits the activity of methanogens. Nevertheless, after a period of acclimatisation, the cumulative gas production of each NP sample matches the amount of that of the control. Apart from NP, Ejlerstsson *et al.* (1999) reported slight inhibition of 2 mg/L NPEO₁₋₂ on CH₄ production via methanol degradation, and at 60 mg/L and 308 mg/L NPEO₁₋₂, reactions preceded at a slower pace. Although it is clear that NP and short chain NPEOs are significantly and slightly inhibitory to methanogens even at low concentrations, the effect of the intermediates on the overall degradation rate of NPEOs is not well understood. Moreover, Lu *et al.* (2008b) observed a sharp decrease in total NPEOs removal rate only when the spiked NP concentration was increased from 22 mg/L to 220 mg/L. Nevertheless, the NP toxicity on NPEOs degrading bacteria was less pronounced below the aforementioned concentration. At the end of test period (day 28), samples were extracted and analysed by HPLC-UV. From the analysis, most of the NP was associated with the biomass due to its lipophilicity, and the highest NP removal (via biodegradation) of only 16% was recorded for Sample 1 (10 mg/L NP). It has also been observed that this concentration was low enough to cause significant toxicity to methanogens (Table 5.2). Based on molarity, 10 mg/L NP is equivalent to about 28 mg/L NPEO_{ave9}. In other words, complete microbial deethoxylation of 28 mg/L NPEO_{ave9} will give rise to 10 mg/L NP, provided that NP is the final end product. For ease of quantification, NPEO_{ave9} concentration of about 20 mg/L was therefore used for subsequent biodegradation study. It was assumed that the stipulated concentration induces no significant toxicity to the methanogens, and NPEOs and co-substrates degrading bacteria. Nevertheless, it was difficult to assess the biodegradation of low concentrations of substrate from its corresponding gas production. Thus, a better approach would be to follow the progress of biodegradation more closely by chemical analysis of individual compounds.

5.3.2 ANAEROBIC BIODEGRADATION BEHAVIOR OF NPEO_{AVE9} WITH AND WITHOUT CO-SUBSTRATE

Previous studies had found that a high diversity of bacterial species have the ability to grow using NPEOs as their sole source of carbon and energy (Ahel *et al.*, 1994c; Lu *et al.*, 2008b). The interaction with other organic constituents in the environment may, however, influence the degradation rate and pathway of NPEOs. In this current study, the effect of supplying specific amounts of an easily degradable co-substrate (1 g/L glucose and 0.5 g/L yeast extract) on NPEO degradation was assessed. Figures 5.3 and 5.4 show the changes in the molar distribution of NPEO_{ave9} during anaerobic incubation in the presence and absence of additional carbon sources, respectively. Both microcosms exhibited similar trends in which the molar distribution of the oligomers was gradually shifted from longer ethoxylate chain lengths to shorter ones during the biodegradability test. For instance, NPEO₂ and NPEO₃ were observed as the major degradation products for both systems. Both intermediates accounting for about 66% and 46% (molar basis) of the total intermediates formed at the end of the test period, with and without co-substrate, respectively. The observed trend is in agreement with earlier reports, showing that under anaerobic conditions NPEOs with a low number of ethylene oxide (EO) units predominates at the end of the test period, and that these metabolites are far more persistent than their parent compounds. This successive excision of the ethoxylate chain is believed to proceed according to the non-oxidative hydroxyl shift mechanism, as proposed by John and White (1998). A favourable bacterial attack on the molecule's ethoxylate side chain causes the terminal ethoxyl unit to cleave liberating acetaldehyde to give the corresponding shortened NPEO. Nevertheless, the authors noticed no detectable amount of NP and NPEO₁, whereas NPEO₂ accumulated in the system.

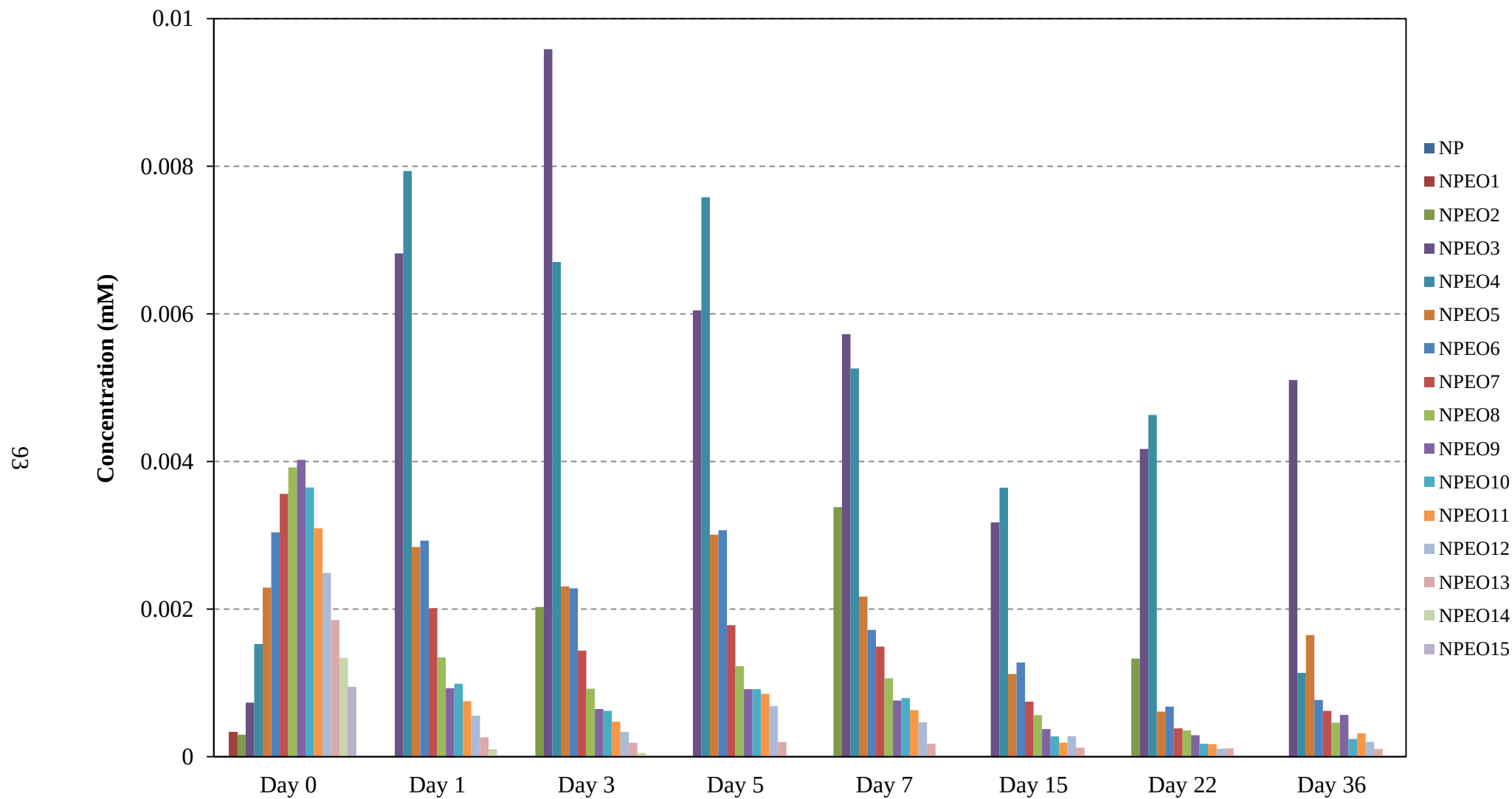


Figure 5.3: Molar distribution of NPEOs during anaerobic incubation without additional carbon sources.

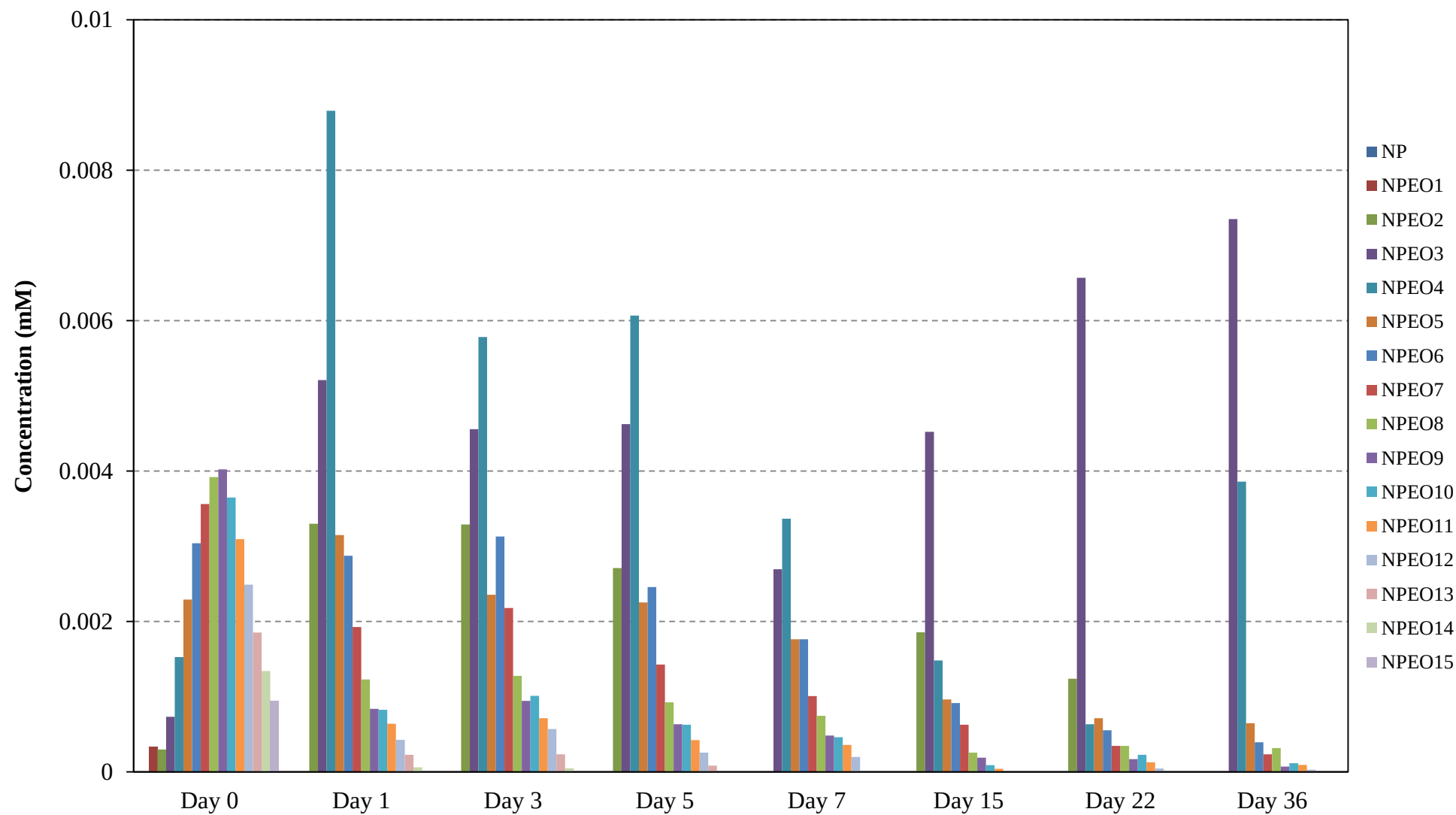


Figure 5.4: Molar distribution of NPEOs during anaerobic incubation with additional carbon sources.

On the other hand, further anaerobic transformation of NPEO₂ to NP and NPEO₁ has been detected under methanogenic conditions in landfill sludge (Ejlertsson *et al.*, 1999), nitrate reducing conditions in activated sludge (Luppi *et al.*, 2007), digester sludge (Lu *et al.*, 2008b), and Iron (Fe(III)) reducing conditions in sediments (Lu *et al.*, 2007). This might be attributed to differences in test set-ups, degradation conditions and source of inoculum. In addition, the physical-chemical behaviour of shorter NPEO molecules such as their bulkiness and hydrophobicity may dampen their affinity for degradation enzymes (Sato *et al.*, 2001). Despite a general assumption of NP being the most persistent and estrogenic of the NPEO metabolites, reported data on the formation of the metabolite NP from NPEOs is surprisingly scarce (Jonkers *et al.*, 2001).

At the end of the test period, accumulation of shortened NPEOs was more significant in the system with a co-substrate, while slower conversion of long chain NPEOs was observed when the compounds acted as a sole carbon source (Figure 5.3 and 5.4). It appears that bacteria are able to co-metabolise NPEOs while degrading energy-rich substrates such as glucose and yeast extract. On the other hand, Lu *et al.* (2008a) reported a slight and significant inhibition by yeast extract and glucose, respectively, on the anaerobic biodegradation of NPEOs in a denitrifying sludge. These authors suggest that the organic matter could compete with NPEOs for electron acceptors during their oxidation process. It is important to note that the concentration of NPEO_{ave9} used in their study was 5 times higher than that of this study, and hence substrate inhibition is not likely to be observed in this study. In addition, the presence of yeast extract promotes relatively good microbial growth and degradation capacity (Yuan *et al.*, 2003). For instance, in a study by Chang *et al.* (2004), the degradation rate of NPEO₁ and NP was enhanced and the microbial population was increased by the addition of yeast extract to the anaerobic media.

Figure 5.5 shows the total NPEOs molar concentration removal for both systems. Based on a paired sample student's t-test, both data set shows no significant difference in total molar concentration; without co-substrates ($M = 0.0218$, $SD = 0.0085$) and with co-substrates ($M = 0.0199$, $SD = 0.0090$); $p = 0.237 > 0.05$. Although the difference between both environments cannot be proven statistically, figure 5.5 has shown that the concentration of NPEOs with supplied co-substrate decreased more rapidly with no observable lag time. Moreover, based on Figure 5.3 and 5.4, the presence of co-substrates may facilitate the shortening of EO chain.

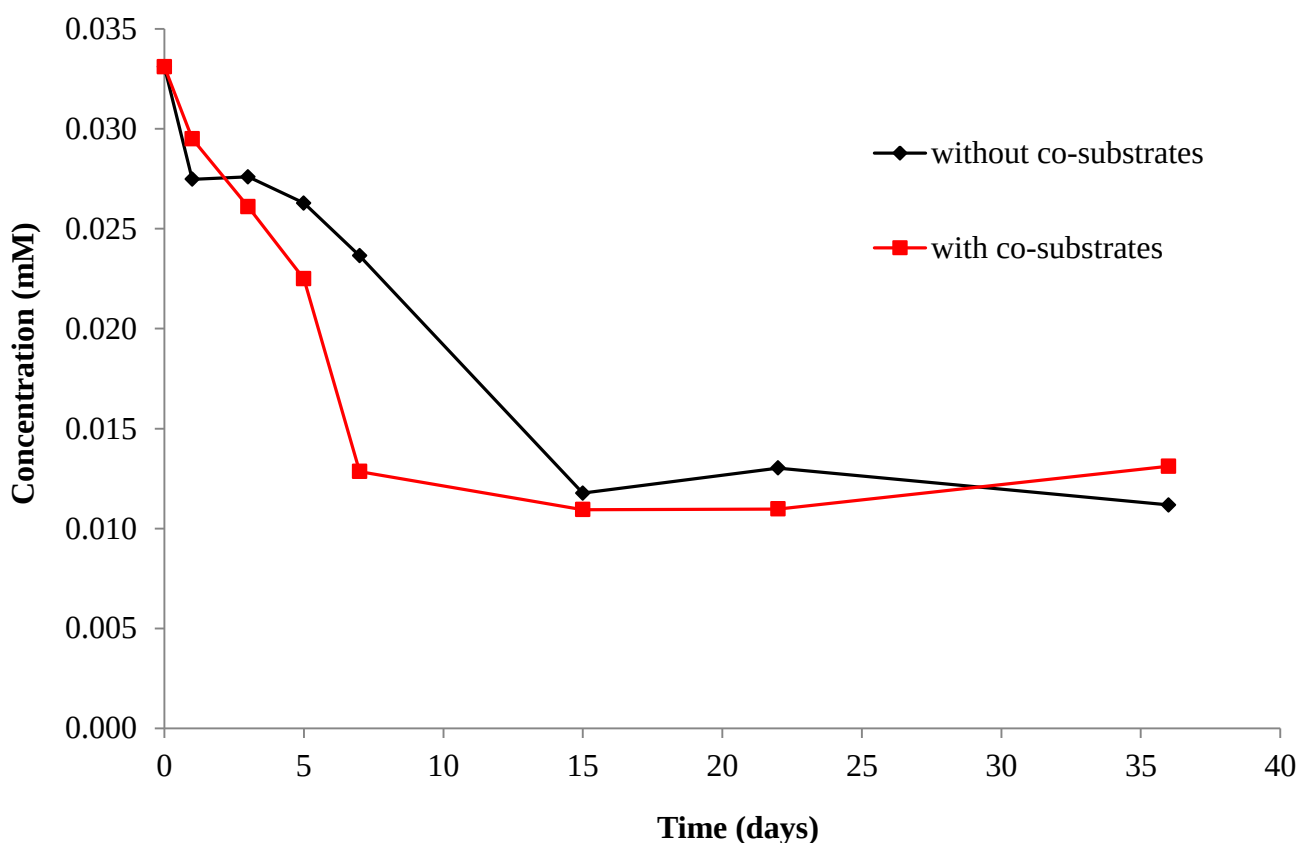


Figure 5.5: Total molar removal of NPEO_{ave9} with and without co-substrates.

It is also evident from Figure 5.5 that complete mineralisation of NPEOs was partially hindered under anaerobic conditions, accounting for about 66% of the total molar reduction.

This confirms the common view on persistency of short chain NPEO oligomers under anaerobic conditions. It is known that NPEOs contain a highly branched alkyl chain (>22 isomers), and some of the isomers containing ternary and quaternary carbon atoms are difficult to degrade and, consequently, these highly branched intermediates resist complete mineralisation (Montgomery-Brown and Reinhard, 2003; Montgomery-Brown *et al.*, 2008). On the other hand, the cleavage of the aromatic moiety and transformation of the alkyl chain has been observed under aerobic conditions (Di Corcia *et al.*, 1998; Jonkers *et al.*, 2001). In fact, most of the biodegradation studies on NPEOs have focused on aerobic treatment of NPEOs, in which the oxidation of EO chains to their corresponding nonylphenol ethoxy carboxylates (NPECs), was extensively monitored. Other than the complexity of its biodegradation pathway, the problem with aerobic metabolism is that low biodegradability and toxicity of its metabolites have not been fully verified by current research. For instance, Gu *et al.* (2010) isolated specific bacterial strains belonging to the genus *Ralstonia*, which are capable of degrading NPEOs by oxidising the EO chain directly without the formation of short chain products. This mechanism has been supported by previous microcosm studies (Di Corcia *et al.*, 1994; Jonkers *et al.*, 2001), while the slow conversion of high molecular weight NPEC₁₀ has also been recorded under aerobic conditions (Hayashi *et al.*, 2005). Hence, it is assumed that a strategy to induce rapid non-oxidative pathways as a primary degradation route, and then oxidation of low molecular weight intermediates might serve as a viable alternative to minimise the production of the unknown high molecular weight aerobic intermediates. To date, no batch study has been conducted on simultaneous anaerobic and aerobic treatment of NPEOs. Thus, the next experiment was aimed at investigating how the presence of oxygen affects the progress of NPEO degradation in anaerobic microcosms.

5.3.3 THE EFFECT OF SUPPLYING OXYGEN ON NPEO_{AVE9} BIODEGRADATION

After 5 days of anaerobic incubation, the bottle headspace (60mL) was replaced with either air or pure oxygen; while another set of microcosms were reserved as anaerobic controls. All bottles were previously fed with the aforementioned co-substrates, and periodic replacement of air and pure oxygen in the headspace was done after each sampling event at Day 7, 15, 22 and 36. After the addition of air and pure oxygen, two layers of biomass settled at the bottom of the bottle, and were visually inspected; they consisted of black and brown layers of anaerobic and aerobic biomass, respectively. The phenomenon of distinct layers of biomass in oxygen spiked microcosm has also been visualised in other studies (Ohandja and Stuckey, 2006). In addition, most of the decomposing bacteria are facultative anaerobes, and hence they are able to grow and degrade organics under aerobic and anaerobic conditions (Berna *et al.*, 2007). The comparison between anaerobic, anaerobic-aerobic (with air) and anaerobic-aerobic (with pure oxygen) microcosm in terms of NPEOs molar distribution are shown in Figure 5.6(a), (b) and (c), respectively. Significant disappearance of NPEOs intermediates were observed in microcosm supplied with oxygen as electron acceptor (both air and pure oxygen), while degradation proceeds at slower rate in anaerobic control with noticeable accumulation of NPEO₃. As can be seen in Figure 5.7, overall molar concentrations of anaerobic, anaerobic-aerobic (with air) and anaerobic-aerobic (with pure oxygen) microcosms reached 60%, 76% and 79% removal, respectively, on day 36. These findings are in-line with previous studies in which the degradation of NPEOs intermediates are more favoured under aerobic conditions (Ying *et al.*, 2002; Montgomery-Brown and Reinhard, 2003).

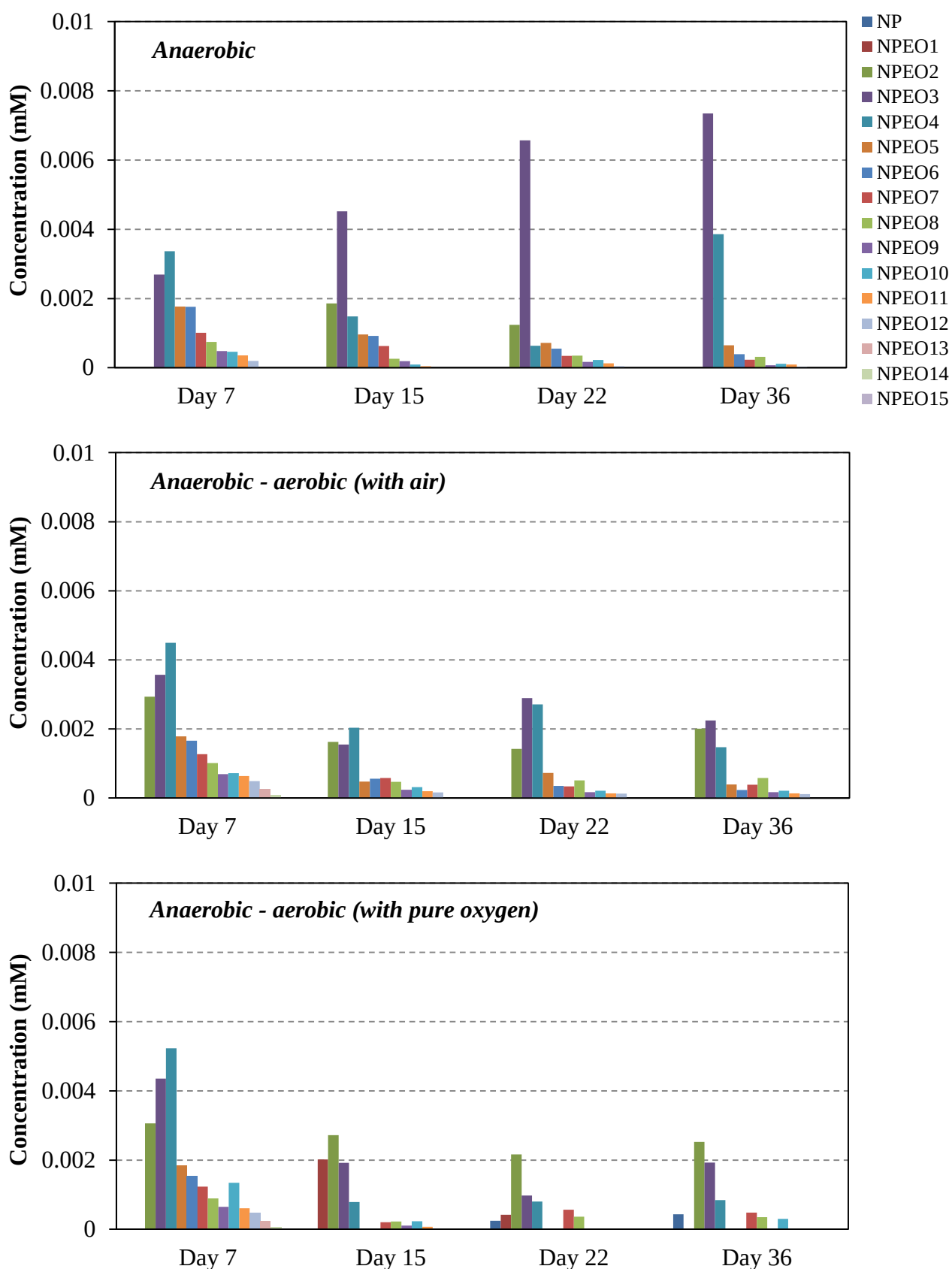


Figure 5.6: Molar distribution of NPEOs under different oxygen supply modes.

It is understood that the higher the redox potential of the environment (i.e. in the presence of oxygen), the more energetically efficient is the reaction (Berna *et al.*, 2007). As a result, facultative anaerobes inoculated into low redox potential environments will preferably respond to oxygen that provides a high energy yield reaction. In addition, a paired sample student's t-test revealed no significant difference between the bottles supplied with air and pure oxygen – air supplied (M = 0.0196, SD = 0.010) and oxygen supplied (M = 0.0197, SD = 0.011); $p = 0.90 > 0.05$. This indicates that a sufficient amount of air had been added to the bottles to boost the growth of aerobic bacteria.

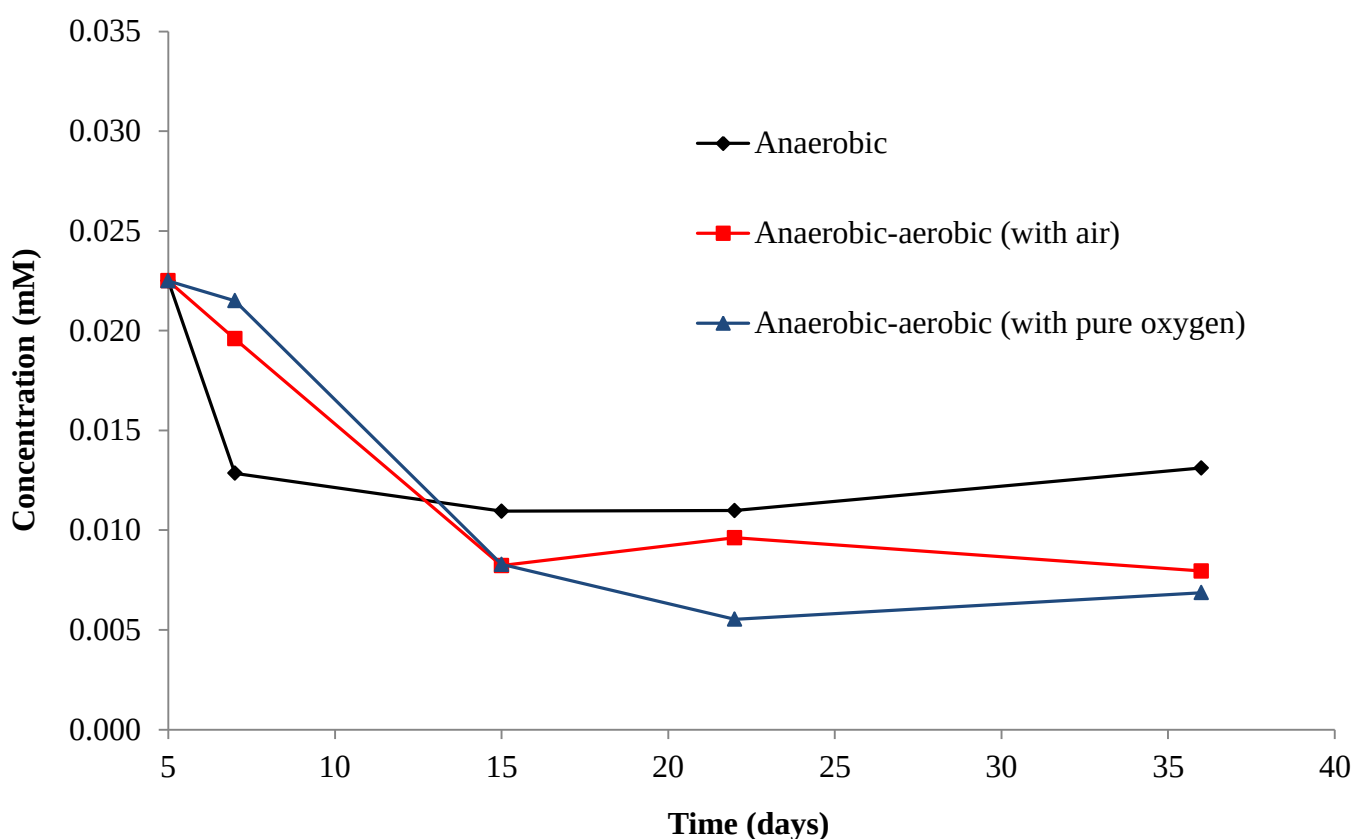


Figure 5.7: Total molar removal of NPEO_{ave9} under different oxygen supply modes.

It is also evident in Figure 5.7 that the extent of biodegradation was slightly delayed just after the first addition of air and pure oxygen, especially the one supplied with pure

oxygen. One possible explanation for this is that the presence of oxygen might facilitate a shift within the anaerobic community, and bacteria had to begin to acclimatise to a new environment. The removal of NPEOs under aerobic conditions suggests either the compounds being mineralised, or the formation of undetected metabolites. Hayashi *et al.* (2005) reported the rapid transformation of NPEOs to corresponding NPECs in the presence of organic matter such as yeast, glucose, and methanol, under aerobic conditions. The authors suggest that NPECs may be generated by cometabolism in the oxidation of methanol. In addition, Jonkers *et al.* (2001) identified the formation of other acidic intermediates of CAPECs (metabolites with oxidized alkyl and ethoxy side chains) in aerobic biodegradation studies of NPEOs in river water by using the powerful technique of LC-MS/MS with an electrospray (ES) source. Furthermore, DiCorcia *et al.* (1998) used LC-ES-MS for the identification of intermediates originating from the biotransformation of branched alkyl side chain, and observed the persistency of CAPECs for more than 5 months after their formation. In this study, identification of unknown peaks was done qualitatively based on the total ion scan of a GC chromatogram and MS fragmentation patterns, and compared with those of previous studies (Maki *et al.*, 1994; 1996). The analysis of all these extracts, however, did not reveal any detectable unknown nonylphenolic metabolites. The lack of a sensitive analytical technique was one the main constraints in the mechanistic studies of NPEO degradation, and only a limited number of studies are available on the characterisation of novel intermediates. In addition, the identification and quantification of the intermediates, especially acidic intermediates of NPECs and CAPECs, has been uncertain due to the lack of pure standards. As a result, optimisation and calibration of analytical methods with respect to these compounds were difficult to obtain in this study. Despite a possibility that the undetected intermediates may form and remained undetected in our system, their toxicity and estrogenicity is not fully understood. In fact, most of the previous studies have been directed

towards eliminating the most potent estrogenic and well-understood nonylphenolic intermediates, such as NP and short chain NPEOs.

Unexpectedly, trace concentrations of NP were detected in the microcosm supplied with pure oxygen on day 22 and 36, at 54.5 and 96.1 µg/L, respectively. The NP, which is expected to be produced only under anaerobic conditions, may be formed in an aerobic system due to the presence of anaerobic microniches in the bottle. A previous study on the composting of wool scour effluent sludge also found a slight increase in NP concentration from the aerobic degradation of NPEO₉ (Jones and Westmoreland, 1998). According to a recent study by Montgomery-Brown *et al.* (2008), NPEC₁ is able to be degraded into NP in aerobic soil microcosms before being rapidly transformed to nonyl alcohols via ipso-hydroxylation. The fate of NP and other intermediates beyond day 36 is unknown. It can be postulated that short chain NPEOs will persist longer in anaerobic microcosms judging by their slow transformation rate over the one month incubation. The disappearance of short chain NPEOs in aerobic microcosms can be either related to the compounds mineralisation, or the formation of aerobic intermediates.

5.4. SUMMARY

This study has shown that the presence of dissolved oxygen is more influential on the progress of NPEOs biodegradation than that of additional co-substrates. Although many previous studies have focused on the aerobic degradation of NPEOs, none them has compared aerobically treated NPEOs with the ones incubated under anaerobic conditions, or under sequential anaerobic-aerobic conditions using the same media and mixed culture. Under anaerobic conditions, NPEOs with a low number of ethylene oxides (EO) predominate

at the end of the test period as a result of the non-oxidative hydroxyl shift mechanism, and these metabolites are far less degradable than the parent compounds. The removal of intermediates was improved when the air/oxygen was introduced into the system. Improved removal of the intermediates under aerobic conditions observed in the system suggests either compounds mineralisation, or unidentified hydrophilic intermediates (i.e. acidic NPECs and CAPECs). It is postulated that by employing sequential anaerobic-aerobic treatment, the formation of highly hydrophilic and refractory aerobic intermediates could be reduced, whereas more hydrophobic anaerobic intermediates are formed and preserved on the surface of the solid biomass. These intermediates will be synergistically removed in the presence of dissolved oxygen. Based on our current knowledge, no study has been performed previously on a simultaneous anaerobic-aerobic metabolism of NPEOs in batch culture. The stipulated metabolic conditions are encouraged to mimic the microenvironments in a MABR, and to investigate the suitability of such conditions for NPEOs biodegradation. In an MABR, spatial stratification of microbial activity exists in the biofilm which permits anaerobic and aerobic catabolism to occur in a single reactor. Hence, it is hypothesised that the MABR environment (in terms of bacterial stratification) might be advantageous for complete mineralisation of NPEOs.

❧ CHAPTER 6 ❧

THE TREATMENT OF NONYLPHENOL ETHOXYLATES (NPEOs) IN A MEMBRANE AERATED BIOFILM REACTOR (MABR) – a preliminary study

Outline:

- 6.1 Introduction
- 6.2 Methods
- 6.3 Results and discussion
 - 6.3.1 Reactor start-up
 - 6.3.2 Preliminary study on NPEO_{ave9} biodegradation in an MABR
 - 6.3.2.1 Description of process parameters and conditions
 - 6.3.2.2 Reactor performance
 - 6.3.2.3 Biodegradation of NPEOs
- 6.4 Summary

6.1 INTRODUCTION

A successful mineralisation of nonylphenol ethoxylates (NPEOs) in a bio-engineered system depends on an understanding of their biodegradation mechanisms, and the effects of manipulating reactor design and operation to enhance their biodegradation. In most conventional STPs, the breakdown of NPEOs starts with a shortening of the EO chain due to non-oxidative pathways until short chain NPEOs (i.e. NPEO₁, NPEO₂) and nonylphenol (NP)

are formed. These intermediates are of greater environmental significance than longer chain NPEOs due to their slow degradation, and their adverse effects on endocrine functions in living organisms (Giger *et al.*, 1984). Most researchers reported that the intermediates are more degradable under aerobic, rather than anaerobic conditions, and this is also in agreement with the results obtained from the batch biodegradation studies in the previous chapter (Brunner *et al.*, 1988; Ying *et al.*, 2002). Besides, they show a strong affinity for sludge and sediments in STPs, which prolongs their persistence under restricted aeration conditions (Thiele *et al.*, 1997). In STPs oxygen is conventionally supplied via bubble aeration, which is often ineffective due to mass transfer limitations. However, the use of membrane aeration via bubbleless or molecular diffusion of oxygen for the treatment of EDCs including NPEOs has not been investigated so far. In a membrane aerated biofilm reactor (MABR), high rates of oxygen transfer can occur, and a biofilm made up of different microenvironments (aerobic/anoxic/anaerobic) can be grown on the membrane surface. It is postulated that this unique stratification of microbial environments in an MABR biofilm may encourage both the anaerobic and aerobic degradation of NPEOs to occur simultaneously in a single reactor. In addition, mixed redox potential biofilm-based treatment has been postulated to be an effective treatment for xenobiotic contaminants due to its excellent retention of slow-growing bacteria (Syron and Casey, 2008). Although a few studies have reported the degradation of NPEOs and their intermediates in a biofilm reactor, none has explored the potential of MABRs to degrade these compounds (Fujii *et al.* 2003; Soares *et al.* 2006; Di Gioia *et al.*, 2009). In this present study, we propose to treat a synthetic wastewater containing NPEO_{ave9} using a dead ended flat sheet MABR. Given the environmental significance of short chain NPEOs and NP, quantification and monitoring the formation of these intermediates will be conducted and overall reactor performance will be assessed under various organic loading rates. The work will provide a preliminary understanding on the

prospect of NPEO_{ave9} mineralisation in an MABR, and on how the presence of this surfactant, and the accumulation of its persistent intermediates, will affect the long term performance of an MABR.

6.2 METHODS

The reactor was inoculated with anaerobic and aerobic sewage sludge (2 gVSS/L); as described earlier. During the start-up, these bacteria were supplemented with readily biodegradable organic substrates (glucose and yeast extract) and a solution of trace elements (listed in Table 3.1), in both batch and continuous modes of operation. The dissolved oxygen (DO) content of the bulk solution was monitored to assess the development of a thick hybrid aerobic-anaerobic biofilm. After this acclimatisation period, 20 mg/L of IGEPAL CO-630 (NPEO_{ave9}) was fed continuously with co-substrates (0.4 to 0.9 g/L and 0.4 to 1.4 g/L of yeast extract and glucose, respectively) at an hydraulic retention time (HRT) of 48 hr. A pressure of 15 mbar was maintained in the gas compartment by a pressure-regulating valve to avoid air bubbling through the membrane. The sample preparation and analytical techniques used in this study were mentioned in Chapter 3 and 4.

6.3 RESULTS AND DISCUSSION

6.3.1 REACTOR START-UP

Reactor start-up aims to develop a stable biofilm containing a diverse microbial species that is capable of degrading a wide range of substrates in the shortest possible time. The first attempt to start up the reactor failed due to the poor quality of the inoculum, and

high substrate loading. The built-up of volatile fatty acids (VFAs) was apparent, inhibiting acetoclastic methanogens, as evidenced by the absence of methane production. Furthermore, the reactor did not recover from the high COD fed within a designated period, and this resulted in very little COD removal. Hence, a new batch of an active inoculum and a synthetic wastewater containing a low concentration of glucose as the only carbon and energy source were added to the reactor. The start-up conditions are listed in Table 6.1. In order to ensure substantial bacterial acclimatisation towards the reactor's environment, a long reactor start-up period of 3 months was used under both batch and continuous conditions.

The initial growth of a biofilm on the membrane was facilitated in batch mode. The dissolved oxygen (DO) content inside the reactor bulk phase was reduced to 0.2 mgO₂/L in a day, suggesting instantaneous biofilm development that created a significant resistance to O₂ mass transfer within the biofilm. This is in line with the phenomenon observed in other biofilm studies, in which organic macromolecules and planktonic cells are adsorbed onto the solid surface rapidly after their inoculation, facilitating the development of an active biofilm (Cresson *et al.*, 2006; Stoodley *et al.* 2001; Ghigo, 2003). The adhesion may be driven by various forces including physico-chemical (van-der-Waals forces, hydrogen bonds), and hydrophobic and electrostatic interactions (Mamatarkova *et al.*, 2002).

As can be seen in Table 6.1, the reactor was unable to cope with a high substrate loading (2.2 g/L of COD as glucose) in a batch mode. About 39% of the COD was removed, compared with 83% when the feeding load was reduced to 0.45 g/L of COD. It is believed that a low initial loading rate is necessary, and that any increase in substrate loading rate should be made gradually, giving the biomass more time to acclimatise, and at the same time prevent the excessive accumulation of VFAs. According to Akram and Stuckey (2008), long

acclimatisation and adaptation times were required in a submerged anaerobic membrane bioreactor (SAMBR) in order to develop strong bacterial cooperative behaviour for interspecies electron transfer; thus allowing them to share energy in the most efficient way.

Table 6.1: MABR start-up operating conditions.

Run	Period (days)	Substrate	Influent COD (g/L)	COD reduction
Batch	40	Glucose	2.2	39% (after 4 days)
		Glucose	1.2	62% (after 4 days)
		Glucose	0.45	83% (after 4 days)
Continuous (72 hr HRT)	20	Glucose	0.53	71% ($\pm$ 6%)
	20	Yeast Extract	0.59	83% ($\pm$ 8%)

After a period of stable COD removal (around $80 \pm 5\%$) and low concentration of VFAs (< 100 mg/L) was obtained, the reactor was then switched to run continuously at a 72 hr Hydraulic Retention Time (HRT). Yeast extract, an easily decomposable substrate and frequently used in culture medium, was chosen as a co-substrate apart from glucose. The highest COD removal was slightly increased from $71\% (\pm 6\%)$ to $83\% (\pm 8\%)$ with the addition of yeast extract. This may represent better bacterial adaptation over the extended operational period, and/or the effect of yeast extract itself; the presence of yeast extract is known to promote relatively good microbial growth and degradation capacity (Yuan *et al.*, 2003). In addition, it also has been proven to enhance the degradation rate of NPEO₁ and NP and increase the microbial population in anaerobic media (Chang *et al.*, 2004). After achieving steady state performance with respect to COD removal ($\pm 5\%$ variation), the reactor was then fed with endocrine disruptor (NPEO_{ave9}) and the performance of the reactor was further evaluated.

6.3.2 PRELIMINARY STUDY ON NPEO_{AVE9} BIODEGRADATION IN AN MABR

6.3.2.1 Description of process parameters and conditions

In a conventional MABR, high oxygen transfer to the biofilm and bulk liquid is desirable to boost organic oxidation and/or nitrification. Thus, the formation of a thin biofilm is maintained in this aerobically oriented system. In a hybrid system, in which both anaerobic and aerobic conditions are desirable for the complete degradation of organics (i.e. xenobiotics), a thick biofilm is grown to induce oxygen limitations in the anaerobic layer and bulk liquid. Since the increase in biofilm thickness was not progressively measured in this study, these assumptions were made based on the production of biogas (i.e. methane) – as an indicator of highly active anaerobic micro-niche. The results from the batch study (see Chapter 5) revealed that the NPEO_{ave9} can be added as a sole carbon source for bacteria, and that the addition of additional substrates slightly enhanced the bacterial attack on EO side chain, in the first week of incubation. In an MABR for xenobiotic treatment, the supply of additional (highly degradable) substrates is required to maintain the biofilm growth and/or as a cometabolite to enhance the degradation of a xenobiotic such as PCE (Ohandja and Stuckey, 2007). It is expected that the level of additional substrates fed to the reactor used in this study was adequate for sustaining biofilm growth without impeding the extent of degradation of the compound of interest, NPEO_{ave9}.

This chapter aims to provide an insight into NPEO_{ave9} biodegradation behaviour in an MABR under constant NPEO_{ave9} loading with a stepwise increase in additional easily degradable substrates loading, over 210 days of operation. Data from the batch biodegradation study in Chapter 5 revealed a step-wise de-ethoxylation of ethylene oxide

(EO) chains on NPEOs, producing corresponding low molecular weight NPEOs and nonylphenol (NP). Hence, the measurement of oligomer distribution is important when dealing with the biodegradation of nonylphenolic wastewater. In the present study, where about 16 known compounds were calibrated, the oligomers will be grouped according to their ethylene oxide (EO) chain length for ease of monitoring. The four main groups are; (1) critical intermediates of NP and NPEO₁, (2) NPEO₂₋₆, (3) NPEO₇₋₁₁, and (4) NPEO₁₂₋₁₅. To further simplify this, the average molecular weight (MW) of the resulting intermediates can be calculated from analytical data and compared with that of the parent compound, IGEPAL CO-630 (NPEO_{ave9}). For instance, the average MW of NPEOs will decline as the molar distribution is shifted towards short chain NPEOs having a lower molar concentration of EOs. The technique was described in Chapter 4, and is according to Wang and Fingas (1993). Nevertheless, the time taken for complete mineralisation of nonylphenolic compounds varied, depending on process conditions – most of which portray slower conversion of low molecular weight intermediates, i.e. NPEO₁ and NP. Thus, a pseudo steady state in this study was declared when higher oligomeric compounds (i.e. NPEO₂ – NPEO₁₄) were successfully removed from the system, in addition to an adequate soluble COD removal of more than 90%.

6.3.2.2 Reactor performance

Figure 6.1 shows the overall performance of the MABR, including soluble COD removal and methane content under different substrate loading rates. The transition between each substrate loading is marked by the vertical lines. From the data, good removal of soluble COD of about 82.7 to 92.9% was achieved at substrate loading rates of 0.54 to 1.07 kgCOD/m³.d with an HRT of 48 hr.

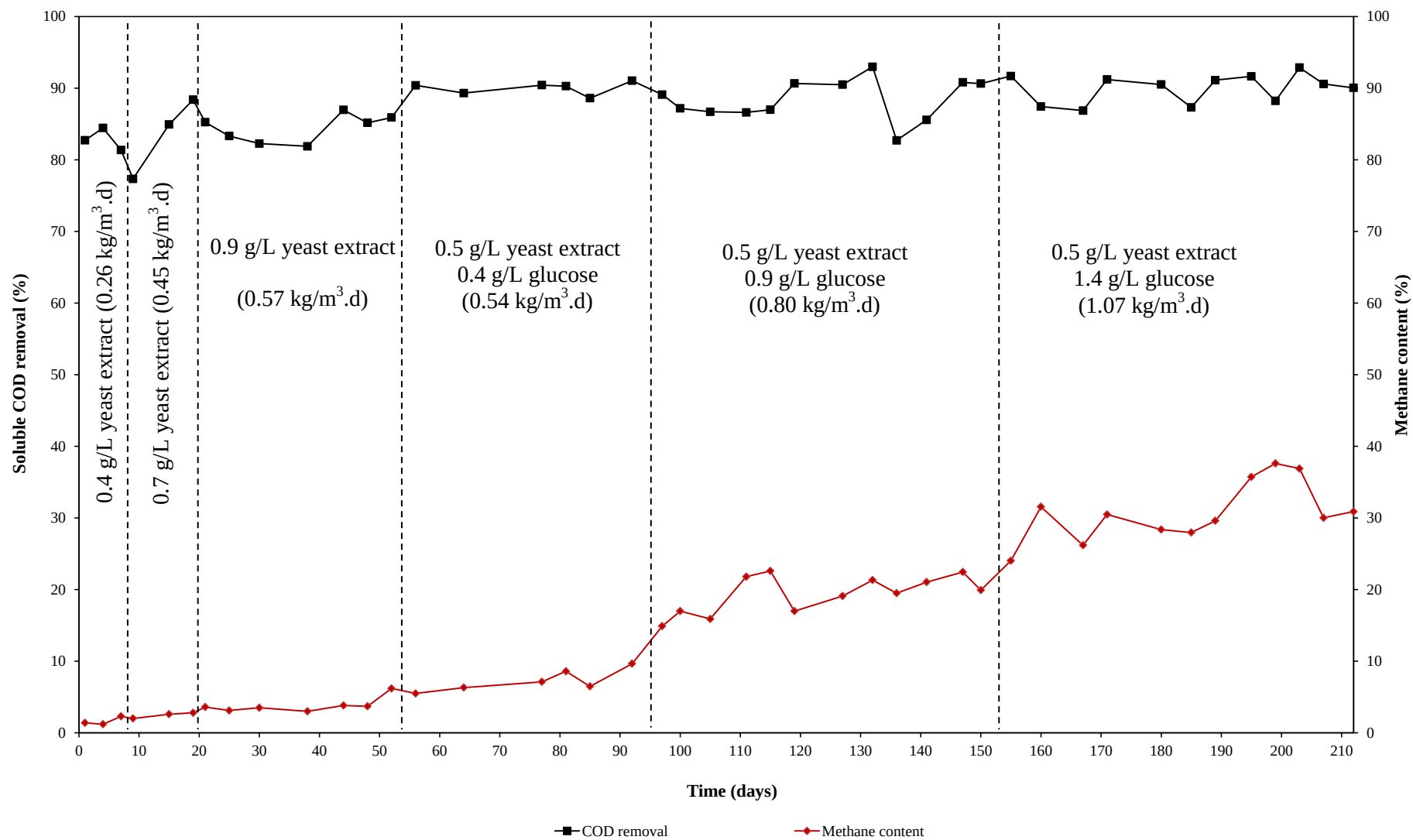


Figure 6.1: Soluble COD removal and methane content in the MABR.

This data is in line with typical values reported in other MABR studies, in which between 63 to 95% of the influent COD was removed at substrate loading rates between 0.06 and 24.5 kg COD/m³.d (Pankhania *et al.*, 1999; Semmens *et al.*, 2003; Liu *et al.*, 2007; Ohandja and Stuckey, 2007). In a hybrid MABR, the breakdown of organic compounds is achieved via both aerobic and anaerobic metabolism. In this present study, the aerobic metabolism of substrates was assumed to occur in the biofilm. This was confirmed by external dissolved oxygen (DO) analysis, where the DO content dropped to zero in the bulk liquid. Moreover, the use of a dead-ended membrane configuration was advantageous in achieving almost 100% oxygen conversion efficiencies for high organic carbon removal rates, as demonstrated by Pankhania *et al.* (1994). A small accumulation of VFAs (data not shown), and the production of biogas indicated the presence of anaerobic fermentative and methanogenic bacteria close to the biofilm-liquid interface (Figure 6.1). The production of biogas in terms of methane content was very low at the start of operation, but increased slowly over time to about 30% of the total gas in the reactor head space. Hybrid MABRs are expected to produce smaller amounts of methane compared to that of fully anaerobic systems, as the substrates in the MABR are partially metabolised by aerobic bacteria. In addition, it is likely that methanotrophic bacteria that oxidise methane to derive energy and carbon for biomass may also have grown in the biofilm. The performance of methanotrophic biodegradation in an MABR has been investigated by Rishell *et al.* (2004) and Casey *et al.* (2004); these studies showed that the MABR promoted a very high metabolic rate of methanotrophic biodegradation. This group of bacteria might be responsible for scavenging the methane produced in the anaerobic layer (Ohandja and Stuckey, 2007). On top of that, the continuous production of methane also underlines the fact that methanogenesis was not inhibited under the continuous supply of oxygen. This is attributed to oxygen diffusional

resistance through the thick biofilm which was constantly depleting oxygen due to metabolism.

The location of the active layers within the biofilm may vary, depending on the intra-membrane oxygen pressure, biofilm thickness, microbial growth rate and stoichiometry. These layers exist either at the biofilm liquid interface (oxygen limitation, where the carbon source fully penetrates), the biofilm membrane interface (substrate limitation, whereby oxygen fully penetrates) or between the interfaces (dual-substrate limitation where the concentration of both carbon source and oxygen reach zero within the biofilm) (Debus, 1995; Rishell *et al.*, 2004; Syron and Casey, 2008). Modelling and experimental studies have ascertained that in thick biofilms, neither organics nor oxygen is capable of complete penetration of the biofilm, and dual substrate limitation prevails (Casey *et al.*, 1999b). With increasing biofilm growth, carbon source diffusional resistance through the oxygen-depleted layer of the biofilm may become prominent; consequently affecting the COD flux and substrate removal. In contrast, in the present study substrate removal improved over the operational period, despite the expected increase in biofilm thickness. This might have occurred because the high substrate loading did not affect oxygen limitations as the thickness of the aerobically active layer in the MABR was significantly greater than in conventional biofilms (Essila *et al.*, 2000; Syron and Casey, 2008). Casey *et al.* (1999b) also found that the acetate removal rate in an MABR was unaffected by the thickness of the oxygen-starved layer, and the active layer remains adjacent to the bulk liquid. Nevertheless, this hypothesis cannot be validated in the present study since the oxygen and substrate concentration profiles through the biofilm depth were not measured. However, higher biomass concentrations with greater species diversity may have helped the reactor to achieve better performance, as observed in previous studies using the membrane augmented anaerobic bioreactor (Kappel *et*

al., 2005). Most importantly, the presence of NPEO_{ave9} (at 20 mg/L) and its possible toxic intermediates did not seem to inhibit the activity of heterotrophic bacteria to degrade carbonaceous pollutants. The behaviour of NPEO_{ave9} biodegradation in this system will be elucidated in the next section.

6.3.2.3 Biodegradation of NPEOs

After the reactor start-up, 0.4-0.9 g/L of yeast extract and 20 mg/L of NPEO_{ave9} were supplied to the reactor for 50 days. During this period, no formation of critical intermediates (NP and NPEO₁) was detected (Figure 6.2). Nevertheless, NPEO₂₋₆ concentrations were almost constant and long chain NPEOs (NPEO₇₋₁₁ and NPEO₁₂₋₁₅) accumulated over time. Surprisingly, the overall NPEO removal data in Figure 6.3 shows that more than 90% of the total mass of NPEO_{ave9} present in the influent was removed in the effluent. This suggests that the source of biomass obtained from the STP was already acclimatised to nonylphenolic compounds, and the enzymes required for NPEOs metabolism were already induced. Apart from biodegradation, the excellent removal of nonylphenolic compounds might have been caused by influential abiotic factors, such as their adsorption onto biomass. NPEOs are amphiphilic compounds in which their hydrophobic moieties are more inclined to adsorb onto solid surfaces with a high organic content, especially organic sewage (John *et al.*, 2000). Adsorption mechanisms on materials of biological origin (i.e. the biofilm) can be described as either biosorption or bioaccumulation. Biosorption is defined as the passive uptake of pollutants by non-growing or non-living microbial biomass, whereas bioaccumulation is defined as a property of living cells in which the pollutants can be transported into the cell across the cell membrane, and accumulate intracellularly through the cell metabolic cycle (Vijayaraghavan and Yun, 2008). If the pollutant accumulation reaches saturation then the

organism's metabolism will cease, resulting in the death of the organism. However, the effect of biotic and abiotic factors on NPEO_{ave5} removal was investigated by Langford *et al.* (2005); these authors found no apparent removal of NPEOs in activated sludge at a temperature of 4°C when compared to its removal at 19°C, demonstrating that degradation is a biological process rather than abiotic one. Despite the compounds susceptibility to adsorption, this reactor could sustain good substrate removal in terms of soluble COD and total NPEOs (Figure 6.1 and 6.2) over long term operation. It can be hypothesised that this occurs because NPEOs initially partition from the bulk aqueous phase onto the bacterial cells before intracellular biodegradation takes place slowly, at a level that induces no significant toxicity to the cells.

The effect of different co-substrate concentrations on NPEO_{ave9} biodegradation was also investigated. The addition of 0.9 g/L yeast extract was inadequate to sustain the removal of long chain NPEOs. Consequently, its concentration was reduced to 0.5 g/L, and glucose was introduced into the reactor at 0.4, 0.9 and 1.4 g/L on day 51, 96 and 154, respectively. As can be seen in Figure 6.4, the average MW of NPEOs reduced rapidly soon after the glucose was supplied. Longer chain NPEOs (NPEO₇₋₁₅) were not detected in the effluent from day 77 to 85 (Figure 6.2). On day 85, a small concentration of NPEO₁ started to appear, and the recorded average NPEOs MW was 424 g/mol. A prominent increase in the concentration of NPEO₁ as well as long chain NPEOs was observed afterwards until day 105 (Figure 6.2). The accumulation of NPEO₁ in the reactor indicated that NPEO_{ave9} were biodegraded through the most common NPEOs pathway, i.e. bacterial attack on molecule's ethoxylate side chain causes the terminal ethoxyl unit to cleave and liberates acetaldehyde to give the corresponding shortened NPEOs.

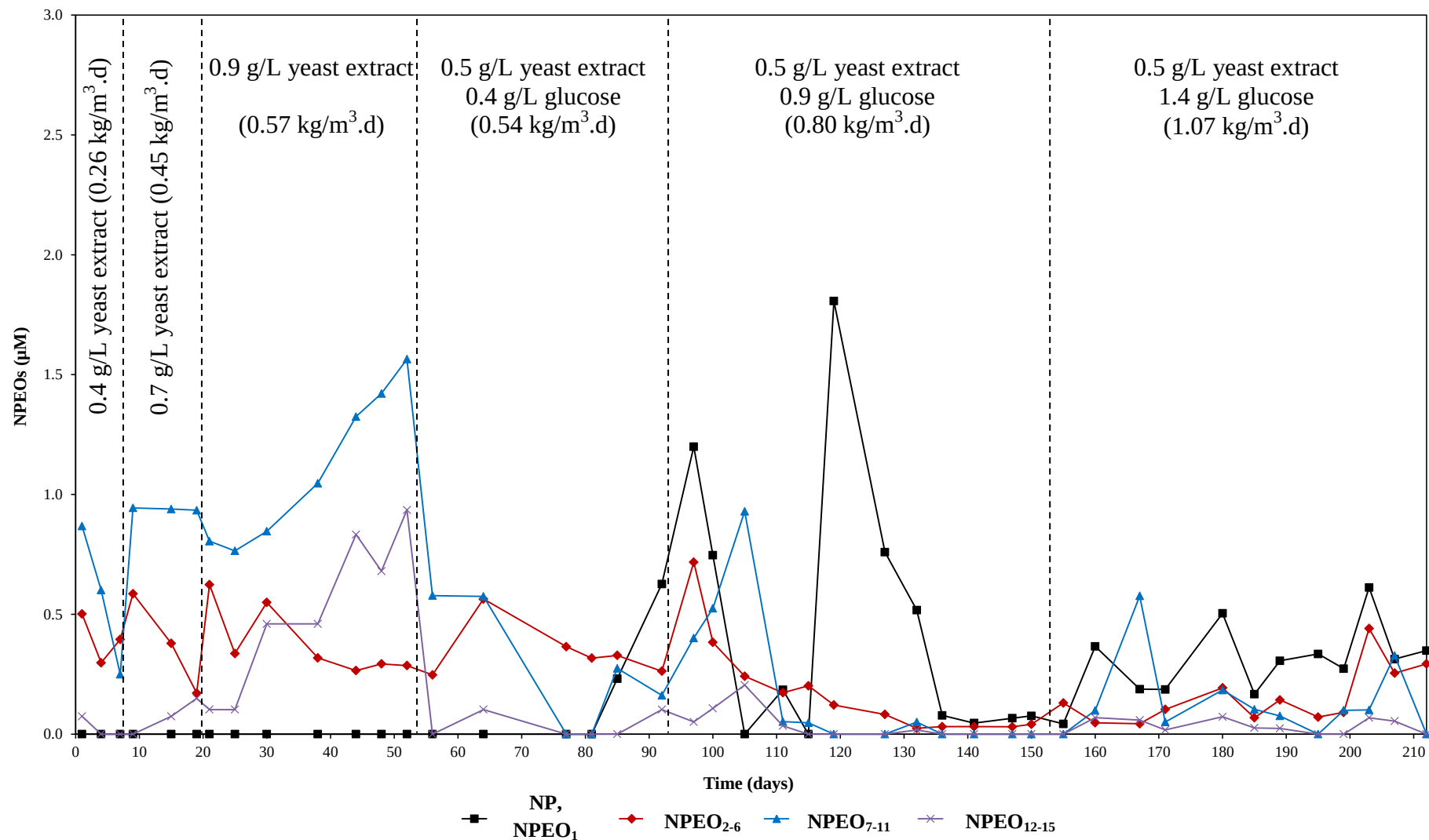


Figure 6.2: Concentration of NPEOs and their intermediates in the MABR.

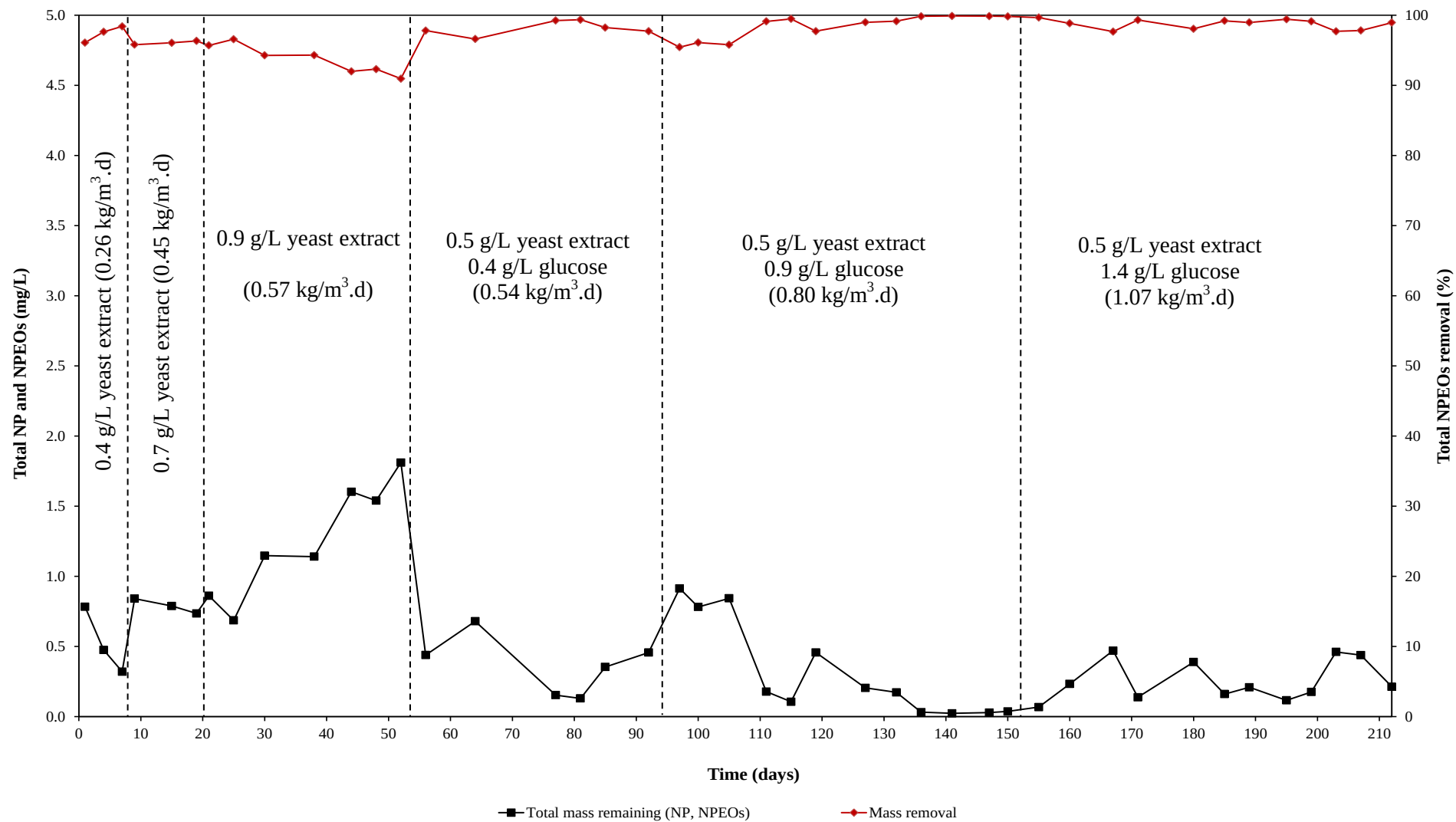


Figure 6.3: Total NP and NPEOs removal in the MABR.

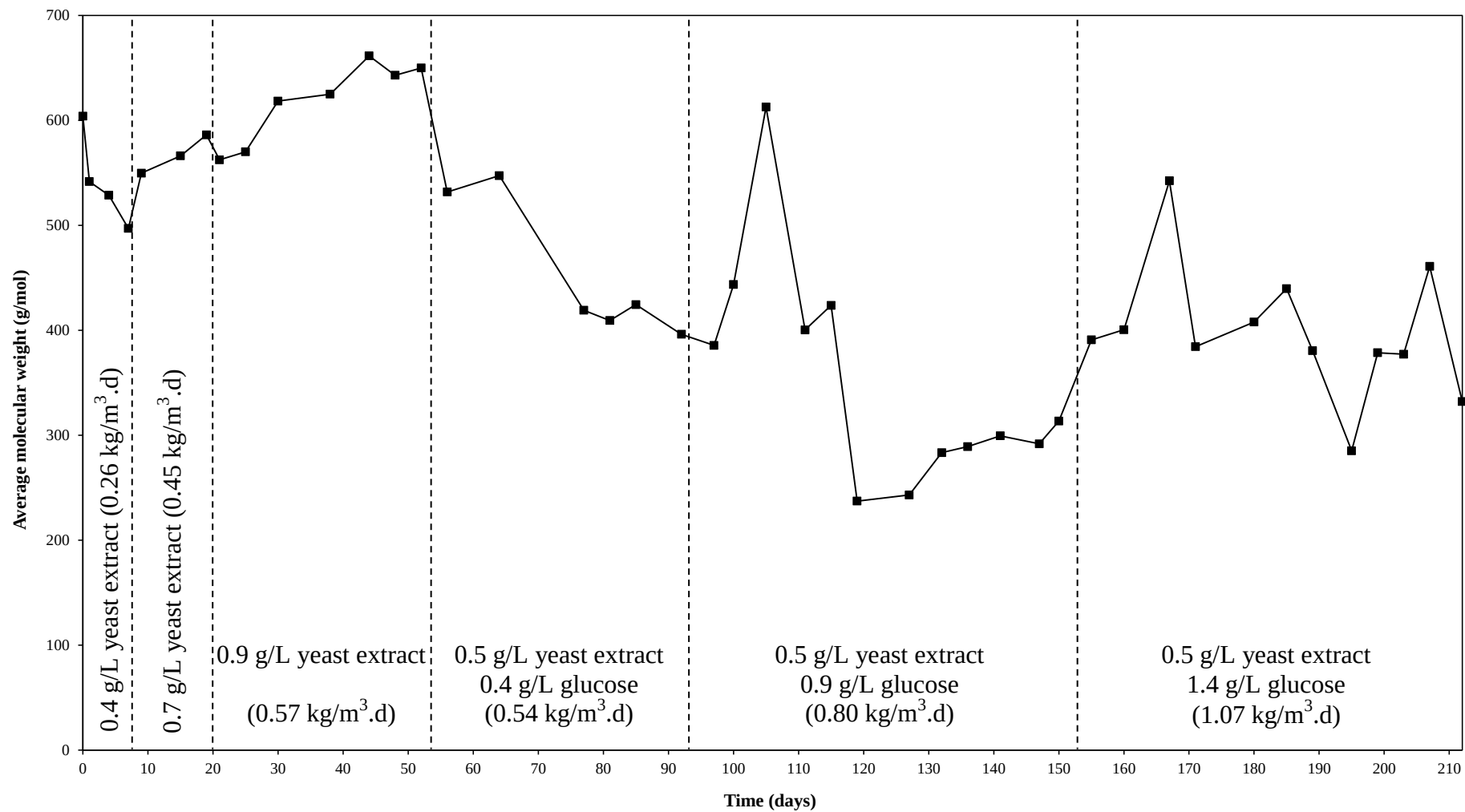


Figure 6.4: Average molecular weight of NPEOs in the MABR.

After the higher glucose concentration of 0.9 g/L was introduced into the reactor, the intermediates were gradually degraded, and the average MW declined drastically to the lowest value ever achieved in this study so far; 273 g/mol (Figure 6.4). According to Hayashi *et al.* (2005), aerobic transformation of NPEOs can be performed rapidly in the presence of organic matter such as yeast, glucose, and methanol, indicating the possibility of cometabolism. In addition, the lowest MW value obtained corresponds to the highest quantified concentration of NP. In fact, the formation of NP was only noticed on day 119 to 132, at a concentration between 0.10 to 0.34 mg/L (0.46 to 1.55 μ M). The NP is mostly produced under anaerobic conditions, although a recent study validated its formation under aerobic conditions (Giger *et al.*, 1984, Montgomery-Brown *et al.*, 2008). The transient appearance of NP could be due to the increase in anaerobic activity in the MABR concomitantly with the degradation of NPEOs. The NP did not persist in the system, and was removed rapidly via the postulated aerobic metabolism. On the other hand, other studies have found that NP was commonly produced by NPEO_{ave5} breakdown in aerobic packed bed biofilm reactors, and persisted in the concentration range of 0.1-0.2 mg/L at the end of the experiments; limited aeration may have caused the NP to accumulate. Fortunately, this toxic EDC was effectively removed in the MABR.

After 136 days of operation, more than 99% of the molar and mass concentration of total NPEOs was removed from the system, and up to day 150 only trace amounts of NPEO₁ were detected (Figure 6.3). During this period the reactor was assumed to have achieved “pseudo steady state”. Although technical constraints made the visual observation and measurement of the biofilm impossible (stainless steel cover over the biofilm), the development of a thick biofilm was evident by a substantial production of biogas. Nevertheless, the compound’s removal was slightly reduced when a higher glucose

concentration (1.4 g/L) was fed into the reactor. NPEO₂₋₁₁ oligomers slowly increased and NPEO₁ was fluctuating in excess of the other intermediates (Figure 6.2). The overall COD removal (Figure 6.1) was almost unaffected, whereas the total NPEOs removal remained more than 97%. This slight disruption of performance from steady state conditions might imply a slight co-substrate inhibition of NPEOs metabolism. In addition, more biomass could have been produced as a result of higher substrate loading. This, in turn, would increase the biofilm thickness and suppress diffusion of NPEOs to the aerobic zone near the membrane-biofilm interface, and consequently disrupt aerobic degradation of the intermediates.

As has been highlighted before, the formation and mineralisation of the acidic intermediates of NPEOs, such as NPECs and CAPECs, is not well understood due to analytical limitations. The analysis of all soluble extracts, however, did not reveal any detectable unknown nonylphenolic metabolites. Hence, the apparent lack of a good mass balance observed in the experiments, and the extent of possible acidic NPEO metabolites could not be elucidated. Although these compounds exhibit a lower toxicity and estrogenicity than that of short chain NPEOs, these compounds are hydrophilic and may be discharged and accumulate in the environment without prior detection at the treatment unit. More study should be conducted in the future to specifically monitor the formation of acidic intermediates, apart from their NPEOs counterparts.

6.4 SUMMARY

The possibility of decontaminating synthetic wastewater containing NPEOs with a continuous MABR was demonstrated in this study. The overall reactor performance was satisfactory with very good removals of soluble COD of between 82.7 and 92.9% at substrate

loading rates of 0.54 to 1.07 kg COD/m³.d, with an HRT of 48 hr. Indeed, more than 90% of total mass of NPEO_{ave9} present in the influent was removed by the reactor. The main intermediates of NPEO₁ accumulated in the system, and the formation of NP occurred occasionally, although the presence of these intermediates did not appear to inhibit the reactor. Their degradation was postulated to proceed under both aerobic and anaerobic conditions. Nonetheless, the main constraint of this NPEOs biodegradation study was the lack of a good mass balance due to the formation of undetected intermediates, and the role of suspected abiotic factors such as adsorption in the removal of NPEOs. In this present study, it was assumed that the NPEO removal was more a biological rather than abiotic process. The compounds partitioning into the bacterial cells might be concomitantly removed by intracellular biodegradation, to a level that induced no significant toxicity to the cells. Because of its efficient removal of NPEOs and COD, the MABR has the potential to be used for wastewater containing nonylphenolic compounds. The effect of different NPEO concentrations and organic shock loads will be investigated in the next chapter.

❧ CHAPTER 7 ❧

THE TREATMENT OF NONYLPHENOL ETHOXYLATES (NPEOs) IN A MEMBRANE AERATED BIOFILM REACTOR (MABR) – The Effect of Organic and Hydraulic Shock Loads

Outline:

- 7.1 Introduction
- 7.2 Methods
- 7.3 Results and discussion
 - 7.3.1 The effect of $\text{NPEO}_{\text{ave9}}$ shock loads
 - 7.3.2 The effect of hydraulic shock loads
 - 7.3.3 Biofilm analysis
- 7.4 Summary

7.1 INTRODUCTION

Studies on NPEO biodegradation have been carried out almost exclusively in batch culture, significantly outnumbering those conducted in a laboratory scale or full scale continuous flow bioreactor. Despite a significant number of biodegradation mechanisms being elucidated, none of these hypotheses are truly comprehensive enough to describe the biodegradation behaviour of NPEOs in “real” treatment systems. The differences in bacterial populations, types of wastewater and variations in process conditions leads to disparities in

the understanding of NPEOs biodegradation behaviour with respect to its biodegradation routes, type of intermediates formed, and its interaction with other organic and inorganic compounds present in the reactor— whether biotic or abiotic. In real treatment plants, temporal changes in the composition and flux of the wastewater are common according to anthropogenic activities. Nevertheless, a deep understanding of NPEO biodegradation behaviour under periodically changing process conditions, especially in terms of hydraulic and organic loads, is still lacking. In the previous chapter we conducted a preliminary study on the use of an MABR for the treatment of NPEOs. Although complete mineralisation of these compounds and their by-products was not evident, the reactor was able to cope with quite high NPEO loading rates, and effectively removed up to 99% of the total NPEO oligomers fed into the reactor. In the present chapter, MABR stability for NPEO removal under different hydraulic and organic loads was investigated.

7.2 METHODS

This chapter is a continuation of Chapter 6, in which the reactor, operational parameters and analytical methodology were as previously mentioned. Any variation in operational conditions from the previous chapter will be highlighted during the description of this work. The reactor was continually fed with readily biodegradable substrates of 0.5 and 1.4 g/L of yeast extract and glucose, respectively, for the whole operational period. In the present study, two parameters were varied which were $\text{NPEO}_{\text{ave9}}$ concentration, and HRT. After the stable accumulation of short chain NPEOs was observed, the concentration of $\text{NPEO}_{\text{ave9}}$ was ramped up from 20 mg/L to 40 mg/L on day 216, and 80 mg/L on day 264; an HRT of 48 hours was maintained during this period. The $\text{NPEO}_{\text{ave9}}$ concentration was reduced back to 20 mg/L on day 290 and sustained for another 10 days to stabilise the

reactor. In order to study the effect of hydraulic load on NPEO biodegradation, the HRT was dropped to 24, 12, and 9 hours from day 300 to 500. It is important to note that the “transition periods”, where the HRT was increased back to 48 hours, was applied between each HRT change. This was to stabilise the reactor before another shock load was induced. At the end of the operational period the reactor was sacrificed, and the membrane and biofilm structure were analysed by Scanning Electron Microscopy (SEM).

7.3. RESULTS AND DISCUSSION

7.3.1 THE EFFECT OF NPEO_{AVE9} SHOCK LOADS

The MABR performance data on the effect of NPEO_{ave9} shock loads are presented in Figures 7.1, 7.2, 7.3, 7.4 and 7.5, looking at; chemical analysis of nonylphenolic intermediates, total NPEOs concentration removal, the average molecular weight of NPEOs, soluble COD removal, and total VFAs and methane content, respectively. As noted earlier, data in the previous chapter (6) provided an overview of the acclimatisation of an MABR to a constant loading of NPEO_{ave9} (20 mg/L; 2.47 g/m².d) under steady state conditions over a period of about 7 months. During the long-term adaptation period, complete mineralisation of the parent compound was difficult due to the persistency of short chain intermediates, i.e. NPEO₁. For instance, 0.349 µM (92 µg/L) of NPEO₁ was still apparent in the reactor at the end of the aforementioned period (on day 212), although 98.9% of the total NPEOs fed to the reactor were successfully eliminated, and none of the long chain oligomers of NPEO₇₋₁₅ were detected in the effluent (Figure 7.1). Accumulation and detection of critical trace intermediates of NPEOs biodegradation is a common problem which occurs in treatment systems. Nevertheless, the MABR used in this study was able to maintain good COD

removals of around 90% over long term operation. Excellent performance of the MABR was due to the retention of a highly diverse aerobic and anaerobic microbial population in the biofilm, and the role of the membrane in providing high rate oxygen transfer into the biofilm. Therefore, from the data it is safe to argue that the level of intermediates produced under the given $\text{NPEO}_{\text{ave9}}$ load in this study was not inhibitory to bacteria at this point.

In a subsequent study, the robustness of the MABR when subjected to sudden changes in the $\text{NPEO}_{\text{ave9}}$ loading rate was investigated under a constant HRT of 48 hrs. Soon after the shock loading of 40 mg/L ($4.94 \text{ g/m}^2\cdot\text{d}$) of $\text{NPEO}_{\text{ave9}}$ took place on day 215, the total NPEO removal was reduced slightly to 89%, and the formation of short and long chain intermediates was more significant, as depicted in Figures 7.1 and 7.2. It took a considerable time for the reactor to recover to its former state. After a month of high loading, total NPEOs removal recuperated to about 98.5%. As expected, NPEO_1 was not totally mineralised, but co-existed with other intermediates, NPEO_{2-11} , thus making an average MW of approximately 320 g/mol (Figure 7.3).

In addition, the soluble COD removal dropped slightly on day 217, but recovered to 92.5% on day 259 (Figure 7.4). The interruption of reactor performance on day 217 occurred concomitantly with an increase in VFA production to 226 mg/L, and a reduction in CH_4 production to about 14.7% (Figure 7.5). The accumulation of VFAs was probably a result of cell impairment, especially by inhibition, or low activity of one or more of the bacterial groups involved in the fermentation process (Ohanjda and Stuckey, 2007). The VFAs produced from the anaerobic degradation of glucose in an MABR will later act as electron donors for either aerobic or anaerobic bacteria present in the biofilm, and concurrently be degraded to relatively low concentrations as the reactor stabilised.

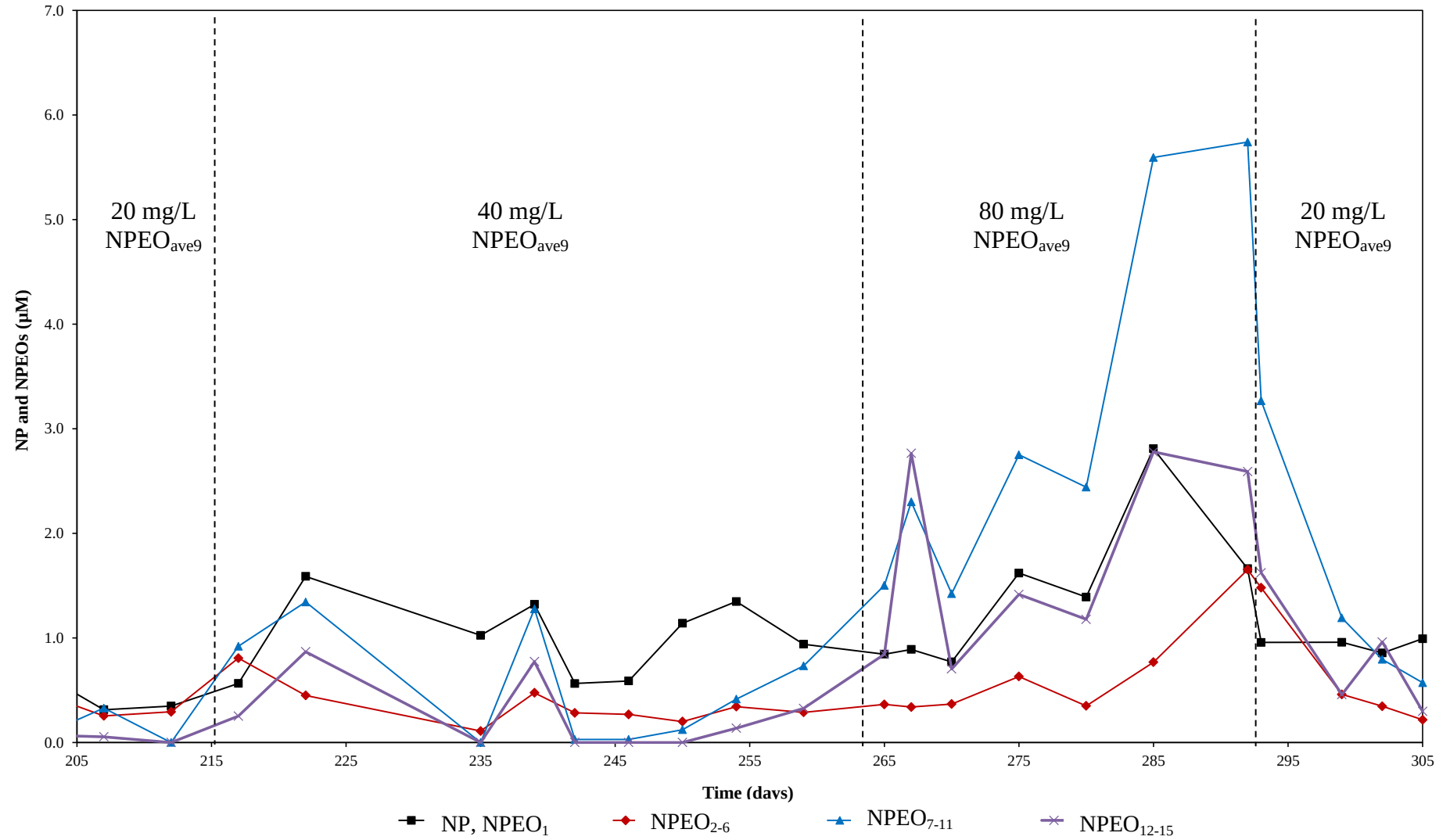


Figure 7.1: Concentration of NPEOs and their intermediates in the MABR under different NPEO_{ave9} loadings.

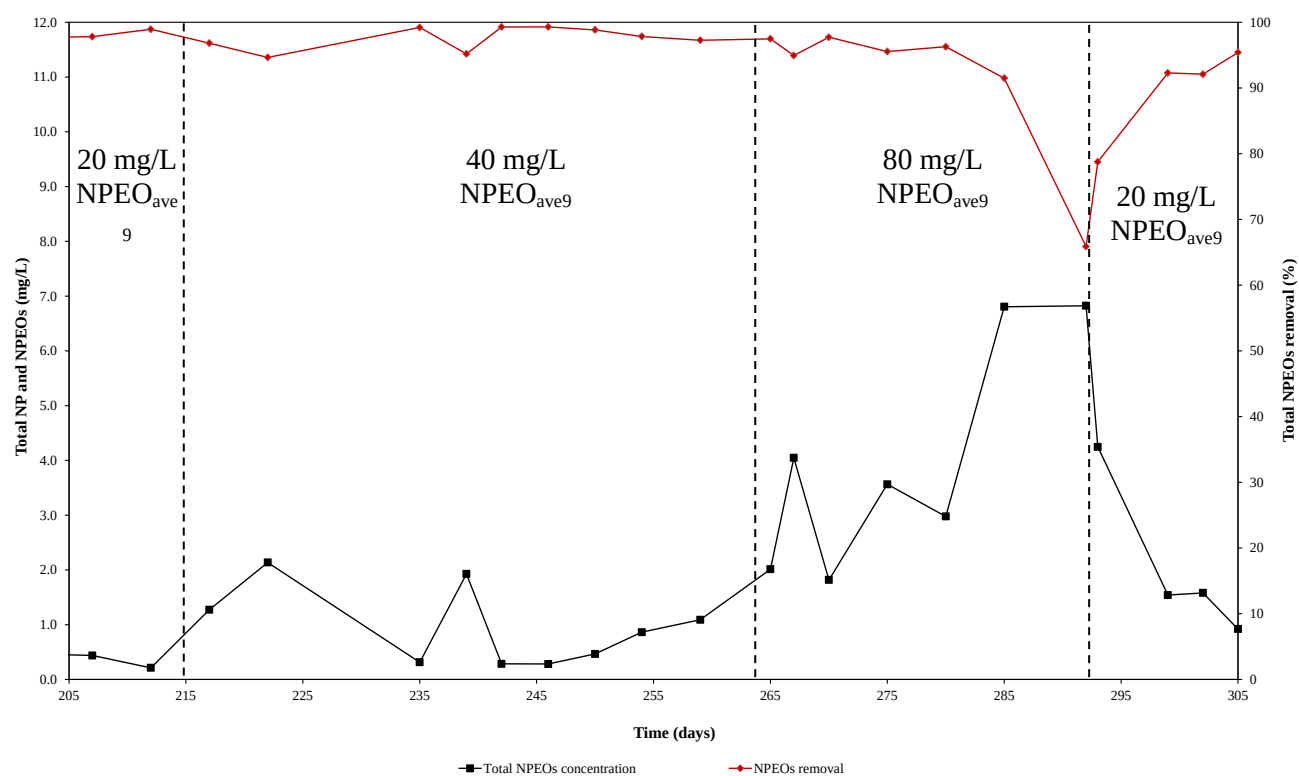


Figure 7.2: Total NP and NPEOs removal in the MABR under different $\text{NPEO}_{\text{ave9}}$ loadings.

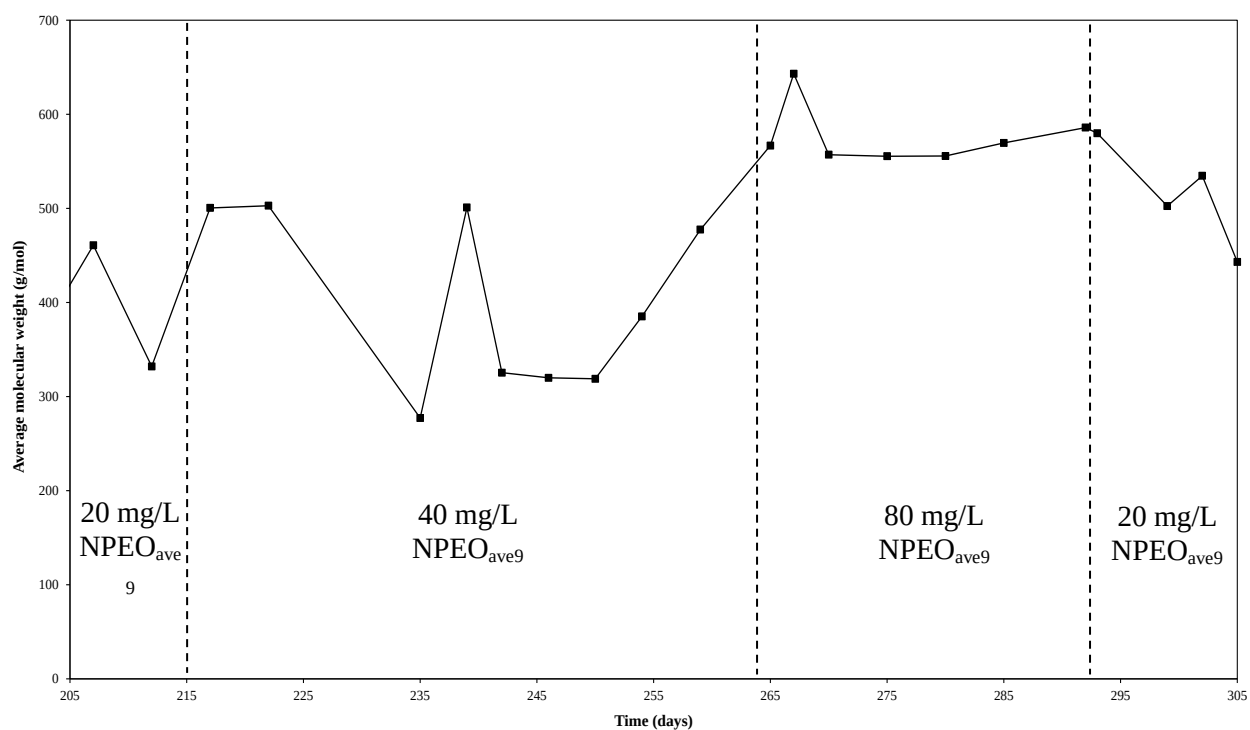


Figure 7.3: Average molecular weight of NPEOs in the MABR under different $\text{NPEO}_{\text{ave9}}$ loadings.

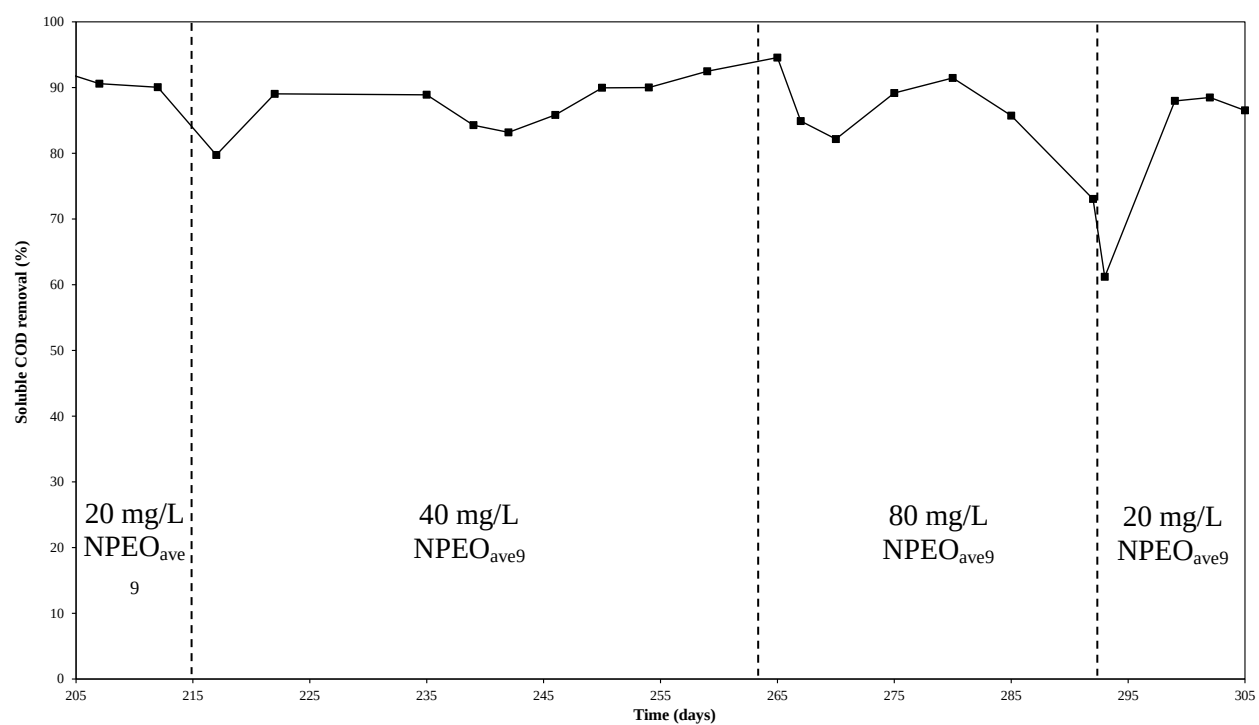


Figure 7.4: Soluble COD removal in the MABR under different $\text{NPEO}_{\text{ave9}}$ loadings.

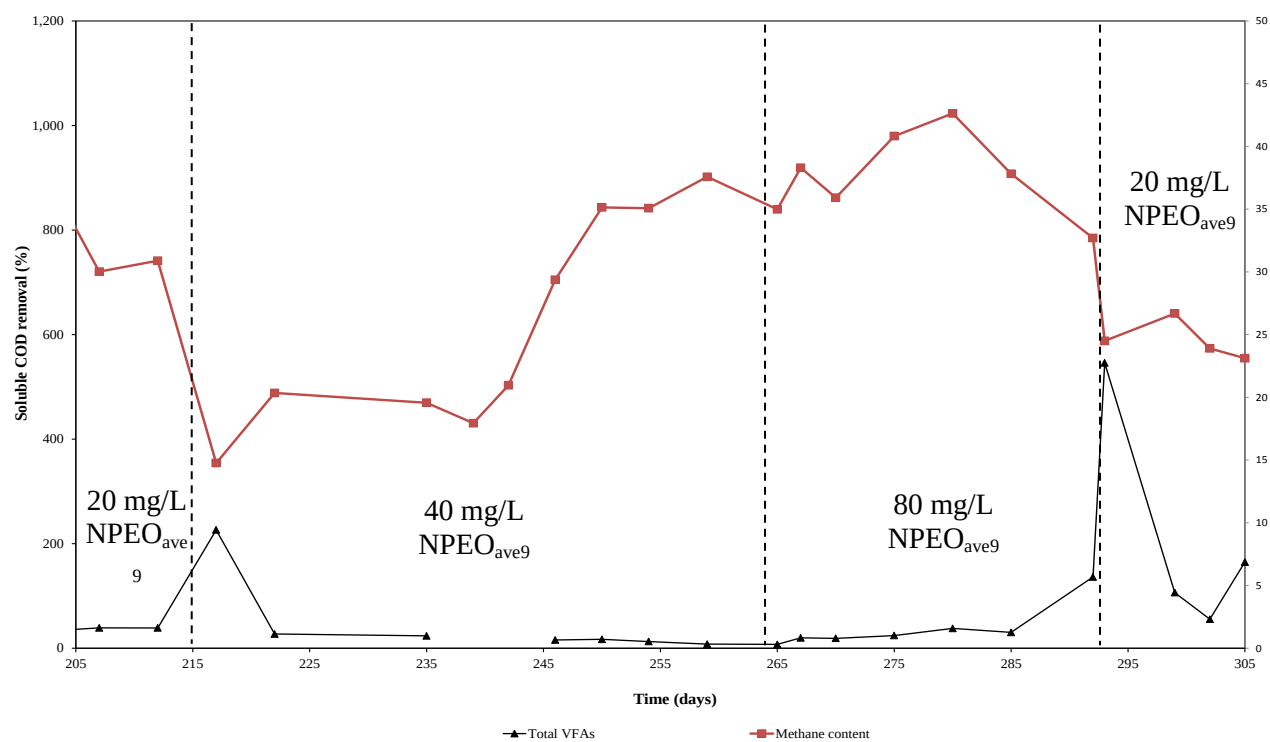


Figure 7.5: Total VFAs and methane content in the MABR under different $\text{NPEO}_{\text{ave9}}$ loadings.

After stabilisation, the methane content increased to 37.5% at the end of this period (i.e. under 40 mg/L NPEOs in the feed); the highest recorded methane content in this study so far. It is also important to recognise a sudden increase in long chain NPEOs concentration, as well as total NPEOs on day 239 (Figure 7.1 and 7.2); the reason for this was not clear. However, it is conceivable that it was caused by a temporary disruption of the compressed air supplied which occurred around then. Nonetheless, the reactor achieved an excellent reduction in total NPEOs (>95%) during this entire period.

On day 264, 80 mg/L (9.88 g/m².d) NPEO_{ave9} was fed into the reactor; unfortunately, the reactor experienced a significant decrease in performance. For instance, soluble COD removal plummeted to about 61% (Figure 7.4), total VFA concentration accumulated to about 546 mg/L, and methane content reduced to 24.5% (Figure 7.5). Propionate was the most detected VFA in the system, and although it has been proven that high concentration of propionate (about 5000 mg/L) can inhibit the activity of methanogens (Hobson and Shaw, 1976), the amount of propionate generated in this study was, however, too low to induce such inhibition. It is therefore postulated that in the present study, the activity of methanogens was not largely inhibited by the increased of VFAs. Since VFAs inhibition is pH related, the undissociated fraction of the VFAs is believed to be the main inhibitor of methanogenesis in a system with a low pH. Nevertheless, this reactor was able to maintain an adequate buffer capacity, at pH around 6.5 to 7.5 (data not shown), thus minimising the formation of undissociated acids (Babel *et al.*, 2004). On top of that, surfactants were also found to be relatively toxic to CH₄ production compared to other organic chemicals (Shcherbakova *et al.*, 1999). Hence, the NPEOs inhibitory nature at high concentrations can largely influence process performance, such as total substrates removal and biogas production.

As can be seen in Figure 7.1, after 80 mg/L of NPEO_{ave9} was introduced into the reactor, NPEO₇₋₁₁ were the most significantly accumulated intermediates, and these were followed by NPEO₁₂₋₁₅. Moreover, only 66% of the total molar NPEOs were removed after 3 weeks of a shock load (Figure 7.2). The result signifies an inhibition of the primary biodegradation of NPEO, and the inability of bacteria to cope with a sudden increase in NPEOs. During this period, NP was detected in the reactor and its concentration increased up to 110µg/L (0.5µM), approximately 19 days after its first detection. The presence of NP during high NPEO loading suggested that the NP was formed and degraded rapidly under low NPEOs loading (i.e. 20 mg/L) to a level below the analytical detection limits. A fast NP degradation route in the aerobic microcosm has been proposed by Montgomery-Brown *et al.* (2008); these authors observed that in acclimatised systems, acidic intermediates of NPEC₁ were broken down to NP before being rapidly transformed to nonyl alcohols via ipso-hydroxylation. This rapidly transformed NP could explain why the compound was rarely detected in some of the studies under aerobic conditions (Kvestak and Ahel, 1995). In a hybrid MABR, biodegradation of NPEOs occurs via both aerobic and anaerobic metabolic routes. The distinction between aerobic and anaerobic intermediates is often difficult to draw due to conflicting results obtained from the literature regarding the biodegradation pathway of these compounds. In this study, conclusions were drawn based on well accepted hypotheses. For instance, complete de-ethoxylation of NPEOs to NP was assumed to proceed under anaerobic conditions, although in a study by Montgomery-Brown *et al.* (2008), NP can also be produced under aerobic conditions. In addition, aerobic biodegradation of NP facilitates its removal in an MABR, in agreement with a typical view that NP degradation rarely occurs in anaerobic systems with high organic carbon available (Giger *et al.*, 1984). In contrast, a claim based on NP degradation under anaerobic conditions has also been made, but the process proceeded at a very slow pace (Chang *et al.*, 2005).

In order to avoid further disruption to the reactor, and a possible bioaccumulation of toxic intermediates in the biofilm, the NPEO concentration was decreased down to 20 mg/L; as a result, the reactor started to recover slowly. Up to this point in this research, we have seen that the critical intermediate of NPEO₁ was not completely mineralised within the stipulated period. Although a number of bacteria with the ability to degrade short chain NPEOs and NP have been isolated and characterised from STPs, these degradation intermediates have also been widely observed in the effluent leaving treatment plants, as well as in natural water bodies (Ying *et al.*, 2002; Chang *et al.*, 2005; Gu *et al.*, 2010). This implies that NPEO-degrading bacterial populations isolated from laboratory cultures may be different to, or may not even represent the actual distribution of the NPEO-degrading bacteria present in natural or engineered environments (Lozada *et al.*, 2004; Gu *et al.*, 2010). An improvement in process conditions, especially a better control of biofilm thickness may help to mitigate these problems.

Inhibition indicates a detrimental effect that a test substance causes on the activity of a microbial population, especially during the shock loads, where the high concentration of a test substance is beyond the microorganism's ability to acclimatise or adapt. Syron *et al.* (2009) proposed a concept of diffusional resistance in the MABR with respect to organic shock load, based on a mathematical model comparing an MABR to a conventionally aerated biofilm reactor (CABR). They found that the shock load resulted in a 40% increase in the phenol utilization rate in the MABR, but a 40% decrease in the utilisation rate in the CABR. The significant difference was assumed to be due to the protection of the active region of the biofilm from high concentrations of toxic substances present in the bulk liquid; by the effect of oxygen depleted layer in the biofilm that acts as a diffusional barrier to overcome shock loads. In addition, the mass transfer of a substrate is largely influenced by the mass transfer

resistances along the biofilm depth, and this is controlled by the thickness and the effective diffusivity of the biofilm. Unfortunately, no experimental data has been presented to validate the model. The model also predicts a lower concentration of phenol in the biofilm than that present in the bulk liquid under the shock load. This contradicts our early hypothesis in which we believed that high concentrations of nonylphenolic intermediates might have been bioaccumulated in the biofilm prior to their breakdown. The compound's concentration in the biofilm was, however, not measured in the present study to validate the hypothesis. Nevertheless, it is expected that the role of the substrate's diffusional resistance might have been more prominent under high organic loading. For instance, higher $\text{NPEO}_{\text{ave9}}$ loading limits the penetration of the parent compounds into the biofilm, thus preventing the biofilm from excessive accumulation of resulting toxic intermediates. This phenomenon might protect the bacteria from oversaturation of toxicants (i.e. nonylphenolic intermediates).

7.3.2 THE EFFECT OF HYDRAULIC SHOCK LOADS

The MABR performance data on the effect of hydraulic shock loads (different HRTs) are depicted in Figure 7.6 (soluble COD removal, CH_4 content and total VFAs), Figure 7.7 (concentration of NPEOs and intermediates), Figure 7.8 (total NP and NPEOs concentration and removal percentage) and Figure 7.9 (average molecular weight of NPEOs). By analysing each graph in this section, and comparing this to the data presented in the previous section (MABR under $\text{NPEO}_{\text{ave9}}$ shock loads), the resulting trends were very similar. For instance, the trend of soluble COD removal, CH_4 content and VFA production under each hydraulic shock load can be generalised in Figure 7.6. A significant deterioration in performance can be observed when the hydraulic shock loads of 12 hours HRT ($1.98 \text{ g/m}^2\cdot\text{d}$) and 9 hours HRT ($2.62 \text{ g/m}^2\cdot\text{d}$) were applied on days 341 and 460, respectively. When the stress conditions

started to occur, a significant drop in COD removal and CH₄ content were evident, while the VFA concentration increased substantially. For instance, after the first load of 12 hours HRT, VFAs increased to 509 mg/L and COD removal reduced to only 66.6%. In fact, the VFAs contributed to about 86% of the total effluent COD. The increase in VFAs was more significant during high hydraulic loads, where the bacteria were struggled to adapt within the short HRT. A similar phenomenon has been also observed by Ohandja and Stuckey (2010) in an MABR treating perchloroethylene (PCE) under hydraulic shock loading. Rapid and high biodegradability of substrate, i.e. glucose, in an anaerobic environment results in a sudden increase in VFA production. These VFAs will in turn diminish over time provided that the population of methanogens is adequate to degrade the VFAs, and the process conditions are not inhibitory to the methanogens well-being (Akram and Stuckey, 2008). An inability of the reactor to recover from stress will lead to significant VFAs accumulation, which will also contribute to high COD content in the bulk liquid.

In this study, a recovery period was used to enable the reactor to recover from high stress conditions, and a period of about a month was required to increase COD removal back up to 90%. Apart from VFAs, low COD removal could also have been due to the excessive production of soluble microbial products (SMP) in the system. SMPs are defined as soluble cellular components released during substrate metabolism and cell lysis (Jang *et al.*, 2006). According to Aquino and Stuckey (2004), the production of SMPs is elevated in the presence of toxic compounds. In the MABR during the shock load, increased production of both biomass and biofilm thickness may be pronounced, and this may be associated with the excessive excretion of extracellular polymeric substances (EPS) by bacteria to protect themselves from the toxicants.

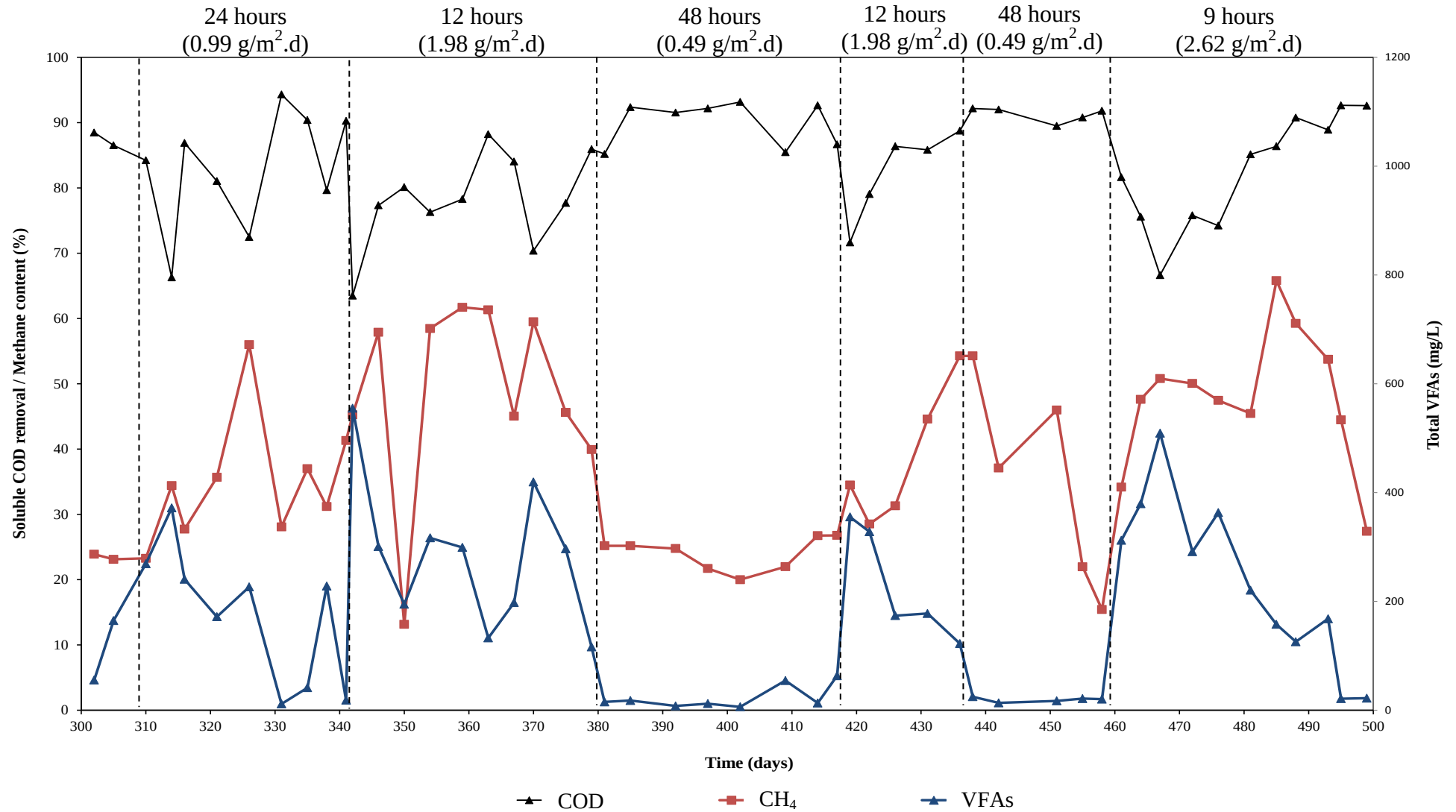


Figure 7.6: Soluble COD removal, CH₄ content and total VFAs concentration during hydraulic shock loads.

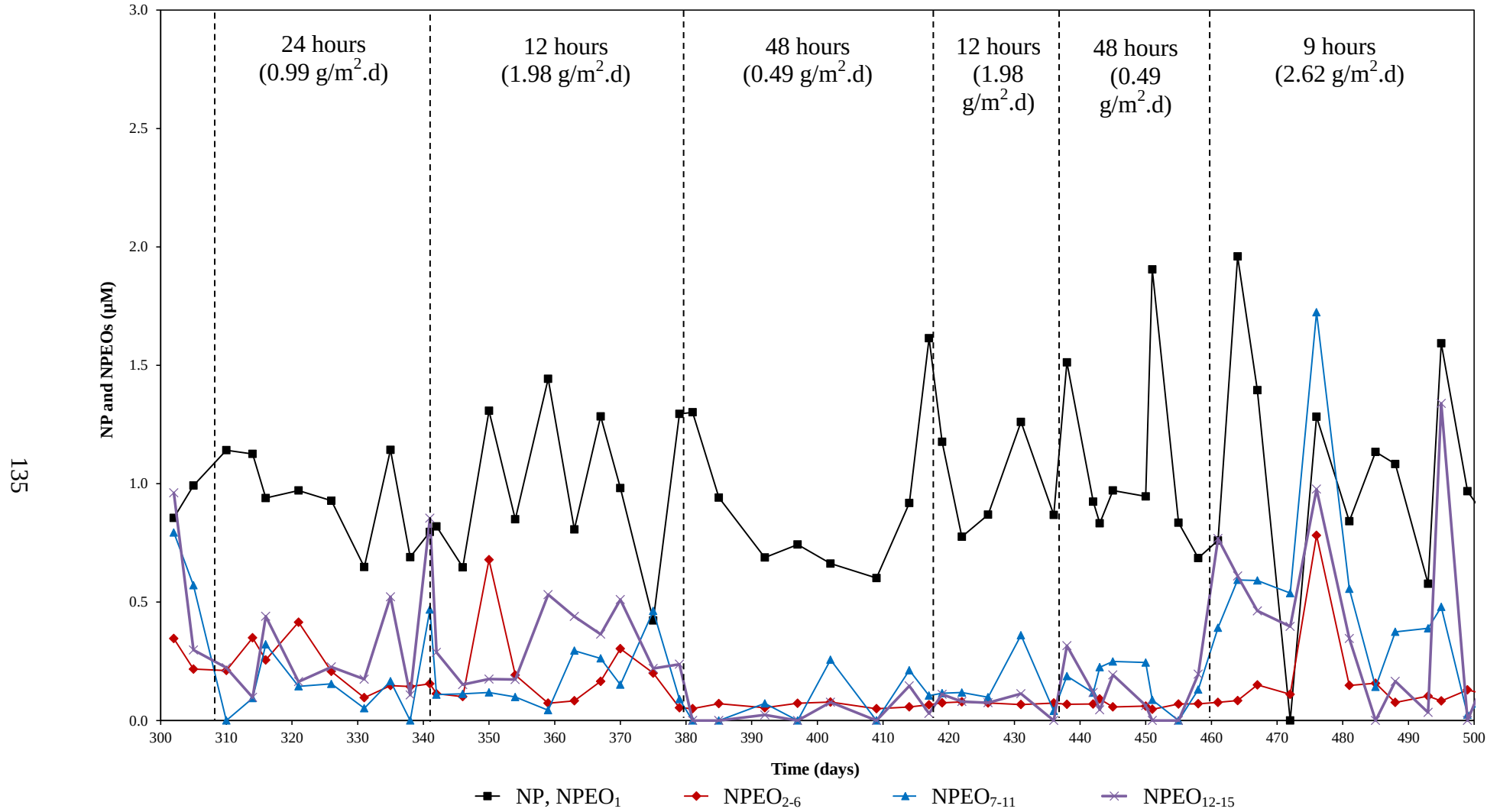


Figure 7.7: Concentration of NPEOs and their intermediates in the MABR during hydraulic shock loads.

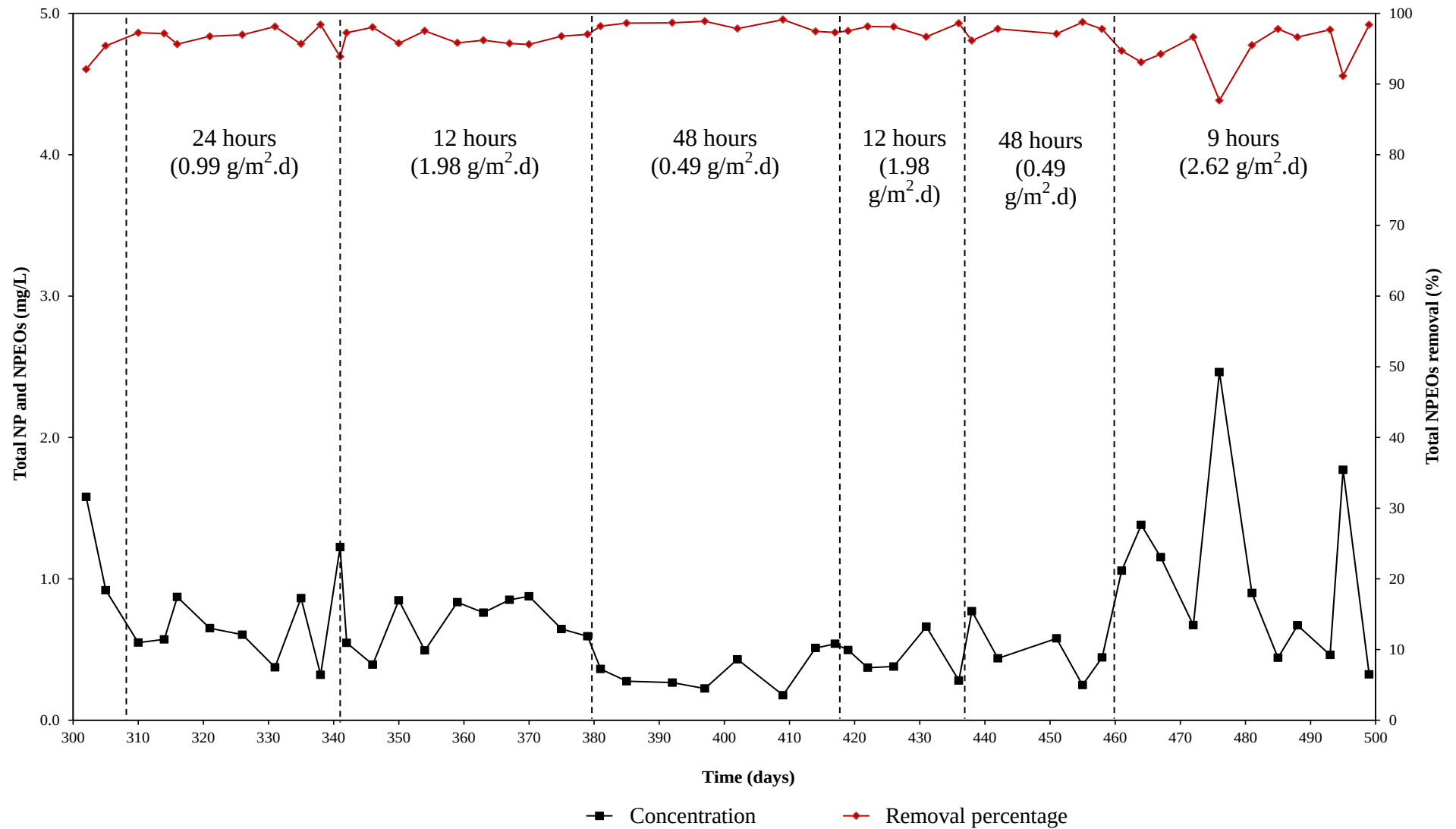


Figure 7.8: Total NP and NPEOs concentrations and removal percentage in the MABR during hydraulic shock loads.

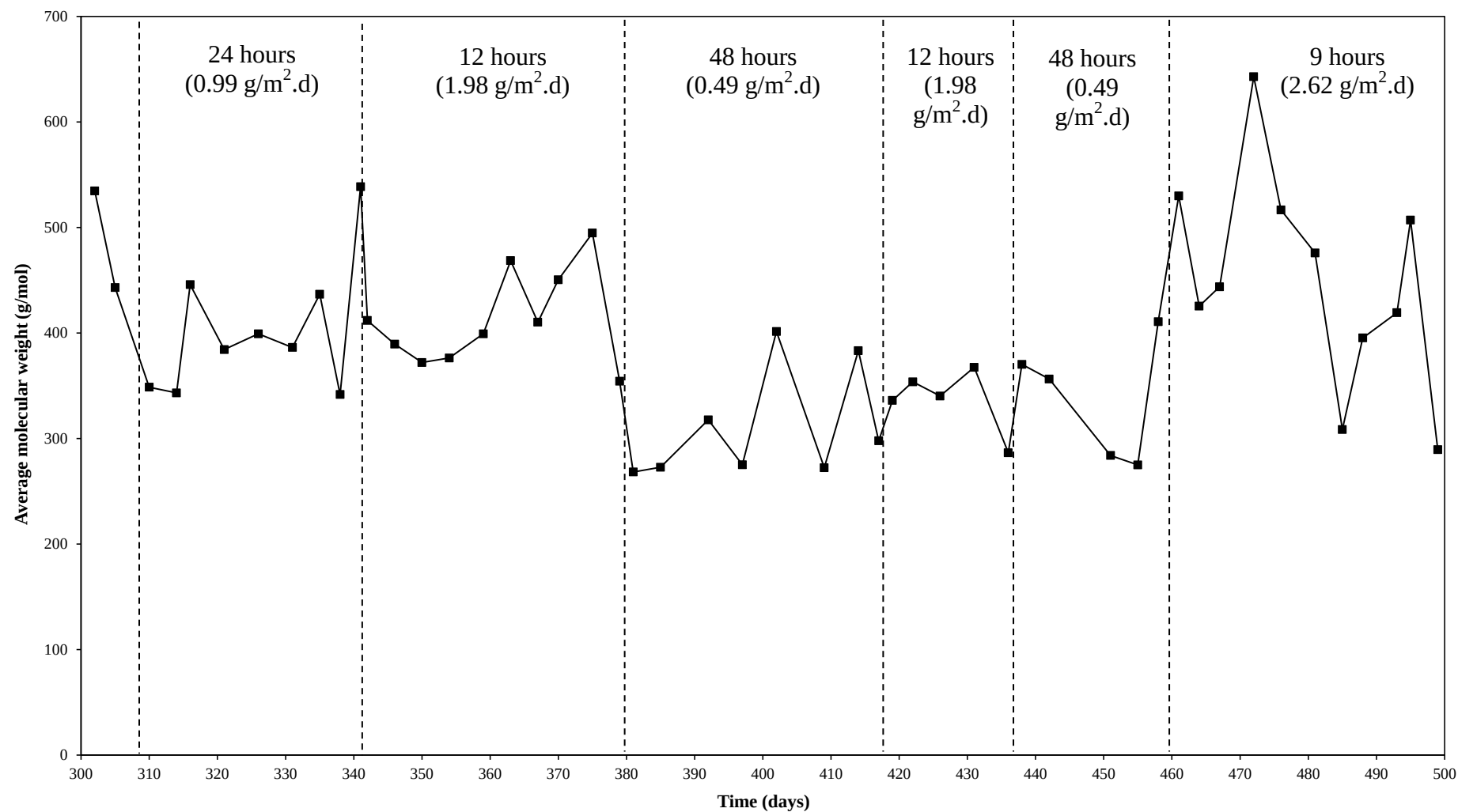


Figure 7.9: Average molecular weight of NPEOs in the MABR during hydraulic shock loads.

The release or hydrolysis of this material into the bulk liquid may subsequently account for the increase in SMP production, and the decrease in COD removal (Akram and Stuckey, 2008; Ohandja and Stuckey, 2010). In addition, the occurrence of endogenous decay and cell lysis may be more significant in a reactor with a high sludge age (SRT) resulting in an enhanced accumulation of SMPs, as predicted to occur in this reactor under long-term operation (Akram and Stuckey, 2008). The stability of the reactor was, however, restored after the HRT was increased back to 48 hours ($0.49 \text{ g/m}^2\cdot\text{d}$).

As can be seen in Figure 7.8, the total removal of NPEOs was affected less by short HRTs, and more than 94% of the NPEOs were removed by the reactor at HRTs of 48, 24 and 12 hours. However, at 9 hours HRT the total removal dropped slightly to about 87%, and there was a significant accumulation of long chain NPEOs in the system, while the average MW increased to about 643 g/mol (Figure 7.9). Nevertheless, NPEO₁ still persisted in the system during the hydraulic shock loads, but its abundance was lower than under the effect of NPEO_{ave9} shock loads (in Section 7.3.1). Besides, given the same surface loading rate, the reactor achieved better effluent quality under hydraulic shock loads than that of organic (NPEO_{ave9}) shock loads. This is possibly due to the higher fluid velocity and improved mixing in the reactor at shorter HRT, thus improving the mass transfer of NPEO_{ave9} into the biofilm. Previous studies have demonstrated changes in the stratification of bacterial activity and community structure in an MABR under different fluid velocities (Cole *et al.*, 2004). Similarly, Li *et al.* (2008a) found that at higher up-flow fluid velocities in a hollow fibre MABR acetonitrile removal was enhanced as a result of improved mass transfer and biofilm activity.

Despite the excellent removal of NPEO_{ave9} achieved in this study, the intermediates of NPEO₁ still persisted over the long term. It is known that commercial mixtures of NPEOs contain various isomers of NP having a highly branched alkyl chain. The branched alkyl chain, especially the quaternary carbon (tert-butyl) structure at the end of the alkyl group, could lead steric hindrance or a shielding effect that inhibits biological attack and biotransformation of the alkyl group (Tanghe *et al.*, 1999). On the other hand, the EO counterparts are far more susceptible to bacterial attack and biodegraded rapidly. In an aerobic environment, oxidation/carboxylation of EO counterparts may produce acidic NPECs. In some cases, both the alkyl group and EO side chain can be oxidised. These dicarboxylic intermediates are referred to carboxyalkylphenoxy ethoxy carboxylates (CAPECs). Indeed, the apparent detection of CAPECs which represented up to 63% of the NPEO breakdown products in sewage effluent by Ding *et al.* (1996) has prompted a new understanding that perhaps there are still more intermediates of NPEOs to be identified. In addition, CAPECs were also shown to be highly persistence in a biodegradation study (Di Corcia *et al.*, 1998). Unfortunately, the NPECs and CAPECs were not detected or quantified by our current analytical techniques. The identification and quantification of other intermediates, including CAPECs, has been uncertain and difficult to achieve due to the lack of pure standards. Although the synthesis of authentic standards of CAPECs have been carried out in previous studies, the whole reaction scheme was chemical intensive and time consuming (Cheng *et al.*, 2006). In addition, a successful identification of CAPECs was normally performed using high sensitivity analytical techniques such as LC-MS/MS with an electrospray (ES) source (Jonkers *et al.*, 2001). Despite its recognisable persistency, the toxicity and estrogenicity of this group of intermediates are still not fully understood. However, it is safe to argue that the CAPECs, if present in the present MABR, would not impede the overall reactor performance (i.e. based on stable reactor operation in terms of total

NPEOs and soluble COD removal over long term operation). Similarly, Lozada *et al.* (2004) found that constant feeding of NPEO₁₀ to a laboratory scale activated sludge reactor did not largely affect organic carbon removal, and the formation of intermediates (NPEO₁₋₃ and NP) were not toxic to bacteria. In fact, Fluorescence In situ Hybridization (FISH) analysis revealed that bacterial community composition was significantly shifted, and they suggested that the beta subclass of Proteobacteria may have a specific role in NPEOs biodegradation. In agreement with our present study, carboxylic acid analogs of NPEOs were also not detected. Unfortunately, the authors (Lozada *et al.*, 2004) did not monitor in depth the evolution of intermediates over reaction courses. The biofilm population was, however, not analysed in the present study. A qualitative analysis of the membrane biofilm, based on scanning electron microscopy (SEM), will be discussed in the next section.

7.3.3 BIOFILM ANALYSIS

Bacterial attachment to a membrane surface is enhanced by modification of the membrane surface. In the literature this can be done either by the grafting of functional groups (e.g. tertiary amino groups), or by attachment of porous structures (e.g. non-woven material) onto the membrane surface (Terada *et al.*, 2004; Gong *et al.*, 2007; Alvarez *et al.*, 2008). Non-woven material, for instance, provides a large specific surface area and porosity, high immobilization potential and diffusivity of substrate, thus protecting the biofilm from shear force and complete sloughing (Gong *et al.*, 2007). In the present study, a microporous polypropylene membrane with a non-woven polypropylene scrim heat-sealed to the surface (made by 3M Corporation, St Paul, MN) was used as a biofilm support and aeration device in the MABR. Figure 7.10 contains images of the clean scrim structure and a close up of the non-woven scrim.

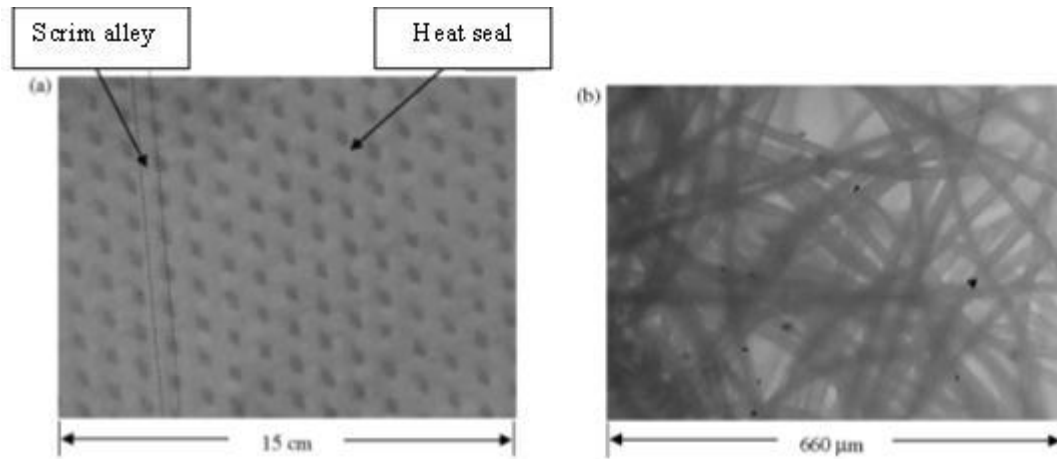
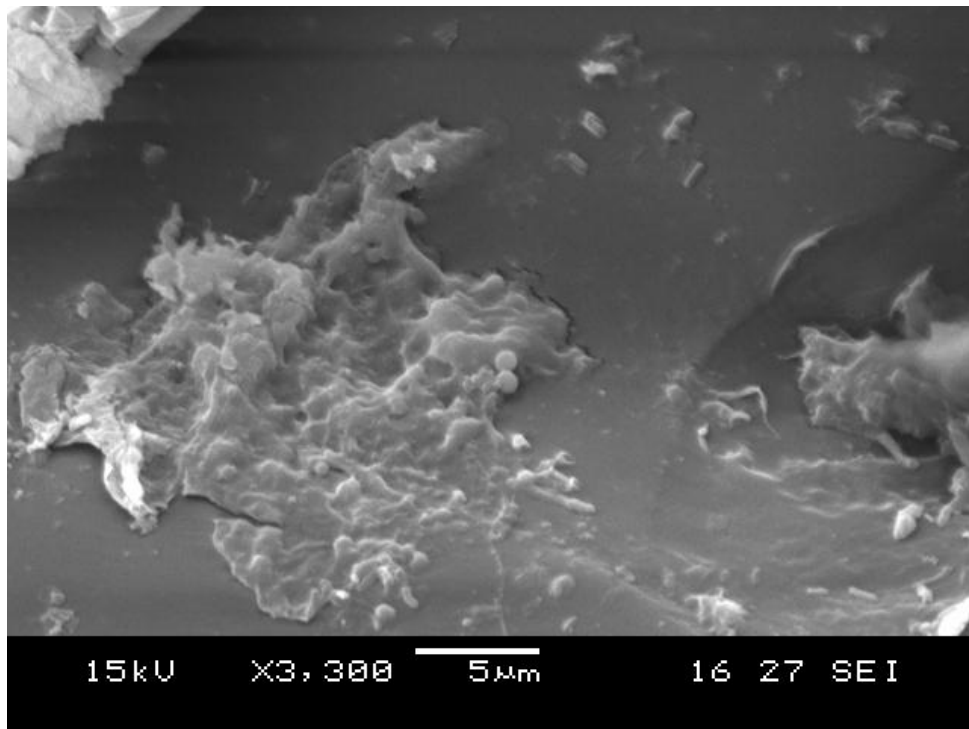


Figure 7.10: Pictures illustrating (a) the scrim/heat seal patterning of the membrane, and (b) the scrim structure (obtained from Alvarez *et al.*, (2008)).

The oxygen mass transfer behaviour of this membrane was characterised by Alvarez *et al.*, (2008), and according to the authors, the presence of the scrim did not significantly impede gas transfer. The scrim, with an average height of $470 \pm 40 \mu\text{m}$; would be ideal for bacterial immobilisation on the membrane surface. A visual observation of biofilm formation during reactor operation was not possible due to technical constraints, ie. a solid metal reactor top. In this study, a qualitative analysis of the biofilm attached to the membrane surface after 500 days of operation was carried out by SEM, and the images are shown in Figure 7.11.

It is important to note that the procedure, which involved dehydration and fixation of the biological sample, may change some of the characteristics of the initial sample. In addition, the loss of biomass because of the preliminary washing step was unavoidable. Hence, quite significant amounts of the biofilm were lost during the process. Based on visual observation after the reactor was sacrificed, the biofilm coverage of the membrane was greater than the scrim height.

(a)



(b)

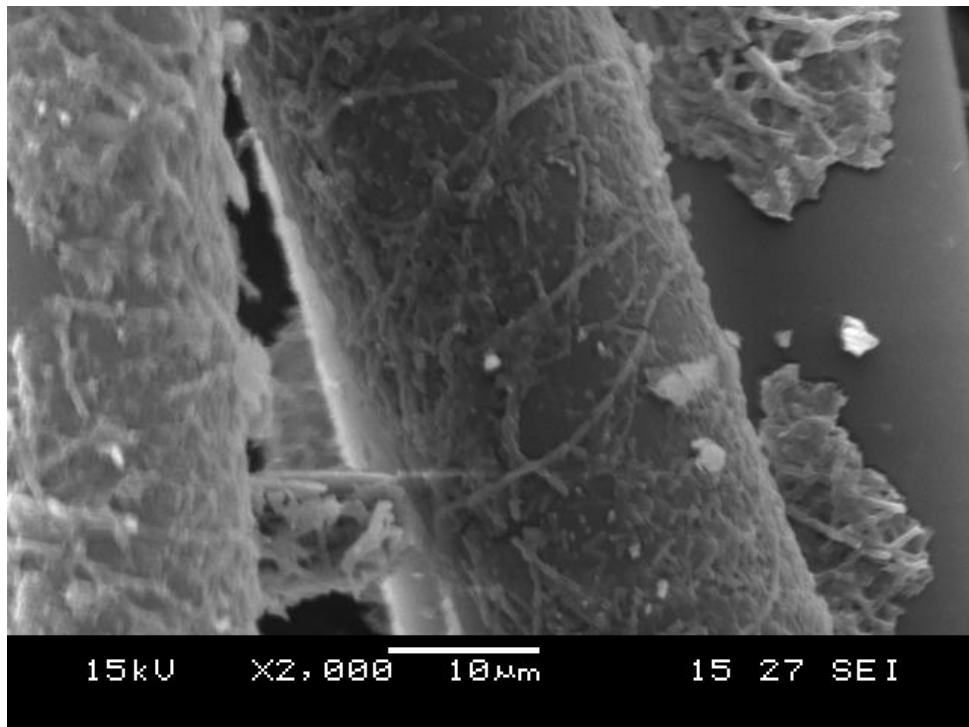


Figure 7.11: SEM images of the biofilm forming after 500 days of operation. (a) Biofilm formed on the heat seal; (b) Biofilm attached on the non-woven scrim.

From the microscopic observation it was observed that smaller amounts of biofilm were formed on the heat seal surface, probably due to its limited oxygen transfer (Figure 7.11(a)). In contrast, as can be seen in Figure 7.11(b), most of the biofilm is trapped within non-woven fibres, or bound to the fibres. These observations are in accordance with Gong *et al.* (2007), in which the authors found that the bacteria adhere densely on the non-woven material (porous polyester with a pyridinium-type polymer), suggesting that the fibrous layer is favourable for bacterial immobilisation. This is because the presence of the scrim significantly improved the specific surface area for biofilm attachment, as well as the porosity for efficient mass transfer.

7.4 SUMMARY

In this study, the MABR was found not to be permanently inhibited by quite large concentration shocks of $\text{NPEO}_{\text{ave9}}$ and hydraulic shock loads, but the reactor took quite a long time to recover. The disruption of performance was less pronounced under the hydraulic shock loads, and this was assumed to be due to improved $\text{NPEO}_{\text{ave9}}$ mass transfer into the biofilm. An exact mass balance for the system was not able to be carried out due to the production of unknown intermediates, an inability to measure the amount of substrates associated with the biofilm, and the lack of a gas capturing device to measure the mass of biogas produced. The critical intermediate, NPEO_1 , accumulated in the system, but its presence did not severely affect the overall reactor performances (i.e. degradation of co-substrates and methane production). Although reactor performance under shock loads was slightly lower than under steady state conditions (i.e. 20 mg/L, 48 hours HRT), the removal of at least 95% of the total NPEOs was easily achieved throughout the 500 days of operation. Furthermore, at 12 hours HRT and 20 mg/L of $\text{NPEO}_{\text{ave9}}$ loading ($1.98 \text{ g/m}^2\cdot\text{d}$), the reactor

was able to achieve more than 96% and 90% of total $\text{NPEO}_{\text{ave9}}$ and COD removal, respectively. Hence, this study has proved the potential of MABRs for the treatment of wastewater containing NPEOs under the long term operation.

CHAPTER 8

MABR CONCLUDING DISCUSSION

NPEO_{ave9} has been fed continuously to an MABR for 500 days. This was done to fulfill the two objectives of this study which are associated with MABR operation and performance. The objectives were:

1. To design and operate an MABR using a gas permeable membrane to achieve complete mineralisation of NPEO_{ave9}, and to monitor the biodegradation intermediates under long term operation.
2. To study MABR stability for NPEO_{ave9} removal under different organic and hydraulic shock loads.

The former objective was designed to establish a pseudo steady state condition of the reactor at a constant 48 hr HRT, whereas the latter objective was aimed at examining reactor recovery under stress conditions. A summary of the reactor's operational parameters is shown in Table 8.1. The pseudo steady state was achieved on day 136, at a 0.49 g/m²/day and 0.80 kgCOD/m³.d of NPEO_{ave9} surface loading rate and substrate loading rate, respectively. A total NPEOs removal of 99.8% was recorded, and a very low level of NPEO₁ was detected and good removal of soluble COD of at least 82.7% was attained under these conditions. Figure 8.1 shows the distribution of NP and NPEOs oligomers during the 217 days of operation, in which the pseudo steady state zone was highlighted. While prolonging the operational period with an increase in NPEO_{ave9} and hydraulic loading, significant accumulation of NPEO₁ was evident; as depicted in Figure 8.2.

Table 8.1: The summary of reactor's operational parameters.

Chapter	Parameters Varied	Glucose concentration (mg/L)	Yeast extract concentration (mg/L)	NPEO_{ave9} concentration (mg/L)	HRT (hrs)	NPEO_{ave9} surface loading rate (g/m².d)	Time
Chapter 6	Yeast extract concentration	-	0.4	20	48	0.494	Day 1
		-	0.7	20	48	0.494	Day 8
		-	0.9	20	48	0.494	Day 20
	Glucose concentration	0.4	0.5	20	48	0.494	Day 51
		0.9	0.5	20	48	0.494	Day 91
		1.4	0.5	20	48	0.494	Day 154
Chapter 7	NPEO _{ave9} concentration	1.4	0.5	40	48	0.988	Day 216
		1.4	0.5	80	48	1.975	Day 260
		1.4	0.5	20	48	0.494	Day 290
	HRT	1.4	0.5	20	24	0.988	Day 308
		1.4	0.5	20	12	1.975	Day 341
		1.4	0.5	20	48	0.494	Day 380
		1.4	0.5	20	12	1.975	Day 418
		1.4	0.5	20	48	0.494	Day 437
		1.4	0.5	20	9	2.617	Day 460 - 500

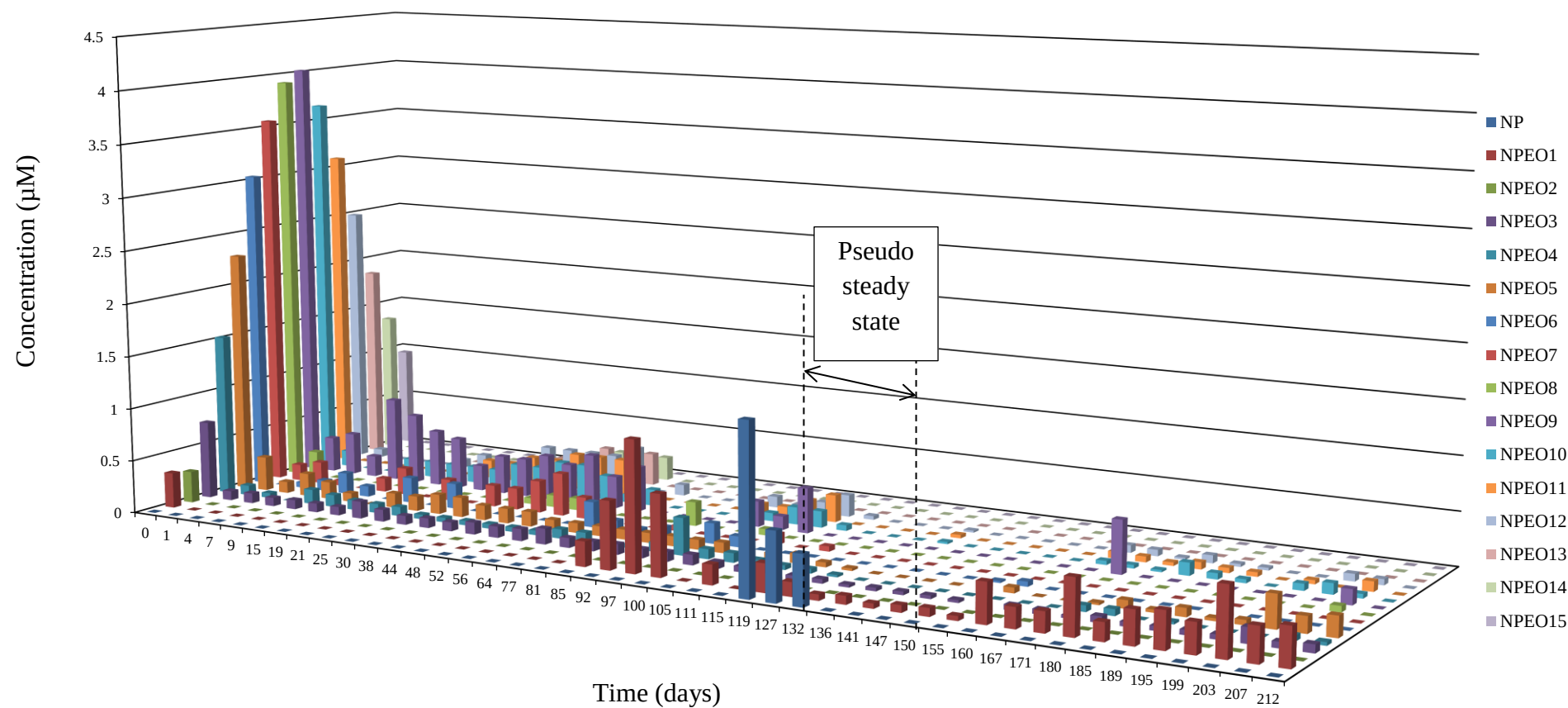


Figure 8.1: NP and NPEOs oligomers distribution at constant $\text{NPEO}_{\text{ave9}}$ loading.

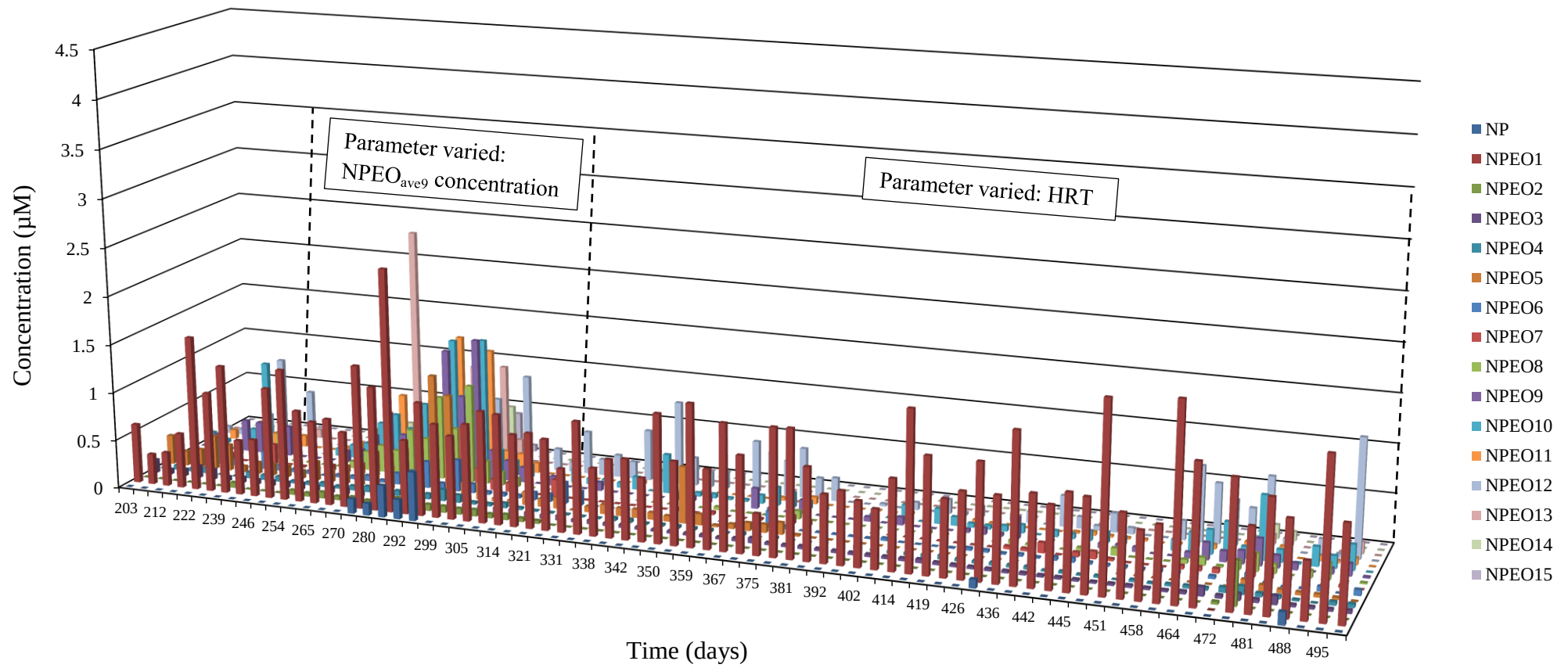


Figure 8.2: NP and NPEOs oligomers distribution at different NPEO_{ave9} loadings.

To simplify the discussion, the average concentration of all nonylphenolic intermediates were calculated over a 20 day period after each parameter change, and the results are summarised in Figure 8.3. In the figure, the average total concentration of NPEOs in the effluent, and the average concentration of its four main groups including (1) critical intermediates of NP and NPEO₁, (2) NPEO₂₋₆, (3) NPEO₇₋₁₁, and (4) NPEO₁₂₋₁₅ are illustrated at different operational periods, at varying NPEO_{ave9} concentrations and HRT. The total removal of NPEOs largely declined with shock loadings of NPEO_{ave9} at long HRTs of 48hr (period I; surface loading rate of 1.98 g/m².d). Nevertheless, given the same surface loading rate, the impact was less pronounced at a lower HRT, as featured in period K and M in the figure. This was assumed to be due to improved NPEO_{ave9} mixing and mass transfer into the biofilm. Despite the slow MABR recovery from stress conditions (shock loads), the reactor achieved more than 95% total NPEOs removal (except for one period of disruption in which only 66% removal was achieved with a 80 mg/L organic shock load) over the 500 days of operation. This shows that the reactor was not permanently inhibited by NPEO_{ave9} shock loads, thus demonstrating the potential of MABRs for the treatment of wastewater containing NPEOs under long term operation. The good MABR performance can be attributed to:

1. The source of biomass obtained from the STP being already acclimatised to nonylphenolic compounds, and the enzymes required for NPEOs metabolism were already induced. Results from our batch tests (in Chapter 5) have also shown the rapid degradation of long chain NPEOs after one day of incubation. Nevertheless, the batch system with anaerobic-aerobic (with air) conditions only achieved about 89.5% of total NPEOs mass removal after 36 days of incubation, compared to 96.1% (on Day 1) and 99.8% (at pseudo steady state) in the MABR. This shows that the MABR can enhance biodegradability in compared with batch tests.

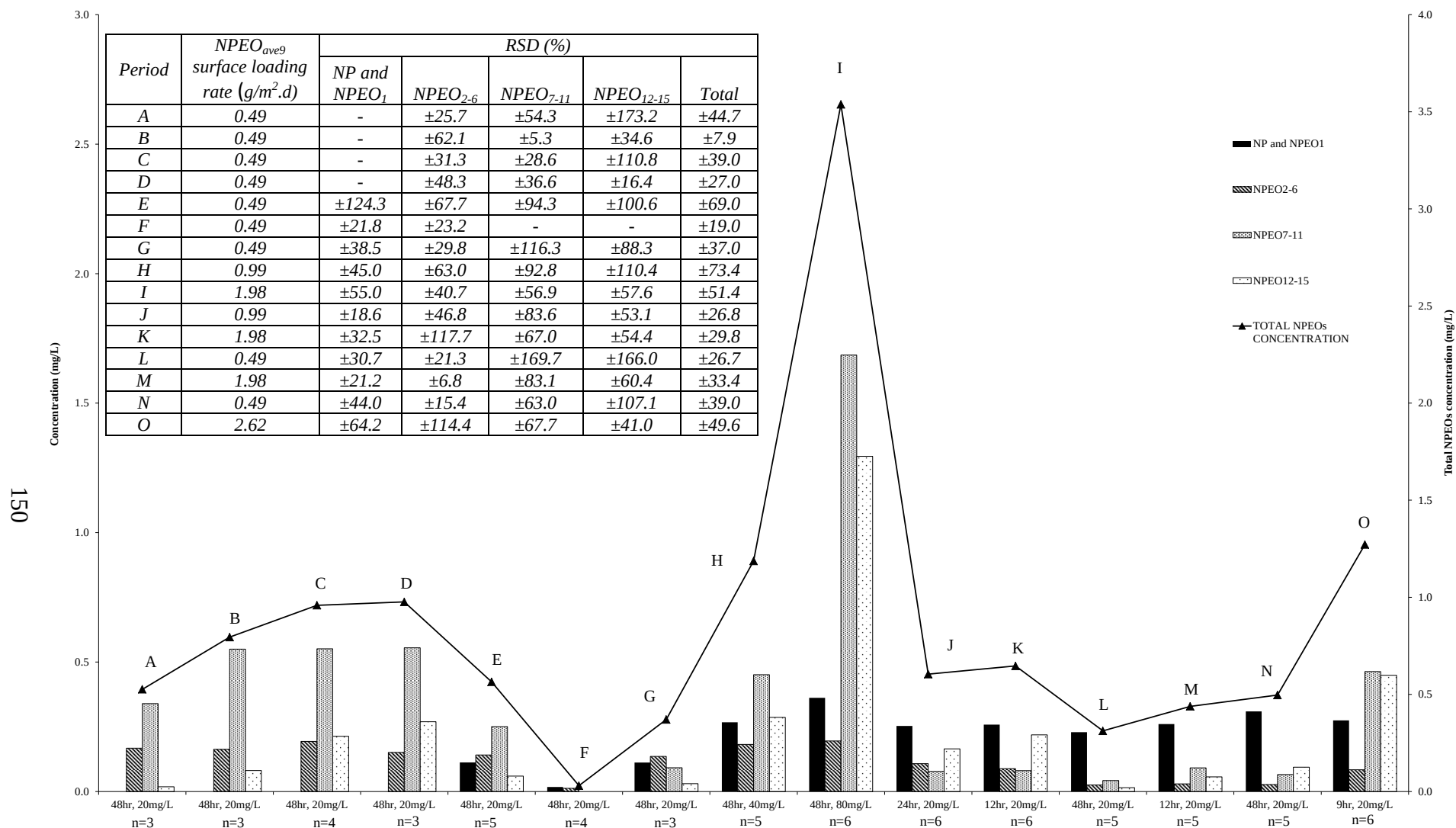


Figure 8.3: The average concentration of NPEOs intermediates over a 20 day period after each parameter change (insert: %RSD).

2. Efficient oxygen transfer into the biofilm that enhanced the aerobic degradation of intermediates. Compressed air was introduced at a constant pressure of 15mbar. Although the rate of oxygen transfer through the biofilm was not measured in this study, it was observed that a temporary disruption of the compressed air supplied on Day 239 reduced the removal of long chain NPEOs.
3. Bioaccumulation, in which NPEOs initially partition from the bulk aqueous phase onto the bacterial cells before intracellular biodegradation takes place slowly, at a level that induces no significant toxicity to the cells. The accumulation of NPEO₁ in the reactor indicated that NPEO_{ave9} were biodegraded through the most common NPEOs pathway (hydroxyl shift mechanism) in which bacterial attack on a molecule's ethoxylate side chain forming corresponding shortened NPEOs. Nevertheless, the presence of these intermediates did not appear to inhibit overall reactor performance. Provided that the bioaccumulation ends up with compounds being metabolised, it is hypothesised that the NPEOs removal in our MABR is more biological than abiotic.
4. The NPEOs were synergistically degraded in the presence of additional organic matter such as yeast extract and glucose, indicating the possibility of cometabolism.
5. The biofilm acts as a protective barrier to bacteria within the biofilm from liquid shear, and allows stable process performance over the long term and during a sudden decrease in the HRT. In the hybrid MABR, mixed bacterial stratification (aerobic/anaerobic) occurred, and this adhered densely to the non-woven scrim. The presence of the scrim significantly improved the specific surface area for biofilm attachment, as well as the porosity for efficient mass transfer. The immobilisation of bacteria on the membrane prevented an excessive sloughing of the biofilm during a sudden decrease in HRT. In addition, given the highest influent and recirculation flow

rate of 0.36 mL/min and 70 mL/min, respectively, influent by-pass was assumed to be negligible. The substrates diffused into the biofilm from the bulk liquid via a concentration gradient, and degradation products diffused back into the bulk liquid for discharge with the effluent.

It is known that EDCs and toxic compounds can pose a threat to the environment, even at very low concentrations, and hence in bioreactor studies very high levels of removal (i.e. > 99%), are desired. On top of that, the proposed Environmental Quality Standards (EQS) for NP based on Directive 2008/105/EC are 0.3 µg/L and 2.0 µg/L for annual average (AA) and maximum allowable concentration (MAC), respectively. By analysing Figure 8.1 and 8.2, the frequency of detectable NP in this study was only 8.8% of all data points, but at concentrations of 19 to 340 µg/L which exceed the proposed standard. However, the method detection limit (MDL) and method quantification limit (MQL) developed for NP in this work was only 1.6 µg/L and 5.6 µg/L, respectively. Thus, our analytical methods were not adequate enough to detect the NP at very low levels in order to meet the stringent EC standards. The analyses, however, were sufficient for monitoring the formation of intermediates in our biodegradation studies, to meet the objective of this study. Besides, for ease of monitoring, high concentrations of NPEO_{ave9} were fed in the reactor (20 to 80 mg/L), which were much higher than the NPEOs concentrations found in the environment.

Nevertheless, the bioreactor performance is frequently limited by various interrelated factors, including biomass, mass transfer, aeration, temperature, toxicity of the compounds fed, and reactor design. Table 8.2 shows studies on NPEOs removal in bio-engineered systems; as can be seen, the degree of NPEOs removal varies between different types of reactors. Apart from the reactor design and process conditions, one of the main reasons for

this is the use of different group of NPEOs oligomers, either the long chain (i.e. NPEO₁₀), middle length (i.e. NPEO_{ave5}), or short chain (i.e. NPEO₁₋₃) oligomers. The MABR performed better than the conventional biofilm reactor by Di Gioia *et al.*, (2009) and Goel *et al.* (2003), while the superior performance observed by Bertin *et al.* (2007) was due to the use of NPEOs as a sole carbon source. To date, none of studies has scrutinised or monitored the biodegradation intermediates of NPEOs in bio-engineered systems.

Table 8.2: Laboratory scale bioreactor studies on NPEOs.

Type of bioreactors	Compounds	Compounds removal	References
Semi-Continuous Activated Sludge (SCAS)	NPEO ₁₀ : 60 mg/L	Total organic: < 96%	Lozada <i>et al.</i> , 2004
Activated sludge	NPEO ₄ : 5 and 10 mg/L	COD: 70-85% NPEOs: 97%	Coello et al., 2009
Upflow Anaerobic Sludge Blanket (UASB)	NP, NPEO ₁₋₃ : 2.49-5.45 mg/L (as NP)	Total NP: 97.9% - 99.9%	Tham and Kennedy, 2005
Sequencing batch reactor (SBR) and Membrane bioreactor (MBR)	NP, NPEO ₁₋₂	NP removal: 85%	Zhou <i>et al.</i> , 2011
Packed-Bed Biofilm Reactors (PBBR)	NPEO _{ave5} and NPEO _{ave1.5} : 30-90 mg/L	NPEO _{ave5} : 77-99% NPEO _{ave1.5} : 97-98%	Bertin <i>et al.</i> , 2007
Packed-Bed Biofilm Reactor (PBBR)	NPEO _{ave5} : 60 mg/L	NPEO _{ave5} : 48-74%	Di Gioia <i>et al.</i> , 2009
Biofilm reactor	NPEO ₁₀ : 5 mg/L	NPEO ₁₀ ; oxic: 50-70% , and anoxic: 30 to 50%	Goel <i>et al.</i> , 2003
MABR	NPEO_{ave9}: 20-80 mg/L	NPEOs: 66-99.8% COD: 61-94%	Current study

Although the current MABR study is not comprehensive enough to identify some of the suspected intermediates, and to propose a novel biodegradation pathway, the results have shown that the reactor was stable under the continuous loading of NPEOs, and achieved a high degree of carbonaceous and NPEOs removal, and produced a significant amount of biogas. In fact, the MABR is a good reactor that provides a multilayered biofilm to degrade refractory compounds under both anaerobic and aerobic conditions. Nevertheless, the reactor deserves further modification and optimisation to overcome some of its main issues, such as the recovery time after shock loads and accumulation of NPEO₁. Future recommendations for further work will be addressed in the following Chapter.

❧ CHAPTER 9 ❧

CONCLUSIONS AND RECOMMENDATIONS

This study was conducted primarily to investigate the biodegradation of nonylphenol ethoxylates (NPEOs) in a laboratory scale flat sheet membrane aerated biofilm reactor (MABR). Prior to this, considerable effort was expended on developing comprehensive analytical techniques to detect and quantifying compounds which may have been present in the reactor. In addition, batch microcosm studies were also carried out on the compounds of interest to screen and evaluate their biodegradation behaviour in batch environments, and to assess their suitability for simultaneous anaerobic and aerobic metabolism in an MABR. In this chapter, the study objectives will be evaluated in terms of what has been achieved, and some of key findings of this study will be discussed. In addition, the limitations of the study and recommendations for future work will be highlighted.

9.1 CONCLUSIONS

9.1.1 ANALYTICAL METHOD DEVELOPMENT FOR NPEOs AND INTERMEDIATES

The first objective of this study was to develop comprehensive analytical methods for the determination of NPEOs and their intermediates in solid and aqueous samples, as depicted in Figure 9.1. A commercial sample of IGEPAL CO-630 (denoted as NPEO_{ave9}, because it had an average of 9 ethylene oxide (EO) units amongst its oligomers) was used as a primary compound. The mixture of oligomers was successfully separated according to their

respective EO units (from 1 to 15 units) on a TSK GEL Amide-80 HPLC column (Tosoh) under normal phase elution, and determined by UV detection at a 277nm wavelength in less than 27 min of retention time. This served as a basis to determine the individual oligomers distribution, and average moles of EO in standard solution, calculated according to Wang and Fingas (1993). The average EO number obtained, 8.73 (0.14% RSD; n=3), was close to the values stated by the manufacturer (i.e. 9) suggesting that the method was capable of not only separating and determining the EO chain length distribution of NPEO_{ave9}, but also analysing for the formation of degradation intermediates (i.e. shorter chain NPEOs).

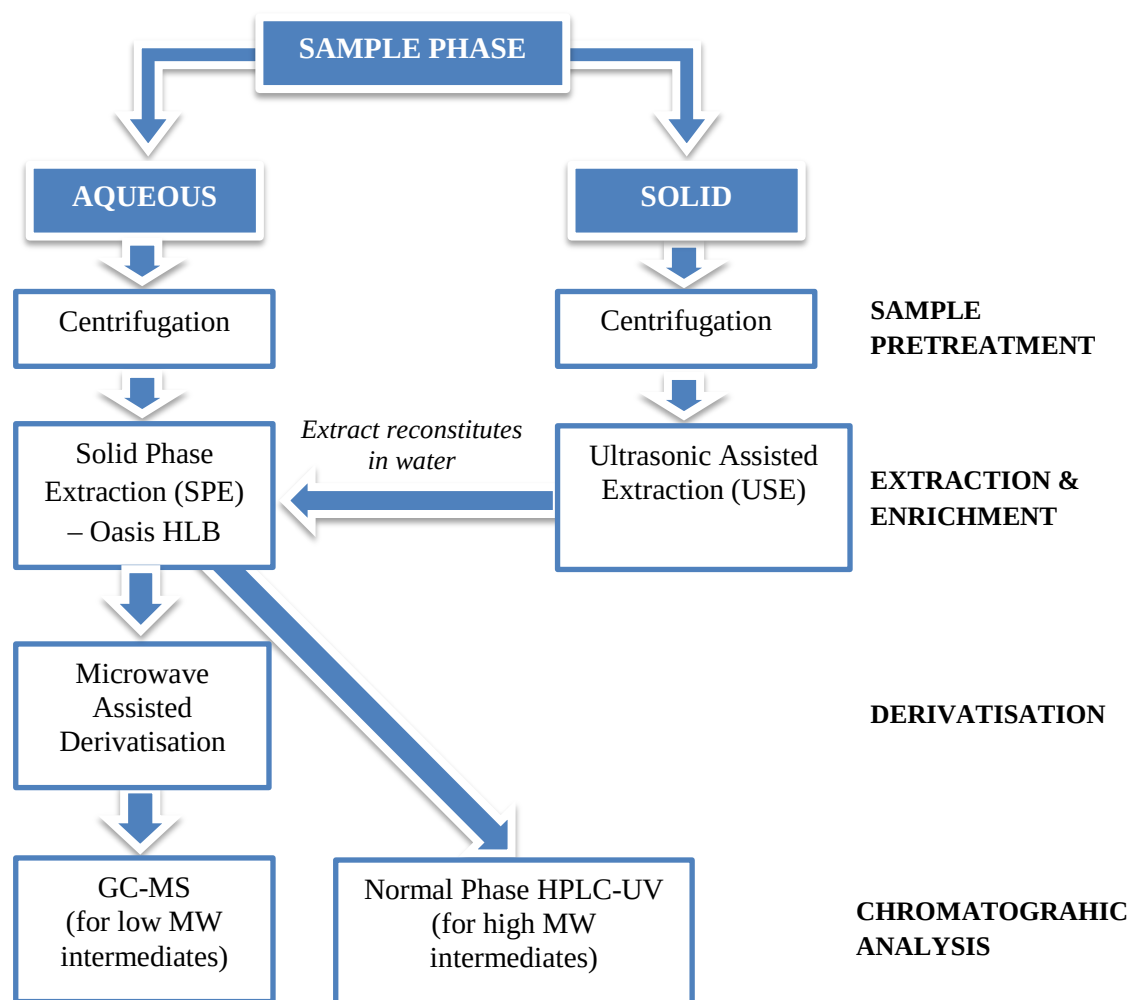


Figure 9.1: Summary of method development.

For the determination of low molecular weight intermediates, such as NP, NPEO₁ and NPEO₂, a GC-MS method was successfully developed. The analysis offered high sensitivity and selectivity provided that sample preparation, extraction and derivatisation were carried out carefully and evaluated prior to the analysis.

9.1.2 BATCH MICROCOSM STUDIES OF NPEOs

The second objective was to investigate NPEO_{ave9} biodegradability in a batch microcosm based on the effect of supplying additional easily biodegradable substrates (glucose and yeast extract), and oxygen. All systems exhibited a similar trend with respect to the changes in oligomeric distribution, in which the molar distribution of the oligomers was gradually shifted from longer ethoxylate chain lengths to shorter ones during the study period. As a result, NPEOs with a low number of ethylene oxide (EO) units, especially NPEO₂ and NPEO₃, predominated at the end of the test period, and these metabolites were far more persistent than their parent compound. This successive excision of the ethoxylate chain is believed to proceed according to the non-oxidative hydroxyl shift mechanism, as proposed by John and White (1998). After 5 days of incubation under anaerobic conditions, oxygen was introduced to simulate an aerobic environment in the inocula. This was also commensurate with MABR microenvironments (simultaneous anaerobic-aerobic conditions), and to investigate the suitability of such conditions for NPEOs biodegradation. A significant disappearance of NPEOs intermediates was observed in the microcosm supplied with oxygen as an electron acceptor, accounting for 76% (air-fed) and 79% (pure oxygen-fed) total molar removal, as compared to 60% in a fully anaerobic system. This confirms previous studies which observed that the degradation of NPEOs intermediates were more favorable under aerobic conditions (Ying *et al.*, 2002; Montgomery-Brown and Reinhard, 2003). Based on

our current knowledge, no study has been performed previously on the simultaneous anaerobic-aerobic metabolism of NPEOs in batch culture. Moreover, no significant difference was noticed between the inoculum supplied with air and pure oxygen, indicating that a sufficient amount of oxygen had been added to the bottles to boost the growth of aerobic bacteria.

9.1.3 THE TREATMENT OF NPEOs IN AN MABR – A PRELIMINARY STUDY

The third objective was to design and operate an MABR using a gas permeable membrane to achieve complete mineralisation of $\text{NPEO}_{\text{ave9}}$, and to monitor the biodegradation intermediates under long term operation. The overall reactor performance was satisfactory. In the hybrid MABR, mixed bacterial stratification (aerobic/anaerobic) was developed in a thick biofilm. Progressive production of CH_4 was evident over the operational period, which corresponded to the growth of slow growing methanogens and the increase in biofilm thickness. In addition, total NPEOs removal of more than 90% was observed in the reactor throughout the operational period, even in the first week of operation. A brief discussion on overall reactor performance was presented in Chapter 8, under the MABR concluding discussion.

9.1.4 THE TREATMENT OF NPEOs IN AN MABR – THE EFFECT OF ORGANIC AND HYDRAULIC SHOCK LOADS

The final objective of this study was to study MABR stability for $\text{NPEO}_{\text{ave9}}$ removal under different organic and hydraulic shock loads. Despite its slow recovery from stress conditions (shock loads), the reactor achieved more than 95% total NPEOs removal (except

for one period of disruption, in which only 66% removal occurred under a 80 mg/L organic shock load) over the 500 days of operation. For instance, at 12 hours HRT and 20 mg/L of $\text{NPEO}_{\text{ave9}}$ loading ($9.88 \text{ g/m}^2\cdot\text{d}$), the reactor was able to achieve more than 96% and 90% of total $\text{NPEO}_{\text{ave9}}$ and COD removal, respectively. It was also shown that the disruption of performance was less pronounced under the hydraulic shock loads, and this was assumed to be due to improved $\text{NPEO}_{\text{ave9}}$ mass transfer into the biofilm. On top of that, the MABR was found not to be permanently inhibited by $\text{NPEO}_{\text{ave9}}$ shock loads, and this study has proved the potential of MABRs for the treatment of wastewater containing NPEOs under long term operation. From the SEM analysis, bacteria were seen to be adhering densely to the non-woven scrim. The presence of the scrim significantly improved the specific surface area for biofilm attachment, as well as the porosity for efficient mass transfer. The immobilisation of bacteria on the membrane prevents an excessive sloughing of the biofilm during a sudden decrease in the HRT.

9.2 RECOMMENDATIONS FOR FUTURE WORK

9.2.1 IDENTIFICATION OF TRACE INTERMEDIATES

The study would have led to more insightful results if the degradation routes of NPEO_1 and NP could have been monitored more in depth. It had been previously shown in past studies that NPEO_1 can be aerobically degraded to NPEC_1 , and finally, to CAPEC_1 ; whereas the aromatic ring on NP can be cleaved to produce hydroquinone and nonylalcohol (NOH). The identification of these intermediates could be possible by careful evaluation of extraction strategies and the use high sensitivity analytical instruments. It was learnt that purification of extracts is important to improve detection sensitivity of subsequent analysis.

An alternative SPE sorbent, the Oasis MAX, is a strong anion exchange sorbent that has been successfully used to separate a group of phenols and selected acidic pharmaceuticals. This new generation of sorbent could be evaluated to separate the acidic and alcoholic nonylphenolic intermediates, and then the analytical strategies for each fraction could be optimised differently. GC-MS is a powerful tool to determine the low MW intermediates of NPEOs. However, the equipment is inadequate to detect and quantify the trace aerobic degradation products. This can be overcome by the use of higher sensitivity equipment, such as GC-MS/MS, as well as working closely with an analytical chemist. Proper characterisation of these intermediates enables researchers to understand the extent of NPEOs mineralisation, to ascertain the degree of their persistency and toxicity, and to determine the role of the aerobic layer in degrading those intermediates. In addition, radioactive labelling on the phenol moiety could also provide information on aromatic ring cleavage. This is useful in investigating the role of oxygen in NPEOs mineralisation, especially in batch microcosm studies.

9.2.2 BIOFILM CHARACTERISATION

The activity of the biofilm in the MABR, such as the location of the active layer and oxygen concentration profiles within the biofilm depth, was not determined in this study. Dissolved oxygen profiles within the biofilm can be measured using a Clark type microelectrode. This will enable researchers to gain insights into the biofilm, manipulate the oxygen intra-membrane pressure and the location of active layer, and obtain a complete mass balance for developing a membrane biofilm model based on NPEOs breakdown.

Qualitative analysis on biofilm structure was carried out using Scanning Electron Microscopy (SEM). The working procedure, however, failed to preserve the integrity of the biofilm. The characterisation of biofilm microbial populations was also not possible using this technique. The fluorescent in situ hybridization (FISH) technique can be used for analysing and quantifying the microorganisms involved in NPEOs degradation. The combination of FISH with a confocal laser scanning microscope (CLSM) permits visualisation of the three-dimensional structure of the biofilm, in other words, the spatial distribution of microbial populations in the biofilm. Other than as a prospect for bio-augmentation, molecular biology techniques can help a researcher to monitor the shifts in microbial population under different process conditions. The dynamic changes in the microbial population in the bioreactor over the operational period can be elucidated by polymerase chain reaction (PCR) and denaturant gradient gel electrophoresis (DGGE) techniques.

Nevertheless, the frequent sampling of the biofilm during the operation proved to be difficult due to technical constraints. Thus, specialised techniques have to be developed that enable direct sampling of the biofilm, without disrupting the biofilm structure. Another crucial parameter is the measurement of biofilm thickness; in the present study the biofilm thickness was measured using direct visualisation at the end of the reactor operation. The measurement could have been more accurate under the microscope. In addition, the evolution of biofilm thickness over time can be monitored through a transparent reactor, in which the membrane-attached biofilm image is magnified by projector system. Nevertheless, this will involve a new reactor design issue. The modification of the reactor design should be made based on the studies' objectives.

9.2.3 MEMBRANE MODULE AND REACTOR DESIGN

A hollow fibre membrane module conveys superior surface area per unit of volume compared to that of a flat sheet membrane module. A higher oxygen transfer rate is desirable to enhance the aerobic degradation of NPEOs intermediates. Hence, the current design of the flat sheet membrane can be switched to a hollow fibre configuration, to achieve higher intermediates removal.

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