Unravelling the chemistry behind the toxicity of oil refining effluents: from characterisation to treatment

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UNRAVELLING THE CHEMISTRY BEHIND THE TOXICITY OF OIL REFINING EFFLUENTS: FROM CHARACTERISATION TO TREATMENT

A Thesis Submitted for the Degree of Doctor of Philosophy

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ABSTRACT

Adequate wastewater management is a crucial element to achieve water sustainability in the petroleum refining sector, as their operations produce vast quantities of wastewater with potentially harmful contaminants. Treatment technologies are therefore pivotal for stopping these chemicals from entering the environment and protecting receiving environments. However, refining effluents are still linked to serious pollution problems, partly because little progress has been made in determining the causative agents of the observed biological effects, resulting in non-targeted treatment. Here it is shown that naphthenic acids, which have been reported as toxic and recalcitrant, are important components of refining wastewater resulting from the processing of heavy crude oil and that they have a significant contribution to the toxic effects exerted by these effluents. Furthermore, it was found that their chemical stability makes them highly resistant to remediation using *Pseudomonas putida* and H_2O_2/Fe-TAML (TetraAmido Macrocyclic Ligands) systems under laboratory conditions, and only sequential aliquots of Fe-TAML catalysts and H_2O_2 showed to partially degrade naphthenic acids (50 mg/L) within 72 hours. Results suggest that a combinatorial approach of Fe-TAML/H_2O_2 followed by biodegradation might improve current treatment options, but further optimisation is required for the biological treatment. These results can serve as a starting point for better environmental regulations relevant to oil refining wastewater resulting from heavy crude oil, as naphthenic acids are not currently considered in the effluent guidelines for the refining sector. Furthermore, the degradation of naphthenic acids under mild conditions using Fe-TAML/H_2O_2 systems indicates that these catalysts hold promise for the remediation of refining wastewater in real-life scenarios.

Key words: petroleum refining, toxicity, wastewater treatment, advanced treatment, effluents, effect-directed analysis, naphthenic acids, Fe-TAML, biodegradation, *Pseudomonas putida*

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ABBREVIATIONS AND ACRONYMS

1-EDC·HCl	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
2-NPH	2-nitrophenylhydrazine
AC	Activated carbon
ADME	Absorption, distribution, metabolism, and excretion
AGP	Attached growth processes
amu	Atomic mass units
ANOVA	Two-way analysis of variance
AOP	Advanced oxidation process
API	American Petroleum Institute
AQC	Analytical quality control
ASE	Accelerated solvent extraction
BAF	Bioaccumulation factor
BAT	Best available technologies
BHM	Bushnell-Haas medium
BNA	Basic, neutral, acidic
BOD	Biological oxygen demand
BPBA	Butylphenyl-butanoic acid
BTEX	Benzene, toluene, xylene
CHCA	Cyclohexanecarboxylic acid
COD	Chemical oxygen demand
CONCAWE	Conservation of clean air and water in Europe
CPI	Coalescing plate interceptors
CWA	Clean water act
DAD	Diode array detector
DAF	Dissolved air flotation
DCM	Dichloromethane
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
EC_{50}	Half maximal effective concentration
ECETOC	European centre for ecotoxicology and toxicology of chemicals
EDA	Effect-directed analysis

EE2	17α-ethinylestradiol
EI	Electron ionization
EI-MS	Electron ionisation mass spectrometry
EQS	Environmental quality standard
ESI FT-ICR MS	Electrospray ionisation coupled to Fourier transform ion cyclotron resonance mass spectrometry
ESI-MS	Electrospray ionisation mass spectrometry
EU	European Union
FTIR	Fourier-transform infrared spectroscopy
GAC	Granular activated carbon
GC-FID	Gas chromatography coupled with flame ionisation detector
GC-MS	Gas chromatography coupled with mass spectrometry
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
I.D.	Internal diameter
IAF	Induced air flotation
ICP-OES	Inductively coupled plasma-optical emission spectroscopy
IED	Industrial emissions directive
IPA	Iso propyl alcohol (IUPAC Propan-2-ol)
IPCC	Integrated Pollution Prevention and Control
IS	Internal standard
ISO	International organization for standardization
K_{ow}	Octanol/water partition coefficient
L	Litre
LBT	Luminescent bacteria test
LC-MS	Liquid chromatography coupled with mass spectrometry
LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometry
LLE	Liquid-liquid extraction
LoD	Limit of detection
LoQ	Limit of quantification
m/z	Mass-to-charge ratio
MAC	Maximum allowable concentration
MCL	Maximum contaminant level

min	Minutes
MLLE	Micro liquid-liquid extraction
MOA	Mode of action
MSM	Minimal salt medium
MTBSTFA	N-methyl-N-t-butyldimethylsilyltrifluoroacetamide
MW	Molecular weight
NA	Naphthenic acid
NDIR	Non-dispersive infrared
NIST	National Institute of Standards and Technology
NOx	Nitrogen oxides
NP	Normal phase
NP-HPLC	Normal phase-high performance liquid chromatography
OD	Optical density
OSPW	Oil sands process waters
PAC	Powdered activated carbon
PAH	Polycyclic aromatic hydrocarbons
PBT	Persistence, bioaccumulation, and toxicity
PCB	polychlorinated biphenyls
PFTBA	Perfluorotributyl-amine
PNEC	Predicted no effect concentration
ppt	Parts per trillion
REF	Relative enrichment factor
RP	Reversed phase
RSD	Relative standard deviation
RT	Retention time
RWW	Refining wastewater
SOx	Sulphur oxides
SPE	Solid phase extraction
SS	Suspended solids
TAML	TetraAmido Macrocyclic Ligands
t-BDMS	Tert-butyldimethylsilyl ether
TDS	Total dissolved solids
TEU	Toxicity equivalent units

TIC	Total ion chromatogram
TIE	Toxicity identification evaluation
TOC	Total organic carbon
TSS	Total suspended solids
TTC	Triphenyl tetrazolium chloride
TU	Toxicity units
TW	Tailing water
UCM	Unresolved complex mixture
US EPA	Environmental protection agency of the United States of America
UV	Ultraviolet
VOC	Volatile organic compounds
WEA	Whole effluent assessment
WET	Whole effluent toxicity
WFD	Water framework directive
WHO	World health organization
WWT	Wastewater treatment

CHAPTER 1
INTRODUCTION

1.1 Background of the study

Beyond fuels for cars, trucks, and planes, it can be easy to overlook how many petroleum-derived products exist. But there are thousands of products made from petroleum with essential functions in our daily lives, including plastics, synthetic rubber, asphalt, and dyes. Petroleum refineries play a central, yet invisible role in our lifestyle by transforming crude oil, which has no practical value, into energy and raw materials that serve as the basis for our daily activities. Numbers can better explain such dependency: by 2016, an average of 15 million $m^3$of crude oil was consumed per day around the world[1], and sectors like transportation and fishing have a >90% dependency on oil in relation to their total fuel consumption[2]. However, petroleum refining is in the centre of discussion when it comes to sustainable practices because of the high water consumption and generation of vast amounts of waste, especially wastewater containing potentially toxic substances. It is estimated that the processing of each m^3 of feedstock requires between 2.0 and 2.5 m^3 of water[3,4], for a total of 5 million m^3 of RWW produced per day globally[5]. In the US alone, refineries reported discharge volumes of wastewater ranging from 5 to 72 million m^3 per year[6], and there is a substantial body of evidence that suggests that refining effluents can have harmful effects on different aquatic species at various trophic levels[7–14]. In theory, wastewater treatment should prevent biological effects on receiving environments resulting from the discharge of refining effluents by functioning as a barrier of contaminants; however, traditional treatment processes have failed to remove pollutants efficiently. The same characteristics that make petroleum so valuable are the ones that make it a struggle to tackle: chemical diversity and resistance to degradation. Modern refinery wastewater treatment plants are generally effective in removing suspended oil and suspended solids, but toxic and hydrophilic contaminants are likely to resist treatment and reach waterways[15]. It is, therefore, necessary to redesign treatment plants for these to reduce the concentration of toxic chemicals to non-hazardous levels, but a good understanding of the chemical and toxicological properties of the constituents of wastewater is essential to develop effective treatment systems.

A problem arises at this point. Harmful effects have been reported for refining effluents in different geographical areas[7,8,12,13,16–18], but it is not fully understood what exactly is causing these biological effects on receiving environments. When toxic effluents are investigated, a range of contaminants appear to be involved, including metals and polycyclic aromatic hydrocarbons (PAHs), but the extensive report of "organics" as essential contributors to overall toxicity demonstrates that there is still a gap in knowledge that hampers the development of

effective treatment plants. Figure 1 provides an overview of some of the previous studies characterising refining effluents beyond bulk parameters and linking toxic effects with chemical components, suggesting that a more in-depth look at organics might reveal toxicants not reported in the past.

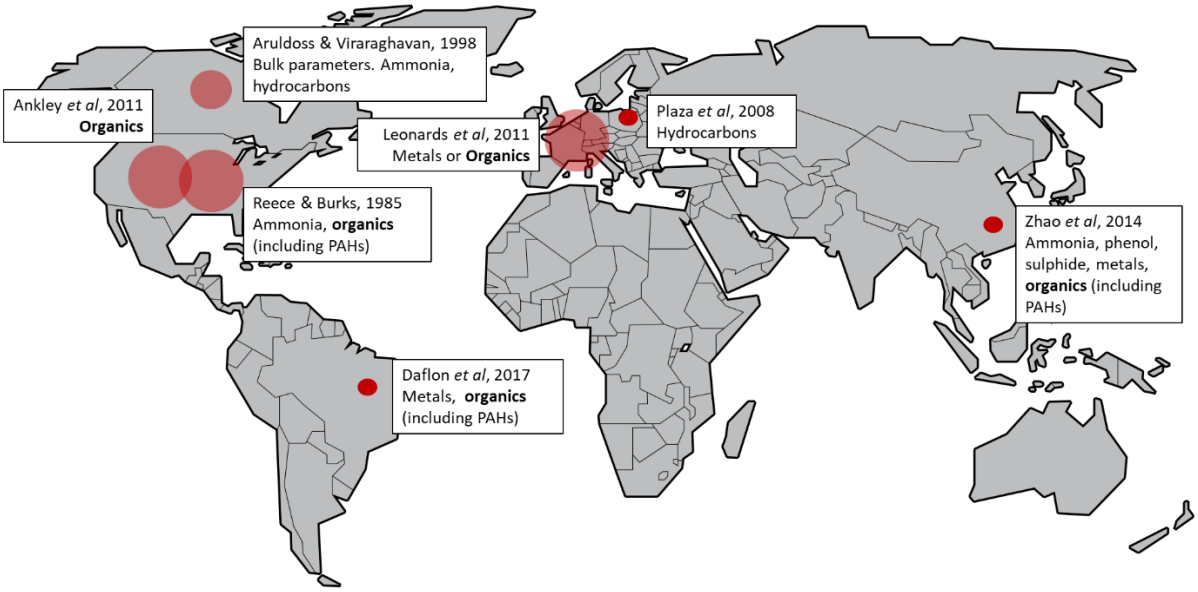


Figure 1. Previous studies linking biological effects of refining effluents to chemical components and their corresponding findings

From an ecological perspective, the interaction between contaminants is critical to understand the biological effects exerted by industrial effluents, therefore an investigation of individual chemicals does not necessarily provide all the information needed for a complete risk assessment. From a wastewater treatment perspective, however, it is an advantageous approach because treatment technologies are developed based on the physicochemical characteristics of contaminants to be removed, and different techniques are assembled in specific sequences within treatment plants to maximise removal efficiency. Hence, it is critical to identify the chemicals behind the biological effects exerted by refining effluents to develop effective treatment technologies.

Removal efficiency, however, is not the only aspect needed from wastewater treatment technologies. Low-cost and sustainability are essential characteristics of treatment technologies for these to be implemented and maintained in the long term. Research in treatment

technologies is continuously proposing emerging treatment methods using a wide range of approaches, but many of these are cost-prohibitive, particularly for low- or middle-income countries, which results in unequal access to safe water. It is estimated that 90% of all wastewater in these countries is discharged to waterways without any treatment, thus threatening food and water security[19]. Therefore, the role of research is crucial for the development of treatment technologies with upscaling potential that can be viable for all. In this context, treatment technologies conducted under simple and mild conditions (ambient temperature, neutral pH) are ideal; as catalysts can help achieving such conditions, they are seen as important energy savers that translate into lower costs. Bacteria are essential biocatalysts within this field because they are considered environmentally friendly, and the flexibility of microbial metabolism allows the cost-effective treatment of polluted water with a variety of contaminants. The use of microbial consortia for the biotransformation of pollutants has provided promising results for the degradation of petrochemicals[20–24], with some species showing exceptional metabolic potential for bioremediation purposes. *Pseudomonas putida*, for instance, is considered a paradigm of metabolically versatile microorganisms and has the advantage of easy handling and low nutritional requirements under laboratory conditions[25–28], which increases its upscaling potential.

Alternatively, advanced oxidation processes (AOPs) are considered the most promising and competitive methods for advanced treatment of industrial effluents because they can destroy recalcitrant compounds[29–31]. Two aspects make AOPs especially attractive: (i) H_2O_2 can be used as oxidant, which has a high active oxygen content (47% compared to 33% of ozone) and is inexpensive and applicable in industrial settings[32]; and (ii) catalysts can be used for more efficient oxidation reactions, improving operating times, capital costs, and overall economic and environmental features of treatment technologies[33]. When combined, catalyst-mediated oxidation processes using hydrogen peroxide as oxidant have great potential for application in large-scale, especially when the amount of catalyst required for successful oxidation is low. For example, Fe-TAML (TetraAmido Macrocyclic Ligands) catalysts were developed as synthetic enzymes that mimic peroxidase enzymes[34] and have been successfully used for the removal of a variety of refractory organics in wastewater, including pharmaceuticals[35], nitrophenols[36], halogenated phenols[36,37], estrogenic compounds[38,39], molluscicides[40,41], and dyes[42–45]. The catalytic cycle of TAML molecules initiates with their activation in the presence of H_2O_2 (oxidant); the active catalyst then oxidises a substrate (*i.e.* contaminant) and, after a number of oxidation reactions, undergoes suicidal inactivation. Fe-TAML catalysts are used at

concentrations that range from nM to µM, which makes them attractive for upscaling purposes as an advanced treatment with potential application in the refining sector.

1.2 The case study

Our study site for the collection of refining effluents was an oil refinery located in the city of Barrancabermeja, Colombia. The site has an installed capacity for processing nearly 40 000 m^3 of feedstock per day, and is equipped with an on-site wastewater treatment plant. The refinery discharges treated effluents directly into Magdalena River via pipelines, three of which were sampled for this study (Figure 2). The selection of this site as the case study stems from the occurrence of several fish kills downstream of the oil refinery that might be an indication of the discharge of refining effluents of concern. This pollution problem has had significant economic consequences for the local population because numerous fishing communities are settled in the rural areas of Barrancabermeja, and there are potential health risks because the Magdalena River is a major source of drinking water and fish for human consumption. Previous studies have reported the presence of metals and PAHs in sediments from the Magdalena river, which exerted various toxic effects on *Caenorhabditis elegans*[46,47]. In particular, a study showed that sediments collected in the surrounding area of this petroleum refinery presented exceptionally high pollution risk for metals, and high expression of stress response genes and high affectation of biological parameters (lethality, growth, and locomotion) in *C. elegans*[46], suggesting that refining effluents are an important source of pollution in the area. However, despite these findings and the recurrent fish kills in the area, there are no previous studies on the toxicity and chemistry of refining effluents from this refining site, making this investigation the first one addressing refining effluents in Colombia. It is noteworthy that the multifactorial nature of the quality of refining effluents makes site-specific investigations necessary to reach an appropriate diagnosis, as many factors are known to be partially responsible for the potential toxic effects of refining effluents, including feedstock characteristics, operational units, wastewater treatment technologies, and local environmental regulations.

1.3 Problem statement

The traditional approach for the regulation and control of industrial effluents uses bulk parameters for enforcement purposes, but this approach has failed to provide safe effluents for human populations and wildlife. It has been demonstrated that effluents complying with discharge regulations based on physicochemical standards (*e.g.* conductivity, pH, temperature, total petroleum hydrocarbons) can still exert biological effects on the receiving environment[48],

A.

B.

C.

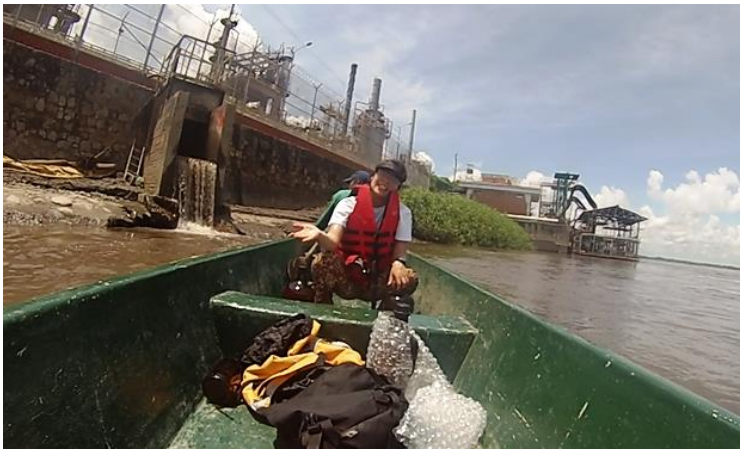


Figure 2. Sampling of refining wastewater discharged into River Magdalena in Barrancabermeja, Colombia. (A) Fishing boats used to locate pipelines for the collection of wastewater samples; (B) Journey upstream of Magdalena River to locate discharge pipelines; (C) One of the three discharge pipelines sampled.

highlighting the need for implementing biological assessments to provide a more holistic characterisation of effluents. Toxicity tests used in combination with chemical characterisation are powerful tools to monitor the quality of effluents, but sometimes the link between these two sets of information is unclear, or simply difficult to establish. In the refining industry, the chemical complexity of effluents has resulted in a weak link between biological effects and causative effects, which in turn has resulted in generic wastewater treatment plants that target mostly suspended oil and suspended particles. The current situation is that toxic hydrophilic chemicals are likely to resist treatment. Very little is currently known about polar organics in refining effluents, hence studying these contaminants could help to establish a stronger link between biological quality and chemical composition.

Numerous emerging technologies have been developed to improve the quality of refining effluents by removing recalcitrant organic pollutants using a variety of approaches, including advanced oxidation processes and adsorption-based technologies. Many of these technologies, however, are cost prohibitive for implementation at large scale. Hence, it is of great interest and vital importance to develop effective and economical methods for the detoxification and removal of toxic refining chemicals from effluents to avoid their discharge into the environment.

1.4 Research Questions

This study aimed to answer the following questions:

- → Can an effect-directed approach help to establish a link between biological effects and causative agents in refining effluents?
- → Is *Pseudomonas putida* suitable for the biotransformation of petrochemicals in laboratory conditions?
- → Does *Pseudomonas putida* have potential for the advanced treatment of refining effluents?
- → Are Fe-TAML/H_2O_2 systems suitable for the advanced treatment of refining effluents to reduce toxicity?
- → What are the conditions required for the oxidation of petrochemicals using Fe-TAML/H_2O_2 systems under laboratory conditions?

1.5 Research objectives

This research aimed to identify toxic organic contaminants in petroleum refining effluents and

to evaluate advanced treatment technologies to reduce acute toxicity of refining effluents.

The specific objectives were to:

1. Characterise refining effluents from the case study site.
2. Identify toxic organics in petroleum refining effluents following an effect-directed analysis using *Vibrio fischeri* as a biosensor.
3. Develop a method to screen for biodegradation potential of petrochemicals in *Pseudomonas putida*.
4. Screen a pool of 86 strains of *Pseudomonas putida* for biodegradation potential of petrochemicals.
5. Develop a method to detect and quantify naphthenic acids in microbiological medium (M9).
6. Evaluate the biotransformation potential of a consortium of *Pseudomonas putida* for the degradation of model naphthenic acids.
7. Evaluate TAML/H_2O_2 systems for the oxidation of model naphthenic acids.
8. Assess the effectiveness of TAML/H_2O_2 systems to clean-up real refining effluents.

1.6 Structure of the thesis

The thesis is divided into six chapters, as follows:

Chapter 1 provides the background of the study, describes the study site, presents the objectives and research questions to be addressed, and outlines the thesis.

Chapter 2 reviews the relevant literature on the processes involved in petroleum refining and the characteristics of refining wastewater, including the main organic contaminants reported in the scientific literature. This chapter also describes the biological assessment of refining effluents and the current and emerging treatment technologies to tackle toxic refining wastewater, and how regulatory systems have evolved towards a more comprehensive approach that led to the inclusion of biological tests to assess the quality of effluents. The missing link between toxicity and treatment of refining effluents is discussed herein.

Chapter 3 describes the characterisation (total organic carbon, pH, metals content) of refining effluents from the study site and the identification of toxic organics following an effect-directed approach using *Vibrio fischeri* as a biosensor and several analytical techniques, such as gas

chromatography-mass spectrometry, high performance liquid chromatography and liquid chromatography-mass spectrometry. Based on the findings of this chapter, naphthenic acids were selected as target contaminants for Chapter 4 and 5. This chapter addresses the specific objectives No. 1 and 2.

Chapter 4 presents the screening for degradation potential of petrochemicals in a pool of 86 strains of *Pseudomonas putida*, which were previously isolated from different environmental sources and identified by the Centre for Systems and Synthetic Biology at Brunel University London. Furthermore, this chapter presents the evaluation of a 3-strain consortium of *Pseudomonas putida* for the degradation of model naphthenic acids. The specific objectives No. 3 to 6 are addressed herein.

Chapter 5 describes the design, experimental setup, and assessment of H_2O_2/Fe-TAML systems for the degradation of model naphthenic acids and the clean-up of real refining effluents, addressing specific objectives No. 7 and 8.

Finally, Chapter 6 provides the general conclusions for this study and some recommendations for further research.

CHAPTER 2

COMPOSITION, TOXICITY, AND TREATMENT OF PETROLEUM REFINING WASTEWATER: A REVIEW

2.1 Introduction

Petroleum-derived products have revolutionised the modern lifestyle by penetrating the market and shaping daily activities, providing 33% of the global energy requirements[49] and numerous raw materials that constitute the feedstock for chemical industries to produce a variety of daily-use goods. Refining separates and transforms chemicals in crude oil into molecules with a practical and economic value by means of multiple transformation and separation processes. However, the collateral damages of refining are significant in relation to the emission of vast amounts of wastewater that can have harmful effects on wildlife and human health. Many factors are known to be partially responsible for the potential biological impacts of refining effluents, such as feedstock characteristics, site-specific operational units and water management practices, wastewater treatment technologies, and local environmental regulations, so the multifactorial nature of the problem requires a multidisciplinary approach.

So far, research on refining wastewater and its impact on aquatic ecosystems has significant gaps that reflect on current wastewater management practices, even though pollution problems linked to the petroleum industry have been on the spotlight for a long time. The growing public awareness of industrial pollution since the 1930s triggered scientific studies aimed at understanding the nature of industrial waste[50], including petrochemical wastewater, but the complex nature of refining wastewater (RWW) was still seen as "impossible to characterise and measure" in the 1960s[51]. Progress in analytical chemistry and improvements in *in vitro* and *in vivo* tests have helped to develop better tools to assess the quality of RWW and its effect on different species, gradually unveiling many aspects that remained unseen in the past. Oil, sulphuric acid, and hydrocarbons were initially identified as the main threats for aquatic ecosystems coming from petroleum refining effluents, but our understanding of RWW has expanded over time to a broader range of toxic chemicals, including phenols, PAHs, metals, and benzene, toluene, and xylene (BTEX). Equally, there is now strong evidence that indicates that RWW can exert a variety of toxic effects, including genotoxicity, carcinogenicity, mutagenicity, and endocrine disruption[7,13,17,52,53]. However, the link between chemistry and observed toxicity is fragile because little progress has been made in determining causative agents.

The regulation and control of industrial effluents have traditionally focused on conventional pollutants, such as chemical oxygen demand (COD), biochemical oxygen demand (BOD), and suspended solids (SS); the allowable discharge limits for these parameters used to be

established based on the treatment technology used. This approach, however, did not stop pollution entering waterways and fish kills were common in lakes and rivers in industrialised areas[54]. The implementation of biological methods in Europe and the United States of America (USA) in the 1980s to help establishing limits for toxic chemicals acknowledged the limitations of traditional physical and chemical-based effluent quality criteria, as a complete characterisation of effluents is analytically challenging, and the prediction of toxic effects from complex combinations of chemicals is currently not possible. The use of whole effluent toxicity (WET) tests on RWW showed that the compliance of chemical parameters did not necessarily correlate with biological quality[48], and made evident that there was a knowledge gap in the components that could be contributing to the observed biological effects.

The missing link between chemistry and observed toxicity has resulted in the use of treatment technologies that do not target toxic refining chemicals. Consequently, treated RWW are not always safe for the receiving environment. An in-depth knowledge of the composition and biological effects of RWW is essential for appropriate design, implementation, and monitoring of wastewater treatment, but such knowledge is not always available. Still, the role of treatment technologies is pivotal to reduce the environmental impact of petroleum refining because they have the potential to provide high-quality effluents that can be either recycled or safely discharged into the environment. Within this context, the biological effects of refining wastewater cannot be discussed in isolation but rather as an element of a multifactorial problem that includes the chemicals in refining wastewater as causative agents and treatment technologies as a final barrier to protect aquatic ecosystems and human health from the potential biological effects of RWW. These three elements are intertwined in a wastewater quality triad (Figure 3) that provide a holistic frame for the study of RWW.

Therefore, this chapter presents a review of the chemistry and toxicity of refining effluents, the treatment technologies currently applied, and the emerging technologies aiming to provide safe refining effluents. Finally, a brief analysis is presented of how environmental regulations have addressed refining effluents over time, and how they function as technical drivers depending on their approach to industrial effluents.

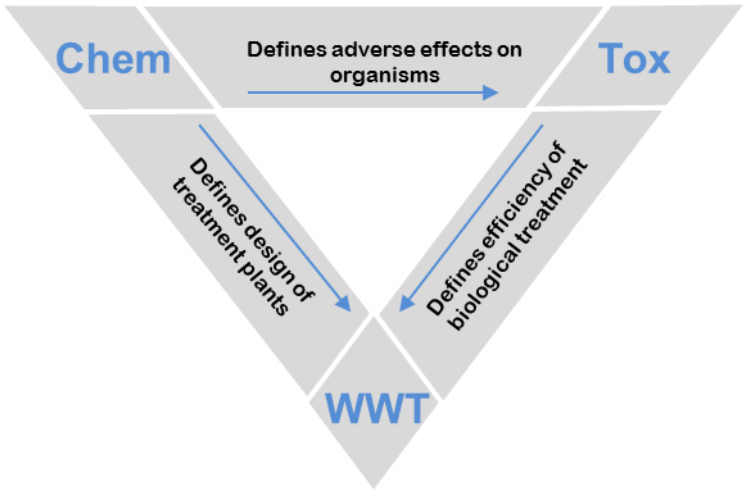


Figure 3. Wastewater quality triad: interaction between chemistry, toxicity, and wastewater treatment (WWT)

2.2 The Refining Process

Petroleum refining is a combination of physical and chemical processes aimed at desalting and separating crude oil into different fractions, and transforming these fractions into marketable products using combination, breaking, and reshaping processes[55]. The nature of the processes involved falls into three main categories: **separation**, **conversion** and **chemical treatment** processes. **Separation** processes lead to separation of different constituents, either by differences in boiling point or solubility. A classic example is distillation, or solvent extraction[56,57]. After separation via distillation, each fraction constitutes a stream which undergoes different **conversion** reactions resulting in transformation either of the structure, the arrangement, or the size of the molecules. Such conversion reactions include (i) decomposition: the breakdown of large molecules into smaller molecules with different processes, (*e.g.* thermal cracking, catalytic cracking) (ii) unification: combination of small molecules to build larger molecules (*e.g.* alkylation, polymerization), and (iii) reforming: alteration of the arrangement of molecules (*e.g.* isomerization, catalytic reforming). The resulting products are further processed by treatment and separation techniques[58]. **Chemical treatment** processes stabilise and upgrade petroleum products by removing undesirable components. For instance, sulphur removal is necessary when sour crude is processed, and it is achieved with the use of hydrogen, forming hydrogen sulphide, which can be easily removed as a gas[57].

The environmental impact of the refining process is caused by two major aspects: the high requirements of water and energy, and the production of vast amounts of potentially hazardous

waste, including wastewater. It is estimated that the processing of a m^3 of feedstock requires between 2.0 and 2.5 m^3 of water[3,4], for a total of 5 million m^3 of RWW produced per day globally[5]. Atmospheric and vacuum distillations alone can produce up to 1.3 m^3 of wastewater per m^3 of feedstock, and catalytic cracking and catalytic reforming produce approximately 0.6 m^3 combined[5]. The RWW generated has a very complex nature stemming from the chemical diversity in petroleum feedstock and the use of operational processes involving various chemicals, such as alkali washes to remove acidic contaminants from distillates, treatment of unfinished products with inorganic acids to improve colour and odour, and metal-containing catalysts[56,59]. Table 1 provides an overview of the principal reactions involved in petroleum refining, including the characteristics of the wastewater generated at each stage, and relevant information about gas emissions and solid waste.

Best practices in the refining sector are aimed at reducing water use, increasing water reuse, and recycling wastewater after treatment, all of which minimise environmental impact and operational costs. Therefore, the role of effective treatment technologies is central to achieving high-quality effluents that are suitable for recycling or discharge, although the use of a combination of at least two advanced treatments is often required for recycling purposes[60,61]. In the past, advances in wastewater treatment have minimised the discharge of priority pollutants into water bodies (*e.g.* chlorobenzenes, PAHs) and reduced wastewater-related human health risks, but the occurrence of contaminants of emerging concern demands ongoing research for the development of efficient and safe technologies[62].

2.3 Oil Refining Wastewater: Characteristics and Components

Undissolved oil, suspended solids, and dissolved organics and inorganics (which contribute to COD and BOD) are the main constituents of untreated refining wastewater[63], as provided in Figure 4[64,65]. Dissolved contaminants in RWW include hydrocarbons[48], metals, phenols, sulphides, chlorides, and ammonia[15], but the range of chemicals goes beyond these groups of pollutants. It is estimated that dissolved organics in wastewater range between thousands and millions of compounds, making it impossible to generate detailed chemical profiles from RWW. Analyses by electrospray ionisation coupled to Fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR MS) have revealed that most organics in RWW range from 150 to 450 Da, including numerous acids (probably naphthenic acids, alkylbenzenesulfonic acids, and other non-identified species), other oxygen containing species (O_1 - O_{10}), nitrogen-containing ($N_1O_{1\text{-}9}$) and sulphur-containing compounds ($O_{1\text{-}8}S_1$ – $O_{1\text{-}7}S_2$,

$O_{1-7}S_3$, $O_{1-7}S_4$, $N_1O_{1-7}S_1$, $N_1O_{1-7}S_2$, $N_1O_{1-7}S_3$, $N_1O_{1-7}S_4$)[15]. Many of these chemicals are suspected to originate from the refining process itself, whereas others are transferred directly from the feedstock. Therefore, a "typical" RWW is challenging to define because its composition and complexity vary greatly among sites. For instance, refining of heavy oil generates wastewater with higher content of polar organics and dissolved recalcitrant compounds in comparison to wastewater obtained from the refining of light petroleum[66]. Also, the wastewater from separate processes is combined before wastewater treatment, in some cases combining streams from up to 15 different working units, which contributes to its uniqueness and variability[67].

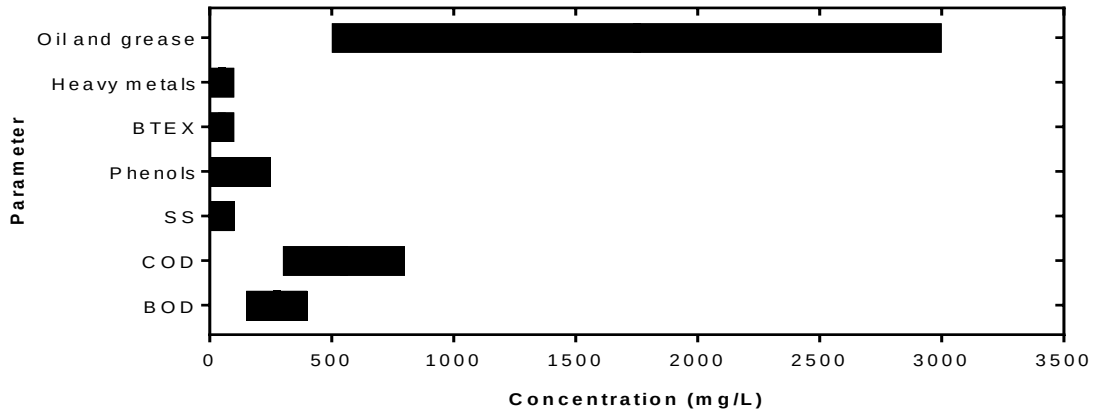


Figure 4. Generic composition of untreated petroleum refining wastewater[68]

Table 1. Principal reactions during petroleum refining and known components of wastewater produced at each stage [3,6,56–58,64,69–71]

Refining process	Aim	Characteristics of wastewater	Other waste generated	Additional remarks
Desalting	Removal of water-soluble impurities and water from crude oil	Suspended solids, metals, ammonia, hydrocarbons, phenols, chlorides, sulphides, bicarbonates.	Emissions: H_2S	
Distillation	Separation of crude oil into fractions based on differences on boiling point	Sulphides, ammonia, chlorides, phenol, mercaptans, suspended solids, hydrocarbons, antifoam, anticorrosion additives	Emissions: SO_x, NO_x, CO, H_2S and VOC	Hotspot for production of wastewater
Thermal cracking	Conversion of high-boiling, high-molecular-weight fractions into lighter products	Sulphides, phenols, ammonia, hydrocarbons		Being replaced in most refineries with catalytic cracking
Catalytic cracking	Conversion of high-boiling, high-molecular-weight fractions into lighter products	Alkaline wastewater with a high BOD and COD, containing metals, sulphides, phenols (40 to 50 mg/L), hydrocarbons, suspended solids, cyanides, ammonia.	Solid waste: catalyst Emissions: CO, NO_x	One of the major sources of sour and phenolic wastewater

Refining process	Aim	Characteristics of wastewater	Other waste generated	Additional remarks
Hydrocracking	Conversion of high-boiling, high-molecular-weight fractions into lighter products. Removal of S, N, and metals from the feedstock	Sulphides, ammonia, hydrocarbons	Solid waste: catalyst	Recovery and regeneration of catalyst generates sour wastewater containing ammonia
Catalytic reforming	Conversion of low-octane naphthas into high-octane products	Sulphides	Solid waste: catalyst Emissions: BTEX, SO_x, NO_x, CO, particulate matter, VOC	
Isomerization	Conversion of straight-chain paraffin into branched isomers, providing additional feedstock for alkylation and gasoline blending	Caustic and sour wastewaters containing sulphides and low levels of phenols	Solid waste: catalyst, sludge containing $CaCl_2$ Emissions: sour gases	
Alkylation	Combination of *iso*-butane and light olefins to produce high-octane branched-chain paraffinic hydrocarbons for gasoline blending	Spent caustic, hydrocarbons, sulphides	Emissions: acidic and non-acidic hydrocarbon gases, sulphonated organic compounds, and organic acids	It does not generate significant volumes of wastewater

Refining process	Aim	Characteristics of wastewater	Other waste generated	Additional remarks
Polymerization	Conversion of short-chain olefins into polymer gasoline for gasoline blending	Mercaptans, amines, sulphides	Solid waste: catalyst	A significant amount of cooling water is required
De-asphalting (lube oil processing)	Processing of vacuum distillation distillates to obtain a variety of lubricants	Solvents (mainly propane), hydrocarbons	Emissions: solvents (primarily propane), hydrocarbons, CO, SO_x, NO_x, particulate matter	
Refining (lube oil processing)	Removal of aromatics and improvement of the viscosity index and quality of lube base stock	Hydrocarbons	Emissions: solvents Solid waste: catalyst containing Co, Ni, Mo, W	Wastewater generated during fractionation
Dewaxing (lube oil processing)	Removal of wax of lube base stock	Solvents, hydrocarbons	Emissions: CO, SO_x, NO_x, particulate matter, hydrocarbons, solvents	
Storage		High COD and relatively high BOD. Suspended solids, emulsified oil. It may also be alkaline and contain Pb		High COD and BOD
Sulphur recovery		Sulphides, Se, As		

After treatment, the presence and abundance of dissolved organics somehow reflect the treatment technologies used. The concentration of oil and grease and phenols tend to decrease significantly after primary and secondary treatment, respectively. COD usually becomes the major parameter in treated effluents, containing mainly hydrophilic compounds because these tend to be less vulnerable to removal during wastewater treatment[15]. The identity of these recalcitrant contaminants, however, is not fully understood because the monitoring of treatment is normally conducted using only bulk parameters, such as total organic carbon (TOC), BOD, COD, heavy metals, and certain classes of organic pollutants (*e.g.* phenols, PAHs). Consequently, most of the research up to now to characterise RWW and develop emerging treatment technologies follow-up these generic parameters, but this approach has a significant drawback. Besides the fact that it does not provide information about specific contaminants that resist treatment, this approach does not indicate whether chemicals that are critical in the toxicity of the whole effluent have been removed. For example, process chemicals used throughout refining are usually included as unspecified fractions of TOC, BOD, and COD, irrespective of their contribution to the toxicity of effluents[72]. Therefore, if improvements are to be made to wastewater treatment, this traditional approach fails to identify target hazardous chemicals[65].

The lack of specificity and efficiency in wastewater treatment in the refining sector has moved recent research towards the identification of critical groups of pollutants that can lead to the selection of chemical indicators for monitoring purposes, and the number of studies going beyond bulk parameters has increased over time. It is noteworthy, however, that such studies tended to focus on non-polar compounds rather than on polar compounds, even though polar chemicals can represent more than half of effluents mass. Their polarity can decrease their treatability in traditional wastewater treatment as these are more resistant to activated sludge processes, and polar toxic chemicals impact the viability of microorganisms used during biological treatment[72,73]. In fact, there is an increase in the proportion of polar compounds throughout treatment, which means that evaporation and dilution are key to decrease their concentration in the environment when these are not entirely removed in biologically-mediated processes[74,75].

Chemometric studies of treated RWW[64,65,76–80] have identified hydrocarbons (aliphatics, BTEX, PAHs), heavy metals, naphthenic acids, and phenols, which are relevant from the environmental perspective because of their toxicological properties, but mostly because some

are also known to resist all types of treatment traditionally used in plants in the refining sector. However, it is vital to keep in mind that their effects on biological systems depend on a number of environmental conditions, including pH and temperature, and the interaction between individual compounds, also known as mixture effects. Previous studies aiming at linking biological effects and specific groups of chemicals have evaluated toxicity of known specific components, such as phenol, chromium, and kalinite (used as catalyst)[9,10], or followed the toxicity identification evaluation approach (TIE)[54,79,81], which has mainly shown that toxicity is linked to miscellaneous organics but failed to identify chemicals that account for the toxic effects observed. This suggests that an emphasis in organics, especially polar organics, could fill the gap between observed toxicity and chemical composition of RWW[82,83] but analytical limitations may have been an significant factor stopping researchers from addressing polar chemicals. Lipophilic organic compounds are easily extracted from aqueous samples by liquid/liquid extraction and solid phase extraction (SPE) using C18 phases or XAD® resins, but the range of hydrophilic compounds extracted has only recently expanded by using sequential SPE at different pH values.

A more in-depth description of the chemical classes reported in RWW is found below.

2.3.1 Aliphatic hydrocarbons

Aliphatic hydrocarbons are straight, branched, or cyclic hydrocarbons that may be saturated (alkanes) or unsaturated (alkenes, alkynes). Aliphatics are major components in RWW[78,80] and their chain length ranges from C_{10} to C_{40}[84]; cyclic aliphatic compounds are mainly alkyl derivatives of cyclohexane[80]. Their solubility in water is low compared to aromatic hydrocarbons, hence primary treatment is more effective removing aliphatics (up to 90%) than non-volatile aromatics, which remain dissolved in wastewater[65,80]. As aliphatics co-occur with PAHs, which are more toxic and persistent[16,85], the apparent lack of toxicity of aliphatics is reflected in a low number of studies addressing this group of chemicals in RWW. In general, alkanes are depressants of the central nervous system, but high molecular weight alkanes ($>C_{20}$) are considered virtually non-toxic because they are completely insoluble in water[86,87]. Previous research have proposed that the presence of mixtures of aliphatic hydrocarbons are central in the biological effects exerted by RWW, mainly by non-polar narcosis[83,88].

Different contents of aliphatics have been reported in the influent and effluent from treatment systems, ranging from trace (10 – 100 µg/L) to high levels depending on the chain length[89],

which suggests that some aliphatic hydrocarbons are resistant to treatment. Biodegradability of alkanes correlates with their bioavailability and solubility in water, suggesting that biodegradation of short-chain alkanes is easier than that of long-chain alkanes (>C_{10}), which are significantly less bioavailable [89–91]. The removal of short-chain aliphatic hydrocarbons is estimated to be in the range of 80 to 90%[80], and therefore their presence in effluents at high concentrations is an indication of insufficient wastewater treatment[78]. Branched-chain alkanes and cycloalkanes are more resistant to biodegradation than *n*-alkanes[87]. The differential loss rates within the group and in relation to other groups of hydrocarbons has led to the use of *iso*-alkanes, *n*-alkanes, and isoprenoids as biomarkers for fingerprinting purposes and as oil weathering indicators[86]. For example, the resistance to degradation of pristane and phytane causes their accumulation in mature oil or in the treated effluent. Therefore, the ratios of pristane/phytane, pristane/n-C_{17}, and phytane/n-C_{18} can be used to monitor the extent of biodegradation[92].

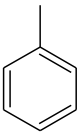
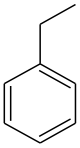
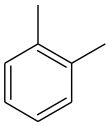
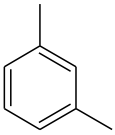
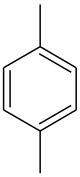
2.3.2 BTEX

BTEX compounds (benzene, toluene, ethylbenzene, and *o*-, *m*-, and *p*-xylenes; see Table 2) are monoaromatic hydrocarbons co-occurring naturally in petroleum and petroleum-derived products, including RWW. BTEX compounds are highly water-soluble and usually represent a significant portion of organic pollutants in RWW[93]. These chemicals are highly volatile thus the evaporative loss is a crucial factor shaping their removal from surface water, usually resulting in trace levels. In groundwater, however, BTEX can be found in higher concentrations because of the reduced evaporation rate[21].

BTEX are an important class on environmental contaminants classified as priority pollutants by the Environmental protection agency of the United States of America (US EPA), and exposure to benzene was defined as a significant public health concern by the World Health Organisation (WHO). As provided in Table 2, maximum allowable concentrations (MAC) have been established for drinking water (ranging from 7.4 to 10 µg/L by the WHO and from 5 to 500 µg/L by the US EPA) and for environmental quality standards (EQS) (ranging from 50 to 370 µg/L). Clear human health hazards from exposure to BTEX have been reported; all individual components can exert neurological effects on humans, stemming from changes in neuronal membranes. Moreover, benzene can cause haematological effects, such as aplastic anaemia and acute myelogenous leukaemia, and ethylbenzene is carcinogenic[94]. BTEX have also been reported to exert toxic effects on algae, which are usually observed as inhibition of

growth and photosynthesis. Individual BTEX showed growth inhibition EC_{50} values ranging from 3.9 to 41 mg/L for *Selenastrum capricornutum*[95], whereas chlorophyll inhibition for *Euglena gracilis* had EC_{50} values from 8.7 to 91 mg/L[96], *p*-xylene being consistently the most toxic compound. A synergistic effect was reported for some BTEX components in both studies.

Table 2. Structure and maximum allowable concentration of BTEX in water

Compound	Structure	MAC for drinking water (µg/L)		MAC for EQS (µg/L)	
		WHO[97]	US EPA MCL[98]	Freshwater	Marine water
Benzene		10	5	50[99]	50[99]
Toluene		700	1000	380[100]	370[100]
Ethylbenzene		300	700	200[101]	200[101]
o-xylene		500	10000	Not considered under the WFD	
m-xylene		500	10000	Not considered under the WFD	
p-xylene		500	10000	Not considered under the WFD	

MAC: Maximum allowable concentration

MCL: Maximum contaminant level

EQS: Environmental quality standard

2.3.3 Polycyclic aromatic hydrocarbons

PAHs are hydrocarbons with two or more fused aromatic rings whose concentration is markedly high in industrial effluents, including RWW[102]; during refining, these originate from catalytic cracking and oil desalting. Their environmental relevance stems from their resistance to treatment and their wide range of deleterious effects on biological systems[81]. PAHs are

nonpolar and hydrophobic, which makes them highly persistent in the environment because they tend to adsorb onto particles that are then deposited in sediments, reducing their bioavailability for biotransformation but increasing the risk of exposure to benthic organisms[103]. To date, several studies have linked discharges of RWW with high concentrations of PAHs in surrounding sediments. Concentrations as high as 4,163 ng of total PAHs per g of sediment have been detected in sites adjacent to RWW discharges, in contrast to sites with no influence of such effluents, where total PAHs can range from 8 to 464 ng/g[11,104].

PAHs are vulnerable to photodecomposition when dissolved in water or adsorbed on particulate matter, and many microorganisms have been reported to degrade, at least partially, many compounds within this group under both aerobic and anaerobic conditions. Nonetheless, bioavailability is always a limitation for biodegradation due to sorption (adsorption and/or partitioning) in micropores of particulate matter that are too small to allow microorganisms to settle[105], especially for PAHs with high molecular weight. In particular, PAHs with 4 to 6 rings are often resistant to complete biodegradation, which explains their persistence[106,107]. As for their effect on biological systems, these have been reported as toxic[107–109], mutagenic, and carcinogenic[110,111]. The group as a whole is classified as priority hazardous substances in the European Union (EU) Directive 2008/105/EC, and a total of 16 PAHs have been classified as priority pollutants by the US EPA since 1979[102,112]. MAC-EQS values under the WFD for some PAHs is provided in Table 3.

Table 3. Maximum allowable concentration of some PAHs in water[113]

Compound	**MAC for EQS (µg/L)**	
	Freshwater	**Marine water**
Benzo[*a*]pyrene	0.27	0.027
Benzo[*b*]fluoranthene Benzo[*k*]fluoranthene	0.017	0.017
Indeno[1,2,3-cd]pyrene	No sufficient data available	No sufficient data available
Benzo[*g,h,i*]perylene	8.2×10^{-3}	8.2×10^{-4}

Naphthalene is generally the predominant PAH found in refining discharges because its bi-cyclic aromatic structure confers high stability and resistance to treatment and biodegradation. Other PAHs considered priority pollutants, such as fluorene, pyrene, fluoranthene,

benzo(*a*)pyrene, and acenaphthene, have also been repeatedly reported in RWW[65,81,112,114]. In some cases, biotransformation of parent PAHs can generate metabolites that are toxic or that can contribute to the overall toxicity[108].

Alkylated PAHs are considered a special group within PAHs because alkyl substitutions often confer or enhance toxicity, phototoxicity, and carcinogenic potential of PAHs[115]. Similarly, alkylated PAHs pose a higher risk than non-alkylated PAHs as their concentration in petroleum is usually higher and these tend to be less volatile, more persistent, less mobile, and more bioaccumulated than parent PAHs. Once in the environment, the proportion of alkyl PAHs to PAHs increases over time, so it is common to find high concentrations of alkyl PAHs in aquatic ecosystems after the discharge of refining effluents[115]. It has been calculated that the contribution of alkyl PAHs to the baseline toxicity of petroleum components is approximately 20%, followed by BTEX, which account for more than 75%. However, the contribution of BTEX to the overall toxicity decreases rapidly because of their high volatility, making alkylated PAHs a key group of pollutants[82]. In particular, numerous studies have reported C_3- and C_4-naphthalenes in oil refinery discharges and these are considered among the most toxic of all PAHs[82,89,115].

PAHs have various mechanisms of toxicity, but some are easier to detect in laboratory conditions. It is likely to observe non-polar narcosis in short-term toxicity tests, which is caused by their accumulation in biological membranes and subsequent disruption, whereas specific modes of action are more likely to occur in natural environments after prolonged exposures to low concentrations[116]. Toxicity assessment of mixtures of PAHs can be challenging because these have an extensive range of physicochemical characteristics; some compounds can be hard to dissolve in water, and some others can be lost by volatilisation or sorption, depending on specific structural characteristics. Bioconcentration kinetics can be significantly different for highly hydrophobic PAHs, complicating the implementation of one standard toxicity test for all PAHs. For instance, single-compound experiments with the benthic amphipod *Corophium volutator* have shown that the uptake of highly hydrophobic PAHs, such as fluoranthene, is slower when compared to less hydrophobic PAHs, and therefore short-term toxicity tests (24 – 96 hours) could underestimate the actual toxicity because there is not enough time to reach maximum internal concentrations[108].

2.3.4 Heavy metals

Heavy metals are metallic elements with relatively high atomic weight and density that occur naturally in the environment[117] and are present in industrial wastewater, including RWW. Cadmium, nickel, chromium, arsenic (a metalloid), lead, zinc, copper, and mercury are especially relevant in the environmental context when their concentration exceeds trace levels due to their biological effects. Some reported concentrations of heavy metals in RWW are shown in Table 4. Many of these metals are essential elements required for a normal biological functioning, for instance as cofactors of a variety of enzymes. However, for some, such as Cr and Cu, the concentration boundary between beneficial and toxic effects is very narrow[118]. The affectation of metabolic functions is caused mainly by the displacement of other metals that are vital for a normal biological functioning, and by the accumulation of metals and resulting disruption of normal metabolism[118,119].

Table 4. Reported concentrations of heavy metals in refining effluents

Metal	Previous Studies			
	Daflon *et al.*, 2017[79]	**Gillenwater *et al.*, 2012[120]**	**Hoshina *et al.*, 2008[121]**	**Ismail & Beddri, 2009[122]**
As (mg/L)	NR	0.007 – 0.017	NR	<0.001 – 0.014
Se (mg/L)	NR	0.025 – 0.045	NR	NR
Pb (mg/L)	<0.01 – 0.05	0.008 – 0.098	0.02	<0.006
Zn (mg/L)	0.04 – 0.200	0.020 – 0.280	0.04 – 0.078	<0.002 – 0.018
Ni (mg/L)	<0.006 – 0.015	NR	0.0056 – 0.0075	<0.001 – 0.501
Cd (mg/L)	NR	NR	0.003	<0.001
Cr (mg/L)	NR	NR	0.005	<0.002 – 0.029

NR: Not reported

Heavy metals in RWW come mainly from metal-containing catalysts that release Ni, V, Cr, and Se, the latter also being transferred from crude oil into wastewater [120,123]. Similarly, Cd, As, Cu, and Zn can be transferred from the feedstock to RWW. Their concentrations in crude oil have been reported to range from 0.4 to 5.3 μg kg^{-1} for Cd, from <10 to 26.2 μg kg^{-1} for As, from 10 to 195 μg kg^{-1} for Cu, and from 63 to 1090 μg kg^{-1} for Zn[124]. In particular, wastewater from sour water strippers is enriched in Se and As, as these show high affinity to sulphur and bind to it, ending up in the wastewater generated at this stage[125,126].

Nevertheless, the hazardous potential to aquatic ecosystems cannot be drawn from total metal

concentrations because their bioavailability can be affected by several factors, such as the presence of organic matter, the hardness of water, which can have a short-term protective effect for some metals, and pH. Similarly, speciation cannot be overlooked, as it defines the distribution of a given metal among the various possible chemical forms. The free ion, on the contrary, is a good indicator of bioavailability because complexed metals are not competing for a biotic ligand[127]. Mixtures of metals can have antagonistic, synergistic, or additive effects, depending on the nature of individual metal ions[128].

On the question of wastewater treatment, the aim is removal, as metals are not biodegradable. Various technologies have been successfully applied, including chemical precipitation, membrane filtration, flotation, adsorption, ion exchange, and electrochemical deposition[63, 64]. Remediation using aquatic plants have received considerable attention due to their fast growth rate and simple, low-cost growth requirements[128].

2.3.5 Phenols

Phenols are hydroxy derivatives of benzene and one of the dominant groups of pollutants in refining effluents. Phenolics originate mainly from the catalytic cracking process and, in a lesser extent, the caustic treatment applied to gasoline to remove sulphur and phenolic compounds[64,129]. Substituted phenols in RWW can be found mainly as alkylphenols, nitrophenols, and chlorophenols, all of which include numerous isomers with either linear or branched alkyl radicals, different substitution patterns, one or more nitro groups, and one or more covalently bonded chlorine atoms. All three groups are considered priority pollutants by the US EPA[130] and the EU classified pentachlorophenol, nonylphenols, and octylphenols as priority hazardous substances, as stated in the Directive 2008/105/EC. Previous studies have reported high concentrations of phenols in RWW, including nitrophenols and alkylphenols[78,131], although their concentration varies greatly depending on the plant configuration, with reports ranging from 2 to 200 mg/L[64,65,69,80,132].

Phenols are considered pollutants of concern due to their persistence and toxicity at low concentrations, with hazardous effects that can be both acute and chronic for humans and aquatic life, as presented in Table 5. Toxicity of phenols is related to the lipophilicity of the phenolic compound[133] and the formation of free (phenoxyl) radicals and electrophilic metabolites, such as semiquinones and quinones, which bind to thiol groups in proteins and deoxyribonucleic acid (DNA)[134]. Likewise, many substituted phenols, especially

chlorophenols, tend to bioconcentrate and biomagnify. Toxicity and bioaccumulation potential increase with the degree of chlorination and with lipophilicity[68,135,136]. Alkylphenols, considered among the so-called emerging contaminants, exert estrogenic and anti-androgenic activity[137]; some isomers are more active than others, and a branched aliphatic side chain together with a hydroxyl group in the *para* position seems to enhance such activity[138].

Table 5. Acute and chronic health hazardous effects of phenols[130]

Hazardous effects	**Organism**	
	Humans	**Other animals**
Acute	Irritation of the skin, eyes, mucous membranes, irregular breathing, muscle weakness, tremor, coma, respiratory arrest.	High acute effects after oral exposure.
Chronic	Anorexia, weight loss, diarrhoea, vertigo, salivation, dark colouration of urine, gastrointestinal irritation, blood and liver effects.	Foetal body weight reduction, growth retardation, abnormal development in the offspring after oral exposure, decreased maternal weight gain and increased maternal mortality, effects on the central nervous system, kidney, liver, respiratory, and cardiovascular system.

It is calculated that at least 70% of phenols released into the environment via wastewater end up in water, approximately 25% in air, and nearly 1% in soil and sediments[135]. Once in the environment, phenols impose high oxygen demands on receiving water bodies[129]. Non-halogenated phenols can be biodegraded in both aerobic and anaerobic environments by a wide range of microorganisms (e.g. *Pseudomonas* spp., *Corynebacterium* sp., *Proteus,* sp., *Klebsiella* sp., *Candida* spp., *Bacillus* spp., *Aspergillus* sp.), provided their concentration is not inhibitory[15,139]. However, the fact that phenols are still present in the environment despite their biodegradability indicates that the conditions for degradation are not always achieved. Halogenated phenols, on the other hand, have proven to be more difficult to degrade. Their partial biodegradation generates chlorocatechols and other chloro-substituted products capable of inhibiting microbial growth[136]. The problem of substrate inhibition is addressed in

engineered systems with preadaptation of sludge to higher phenol concentrations, cell immobilisation, or metabolic enhancing[139].

Successful lab-scale removal of phenols has been achieved using activated carbons — in many cases deriving from agricultural waste such as apple pulp, coconut shell, pine bark, and straw — membrane technologies, ozonation, photocatalysis, and solvent extraction[140]. Usually, a combination of methods shows better results than separate technologies; however, it should be noted that no appropriate methods have been developed at industrial scale to eliminate phenols from RWW[138] fully.

2.3.6 Naphthenic acids

"Naphthenic acids" (NAs) is an umbrella term used to refer to a complex mixture of cyclic and acyclic carboxylic acids present in crude oil and bitumen. The traditional, strict definition of NAs refers only to alkyl-substituted acyclic and cycloaliphatic carboxylic acids with the general chemical formula $C_nH_{2n+z}O_2$, where *n* is the number of carbon atoms, and *z* is a negative even integer known as hydrogen deficiency[141]. However, the range of carboxylic acids considered NAs has expanded over the years; compounds containing sulphur and nitrogen have been included in the definition[142], as well as aromatic species and oxidised NAs fitting the formula $C_nH_{2n+z}O_x$[143], all of which have been reported in both environmental[144] and commercial samples[145]. Due to this structural diversity, alternative names have been proposed to refer to NAs, such as acid extractable organics or naphthenic acid fraction compounds[146]. Some examples of NAs are provided in Figure 5.

NAs are natural constituents of crude oil (ranging from undetectable to 3%[147]) and transferred from the feedstock to RWW, although only a few reports discuss these acids in refinery wastewater[15,148–151]; most reports are in relation to the process water generated during the extraction of bitumen from the oil sands of northern Alberta, Canada, mainly driven by an increment in environmental monitoring programs in Canada. Bitumen ore in the Athabasca oil sands surface mining operations is extracted using hot caustic water, washing the bitumen from the sand and generating water with high contents of NAs, as these partition to the water phase at pH above their p*K*a, which ranges from 5 to 6[152]. NAs play a significant role in the corrosion of process equipment[153] and are known to be toxic to aquatic organisms[144,154–156], thus wastewater management practices for oil sands process-affected waters (OSPW) avoid their recycling and discharge into the environment, resulting in tailing ponds storing OSPW for years

where the concentration of NAs ranges between 40 and 120 mg/L[157]. However, in the case of RWW, NAs are not considered in wastewater management practices nor targeted during wastewater treatment, making RWW an important source of NAs into the environment. The exact composition and concentration of NAs in RWW depend on the type of feedstock, differences in extraction processes, and degradation over space and time[141]. NAs in RWW have been reported between 4.2 and 40.4 mg/L in refinery desalter brine, between 4.5 and 16.6 mg/L in the influent of refinery wastewater treatment systems, and between 2.8 and 11.6 mg/L in the effluent[150].

Many NAs are recalcitrant due to their chemical stability, resistance to photolytic degradation, and low Henry's constants[158]. Only some NAs are susceptible to biodegradation under aerobic conditions, which has proven to be the most cost-effective technique for degradation so far[147]. Because NAs are present as a complex mixture that contains a variety of acids, complete mineralisation of environmental mixtures has not been achieved because recalcitrant species remain after treatment[142]. Biodegradation rates decrease with increasing number of carbon atoms and degree of cyclization and branching. Similarly, molecular weight is a key factor in biological degradation, as bigger molecules are more difficult to degrade[158]. Their amphipathic nature might also impact their availability for microorganisms[141].

There has also been extensive research in non-biological treatment for NAs, including studies on adsorption, phytodegradation, and advanced oxidation processes, including ozonation. Activated carbon-based adsorption has proven to remove NAs with high structural complexity, ranging from 12 to 18 *n* and between -10 and -12 *Z*, as a consequence of higher hydrophobicity and less solubility in the aqueous phase, causing a higher affinity to adsorption substrates[159,160]. Ozonation has been suggested as a pre-treatment to biological treatment, as it helps to increase biodegradability because ozone attacks preferentially high-molecular-weight congeners and species with high degrees of cyclicity. Consequently, ozone-treated OSPW has been shown to contain NA species that are more biodegradable[142,161–163].

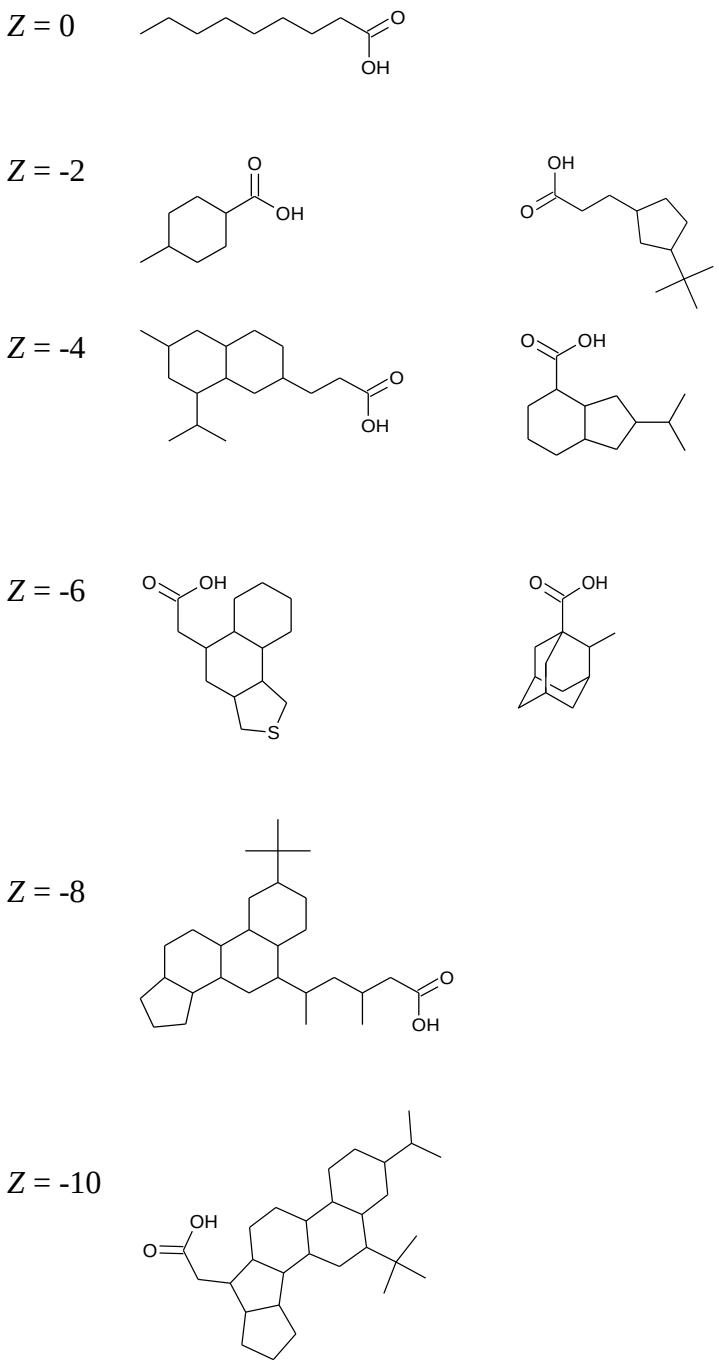


Figure 5. Example of NAs with the general formula $C_nH_{2n+Z}O_2$ where *Z* indicates homologous series based on hydrogen deficiency and *n* is the number of carbon atoms

Once discharged into the environment, NAs remain in the water column of receiving bodies for considerable periods because they are chemically stable and non-volatile, with an estimated half-life in nature of 12.8 – 13.6 years[164]. Due to the presence of both hydrophilic (carboxyl group) and hydrophobic (aliphatic) moieties, NAs have high solubility in water but may accumulate in sediments by adsorption to particles, which will end up incorporated into the sediment bed[160,165]. In the form of naphthenates, NAs are water-soluble at a wide range of pH (neutral to alkaline), making them quite mobile in aquatic ecosystems[141,147]. Their impact on a variety of organisms has been described in numerous publications and reviews[156,160,165,166], with reported endocrine disruption activity[167] and toxicity to plants[168], fish[169,170], mammals[171], and amphibians[172]. However, as NAs refer to a vast range of acids that are structurally different, it cannot be assumed that all NAs are persistent or toxic[173]. As NAs are always found in highly complex and variable mixtures, it has not been established which individual acids are the most bioactive, but evidence suggests that NAs with fewer rings (lower molecular weight) are more probable to exert toxicity[153].

The fundamental mechanisms of NA toxicity are not well understood, possibly as a result of the structural diversity and variability within the complex mixtures of NAs. Evidence suggests that their cytotoxicity derives from the disruption of biological membranes and the resulting alteration of fluidity, thickness, and surface tension[173]. Additionally, oxidative stress [170] and endocrine disruption (antiestrogenic and antiandrogenic potency)[167] have been proposed as a possible mode of action for NAs. A study using an effect-directed analysis of produced water showed that NAs act as estrogen receptor agonists and aryl hydrocarbon receptor antagonists[154]. As for bioaccumulation, their potential is low, with estimated log K_{ow} between 0 and 1.

2.4 Biological assessment of petroleum refining effluents

The petroleum refining industry ranked fourth among the highest producers of toxic and non-conventional pollutant discharges, and fourteenth in top dischargers of pollutants in a study carried out in 2000 by the US EPA[6]. There is also a substantial body of evidence that shows that RWW causes harmful effects on different aquatic species at various trophic levels[7,10,67,174]. A biological assessment is therefore essential to complement the currently available chemical data because the existing treatment facilities in refineries may comply with specific chemical and physical criteria, but biological tests may show that effluents are still hazardous[48]. This is explained by the fact that treatment plants remove mainly highly hydrophobic compounds (via

adsorption onto sludge) and non-recalcitrant organics (via biodegradation) but hydrophilic, toxic, and recalcitrant compounds are discharged into the environment[175]. In this context, it is essential to examine causal linkages between observed biological effects and refining chemicals to propose effective treatment solutions and prevention measures, but the establishment of such a link has proven to be challenging.

The usual components of any biological assessment are persistence, bioaccumulation, and toxicity (PBT); mutagenicity and endocrine effects have recently been included in some approaches[176]. However, the biological assessment of RWW is usually conducted using toxicity tests only because of the limitations for measuring persistence and bioaccumulation. Persistence refers to the resistance of a chemical to degradation (chemical, physical or biological), which increases the potential for long-term exposure and adverse effects[177]. As such, persistence cannot be measured directly, and only the continued presence of a certain chemical in the environment, or the systematic resistance to degradation under laboratory conditions can suggest its persistence. The experimental approach estimates half-lives of chemicals in individual environmental compartments, which does not represent real scenarios. *In silico* approaches can include information about the multi-phase partitioning and fate of compounds, but there are still considerable uncertainties in many parameters[48,178]. In the regulatory context, a chemical is classified as persistent based on its half-life, which is derived from the potential occurrence of biodegradation and the abiotic half-life. Persistence criteria for chemicals in water vary among different organisations, ranging from >50 days to >182 days[178].

Bioaccumulation can be experimentally determined in laboratory feeding experiments or field conditions by quantifying the concentration of the chemical in tissue and water. It is expressed as bioaccumulation factor (BAF), which refers to the ratio of the concentration in the organism and water[179]. A predictive correlation can be drawn from plotting Log BAF versus Log K_{ow}[180]. However, chemicals with high Henry's constant easily partition into the air, and hydrophobic compounds might adsorb onto organic matter in the water phase or surfaces of testing equipment, leading to an underestimation of BAF; all these variables add significant error to experimental determinations[181]. Alternatively, an *in silico* approach can also be used to determine bioaccumulation; although it requires more data, it includes chemical-specific metabolism rates that the experimental approach does not provide[180]. However, precise *in vitro* experimental data for absorption, distribution, metabolism, and excretion (ADME) are

required, with absorption and metabolism representing the most significant source of uncertainty. Detailed ADME information is available only for a small number of chemicals, which limits the extrapolation of *in vitro* results to real-life contexts[182]. In short, the testing conditions and interpretation of results for bioaccumulation and persistence are not reliable enough to incorporate these parameters in standard effluent assessments[177].

On the contrary, there is considerable robustness in numerous tests to assess the environmental toxicity of a sample, which means that such assays can provide a reliable assessment of the biological quality of the effluent[175]. *In vivo* tests, sometimes referred to as whole-organisms tests, use surrogate organisms that are representative of those found in the receiving environment to determine the extent of toxic effects, and how time- and dose-dependent these effects are. These assays, which can also be performed *in situ*, are useful when it comes to detection of "symptoms" of toxicity (*e.g.* growth impairment, mortality, reproductive alterations) but fail to provide an insight into underlying modes of action[62]. Routine assessment of effluents generally uses standardised *in vivo* tests, such as those using daphnids, luminescent bacteria, and green algae, all of which are described in detail in ISO guidelines (ISO 8692, ISO 11348, ISO 6341, respectively)[183]. *In vitro* bioassays are based on the use of biomarkers (measuring biochemical, cellular, or molecular responses), which makes them more sensitive, simpler, less expensive and easier to handle[177,184]. Such assays are useful to determine biological activity and modes of action and can detect and measure different responses, such as cytotoxicity, genotoxicity/mutagenicity, endocrine disruption, and adaptative stress response induction[62]. However, when used alone, results from these type of tests cannot be extrapolated to real-life scenarios because the induction of biomarker stress responses is not necessarily caused by a toxic contaminant but by other factors, such as salinity or hardness[177]. For this reason, it is recommended that results obtained from *in vitro* toxicity tests are used only to assess wastewater quality and wastewater treatment technologies, and not to draw conclusions on ecological relevance[48].

Bacteria-based tests have been widely used to perform toxicity assessment of RWW, especially Microtox®, which has shown to be generally more sensitive than *D. magna* to detect toxicity of refining effluents[48]. The use of this standardised test has provided a comparable, yet wide range of IC_{50} values depending on the type of refining effluent and wastewater treatment technologies applied. For instance, Aruldoss & Viraraghavan (1998) reported EC_{50} values ranging from 0.74% to 73% of RWW, with an average toxicity of 40% ± 16%[174], and Chang

et al. (1981) reported EC_{50} ranging from 1.8% up to 100% with an average value of 66% ± 40%[185] for effluents from 5 different refineries.

A review by Wake (2005) examined toxicity studies of refining effluents using whole-organisms tests, including phytoplankton, algae, several invertebrates, and fish. Overall, the author concluded that RWWs are generally toxic but to varying extents, depending on the species and, in some cases, life cycle. Specifically, there are some points worth highlighting. In the case of algae, evidence suggests that RWW induce sublethal effects (growth rate and germination), almost certainly impacting the whole aquatic food chain. As for crustaceans, these seem to be more sensitive to the acute toxicity of RWW than other aquatic organisms; sublethal effects have been demonstrated in the form of genotoxicity, behavioural changes, and reproductive toxicity. For fish, lethal and sublethal effects have been reported, the latter including respiratory distress, changes in maturity index and fecundity, growth reduction, and behavioural changes[7]. Similarly, there is enough evidence to suggest that sediments contribute significantly to the chronic impacts of RWW on aquatic ecosystems, specifically on the liver size and increased liver detoxification enzyme activity in fish, stemming from the accumulation of PAHs downstream of discrete discharges[103]. However, the causative agents of these observed effects are yet to be established.

Several studies have characterised RWW in an attempt to correlate specific groups of chemicals to toxicity, all of which used the TIE approach developed by the US EPA in the 1980s[54,79,81,83]. PAHs and "other organics" have consistently been identified as the source of toxicity, and metals have been reported in both toxic and non-toxic fractions, suggesting that these are not necessarily involved in toxic effects. The identification of specific toxicants has been unsuccessful partly because there is a broad distribution of toxicity among numerous fractions[54] but also because the TIE approach does not focus on organic chemicals, which have been shown to be behind the observed toxicity. Alternatively, the effect-directed analysis (EDA) approach could help to study in detail the toxic fractions containing organic components because, as opposed to TIE, it requires the extraction of organics from the aqueous matrix and therefore facilitates their detection and identification[186].

2.5 Treatment of RWW

Current wastewater management practices in the refining industry focus on reducing water use, increasing water reuse, and recycling wastewater after treatment. The recycling of treated effluent for cooling systems is feasible but the quality and quantity of the final effluent need to

be closely monitored. The use of low-quality water for cooling towers results in the precipitation of salts on heat exchangers, corrosion due to dissolved solids, and biological and/or organic fouling[61]. Therefore, treatment technologies play a central role in the recycling of effluents. Still, the treatment of RWW is still a technological challenge because of the significant concentrations of recalcitrant organics, the presence of nitrification inhibitors known to impact biological treatment, and the fact that treatment plants are not designed empirically but rather theoretically, based on bulk parameters of a "standard" refining wastewater. Ideally, the selection of treatment technologies and the optimisation of detention times would need to be site-specific and based on actual wastewater.

There are no standard guidelines for the treatment of RWW. The technologies currently used depend on the characteristics of the influent and on local regulations, which vary greatly depending on whether the refinery is located on a high-, medium-, or low-income country. A typical treatment process is composed of 2 stages intended to remove, at first, free oil and gross solids, while the second stage is a biological treatment that aims to remove dispersed oil and fine solids. However, it is now known that there is more to look at than oil and suspended solids, so the complexity of treatment has increased over time aiming to remove a broader range of contaminants. Some advanced methods have become part of some treatment systems, thereby decreasing the final concentration of dissolved organic contaminants[48,60], although these improvements increase the costs of treatment significantly. These stages are known as primary, secondary, and tertiary treatment, which are summarised in Figure 6.

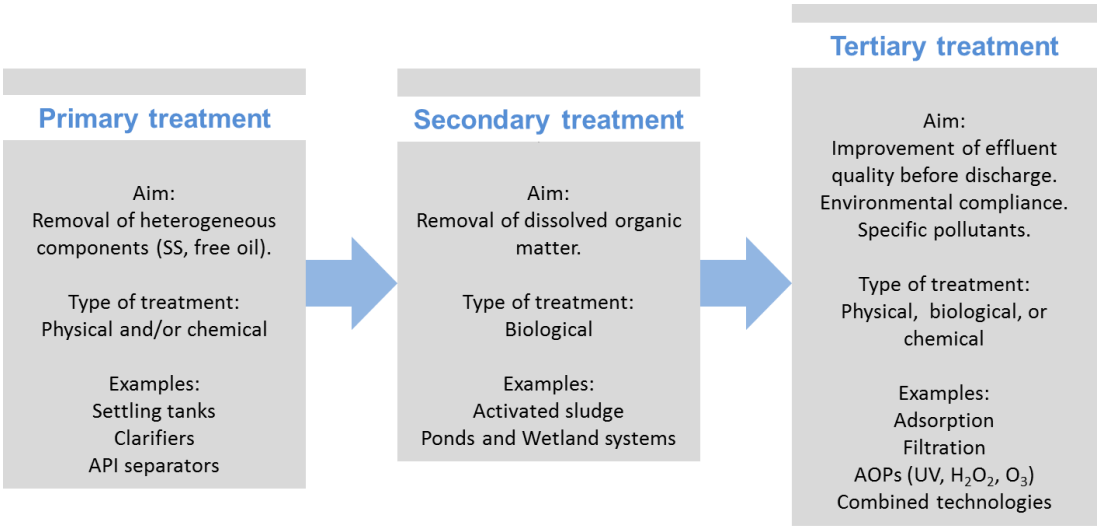


Figure 6. Typical stages of treatment in petroleum refining wastewater treatment plants

2.5.1 Primary Treatment

Primary treatment aims to separate oil from water and remove suspended solids and colloidal materials, simultaneously decreasing the oxygen demand and lowering BOD and/or COD values, depending on the biodegradability of contaminants. It also reduces the concentration of salts and sulphides that would otherwise inhibit subsequent biological treatment. Emulsified and dissolved oil remains, and needs to be removed in the second stage of treatment[187]. The primary treatment uses flotation followed by sedimentation and is based on the difference in density between water, hydrocarbons, and solids, although its efficiency also depends on the size of particles and the viscosity of the liquid. During primary treatment three phases are continuously generated: oil skim, water and sludge[60,67,188].

In petroleum refineries, it is frequent to find two stages of oil/water/solids separation because of the typical high concentration of free oil, which would impact the subsequent biological treatment. However, not all influents undergo two separation steps; depending on the total dissolved solids (TDS) content, it is common to implement a segregated wastewater system with both low-TDS and high-TDS streams, where only high-TDS effluents undergo double oil/water separation. For instance, wastewater from desalting, storage tanks washing and spent caustic solution (resulting from the extraction of acidic components from hydrocarbons stream) have high TDS content, while runoff and stripped sour water are sent to the low-TDS train. This strategy facilitates the reuse of low-TDS water if needed[60].

The first stage of primary treatment leads to the recovery of floating hydrocarbons and starts with a gravity separation of oil, being API separators (name derived from the American Petroleum Institute) the most widely used[70]. Such separators are designed based on the difference between the specific gravity of wastewater and oil and are usually rectangular tanks where detention time is long enough to allow oil flotation and skimming[189]. Most API separators are divided into several bays to maintain the laminar flow of the influent, making recovery of oil more effective. Although part of the suspended solids settles in a pre-separation chamber and sludge has to be removed periodically, the primary objective of API separators is the removal of oil and oil-bearing sludge[190]. API separators cannot remove emulsified or dissolved oil, and if pH is not controlled, emulsions can get stabilised in alkaline conditions[70]. Another technology used in refineries, although not as frequently as API separators, involves the use of coalescing plate interceptors, which consist of stacks of plates or groups of tubes

angled at 60 degrees inside a tank, where the plates are the settling surface with a large surface area in contact with wastewater[189,190].

Once oil has been removed, separation of fine solids and water takes place via coagulation/flocculation or coagulation-dissolved air flotation processes[189]. Clarifiers, for example, are equipped with skimmers to remove the remaining oil, and the addition of coagulants like lime or inorganic salts of aluminium or iron facilitate settlement of particles, removing suspended solids by gravity in the form of sludge. Such chemicals are used when the difference in density between water and particles is negligible, and reduce the electrostatic forces of repulsion among solids, making coagulation easier[60,190]. Alternatively, there are dissolved air flotation (DAF) systems and induced air flotation (IAF) systems. DAF systems include (i) chemical coagulation/flocculation with the use of salts of iron and aluminium, and (ii) recirculation/air pressurization of the effluent, which releases air microbubbles that adhere to oil/solids particles and reduce their apparent density to below that of water, helping these to float for subsequent removal. The selection of coagulation agents is critical to the removal efficiency due to the high complexity of refinery wastewaters, and the control of pH is necessary to achieve high removal rates. In contrast to API separators, DAF systems are useful in breaking down emulsified hydrocarbons[60,189]. Alternatively, the air in IAF units is induced by a rotor-based mechanism that creates vacuum and forces the air and the effluent to mix in special cells[60,67,70].

A number of studies have used lab-scale electrocoagulation for primary treatment of RWW to reduce sulphates, phenols and COD, and reduce the risk of overloading biological treatment. Aluminium has proven to be highly efficient in comparison to other materials for electrodes, such as stainless steel and iron, and the method has shown to successfully remove phenols and even the smallest colloidal particles, which chemical coagulation cannot successfully remove. However, the efficiency of removal is highly dependent on the initial concentration of contaminants, and more research is needed for a full-scale application[191,192]. Similarly, laboratory-scale photocatalysis and photoFenton (oxidation of Fe to Fe^{2+} in aqueous solution under acidic conditions in the presence of short UV light; Fe^{2+} catalyses the decomposition of H_2O_2, generating hydroxyl radicals that oxidise contaminants) have been evaluated as pre-treatment technologies for RWW before biological treatment, showing to reduce COD significantly in short periods. In both cases, the use of UV light makes energy consumption a major limitation for full-scale implementation, and the concentration of reagents needed for an

efficient removal rate in photoFenton remains cost prohibitive[4,193].

2.5.2 Secondary Treatment

After separation of water, solids, and oil, dissolved organic compounds must be reduced to allowable limits for discharge[67,189]. Biological treatment is the most widely used technique because it is highly cost-effective for the removal of a wide range of pollutants, even though not all dissolved organics are eliminated[60,194]. The two main biochemical processes occurring during biological treatment are carbonaceous oxidation and nitrification: organic matter is first transformed into new biomass, carbon dioxide, water, and ammonia, and then ammonia is oxidised to nitrates. However, complete oxidation of all components rarely occurs, and effluents always contain residues and by-products of incomplete oxidation of organic matter. The entire process is highly influenced by aeration and the load and characteristics of the organic matter suspended in the effluent, where a balance of nitrogen and phosphorus is needed to induce bacterial growth[188].

In biological treatment, microorganisms can be either suspended or immobilised on inert materials. Suspended growth processes (SGPs) mix microbial populations with the effluent under aerobic or anaerobic conditions, where bacterial growth leads to active biomass formation after utilising the carbon and nitrogen in wastewater as nutrients[195]. Within SGPs, several alternatives exist, including activated sludge, activated sludge with powdered activated carbon, sequencing batch reactors, membrane bioreactor technology, and aerated lagoons, which are artificial ponds where aeration and settling occurs. These can be aerobic or aerobic-anaerobic / facultative[60,70,188].

Biological treatment with immobilised biomass, also known as attached growth processes (AGP), use inert materials, such as rocks or gravel, which will be eventually covered with a biofilm containing microorganisms embedded in extracellular polymeric substances of microbial origin[60,195]. Tolerance to toxic and organic shock loads is higher in these systems, improving COD removal[67]. There are several methods within AGPs: (i) *trickling filters* consists of a packing material bed where bacteria are immobilised, and wastewater is biodegraded while distributed continuously; (ii) *rotation biological contactor* includes plastic discs where bacterial populations are attached. These plastic discs are submerged in the wastewater and rotate continuously, thereby accumulating a thick biofilm that eventually comes off; (iii) *nitrification* or *nitrification/denitrification* uses an aerated tank with nitrifying bacteria +

methanol (used as carbon source) for nitrification and an anoxic tank with denitrifying bacteria + wastewater organic matter (used as carbon source) for denitrification[60,70].

The microbial composition of biological treatments depends significantly on the nature of wastewater, including the concentration of toxic compounds, pH, availability of petroleum hydrocarbons, dissolved oxygen, etc. Usually, chemoorganotrophic species play a crucial role in the degradation of organic pollutants, using these as carbon sources and electron donors for the generation of energy[194]. Culturing methods have limited for decades the complete characterisation of microorganisms associated with wastewater treatment, although molecular tools have made it possible now to describe the composition of microbial communities without depending on the culturability of the strains. As reported by Juretschko *et al.* (2002)[196], an analysis of microbial communities from the activated sludge of an industrial wastewater treatment plant showed that diversity tends to be lower in comparison with municipal treatment plants, yet *Proteobacteria* remains as the crucial group in wastewater systems. β-*Proteobacteria* were the most abundant subdivision, while α-P*roteobacteria*, *Nitrospira* and *Planctomycetes* were recognised as strategic members of these communities. On the other hand, strains belonging to the genera *Aeromonas, Bacillus, Pseudomonas, Alcaligenes, Acinetobacter, Arthrobacter, Cytophaga, Mycobacterium*, among others, have been reported to successfully remove petrogenic contaminants from wastewater when used in biological treatment, more specifically in suspended-growth methods[195]. The production of biosurfactants has been proposed as the core of biodegradation potential, as these increase the availability of hydrophobic compounds that would otherwise be impossible to degrade[194].

One of the main limitations of biological treatment, however, is the considerable amount of sludge produced, which increases operational costs stemming from its treatment and disposal. The optimisation of operational conditions based on RWW characteristics in a pilot-scale study proved to reduce the production of sludge while maintaining high removal performance (up to 78% of COD), and showed that aeration is a critical factor for both sludge production and removal efficiency. Low concentrations of dissolved oxygen decrease microbial metabolism and increase the generation of sludge[197]. Another technology proposed to address the excessive production of sludge is microalgae biofilm cultivation, where, in addition, a variety of bioproducts can be obtained from nutrients present in RWW. In laboratory conditions, TSS, nitrogen and phosphorus have been successfully removed using this approach, but improvements are needed to increase COD removal[5].

Artificial wetlands have been evaluated in pilot-scale studies as an alternative to treat RWW in low- and middle-income countries due to the low construction and maintenance costs, and the possibility to use local, low-cost substrates and vegetal species. Wetlands can be used either as a secondary or tertiary treatment depending on the discharge limits and characteristics of RWW. Different filter media have been utilised, including sand, gravel, and compost, the latter achieving COD removal of up to 78%, and a reduction of 83% in BOD[198,199]. Constructed wetlands can also remove heavy metals, TSS, ammonia, and phosphates from RWW[200].

2.5.3 Advanced Treatment

Advanced treatment, also known as tertiary treatment, is applied when further polishing of effluents is required for environmental compliance, frequently to meet allowable limits for total suspended solids (TSS), COD, metals, or trace organics[60]. Depending on national legislation, tertiary treatment can also be applied when receiving waters are threatened by eutrophication or are considered sensitive environments[201]. Advanced treatment plays a crucial role in water management practices as it provides high-quality effluents suitable for reuse, eliminating any contaminant promoting corrosion, scaling, fouling, or biological growth[61]. It can be achieved using a variety of technologies, either separately or in combination, whose selection depends on the baseline composition and specific targets. These methods, however, tend to be more expensive than the technologies used during primary and secondary treatment because the remaining contaminants show high stability and tolerance to treatment, which usually implies that their removal requires higher energy consumption and costs. For instance, advanced treatment using solvent extraction costs between 250 and 2500 US dollars per 1000 m^3 of water, as opposed to sedimentation and gravity separation, which cost between 5 and 10 US dollars for the same volume of water (all costs corresponding to 2012)[202].

Current technologies for the advanced treatment of RWW include filtration, microfiltration, and ultrafiltration for the removal of excess SS; adsorption, chemical oxidation, and reverse osmosis to reduce the concentration of residual, refractory dissolved organics; and electrodialysis to decrease levels of dissolved solids[61]. However, studies on pilot or full-scale application of advanced treatment technologies for RWW are scarce; many emerging technologies are still in early stages and subject to optimisation aiming to achieve inexpensive and suitable settings for full-scale implementation. As shown in Table 6, numerous laboratory scale studies have demonstrated good removal rates and efficiencies using diverse removal

processes, usually monitoring COD. The bottom half of the table presents combined methods, which are often more effective because of the diverse nature of contaminants in RWW.

A series of successful full-scale systems for discharge/reuse of refining effluents operating in Beijing, China, were reported by Wang *et al.* (2011). The systems combined biological treatment and membrane technologies, including ultrafiltration, microfiltration, nanofiltration, and reverse osmosis. As one of the most significant limitations of membrane-based technologies is fouling, biological treatment was used as pre-treatment to remove organics and protect membranes. At the same time, ultrafiltration units were used as pre-treatment to protect reverse osmosis systems from suspended solids, colloids, and high-molecular-weight organics. These systems achieved a 34% reduction in TOC with ultrafiltration, and a decrease of 84% and 98% in TOC and salt, respectively, with reverse osmosis, for a final effluent containing approximately 1.1 mg/L TOC, 24 mg/L TDS, 5.9 mg/L Cl^-, 1.0 mg/L Ca^{2+}, and 0.3 mg/L K^{+}[203].

2.5.4 Emerging treatment technologies

Among the numerous emerging technologies explored for advanced treatment of RWW, chemical oxidation, filtration, biological treatment, and adsorption have been successfully used in laboratory studies and, to a lesser extent, pilot studies, as presented in Table 6. Only a few case studies of full-scale implementation systems deriving from laboratory and pilot studies exist, such as that of Wang *et al* (2011). For most technologies, however, optimisation of lab-scale operating conditions is ongoing because one of the main limitations for implementation is related to high energy consumption, use of high concentrations of reagents, or use of high-cost substrates, all deriving in cost-prohibitive settings[4,204]. A brief description of emerging treatments for RWW based on adsorption, microbial metabolism, and filtration is found below, but an emphasis is given to AOPs, which have shown to have the highest potential to mineralise a wide range of recalcitrant contaminants that would otherwise be almost impossible to degrade.

Membrane technologies

The principle of membrane processes is a physical separation of contaminants using permeable membranes with different pore sizes. These technologies are used to remove soluble organics and inorganics, biological agents (bacteria using microfiltration, and virus using ultrafiltration)[202,205] and metals, such as Cu^{2+}, Cd^{2+}, Hg[206,207] from wastewater. Some membrane processes have been successfully up-scaled to pilot and full-scale systems, partly

because the separation process is usually carried out under atmospheric conditions (which reflects in energy savings) and in membrane modules that allows modifying the design based on specific needs[208]. Likewise, constant removal efficiencies are achieved regardless of the feed water characteristics. However, one of the most significant limitations of membrane-based technologies is fouling, which is influenced by membrane type, module configuration, and the presence of high-molecular-weight compounds, all leading to loss of membrane flux and higher operational costs[205]. Fouling-resistant membranes have been developed by increasing hydrophilicity of the surface, either by using hydrophilic coatings or adding amphiphilic copolymers during the manufacturing process of membranes. Consequently, the need for chemical cleaning is reduced, decreasing operational costs[209].

Ultrafiltration can remove hydrocarbons, and free and dispersed oil efficiently, but fails to remove salts. This means that other technologies, such as reversed osmosis, are needed in combination with ultrafiltration when effluents are intended for reuse (because salts induce corrosion) or for discharge in freshwater[209]. Ultrafiltration has also been used to remove Hg to <1.3 ppt in secondary RWW[207].

Adsorption technologies

The removal of contaminants from wastewater using adsorption is based on the transference of chemicals in the water phase onto the solid phase of an adsorbent, stemming from physical and chemical interactions occurring in the interphase[210]. Adsorption-based technologies can remove SS, dissolved organics and inorganics, and biological agents[202]. Its performance depends on a variety of factors, including the quality of pre-treatment, the nature of contaminants, and the nature of the adsorbent, the latter being a key aspect under constant research. The standard for adsorption-based treatment is commercial activated carbon (AC) derived from charcoal, which has proven to be useful for the removal of various organics and inorganics from wastewater. However, its high cost often limits implementation of commercial AC as adsorbent in full-scale systems. Alternatively, a wide range of AC can be developed from other carbonaceous precursors provided the final product has the desired porosity, surface area, and sorption characteristics[211]. Low-cost materials have been evaluated for the production of effective and inexpensive adsorptive materials, as set out in Table 6. For instance, AC derived from date pit and coconut coir pith can successfully remove phenols and COD from secondary RWW after an appropriate production process (carbonisation of substrate + activation of carbonised material) [211,212].

Table 6. Emerging advanced treatment technologies for refining wastewater

Type of treatment	Method	Status	Characteristics of influent	Treatment efficiency	Advantages	Limitations	Reference
Adsorption	Date-pit AC	Lab-scale	COD: 3490 mg/L	COD: 90%	Agricultural waste, such as coconut shell and rice husk, can be used as substrates, reducing operational costs and allowing valorisation of waste. Sludge-free operation.	Activated carbon is the standard substrate used, which is cost prohibitive for full-scale implementation. Adsorbents need frequent regeneration, which usually requires high temperatures or alkali treatment.	El-Naas *et al.*, 2010[212]
	Coconut coir pith AC	Lab-scale	Grease: 145 mg/L Free oil: 1412 mg/L BOD: 106 mg/L COD: 584 mg/L Phenol:0.24 mmol/L *p*-Chlorophenol:0.22 mmol/L *p*-Nitrophenol: 0.21 mmol/L	Phenol: below detection limits with 200 mg/L of AC *p*-Chlorophenol: below detection limits with 175 mg/L of AC *p*-Nitrophenol: below detection limits with 150 mg/L of AC			Anirudhan *et al.*, 2009[211]
	PAC in DAF system	Pilot-scale	BOD: 95 mg/L COD: 198 mg/L	DAF only: BOD: 27 – 70% COD: 19 – 64% DAF + PAC: BOD: 76 – 94% COD: 72 – 92.5%	The method allows improving the performance of an existing system	The study used commercial AC, which might be cost prohibitive for implementation in developing countries	Hami *et al.*, 2007[213]
Filtration	Ultrafiltration as pre-treatment for reverse osmosis	Lab-scale	COD: 70 – 190 mg/L BOD: 5 mg/L TOC: 8.8 – 10.35 mg/L Turbidity: 12.5 – 14.2 NTU	TSS: 98% Turbidity: 98% COD: 30%	Good removal of iron originated during the coagulation-flocculation process. Constant removal efficiencies. Ease of adaptation to existent facilities. Simple maintenance.	Membrane fouling/scaling. Frequent backwashing needed. High capital costs.	Teodosiu *et al.*, 1999[61]

Type of treatment	Method	Status	Characteristics of influent	Treatment efficiency	Advantages	Limitations	Reference
Filtration	Ultrafiltration	Pilot-scale	TOC: 8 – 10 mg/L COD: <10 – 12 mg/L DOC: 7.1 – 8.9 mg/L Hg: 5.5 ppt	Flow rate: 10 – 20 gpm Hg: < 1.3 ppt	Constant removal efficiencies regardless the feed water characteristics.		Urgun-Demirtas *et al.*, 2013[207]
AOPs	Photocatalysis	Lab-scale	COD: 200 DOC: 20 Phenols: 3.7 Oil and grease: 23	Phenols: 93% DOC: 63% Oil and grease: 50%	Short treatment time: 1 hour Regardless of initial pH, final effluent tends to neutrality.	Use of UV light increases energy consumption. Not enough to provide high-quality effluents for reuse.	Santos *et al.*, 2006[214]
	Photocatalysis	Lab-scale	COD: 200 - 240 mg/L BOD: 110 - 230 mg/L Phenols: 9 mg/L TDS: 510 - 620 mg/L TSS: 15 - 27 mg/L	COD: 40.68%	A lower optimum concentration of TiO_2 and ZnO was achieved (1.2 and 0.8 g/l respectively). Use of UV light helps to eliminate biological agents.		Khan *et al.*, 2015[215]
	ElectroFenton (introducing air and iron particles simultaneously into the electrochemical process)	Lab-scale	COD: 4753 mg/L Phenols: 146 mg/L	EC (electrochemical): COD: 57.08% EC + Fe: COD: 68.86% *EC + air:* COD: 77.91% EC + Fe + air: COD: 83.65% *EC + Fe + air + pH 3:* COD: 89.91% Time: 60 min	Relatively low energy requirements. High-efficiency system. In this study all experiments were carried at ambient temperature, reducing energy consumption.	The optimum pH for Fenton reactions is 3, which requires pH adjustment and use of H_2SO_4.	Yan *et al.*, 2014[216]

Type of treatment	Method	Status	Characteristics of influent	Treatment efficiency	Advantages	Limitations	Reference
AOPs	ElectroFenton, electrocoagulation (both using iron electrodes), Ru-MMO electrode, BDD anode	Lab-scale	Phenol: 192.2 mg/L COD: 590 mg/L	*Ru-MMO:* Phenol: 94.5%, COD: 70%, Time: 300 min *Electrocoagulation:* Phenol: 6.27%, COD: 2.26%, Average energy consumption: 31.949 kWh/g Time: 120 min *ElectroFenton:* Phenol: 98.74%, COD: 75.71%, Average energy consumption: 0.143 kWh/g *Diamond:* Phenol: 99.53%, COD: 96.04%, Average energy consumption: 4.05 kWh/g, Time: 75 min	Versatility, energy efficiency, selectivity, amenability to automation, cost-effectiveness. In this study all experiments were carried at ambient temperature, reducing energy consumption.	Performance of electroFenton is heavily affected by feeding type of H_2O_2. Continuous feeding may be necessary if short contact times do not meet discharge limits.	Yavuz *et al.*, 2010[217]
	Fenton-like process	Lab-scale	Raw RWW COD: 1343 mg/L TOC: 398 mg/L BOD: 846 mg/L Oil and grease: 240 mg/L TSS: 74 mg/L	COD: 98% TOC: 70%	Short reaction time (approx. 30 minutes).	Requires strict control of pH	Diya'uddeen et al, 2012[218]

Type of treatment	Method	Status	Characteristics of influent	Treatment efficiency	Advantages	Limitations	Reference
AOPs	Catalytic ozonation using activated carbon-supported manganese oxides as catalysts	Lab-scale	Oil: 71.3 mg/L COD: 3145 mg/L BOD: 341 mg/L TOC: 1812 mg/L Phenols: 105 mg/L	COD: 54% TOC: 49%	Low cost, simple operation. Increase degradability of wastewater.	Most of the catalysts for ozonation are expensive, limiting application in full-scale systems. Catalysts are now being developed based on cheap materials, such as activated carbon.	Chen *et al.*, 2014[219]
Chemical oxidation	Laccase-catalysed oxidation	Lab-scale	Phenols: 16 mg/L and 248 mg/L	Phenols: >98% and 99.8%	Use of agricultural waste as substrates for biotechnological production of the enzyme.	Optimum enzymatic activity at 50°C, increasing operational costs. There is low potential for application in full-scale systems.	Vargas & Ramírez, 2002[220]
Hydrothermal processes	Microwave-assisted catalytic wet air oxidation under low temperature (150°C) and low pressure (0.8 Mpa)	Lab-scale	BOD:COD ratio 0.04 COD: 5500 mg/L	BOD:COD ratio 0.47 COD: 91.8% Time: 30 min	It increases the biodegradability of contaminants. This study developed a catalyst-mediated version that used milder operation conditions, decreasing operational costs. The use of microwave induces heating at the molecular level, leading to fast thermal reactions.	Traditionally, catalytic wet air oxidation operates from 180°C to 315°C and 2 Mpa to 15 Mpa, leading to high energy consumption and expensive running costs. Pre-treatment of catalyst required (using HCl, heating, water rinsing, drying).	Sun *et al.*, 2008[221]

Type of treatment	Method	Status	Characteristics of influent	Treatment efficiency	Advantages	Limitations	Reference
Combined processes	Hollow fibre membrane bioreactor (activated sludge + membrane filtration)	Pilot-scale	BOD: 203 mg/L COD: 250 mg/L TDS: 2100 mg/L TSS: 110 mg/L Turbidity: 40 NTU	COD: 82% BOD5: 89% TSS: 98% VSS: 99% Turbidity: 98%	No chemical additives. High COD removal efficiencies. Treatment tools can be small and completely automatic. It provides high-quality effluent for reuse.	Needs sufficient pre-treatment to protect membranes. Frequent chemical cleaning needed.	Razavi & Miri, 2015[222]
	Microbial fuel cell	Lab scale	Phenols: 60 mg/L Sulphides: 94 mg/L Oil and grease: 720 mg/L COD: 1040 mg/L	COD: 84% Hydrocarbons: 99% removed below detection limits. Oil and grease: 95% Phenols: 85% Sulphides: 79.5% Power output: 225 mW/m^2 (higher than studies using single species) Time: 17 days	Transformation of chemical energy of substrates into electrical energy by microbial metabolism, which translates into valorisation of wastewaters.	It has a high energy consumption that stems from the continuous demand of current.	Srikanth *et al.*, 2016[223]
	Biofiltration + H_2O_2/UV oxidation + reverse osmosis	Lab scale	COD: 18 - 61 mg/L TOC: 6.3 - 17.51 mg/L	Biofiltration: TOC: 65% *H_2O_2/UV:* TOC: 78% Final effluent: NH4 < 10 mg/L COD <50 mg/L	It removes readily, poor, and non-biodegradable compounds. Biofiltration: simple construction, robust operation, low energy requirements. Good retention of suspended particles, which contribute to turbidity.	Potential inhibition of microorganisms due to toxic chemicals in influent.	Nogueira *et al.*, 2016[224]

Type of treatment	Method	Status	Characteristics of influent	Treatment efficiency	Advantages	Limitations	Reference
Combined processes	Filtration + H_2O_2/UV + catalytic wet peroxide oxidation	Lab scale	COD: 128 mg/L TOC: 35 mg/L SS: 20 mg/L Phenols: 570 mg/L	Filtration: Turbidity: 92%; SS: 80% H_2O_2/UV: Phenols: 100%; TOC: 52.3% COD: 84.3% Final effluent: TOC: 94.7%; COD: 92.2%	Provides high-quality effluent for reuse. Filtration decreases hydrophobicity, enhancing oxidation by wet oxidation.	High energy consumption due to UV lamp use, which can be reduced with low-pressure lamps. Potential production of toxic by-products (chlorophenols) depending on the type of lamp used.	Rueda-Márquez *et al.*, 2016[225]

AC can be applied as an advanced treatment for RWW using granular AC (GAC) columns or powdered AC (PAC) integrated into activated sludge. In particular, one pilot-scale study reported the use of PAC (50 mg/L) in a DAF unit to improve removal of BOD, concluding that flow rate and residence time are pivotal for removal efficiency because these define contact time between contaminants and adsorbent[213]. Similarly, another pilot-scale study using GAC units (2 serial activated carbon beds) lowered the concentration of NAs in RWW and significantly reduced toxicity (96-hour flow through bioassay) on rainbow trout, even though chemical analyses indicated that some NAs remained at very low concentrations[149].

Advanced Oxidation Processes

Chemical oxidation aims at complete mineralisation of contaminants into CO_2, water, and inorganic salts, but it is common to find that chemicals that resisted biological treatment have a highly stable chemical structure, limiting their mineralisation. Therefore, it is often necessary to use aggressive reactive systems to achieve more effective oxidations. This is achieved by using simultaneous oxidation processes that promote an accelerated *in situ* generation of chemically reactive species under near-ambient temperature and pressure conditions. These systems are collectively known as AOP and are currently considered the most promising and competitive methods for advanced treatment of industrial wastewater[31,202,226] because these can destroy non-biodegradable structures[31]. Different AOPs have been used to oxidise organic compounds in RWW that resist biological treatment, as provided in Table 6.

AOPs are based on the generation of different chemically reactive species, including sulphate radicals ($SO_4^{\cdot-}$), ozone (O_3), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$), the latest showing little oxidation selectivity and the highest oxidation potential[29], as shown in Table 7. These characteristics make hydroxyl radicals the most common oxidising agent in water treatment[131]. Hydroxyl radicals are highly unstable and reactive species that rapidly attack organic compounds via radical addition, hydrogen abstraction, electron transfer, or radical combination. Abstraction of hydrogen atoms from organic compounds occurs fast and produces H_2O molecules and a carbon-centred radical $RC^{\cdot}$ that typically reacts with oxygen forming a peroxyl radical $ROO^{\cdot}$. In the case of aromatics, radical addition is the most common reaction, and hydroxylated adducts are formed. The successive reaction of radicals forms even more reactive species that eventually leads to the degradation of pollutants, with potential mineralisation[131,227].

The mechanisms to generate hydroxyl radicals vary, including different oxidising agents,

sources of irradiation, and catalysts. The production of hydroxyl radicals can be improved with the addition of other oxidants or under UV irradiation[131,228]. For instance, in UV-based AOPs, hydroxyl radicals are generated through the production of photons in the presence of a variety of catalysts (*e.g.* TiO_2) and oxidants (*e.g.* H_2O_2 and O_3)[131]. The destruction of pollutants is achieved not only by the presence of hydroxyl radicals but also by absorption of UV radiation, in which direct photolysis contributes significantly to degradation, even in cases where absorption is weak. In the case of strong UV absorbing substances, destruction is mainly due to photolysis, and the contribution of other oxidants is low[228]. However, UV/O_3 and UV/H_2O_2 systems consume large amounts of oxidants and operating costs are high[67], limiting their scaling-up. Other AOPs include Fenton and photo-assisted Fenton processes, and photocatalytic oxidations (UV/TiO_2).

Table 7. Redox potential for some oxidising agents used in AOPs[131,229]

Reactive species	Redox potential (V, 25°C)
Chlorine	1.36
Chlorine dioxide	1.57
Permanganate	1.68
Hydrogen peroxide	1.78
Ozone	2.07
Hydroxyl radical	1.95 – 2.8
Fluorine	3.03

It is noteworthy that AOPs may generate effluents that are more harmful after treatment because of the formation of stable intermediates with lower biodegradability and/or higher toxicity, or due to the presence of excess oxidants and/or catalysts that may be toxic to biota[30]. Consequently, the concept of sustainability has slowly integrated into the development of AOPs by eliminating potential hazards from the beginning of the process, resulting in a reduction of environmental impact and overall costs, which in turns leads to safer chemical processes. In this context, AOPs conducted under simple and mild conditions are encouraged, and catalysts are seen as energy savers. Oxidants with higher active oxygen percentage content are preferred because these lead to higher chemical yield, as the mass of oxygen transferred to the substrate with respect to the total mass of oxidant is higher[32]. Under these principles, H_2O_2 is considered the ideal oxidant because (i) its active oxygen content is 47% in comparison with

that of O_3 of 33%. O_2 has a theoretical active oxygen content of 100%, but in most cases only one oxygen atom is transferred to the substrate, meaning that O_2 usually has an active oxygen content of 50%. Besides, extreme conditions (*i.e.* temperature and pressure) are needed to activate O_2; and (ii) industrial production of hydrogen peroxide is significantly high, thus it is considered affordable for most wastewater treatment plants[32]. Still, H_2O_2 alone is a weak oxidant and only oxidise a limited range of organics, such as reduced sulphur compounds, cyanides, and some nitro-organic and sulpho-organic compounds[216]. Some catalysts have been developed to be used in combination with H_2O_2 to increase removal efficiency, and catalytic iron species are especially popular because these activate H_2O_2 to generate hydroxyl radicals. Iron-based catalysts have been developed as metal salts, metal oxides, and zero-valent metal, and have several advantages, including high reactivity, low toxicity, high abundance in earth's crust, and low commercial cost[230]. The Fenton reagent and Fe-TAML (TetraAmido Macrocyclic Ligand) activators are two examples of this type of catalysts. Non-iron catalysts using aluminium, cerium, chromium, cobalt, copper, and manganese have also been developed for catalysing H_2O_2-based AOPs[230].

2.6 Regulatory frameworks for refining effluents

The understanding of environmental quality has progressed overtime for a variety of reasons, including improvements in the ability to measure and control pollution, increased awareness of the impacts of pollution, and the increasing load and diversity of contaminants in the environment resulting from anthropogenic activities[231]. Consequently, environmental regulations are co-evolving with societal and scientific progress, and the regulatory framework for refining effluents has not been an exception. In general, environmental regulatory systems are structured around national legislation and (ratified) international agreements, as both are legally binding. National legislation around the world vary significantly, but regional and international agreements help to bridge gaps. In the case of treatment and discharge of RWW, international conventions aiming at controlling and reducing hazardous substances are relevant, such as the Stockholm convention (dealing with persistent organic pollutants) and the London protocol (Protocol to the convention on the prevention of marine pollution by dumping of waste and other matter).

Environmental regulations are certainly relevant within the context of wastewater management in the refining sector because these are strong technical drivers that define the adoption of new technologies in wastewater treatment plants as legislation become more stringent, thus

regulating the operations and resulting environmental impact of refining sites[232]. Two different regulatory approaches address the discharge of effluents by petroleum refineries. The first approach, also known as "end-of-pipe", aims at controlling wastewater quality by regulating point source discharges. The second approach, commonly referred to as the water quality approach, targets the potential environmental impact of discharges by monitoring receiving water quality. In both cases, the assessment can be carried out considering physicochemical parameters only, or it can also include toxicity-based methods to assess the potential of effluents to cause damage to aquatic ecosystems.

The end-of-pipe approach sets limits on specific parameters of effluents or on the concentration of certain chemicals. It has evolved from a basic chemical analysis of effluents considering general parameters such as COD, BOD, suspended solids, and nutrients (e.g. phosphorus, nitrogen) to the implementation of a chemical-by-chemical control, which developed along the definition of priority pollutants by environmental authorities in the mid-seventies[233]. Emission limit values are based on what the Best Available Technology (BAT) for a specific industry sector can achieve, so there is no guarantee that the environmental concentrations will be safe for the receiving environments[234]. As the tolerance of the receiving environment is not considered, this approach moves towards near-zero environmental concentrations of hazardous chemicals, which may become cost prohibitive or not technically achievable[235,236]. At the same time, this approach can also include biological criteria to be met for discharge, as this helps to (i) assess mixture effects of different individual pollutants, which is particularly useful with RWW given their complexity, and (ii) optimise wastewater treatment plants to minimise the environmental risk of effluents[48]. The strategy of assessing adverse effects of effluents to biological systems receives different names depending on the country or region, including WET, whole effluent assessment (WEA), direct toxicity assessment, assessment of environmental effects, and effluent toxicity test[177,235].

In the water quality approach, the goal of emission limit values is to maintain environmental quality standards, which is achieved by controlling the environmental concentration of certain chemicals so that safe thresholds are not exceeded in receiving waters, taking into account the dilution of the effluent in a particular discharge point[235]. Surveys of receiving water quality include the measurement of certain groups of chemicals in water and sediments, and *in situ*

biomonitoring helps to estimate diversity and abundance of species[48]. Bioassay methods can also help monitor the quality of receiving waters and help to track toxic chemicals[177].

It is to be noted that the end-of-pipe approach leads to unified emission limit values, whereas the second approach favour those refineries discharging to large watercourses, as hazardous chemicals would dilute significantly[234]. Historically, the usual trend for national legislation has been to start with a chemical-based end-of-pipe approach, followed by the addition of acute and then chronic toxicity assessment of effluents. Later on, the environmental quality approach is implemented, and sometimes additional endpoints are included[177]. Most high-income countries require both approaches, so refineries must use the best available technology to meet receiving water quality requirements[56,235].

The US and Europe are perfect examples of case studies for high-income countries. The Clean Water Act (CWA) addresses water pollution in the US and therefore frames the environmental regulation applicable to RWW. The refining industry has specific effluent guidelines based on BAT, with limits on ammonia, 5-day BOD, TOC, hexavalent and total chromium, oil and grease, pH, phenols, sulphide, and TSS[6]. At the same time, the WET assessment is a component of the integrated approach of the US EPA to detect and address toxicity in surface waters[237]. In the case of Europe, the Water Framework Directive (WFD) guides water management in all member states with aquatic ecology as the base for management decisions, and the industrial emissions directive (IED) is the main EU instrument regulating pollutant emissions from industrial installations[238]. The WEA tool within the WFD and IED is comparable to WET within CWA in the sense that it is a tool helping to control discharges of hazardous substances, but it differs in that it considers persistence and bioaccumulation in addition to toxicity[177]. However, WEA approaches are not mandatory, and there are different regulatory strategies in various members of the EU. A survey applied by Conservation of Clean Air and Water in Europe (CONCAWE) to 64 refineries around Europe showed that only 44% of the facilities are required to use some form of biological assessment on their effluents, mainly acute toxicity tests; only four refineries included tests for bioaccumulation. Results from this survey suggest that not all refineries within the same country are required to apply WEA tests, and such requirements are based on a case-specific basis[48].

Latin America provides an excellent example for middle-income countries because the regulatory framework for RWW is evolving following the normal trend of transition from end-

of-pipe approach to environmental quality approach. In the case of Brazil, environmental regulation has become more stringent over the last few years, which has led to the modernisation of existing refineries. The legislation limits effluents release on the basis of inorganic (a range of metals, ammonia, and sulphides) and organic parameters, the latter including specific pollutants, such as benzene, styrene, toluene, xylene, carbon tetrachloride, and dichloroethene. Assessment of ecotoxicity is mandatory using whole-organisms tests from at least two different trophic levels, and in some contexts, the receptor body effluent concentration is taken into account. The efficiency of wastewater treatment has allowed to reuse effluents successfully, and Petrobras (the biggest petroleum company in Brazil) reported to have reused 17 billion litres of water in 2010[239]. Conversely, Colombia recently released new effluent guidelines (Resolution 0631/2015) that were developed on an industry-by-industry basis, although so far the legislation does not consider biological assessments. Physicochemical parameters for RWW include 5-day BOD, oil and grease, pH, phenols, BTEX, PAHs, cyanides, and a variety of metals. Up to 2015, the legislation did not separate industrial effluents by sectors, and the directive focused on the efficiency of the applied wastewater treatment rather than the discharged contaminants. Similarly, the use of pollutant load (Kg/day) as an indicator of wastewater quality was replaced by pollutant concentration (mg/L), as this strategy helps to assess the biological significance of discharges.

2.7 Summary and conclusions

The benefits of petroleum refining for modern societies are shadowed by the production of vast amounts of wastewater with potential biological effects on a wide range of species and trophic levels once discharged into the environment. Within this context, refinery wastewater treatment plants are a critical barrier in limiting the release of harmful toxicants into aquatic ecosystems, but evidence suggests that current treatment technologies do not always provide effluents that are safe for humans and wildlife. The use of bulk parameters (such as COD, BOD, and SS) to assess the quality of refining effluents in environmental regulations is an important driving factor on the use of treatment technologies that provide complying, yet toxic effluents. The limitations of measuring such parameters were recognised decades ago, giving rise to the development of water quality-based discharge consents in various countries to limit the discharge of specific toxic chemicals from industrial wastewater, starting a regulatory process to protect the receiving environment from ecological damage. Whole effluent toxicity testing, which was introduced later, was a significant step forward in protecting the environment from

other components in the effluent and addressing the limitations of measuring specific chemicals.

While whole effluent testing remains an essential tool in environmental testing as it addresses the effect of unknown chemicals and the effect of exposure to chemical mixtures, the generic nature of the test does not allow the identification of the cause of observed effects. This is a limitation with important consequences, as wastewater treatment technologies require an understanding of the chemical composition of wastewater to target removal. Approaches combining chemical analysis and toxicity tests have been used to address these shortfalls, but little progress has been made in determining the causative agents of the toxicity exerted by RWW, thus challenges to treat RWW effectively remain. The quality of effluents from major refineries in Europe and the US has shown significant improvements, but high contents of organic matter other than oil remain. Previously published studies have identified groups of contaminants of toxicological concern in RWW, but there is an apparent lack of research on polar refining pollutants due to analytical limitations, which could explain the gap between observed toxicity and reported chemistry. Further research is needed to identify the constituents of this fraction to determine the causative agents of acute and chronic toxicity. As evidence suggests that organic compounds might be behind the observed effects, an EDA approach could help bridge this gap provided it includes sequential extraction methods targeting polar compounds.

The role of research is crucial. The more it is known about a problem, the easier it is to provide effective solutions. Advances in analytical techniques and biological assays have deepened the knowledge we have on pollution, including pollution derived from petroleum refining. Research on treatment technologies is continuously proposing and optimising emerging methods to provide high-quality effluents for recycling purposes, but only an understanding of the composition of effluents will complement the design, development, and implementation of new wastewater treatment processes to generate effluents that are environmentally safe.

CHAPTER 3

IDENTIFICATION OF NAPHTHENIC ACIDS AS IMPORTANT CONTRIBUTORS TO TOXICITY OF REFINING EFFLUENTS BY AN EFFECT DIRECTED APPROACH

3.1 Introduction

There is a solid link between energy and water. Water is essential for almost all processes in the energy sector, including the processing of petroleum and the generation of electricity, and energy is needed for the delivery and treatment of water[240,241]. Consequently, energy and water security are tightly intertwined, and both the water and energy sector are shifting their policies, technologies, and practices in alignment with the increasing needs of both resources. The water community is carefully looking at their energy consumption and has focused much of their research effort on low-energy treatment technologies, while the energy sector has now focused on their water consumption and impact on water availability and quality. With a current population of 7.6 billion people and an average consumption of 55 kWh/day of primary energy per capita, 15 million m^3 of crude oil need to be refined per day (in 2016)[1] to provide 33% of the global energy requirements[49], consuming approximately 2 to 2.5 million m^3 of water per m^3 of oil refined[3]. In the US alone, refineries produce a discharge volume that ranges from 5 to 72 million m^3 per year[6].

The quality of such discharges has a significant impact on receiving ecosystems and surrounding human populations. Thus continuous monitoring of the quality of effluents gives an indication of the efficacy of wastewater treatment plants and helps to identify effluents of concern. However, there are important discrepancies in relation to quality criteria for refining effluents, which mainly derive from differences in environmental regulations. Some regulations, mostly from middle- and low-income countries, consider only bulk parameters to establish discharge limits and monitor treatment efficacy, whereas regulations in high-income countries generally have a more comprehensive approach that combines physicochemical parameters with biological assessments aiming to protect the receiving environment by addressing chemicals that are not accounted for in physicochemical parameters. This way, the detection of effluents of concern should, in theory, lead to an investigation to determine the causes of biological effects so these can be targeted during treatment. In practice, however, this is not straightforward because toxicity often results from the interaction of different chemicals or stem from chemicals at concentrations hard to detect. Moreover, extraction techniques are not universal, thus excluding groups of compounds that could be essential for understanding the observed effects of RWW. So, what is it known so far from the composition of refining effluents?

A common approach conducted by numerous studies is to characterise chemical components

present in effluents without considering their potential biological effects. Such studies can be either targeted or non-targeted, the latter being expensive and time-consuming, especially if quantification is conducted. In this case, the concentration of contaminants does not necessarily provide an adequate assessment of the risk posed because chemicals are not always bioavailable[242] and can be toxic at very low levels. Also, the interaction between compounds can create mixture effects that are larger than the effects exerted by each component separately, even when these are present at concentrations considered safe[243]. As for targeted studies, their major drawback is that they are limited to known pollutants and therefore fail to address the growing problem of the so-called "emerging contaminants" or "contaminants of emerging concern". These contaminants are always a moving target that reflects the continuous production and release of new chemicals into the environment, making the term "emerging" a rapidly evolving concept that requires non-targeted approaches.

Nonetheless, chemical studies of refining effluents have provided crucial information for the design of current wastewater treatment plants, as these studies have reported the presence of aliphatic and aromatic hydrocarbons, phenols, BTEX, and metals[65,79–81]. The monitoring of bulk parameters have indicated that BOD and COD in RWW are typically high before treatment, and COD usually remains high after treatment[77,80], suggesting the presence of recalcitrant dissolved chemicals. However, current knowledge has proven to be insufficient to provide refining effluents that are environmentally safe, and a significant gap in knowledge has surged in relation to the high COD levels remaining after treatment, which are linked to the observed effects of RWW. The composition of this COD has yet to be revealed, but the combination of chemical analysis and biological assessment can help to simplify this problem.

Two different approaches combining biology and chemistry were developed to address the problem of environmental diagnostics and hazard assessment, namely effect-directed analysis (EDA) and toxicity identification evaluation (TIE). These two approaches can help to identify toxic chemicals from a complex mixture in a cost-effective way, helping to focus analyses on relevant contaminants. The first set of protocols combining toxicity tests and chemical analysis were developed during the late 1970s by different laboratories for the study of ambient air and diesel particulates, and the identification of chemical mutagens in synthetic fuels and drinking water[244], but the approaches as we know them today were developed in the 1980s. Both EDA and TIE aim to reduce the complexity of environmental samples to identify toxicants[186], as the premise behind these two approaches is that the identification of key toxic chemicals is

essential to mitigate risks[245]. However, it is important to highlight the fact that toxic chemicals may be present below detection limits or masked by other compounds, making their identification a challenging task[246].

TIE evolved from the WET program developed by the US EPA to stop toxic chemicals from entering the environment in concentrations exerting biological effects, which was one of the requirements of the CWA as part of pollution control programs. WET is based on whole-organism tests and aims at quantifying toxicity of all constituents together, but this information alone was not leading to concrete programs leading to reduce toxicity from the source, and it became necessary to identify the chemicals causing toxicity, so TIE was developed[186]. Overall, TIE aims for results that are ecologically relevant, and consequently, toxicity is defined as any adverse effect on whole organisms. In the same way, one of the main objectives of TIE is to preserve the link between the original sample and the observed toxicity, avoiding severe manipulation of the original sample that can alter bioavailability, induce loss of some classes of toxicants, or create artifacts[54]. The TIE approach is divided into three phases[247,248], as follows:

- → Phase I is intended to assess potential classes of toxicants by manipulating the sample and determining whether those manipulations have an effect on toxicity, which gives an indication of physicochemical characteristics of toxicants. Sample manipulations include pH changes, aeration, filtration, and the addition of chelating and reducing agents, all of which are conducted together with toxicity testing[247].
- → Phase II aims to identify the type of toxicants present in the sample depending on the results from phase I; it defines whether toxicants are organic, metals, ammonia, or non-polar. Methods for polar organics are not well developed, and only recently methods for identification of specific toxicants, such as organophosphate, carbamate, and pyrethroid pesticides, have been successfully developed and included when needed[186,248].
- → Phase III is designed to confirm the identity of toxicants, and the methodology overlaps with that of phase II[247,248].

TIE was first developed for water samples (effluents, receiving waters, interstitial waters, groundwater), and later applied to whole sediments. One of the drawbacks of the TIE approach is the high sample volumes required for full analysis and the large numbers of test organisms needed, as toxicity tests evaluate adverse effects on whole organisms, usually impacting

survival, growth, reproduction, and development[186].

EDA is the European approach to environmental diagnostics developed to address the problem of both emerging contaminants and legacy pollutants entering European waters[186]. Like the TIE approach, the EDA approach can be applied to water samples and sediments, but also to tissues and biological fluids[186] because the methodology includes an extraction step, which has provided useful information related to bioaccumulation, bioavailability, and metabolization[242]. A big difference between TIE and EDA is that the latter makes an emphasis on organic contaminants as the cause of toxicity, whereas TIE has limitations in the identification of organics[183,242]. The general methodology is composed of four main steps, as follows:

- → Extraction: separates the toxicants from the matrix, so these are available for chemical fractionation and analysis. This step makes possible the application of EDA to a wide range of matrixes, including tissues and biological fluids. Numerous techniques are available for the extraction of chemicals from different environmental matrixes, such as solvent extraction, SPE, accelerated solvent extraction (ASE), soxhlet extraction, and passive sampling.
- → Fractionation: reduces the complexity of the mixture obtained after extraction by removing non-toxic chemicals. Depending on the nature of analytes, fractionation can be based on polarity, molecular size, hydrophobicity, planarity, or the presence of specific functional groups[246].
- → Toxicity testing: determines the toxic potency of samples, extracts and fractions. A wide range of toxicity tests are available for use, and the selection of an appropriate test or set of tests depends on whether the aim is merely to detect toxicants or assess environmental hazards. As opposed to TIE, biotests in EDA can be used just as additional detectors because the approach does not necessarily aim at results that are ecologically relevant[246].
- → Identification of chemicals in toxic fractions: conducted using different analytical techniques, such as liquid chromatography coupled to mass spectrometry (LC-MS) and gas chromatography coupled to mass spectrometry (GC-MS).

EDA is based on the premise that all toxic effects begin by changing structural and/or functional properties of molecules involved in cellular activities, and that these interactions then cascade into effects on upper levels of organization. Consequently, EDA tends to use *in vitro* bioassays rather than whole-organism tests aiming to understand toxic effects at the

biochemical level, although *in vivo* tests are being increasingly used to obtain results with ecological relevance. Some toxicity tests are based on specific modes of action (MOA), such as aryl hydrocarbon receptor-, estrogenic receptor-, and androgenic receptor-mediated activities[242], whereas many others can detect chemicals with several MOAs, such as bacteria-based tests, which assess overall toxicity. This characteristic, and the fact that bacterial bioassays are rapid, easy to handle, and have low requirements of sample make these tests excellent options to screen toxicity[246].

Different endpoints are used to detect toxicity in bacteria, including glucose uptake activity, oxygen consumption, and luminescence output. The latter category, known as luminescent bacteria tests (LBT), has been widely used primarily with *Vibrio fischeri* as a biosensor in the form of standardised, commercial versions readily available, such as Microtox®, BioLuminex™ and Lux-Fluoro. This gives another advantage to LBT, which is its low coefficient of variation compared to other bioassays because bacteria come in the form of a standardised lyophilised reagent[249]. Results obtained from LBT have no ecological relevance when the test is used alone because the exposure time is very short, usually 15 minutes, meaning that chronic effects cannot be determined using this test. However, when the objective is to select target chemicals for water treatment, LBT can spotlight toxic effects, helping to focus on relevant compounds. In other words, luminescent bacteria can be used as an additional detector or toxicity sensor.

Previous studies aiming at linking toxic effects and chemicals present in RWW have evaluated toxicity of known specific components like fuel oil, phenol, and chromium[9,10], or followed the TIE approach[54,79,89,250], which has mainly shown that PAHs and "other organic chemicals"[54,79,89,250] are behind the toxic effects without shedding much light on the identity of these miscellaneous organics. These outcomes are expected, as one of the most significant limitations of TIE is the identification of organic compounds, which seem to play a critical role in refining effluents. Therefore, the objective of this chapter was to identify toxic organics in petroleum refining effluents following a non-targeted EDA procedure using *Vibrio fischeri* as a biosensor. The methodology was designed to cover a broad range of organics because previous research has tended to limit extraction to hydrophobic or weakly polar compounds and evidence suggests that the study of polar compounds could improve the understanding of

refining effluents and their effects on biological systems. The schematic representation of the EDA performed is provided in Figure 7.

3.2 Experimental section

3.2.1 Chemicals and reagents

Solvents (analytical and HPLC grade) were obtained from Fisher Scientific. Sulphuric acid and sodium hydroxide were purchased from Fluka. Oasis® WAX 6cc (150 mg, 30µm particle size) and HLB 6cc (200 mg) extraction cartridges were obtained from Waters. *tert*-Butyldimethylsilyl derivatisations were carried out using N-methyl-N-(*tert*-butyldimethylsilyl)-trifluoroacetamide containing 1% t-BDMS-chloride (MTBSTFA), which was purchased from Sigma-Aldrich. Stock solutions for spiking and fractionation check solutions were purchased from Restek UK, as follows:

i. The aromatic hydrocarbons spiking solution (200 µg/mL in acetone) was composed of acenaphthene, acenaphthylene, anthracene, benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*ghi*]perylene, benzo[*k*]fluoranthene, chrysene, dibenz[*i,h*]anthracene, fluoranthene, indeno(1,2,3-cd)pyrene, 2-methylnaphthalene, naphthalene, phenanthrene, pyrene, and 1,2,3-trimethylbenzene.
ii. The aliphatic hydrocarbons spiking solution (200 µg/mL in acetone) was composed of *n*-nonane, *n*-decane, *n*-dodecane, *n*-tetradecane, *n*-hexadecane, *n*-octadecane, *n*-eicosane, *n*-heneicosane, *n*-docosane, *n*-tetracosane, *n*-hexacosane, *n*-octacosane, *n*-triacontane, *n*-dotriacontane, *n*-tetratriacontane, *n*-hexatriacontane, *n*-octatriacontane, *n*-tetracontane, 2-methylnaphthalene, and naphthalene.
iii. The internal standard (IS) solution (400 µg/mL in acetone) was composed of 1-chlorooctadecane and *o*-terphenyl.
iv. The fractionation check solution (70 mg/L in hexane) was composed of naphthalene, bisphenol A, and phenol.

Storage of solutions was conducted at 4°C in dark and airtight conditions.

For the bioassay, phenol and $K_2Cr_2O_7$ were used as reference substance and positive control, respectively, both of which were purchased from Sigma-Aldrich. NaCl for the preparation of the diluent was purchased from Fischer Scientific. The Microtox® acute reagent was obtained from Modern Water Inc.

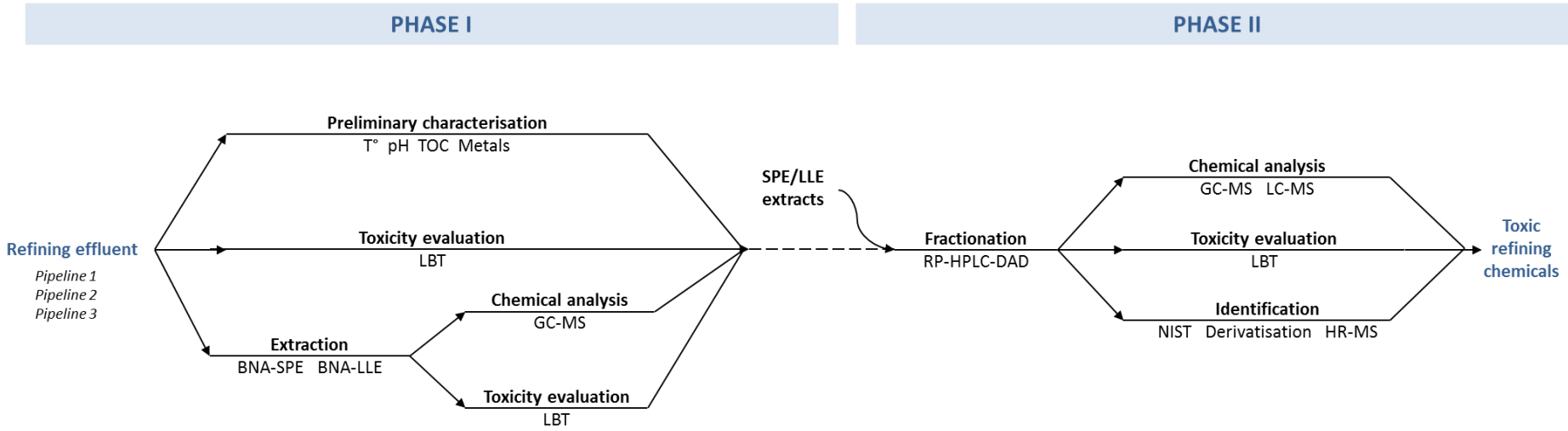


Figure 7. Schematic representation of the EDA performed to identify toxic organics in petroleum refining effluents. T°: Temperature; TOC: Total organic carbon; LBT: Luminescent bacteria test with *Vibrio fischeri*; BNA-SPE: basic/neutral/acidic solid phase extraction; BNA-LLE: basic/neutral/acidic liquid-liquid extraction; GC-MS: gas chromatography coupled to mass spectrometry; RP-HPLC: reverse phase high performance liquid chromatography; LC-MS: liquid chromatography coupled to mass spectrometry; NIST: National Institute of Standards and Technology Mass Spectral Library; HR-MS: high resolution mass spectrometry.

3.2.2 Sampling

Treated petroleum refining effluent samples were collected from 3 pipelines from an oil refinery located in Barrancabermeja, Colombia, directly discharging into River Magdalena. Sampling details are provided in Table 8. The refinery consists of five topping units (units containing both atmospheric and vacuum distillations sections), four catalytic cracking units, two polyethene plants, one alkylation unit, one paraffin unit, one aromatics unit, and a capacity of 250 kbpd.

Grab effluent samples were collected in amber, polyvinyl chloride coated glass bottles previously soaked overnight in Decon® and nitric acid 10% (v/v) to remove any traces of organic matter and metals. The temperature (°C) and pH of samples were measured *in situ* using an Oakton® portable meter. Samples were acidified to pH 2 and stored at 4°C in airtight conditions until analysis.

3.2.3 Sample Preparation

Each sample was divided into two separate sub-batches for chemical analysis and toxicity evaluation. Blanks and analytical quality control (AQC) samples were prepared for quality assurance purposes, as shown in Table 9. Briefly, samples were spiked with aromatic and aliphatic hydrocarbons to a final concentration of 80 µg/L, and with IS (*o*-terphenyl and 1-chlorooctadecane, corresponding to aromatic and aliphatic hydrocarbons, respectively) to 100 µg/L.

Table 8. Sampling of wastewater from an oil refinery located in Barrancabermeja, Colombia

Sample	Location	Date	Sampling point
Pipeline 1	7° 4‘ 15“ N 73° 52‘ 25“ W	20.05.2015	Post-treatment
Pipeline 2	7° 4‘ 1 “ N 73° 53‘ 1“ W	20.05.2015	Post-treatment
Pipeline 3	7° 4‘ 35“ N 73° 53‘ 15“ W	02.06.2015	Post-treatment

Table 9. Preparation of samples for chemical characterisation and toxicity evaluation

Batch	Type of sample	Sample	Spiking		
			Aliphatics	Aromatics	IS
Toxicity evaluation	Effluent	Pipe1	-	-	-
		Pipe2	-	-	-
		Pipe3	-	-	-
	AQC	Blank 1	-	-	-
Chemical characterization	Effluent	Pipe1	-	-	100 µg/L
		Pipe2	-	-	100 µg/L
		Pipe3	-	-	100 µg/L
	AQC	Blank 2	-	-	100 µg/L
		AQC	80 µg/L	80 µg/L	100 µg/L

IS: 1-chlorooctadecane and *o*-terphenyl

3.2.4 Preliminary characterisation of effluents

Samples were filtered using 1.2 µm pore size Whatman® filters to remove suspended solids. Total organic carbon (TOC) of aqueous filtrates was measured by combustion catalytic oxidation/non-dispersive infrared (NDIR) spectrometry using a Shimadzu TOC-VCPN Total organic carbon analyser coupled with a Shimadzu OCT-1 8-port sampler.

The total concentration of V, Ni, Zn, As, Se, Hg, Cr, Pb, and Cd was determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES) using a Perkin Elmer Optima 5300 DV spectrophotometer attached to a Perkin Elmer AS 93plus autosampler. Before analysis, 50-mL aliquots of the samples were transferred to polyethene bottles containing 3.68 mL of 68% nitric acid, for a final concentration of 5% nitric acid. Digestion of organic matter was carried out in a CEM MARS 6 following method 3015A from the US EPA for microwave assisted acid digestion of aqueous samples and extracts.

3.2.5 Sample extraction

Samples were filtered using 1.2 µm pore size Whatman® filters to remove suspended solids and extracted in duplicate using liquid-liquid extraction (LLE) and SPE in basic, neutral, and acidic conditions, as described below.

3.2.5.1 Liquid-liquid extraction

A sequential extraction procedure was applied to sample aliquots of 800 mL at different pH values with three 50-mL portions of dichloromethane (DCM), as described in Appendix I. Briefly, samples were extracted at neutral pH (6 – 8), acidic pH (≤ 2), and basic pH (12) after the corresponding pH adjustments using H_2SO_4:water 1:1 (v/v) or 10N NaOH as required. All three basic, neutral, and acidic (BNA) extracts were combined to obtain a final LLE extract per sample. The combined 450-mL DCM extracts were evaporated to incipient dryness in a TurboVap® LV concentration evaporator workstation. The extracts were then reconstituted into 800 µL in hexane prior to fractionation. AQC and blank samples (Table 9) were prepared using Millipore® deionised water and extracted identically to the samples.

3.2.5.2 Solid phase extraction

A sequential extraction procedure was applied to 800-mL aliquots of samples using Oasis® HLB cartridges (6 mL, 200 mg) and subsequently Oasis® WAX cartridges (6 mL, 200 mg), following the protocol set out in Appendix I. Briefly, each sample was adjusted to neutral pH (6 – 8) and loaded onto a first HLB cartridge; washing was carried out using 10 mL of 5% methanol in water and elution was done using 20 mL of methanol. Afterwards, the pH of samples was adjusted to 12, and each sample was loaded onto a second HLB cartridge; a first wash was carried out using 10 mL of 5% methanol in water, a second wash was performed using 10 mL of 5% NH_4OH in methanol, and elution was carried out using 20 mL of 2% formic acid in methanol. Subsequently, each sample was acidified to pH ≤ 2 and loaded onto a third HLB cartridge; a first wash was carried out using 10 mL of 5% methanol in water, a second wash was performed using 10 mL of 2% formic acid in methanol, and elution was carried out using 20 mL of 5% NH_4OH in methanol. Each sample was then loaded onto a WAX cartridge; 10 mL of 5% ammonia in water were used for the first washing, a second wash was performed using 10 mL of 100% methanol, and elution was done with ethyl acetate containing hydrochloric acid (2 M HCl:ethyl acetate (1:10) v/v). AQC and blank samples (Millipore® deionised water) were extracted using identical conditions to the samples.

A composite SPE extract was obtained for each sample after combining the HLB and WAX extracts, which were evaporated to incipient dryness in a TurboVap® LV concentration evaporator workstation. All extracts were reconstituted to 800 µL in hexane prior to fractionation.

3.2.6 Fractionation

Fractionation of LLE and SPE extracts was performed using normal phase high-performance liquid chromatography (NP-HPLC) using an Agilent 1260 system consisting of a 1260 binary pump, a 1260 high-performance autosampler, a 1296 column compartment, a 1260 diode array detector (DAD), and a 1260 fraction collector. The column used was an analytical Waters® SPHERISORB® silica column (4.6 x 100mm, 3-µm particle size). Mobile phases were (A) hexane 100% and (B) hexane:methanol:IPA 10:25:65 (v/v/v), flowing at 1 mL/min. The gradient started with 100% mobile phase A for 4 minutes, it then decreased to 99% by minute 8, to 96% by minute 20, and finally decreased to 10% by minute 30, and then reset to 100% by minute 35 and for 5 minutes, for a total run time of 40 minutes. The injection volume was 100 µL, and the absorbance of eluate was monitored at 210 nm. Fractionation was time-based with time-slices of 1 minute, and fractions were collected up to minute 32. A total of 400 µL of extract was fractionated per sample, which required 4 injections of 100 µL each. Fractions were evaporated to incipient dryness using a Thermo Reacti-Vap^{TM} evaporator and made up to 100 µL with deionised water prior to toxicity testing using LBT.

For quality control purposes, samples were bracketed by injections of the fractionation check solution to confirm that equivalent fractions were collected, *i.e.* there was no drift in retention times.

3.2.7 Toxicity evaluation

The toxicity assessment was performed using a modified version of the LBT methodology described in BS EN ISO 11348-3:2008 "Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria) – Part 3: Method using freeze-dried bacteria", adapting the procedure to 96-well plates.

3.2.7.1 Method development

The procedure described in BS EN ISO 11348-3:2008 was adapted to 96-well plates aiming to reduce the requirements of sample. Similarly, different contact times between samples and biosensor were evaluated (5 min, 15 min, 30 min, 60 min and 90 min), and the maximum concentration of methanol to use as carrier solvent (1%, 2%, 3%, 4%, 5%, 7.5%, and 10%) was estimated so bioluminescence was not affected. Aqueous samples were used for method development purposes. The experimental conditions used are provided in Table 10.

Table 10. Experimental parameters for LBT

Parameter	Value
Diluent	20% sodium chloride solution
Positive control	Cr(VI) (105.8 mg/L of $K_2Cr_2O_7$ in diluent)
Negative control	Diluent
Reference substance	Phenol (expected EC_{50}: 13 – 26 mg/L)
Carrier solvent	Methanol

The preparation of sample dilutions was carried out in standard transparent microplates, and the measurement of luminescence outputs in white opaque microplates. Light output was measured in a Promega GloMax™ luminometer, and incubation during contact time was performed in an Aqualytic thermostatic cabinet at 25°C ±0.3.

A detailed description of the complete procedure is included in Appendix I. Briefly, the general process is as follows:

a. Test samples were prepared for analysis by adjusting pH to 6 – 8.5, adding NaCl at a final concentration of 2%, and stirring for 3 minutes.
b. Sample(s) dilutions were prepared in *x*% methanol (*x* = 1%, 2%, 3%, 4%, 5%, 7.5%, 10%) using a geometric dilution series in standard transparent microplates.
c. The bacterial stock solution was prepared by removing one reagent vial from the freezer (-20°C) and quickly adding 1 mL of previously cooled water (4°C ± 3°C). A waiting time of 10 minutes was applied before using this suspension, keeping it at 4°C at all times.
d. The bacterial test solution was prepared by transferring the total volume of the bacterial stock suspension into a 10-mL test tube containing 9 mL of diluent and mixing thoroughly.
e. A total of 20 µL of the bacterial test solution and 80 µL of diluent were transferred to each test well in white microplates.
f. Microplates were left to equilibrate for 15 – 20 min at 15°C until luminescence remained stable.
g. Luminescence was read as timepoint 0 (I_0).
h. A total of 100 µL of sample(s) dilutions, controls, and standards were transferred to the test wells containing bacteria, for a final volume of 200 µL.

i. Plates were incubated at 25°C for *t* minutes (5 min, 15 min, 30 min, 60 min, 90 min).
j. Luminescence was read as timepoint *t* (I_t).

EC_{50} values for extracts were determined using a linear regression analysis of the logarithm of the gamma value (ratio of light lost to the amount of light remaining at time *t*) and the logarithm of the concentration of the sample (relative enrichment factor REF, mg/L, as applicable), considering only inhibition values between 10% and 90%. Calculations were carried out in accordance to BS EN ISO 11348-3:2008.

3.2.7.2 Testing of samples

The conditions presented in Table 10 and those established during method development were used for the toxicity evaluation of aqueous samples, extracts, and fractions. The standard assay procedure, where the test solution is composed of 50% diluted bacterial reagent and 50% test sample, was applied to aqueous samples and extracts, which were tested in duplicate. The increased sensitivity assay procedure was used for fractions, where 90% of the test solution corresponds to the sample and 10% corresponds to the diluent/bacterial reagent. Due to limitations in the amount of sample, only one replicate was performed to evaluate the toxicity of fractions; EC_{50} values obtained for phenol, f_{kt} values (between 0.6 and 1.8), and RSD for the positive control (≤ 3%) within each batch were used as criteria of validity. Toxicity was expressed as toxicity units (TU), where $TU = 100/EC_{50}$.

3.2.8 Chemical analyses

3.2.8.1 GC-MS

GC-MS analysis was performed using a Perkin Elmer Clarus® 500 instrument equipped with a DB-5 capillary column (30 m x 0.25 mm I.D) coated with 0.25 µm film 5% phenyl polysilphenylene siloxane. High purity helium was used as carrier gas with flow-rate at 1.0 ml/min. The inlet was held at 250°C, and the injection volume was 1 µL. The column was held at 35°C for 4 minutes, ramped at 8 °C/min to 310°C, and held for 10 minutes, for a total run time of 48 minutes. The mass spectrometer was operated in electron ionisation (EI) mode with an ionization energy of 70 eV. The scan range was 50 to 600 amu. The instrument was mass calibrated using perfluorotributylamine (PFTBA) using the ions *m/z* 69, 131, 219 and 502. The filament emission current was set at 0.06 pA.

Identification of individual compounds was conducted by probability-based matching (match and reversed match ≥ 800) with mass spectra in the National Institute of Standards and

Technology (NIST) Mass Spectral Library database 2014 version 2.4.0. For identification of alkanes, positive EI mass spectra and RT were considered, using the aliphatic hydrocarbons present in the spiking solution as reference.

3.2.8.2 LC-MS/MS

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis was carried out using reversed phase high-performance liquid chromatography (RP-HPLC) and negative-ion electrospray ionisation mass spectrometry (ESI-MS). Chromatographic separation was conducted using an Agilent 1100 HPLC instrument on a Varian Pursuit XRs C18 (100 x 3.0 mm, 3 µm, 100 Å) column. The mobile phase consisted of 0.1% NH_4OH in HPLC water (A) and 0.1% NH_4OH in methanol (B), which was pumped using a quaternary pump at a flow rate of 600 µL min^{-1}. The gradient elution used was 10% B at 0 - 2 min, ramped to 70% B by 2.5 min and held until 6 min, then ramped to 100% B by 6.5 min and held until 7.5 min, returning to 10% B by 9 min and held for 3 minutes, for a total run of 12 minutes. Detection was performed in negative ion mode with a scan range of 50 – 500 Da, and the following ESI inlet conditions were applied: curtain gas 15 psi; nebulizer gas (GS1) 40 psi; auxiliary gas (GS2) 30 psi; ion spray voltage -4500 V; ion source temperature 550 °C; declustering potential -20 V; entrance potential -11V.

3.2.8.3 HRLC-MS

High-resolution mass spectrometry was carried out using a Thermo Accela LC pump and a CTC autosampler interfaced directly to a Thermo Exactive mass spectrometer. Chromatographic separation was conducted using the same column, mobile phase, and gradient elution described above for LC-MS/MS. Detection was performed in negative ion mode with a scan range of 80 – 500 m/z, and the following HESI source conditions were applied: sheath gas flow rate 50 units; spray Voltage 4000 V; capillary temperature 350 °C; capillary voltage 55 V; lens voltage 105 V; skimmer voltage 26 V; heater temperature 300 °C.

3.2.8.4 Derivatisation with MTBDSTFA

Derivatisation of naphthenic acids for GC-MS analysis was performed adding 100 µL of the MTBDSTFA reagent to 100 µL of a 5-mg/L solution of the SPE extract from pipeline 3 in 1.5 mL capacity glass vials, which were sealed and mixed on a vortex for 1 minute. The vials were transferred to an oven at 60°C for 60 minutes to ensure complete ester formation. After this, vials were let to cool to room temperature, and the solvent was evaporated to approximately 10 – 20 µL using a TurboVap® LV concentration evaporator workstation. The volume was then

made-up to 100 µL with DCM and samples were analysed using GC-MS under the conditions described above. 4-Methyl-1-cyclohexanecarboxylic acid (25 mg/mL in DCM) was used as a control to corroborate the derivatisation process and help to identify the ions generated.

3.3 Results and discussion

3.3.1 Phase I of EDA

3.3.1.1 Preliminary characterisation

Table 10 presents the results for the preliminary characterisation of aqueous effluent samples and compares them with the maximum national discharge limits as stated in Resolution 0631/2015 (Ministry of Environment and Sustainable Development of Colombia). Calibration curves for the determination of TOC are provided in Appendix II. Data in Table 10 show that pipeline 3 is different from pipelines 1 and 2 in a number of respects. It was discharged at a significantly higher temperature, which exceeds the maximum national discharge limit of 40°C, and it has by far the greatest TOC content, which is considerably higher than levels previously reported for treated RWW, ranging from 6 to 70 mg/L[79,120,183,187,224]. Moreover, pipeline 3 contains detectable yet legally compliant levels of Ni, Zn, and As, the latter present at levels exceeding the maximum contaminant level of 0.010 mg/L in drinking water established by the US EPA aiming to avoid chronic effects of exposure to low concentrations of arsenic. The EQC for freshwater (0.340 mg/L) and marine water (0.069 mg/L)[251], however, are not exceeded. It is surprising that our samples contain such low levels of heavy metals, which would be expected to be at higher concentrations in refining effluents stemming from the use of metal-containing catalysts and the transference of Se, Cd, As, and Zn from the feedstock to wastewater[120,123]. However, as presented in Figure 8, previous studies have reported a wide range of concentrations for different heavy metals in RWW, as these values are directly linked to the feedstock processed and the treatment technologies used, which in turn depend on local environmental regulations. In other words, there is no such thing as a "typical" concentration of metals in RWW. The results of this study are comparable with previous studies in the sense that various metals were present at concentrations below the limit of detection (LoD), but it is noteworthy that the sensitivity of the methods used in some of the previous studies is higher. Our lowest LoD is 0.01 mg/L, whereas other studies report detection limits of 0.001 mg/L[122,252]. For regulatory purposes, the methods used in this study are sensitive enough to know whether an effluent sample complies with the maximum discharge limits, as provided in Table 11, but only the evaluation of toxicity can provide information regarding the potential biological effects of the metals present in these effluent samples.

Table 11. Results for the preliminary characterisation of effluent samples from Barrancabermeja, Colombia, showing compliance with discharge limits as stated in Resolution 0631/2015 from the Ministry of Environment and Sustainable Development of Colombia

Parameter		Sample			Maximum discharge limits - Resolution 0631/2015 of Colombia
		Pipeline 1	Pipeline 2	Pipeline 3	
pH		7.36	7.30	6.47	6.0 – 9.0
Temperature (°C)		32.30	30.40	60.40	< 40
TOC (mg/L)		39.59	22.65	127.50	Not specified
Total content (mg/L) (*n*=3)	**V**	< 0.02	< 0.02	< 0.02	1.00
	Ni	<0.01	<0.01	0.045 ± 0.001	0.50
	Zn	0.027 ± 0.001	0.078 ± 0.002	0.027 ± 0.004	3.00
	As	< 0.02	< 0.02	0.086 ± 0.000	0.10
	Se	< 0.01	< 0.01	< 0.01	0.20
	Hg	< 0.01	< 0.01	< 0.01	0.01
	Cr	<0.04	<0.04	<0.04	0.50
	Pb	<0.04	<0.04	<0.04	0.10
	Cd	<0.04	<0.04	<0.04	0.10

3.3.1.2 Toxicity Evaluation

3.3.1.2.1 Determination of testing conditions

For LBT, different contact times between the biosensor and the samples were evaluated because light-level time response curves might change depending on the nature of the chemical(s) tested. Sometimes, 5 minutes are enough to evidence the drop in light output because the interaction between the pollutant(s) and the test organism takes seconds, and therefore the effect on light emission is almost immediate. On the contrary, some chemicals, such as metal-containing compounds, take longer times to interact with the test organism, which makes longer exposure times necessary. In the case of organic compounds, 15 minutes of exposure are sufficient, whereas the testing of mixtures whose composition is unknown, or whose behaviour in the test has not been documented, requires multiple light readings over time[253–255]. Consequently, contact times of 5 min, 15 min, 30 min, 60 min, and 90 min were evaluated during method development. As shown in Figure 9, a general trend is observed for

all three samples; light output shows a plateau after 15 minutes of contact time, which suggests that the observed toxicity is caused by organic compounds and not by inorganic chemicals or metals. This indicates that the concentration of metals provided in Table 11 is not high enough to reduce the light output, or that the metals present may not be bioavailable to compete for a biotic ligand. This is because total metal concentrations are not necessarily a good indicator of their hazardous potential because of the protective effect of different factors, such as organic matter, carbonates, and pH[127]. Furthermore, these results indicate that 15 minutes of exposure are sufficient to induce the toxic response in *V. fischeri* when exposed to the test samples.

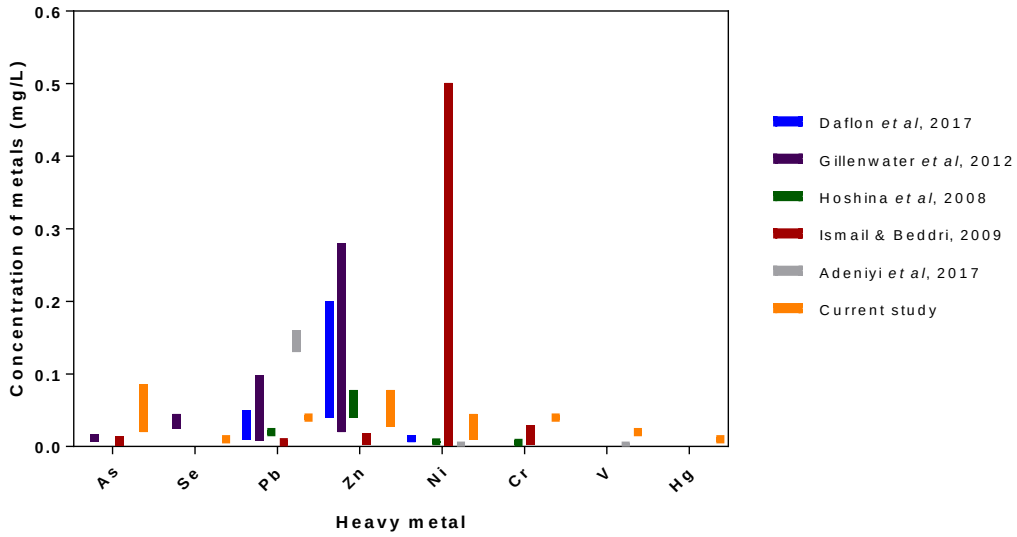


Figure 8. Comparison of current findings with previous reports of metals in RWW

Moreover, the low water-solubility of extracts required the use of methanol as a carrier solvent, which made it necessary to test the toxicity induced by different concentrations of it. As observed in Figure 10, 1% methanol neither inhibits nor induces light output, making it suitable for toxicity testing. Interestingly, levels higher than 5% induced bioluminescence in all time-points, demonstrating that cytotoxicity can also increase bacterial luminescence[185]. In all cases, EC_{50} values obtained for phenol using the adapted test for validation purposes were within the reported range of 13 – 26 mg/L, therefore validating the adaptation of the standard procedure of LBT to a miniaturised version using 96-well plates.

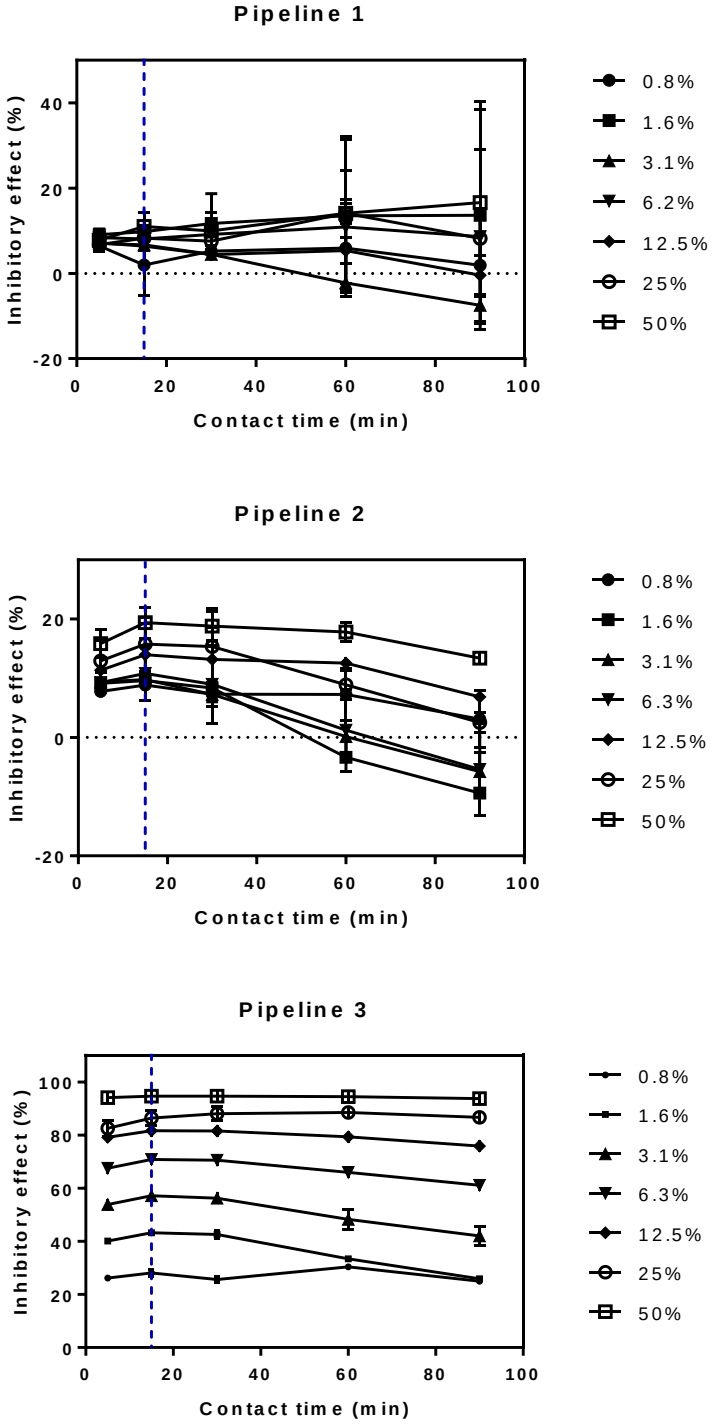


Figure 9. Inhibition of bacterial luminescence (%) by RWW samples at different concentrations (0.8% - 50%) over time (min) during the determination of optimal contact time between *V. fischeri* and samples. The contact time of 15 minutes, which proved to be sufficient to detect toxicity of samples, is indicated with a blue dashed line. Error bars for SD (n=3) shorter than the height of the symbol are not shown.

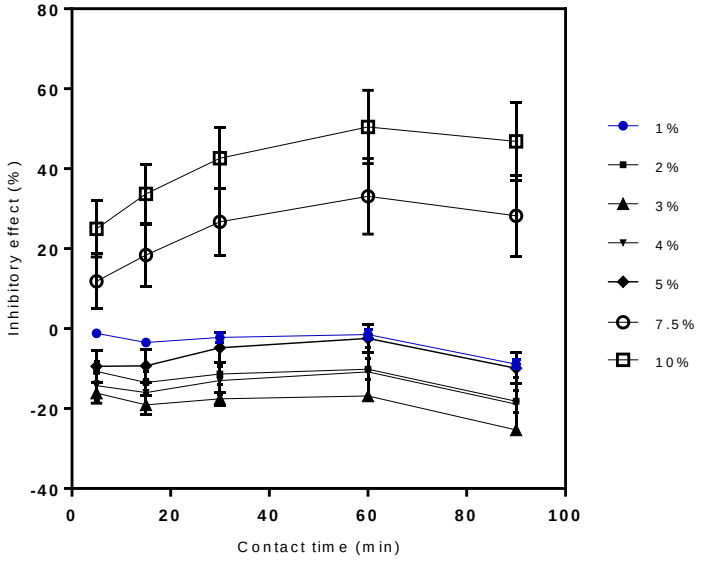


Figure 10. Determination of the concentration of methanol as a carrier solvent for toxicity testing of extracts. The lowest inhibitory/stimulatory effect on bacterial luminescence was provided by 1% methanol, shown in blue. Error bars for SD (*n*=3) shorter than the height of the symbol not are shown.

3.3.1.2.2 Toxicity results

Toxicity of aqueous samples and extracts was tested using a contact time of 15 minutes and 1% methanol as the carrier solvent. Calibration curves for the calculation of EC_{50} values are provided in Appendix II and raw data in Appendix III. The corresponding results are presented in Table 12, expressing toxicity as EC_{50} and TU. It can be seen from the data in the table that samples vary greatly in their inhibition of luminescence, and that results correlate with TOC values provided in Table 11. The toxicity of pipeline 3 (aqueous sample), which had the highest TOC content, was 5.0 TU (EC_{50} = 20%), whereas the TU and EC_{50} values of aqueous samples from pipelines 1 and 2 could not be determined due to their low toxicity to *V. fischeri*. The EC_{50} value obtained for pipeline 3 is comparable to previous reports of RWW, although it is noteworthy that the composition of RWW is highly variable and this is reflected in a wide range of EC_{50} values in the literature. For instance, Aruldoss and Viraraghavan (1998)[174] analysed 33 treated RWW samples (contact time of 20 minutes) and obtained toxicity values ranging from 12% to 72%, whereas Chang *et al.* (1981) obtained EC_{50} ranging from 58 to 100%, with one extremely toxic sample with an EC_{50} value of 1.8%[185]. The toxicity of the aqueous sample indicates that there are hazardous compounds impacting luminescence of *V. fischeri* that resist treatment and could potentially represent a hazard for the receiving

environments, making further treatment crucial to achieve safer effluents with reduced risk of exposure to humans and wildlife.

Table 12. Toxicity results (EC_{50} values and TU) for aqueous samples and extracts

Sample	Type of sample	EC_{50}	TU
Pipeline 1	Aqueous	No inhibition observed	
	SPE	22.6 REF	4.4
	LLE	24.1 REF	4.1
Pipeline 2	Aqueous	No inhibition observed	
	SPE	11.9 REF	8.4
	LLE	33.4 REF	3.0
Pipeline 3	Aqueous	20.0%	5.0
	SPE	0.2 REF	666.6
	LLE	0.1 REF	1000.0

REF: Relative enrichment factor = Enrichment factor (Volume of sample / Volume of extract) x Dilution factor (Volume of extract added to assay / total volume of assay)

Extracts obtained from LLE and SPE from pipeline 1 showed similar toxicity (4.4 vs 4.1 TU), whereas for pipeline 2 the SPE extract showed higher toxicity than the LLE extract (8.4 vs 3.0). Nevertheless, these toxicity values look insignificant when compared to those of pipeline 3, as shown in Figure 11. The LLE extract from pipeline 3 was almost 350 times more toxic than that of pipeline 2, and nearly 250 times more toxic than that of pipeline 1. As for SPE extracts, that of pipeline 3 was nearly 80 times higher than pipeline 2 and 150 times higher than pipeline 1.

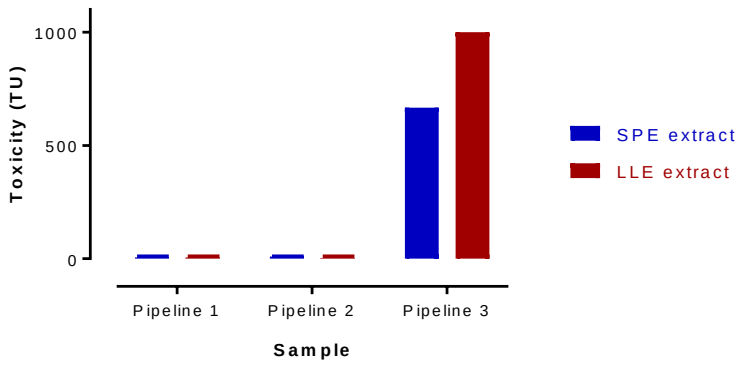


Figure 11. Toxicity expressed as TU for SPE and LLE extracts of pipelines 1, 2 and 3

3.3.1.3 Chemical analysis

Refining effluents contain organic contaminants with a broad range of physico-chemical properties, and it is currently impossible to extract them with a single extraction method. Consequently, LLE and sequential SPE (Oasis® HLB + Oasis® WAX) were used for extraction under basic, neutral, and acidic conditions aiming at a broad extraction of organics with a wide range of polarity. Lipophilic organics are relatively easy to extract using DCM as extracting solvent, whereas some polar and ionic compounds are difficult to extract this way and alternative extractions such as SPE using polar or ionic sorbents are required and thus used in this study. *o*-Terphenyl and 1-chlorooctane were used as internal standards to monitor the extraction of both aliphatics and aromatics; the corresponding peaks are observed in all total ion chromatograms (TICs) at approximately 26.5 and 28.4 minutes, respectively, in Figure 12 and Figure 13. Volatile organic compounds (VOCs), including BTEX, were not targeted during extraction due to the fact that these compounds tend to rapidly evaporate once they reach the environment, leading to reduced exposure of wildlife to these compounds.

Chemical analysis by GC-MS of SPE and LLE blanks revealed that SPE generates more extraction artifacts than LLE (Figure 12 and Figure 13; F), which were predominantly identified as phthalates based on their mass spectra (data not shown) and assumed to originate from the SPE cartridge. This was confirmed with extraction blanks (Figure 13). Moreover, TICs of LLE/SPE extracts (Figure 12 and Figure 13; A to E) showed that pipeline 3 was the most complex sample, regardless of the extraction method. A large unresolved complex mixture (UCM) was observed in both extracts with an RT between 16 and 26 minutes approximately, suggesting that it is composed of multiple co-eluting compounds rather than one compound at a very high concentration. Previous studies have reported 10-minute-long UCMs that correspond to naphthenic acids (NAs)[256–258], which are of toxicological concern due to their endocrine disruption potential and acute and chronic toxicity to a range of species[147,155,171]. The averaged mass spectra for the UCM (Figure 14) evidenced the presence of the ions 41, 55, 69, 81, 95, 109, 123, 135, 150, 164, 181, and 195 m/z, which have been reported for NAs in EI-MS in almost identical relative abundances[257], hence suggesting that the UCM corresponds to NAs. The concentration of the UCM in the aqueous sample was estimated semi-quantitatively following the single point external standard method, using the formula below:

$$Concentration\ of\ UCM = \frac{Area\ of\ UCM}{Area\ of\ IS} x\ Concentration\ of\ IS$$

The concentration was estimated to be roughly between 88.6 and 135.6 mg/L using 1-chlorooctadecane as standard (spiking concentration 100 µg/L; RSD = 14.0% for SPE extracts, RSD = 17.9% for LLE extracts), as peak area for *o*-terphenyl showed a higher variability (spiking concentration 100 µg/L; RSD = 110.4% for SPE extracts, RSD = 65.7% for LLE extracts). This concentration, however, must be interpreted with caution because the detector does not respond identically to 1-chlorooctadecane and NAs, so an accurate quantitation would require a multiple point standard method using known amounts of the NAs present in the UCM.

NAs are naturally present in oil reserves, especially in bitumen[259], so these have been studied in detail in relation to OSPW generated during the extraction of bitumen from the oil sands of northern Alberta, Canada. OSPW are considered an important environmental problem because of the significant health risk they pose to aquatic and mammalian species due to the high contents of NAs when compared to background levels in natural waters, which are typically below 1 mg/L[260]. Consequently, Canada has a zero-discharge policy for OSPW and these must be stored in settling ponds[261], where NAs are present in concentrations up to 120 mg/L[157,160]. However, contents of NAs are also high in heavy crude oil[147,259], which makes these chemicals highly relevant within the context of refining wastewater, especially because NAs are not targeted during treatment of RWW as they are in the treatment of OSPW. Still, only a few publications address these pollutants in RWW[148–151]. In the case of our samples, Colombian petroleum has been classified as heavy crude containing significant levels of naphthenic acids[262], which would explain the considerable contents of NAs in pipeline 3. The estimated concentration of NAs in the aqueous sample (88.6 to 135.6 mg/L) is significantly higher than previous reports of NAs in treated RWW (2.8 to 11.6 mg/L)[150] and more in the range of reported levels in OSPW. However, wastewater management practices are entirely different in these two scenarios because refining effluents are treated and discharged under effluent guidelines that do not require detection, report, or quantification of naphthenic acids, so these are included under the bulk parameters of BOD, COD, or TOC, which means that only toxicity tests can suggest their presence. Furthermore, the presence of NAs in the aqueous sample at high concentrations indicates that they resist treatment, hence confirming the need for further treatment for complete removal.

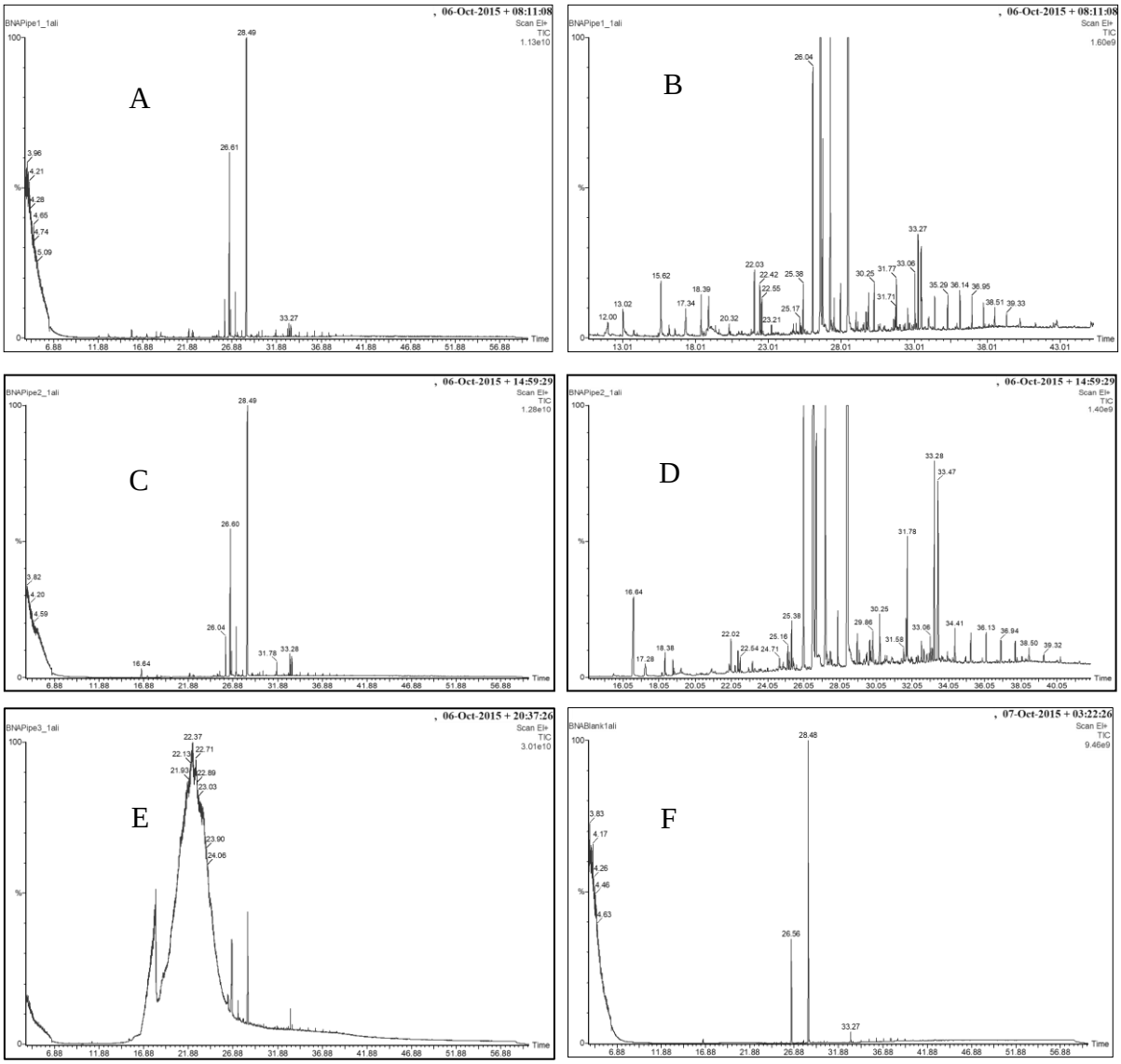


Figure 12. TIC of LLE extracts of RWW samples.

IS are observed at 26.5 and 28.4 minutes. (A) Pipeline 1; (B) Enlargement of A; (C) Pipeline 2; (D) Enlargement of C; (E) Pipeline 3; (F) Extraction Blank.

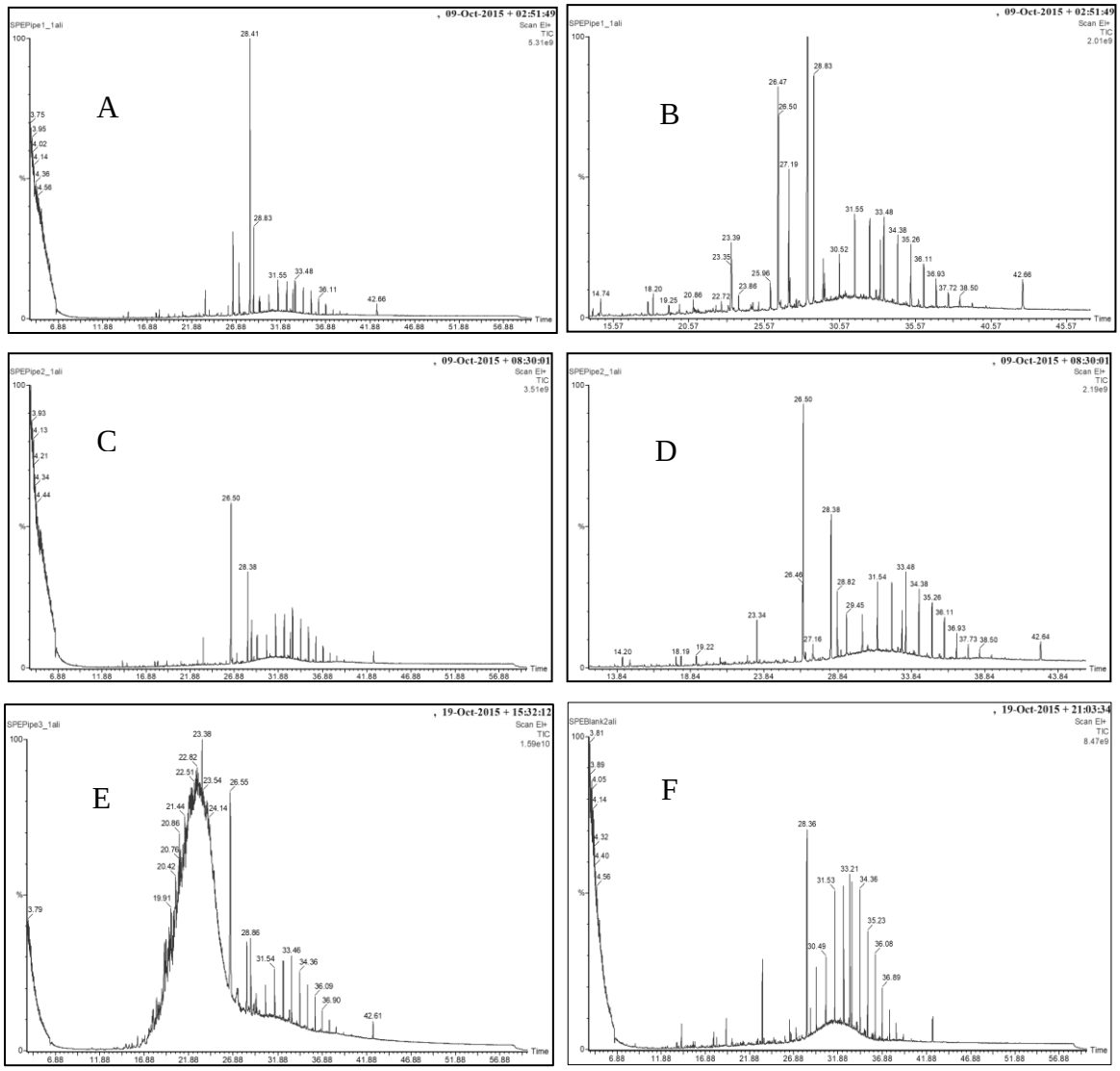


Figure 13. TICs of SPE extracts from RWW samples.

IS are observed at 26.5 and 28.4 minutes. (A) Pipeline 1; (B) Enlargement of A; (C) Pipeline 2; (D) Enlargement of C; (E) Pipeline 3; (F) Extraction Blank.

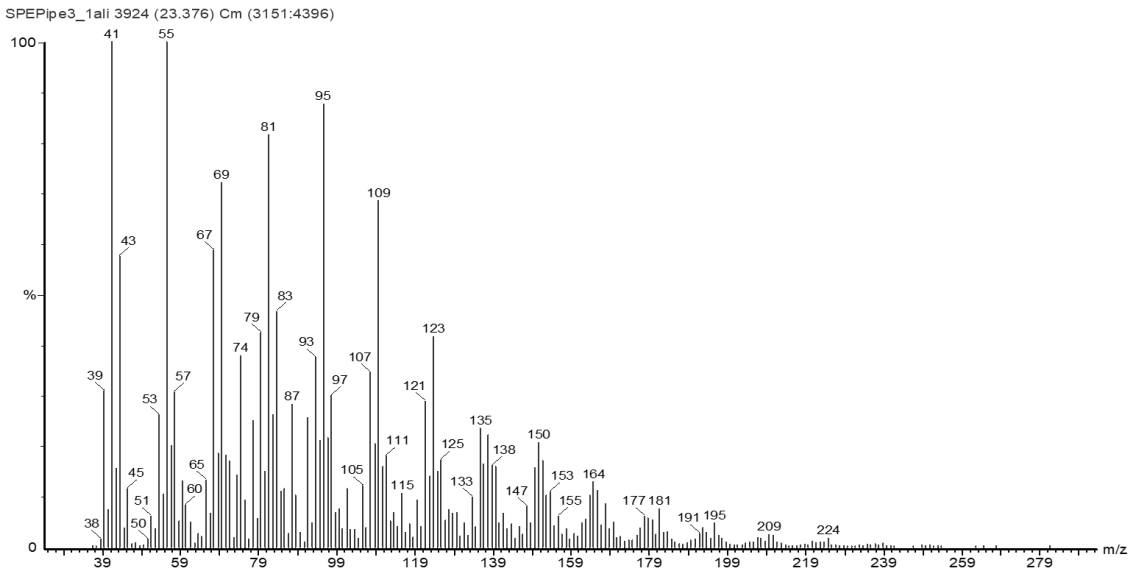


Figure 14. Averaged EI mass spectra of the unresolved hump in extracts from pipeline 3

The analysis of extracts from all three samples by GC-MS revealed the presence of organic acids, esters, phenols, aliphatic and aromatic hydrocarbons, ketones, and miscellaneous organics, as provided in Table 13. The table also shows the corresponding number of reports for single-chemical aquatic toxicity for each compound (including data on algae, bacteria, crustaceans, fish, amphibians, and invertebrates), as reported by the US EPA ECOTOX Knowledgebase. The structural diversity of RWW samples is apparent from the table, but also the fact that only a third of the organics identified have been toxicity-tested as pure substances, with evident differences between types of compounds. Phenols and PAHs have numerous reports of aquatic toxicity, whereas alkanes and carboxylic acids/esters have a significantly lower number. Within alkanes, only C_{22}, C_{23}, and C_{28} had reports of aquatic toxicity, for a total of 18 reports. Ketones, on the other hand, have been barely reported regarding their single-chemical aquatic toxicity, although these are expected to exert baseline toxicity because of the electron-withdrawing carbonyl moiety[263]. Within the miscellaneous organics, which included amides and ethers, among others, no reports were found in the database. This might be the result of methodological challenges to toxicity-test certain compounds, different risks of exposure among chemicals, or simply trends in research. In any case, the lack of ecotoxicological data complicates the establishment of a link between chemistry and observed toxicity, and the selection of target chemicals with environmental relevance for treatment and monitoring purposes, hence reinforcing the need of a toxicity-directed approach.

Table 13. Organic compounds identified in SPE and LLE extracts from RWW samples

Type of compound	Compound	Pipeline	Extract	No. of reports in ECOTOX Knowledgebase
Organic acids and esters	Hexanoic acid	1	LLE	15
	Heptanoic acid	1	LLE	10
	Nonanoic acid	1	LLE	23
	4-acetylbutiric acid	1	SPE	No reports
	Undecanoic acid	2	LLE	5
	9,12-octadecadienoic acid	3	LLE	90
	4-methyl-3-pentenoic acid	3	LLE	No reports
	2,2,4-trimethyl-3-carboxy isopropyl pentanoic acid, isobutyl ester	1	LLE	No reports
	2,2,4-trimethyl-1,3-pentanoic acid, diisobutyl ester	1	LLE	No reports
	Hexanedioic acid, bis(2-ethylhexyl)ester	1	LLE	No reports
	Tetradecanoic acid, methyl ester	1, 2	SPE	No reports
	Pentadecanoic acid, methyl ester	1	SPE	No reports
	Hexadecanoic acid, methyl ester	1	SPE	No reports
	Octadecanoic acid, methyl ester	1	SPE	No reports
	Octadecanoic acid, ethyl ester	1	SPE	No reports
	cis-butenedioic acid, bis(2-ethylhexyl) ester	1	LLE	No reports
	1,2-benzenedicarboxylic acid, bis(2-methylpropyl)ester	3	LLE	2
	Hexanedioic acid, bis(2-ethylhexyl)ester	3	LLE	9

Type of compound	Compound	Pipeline	Extract	No. of reports in ECOTOX Knowledgebase
Organic acids and esters	2-isopropylphenyl oxalic acid, pentyl ester	3	SPE	No reports
Phenols	2,6-dichlorophenol	1	LLE	60
	2,4-dichlorophenol	1	LLE	756
	2,6-dichloro-4-(1,1-dimethylethyl)phenol	1	LLE	No reports
	2,4,6-trichlorophenol	1	LLE	433
	2,4,6-tribromophenol	1	SPE	25
	2,5-dimethylphenol	3	LLE, SPE	21
Hydrocarbons	Alkanes $C_{22} – C_{36}$	1, 2, 3	LLE, SPE	18
	1,2-epoxyhexadecane	1	LLE	No reports
	1,2-epoxynonadecane	1	LLE	No reports
	1,2-dichlorooctane	2	LLE	No reports
	1,5,5-trimethyl-6-acetylmethylcyclohexene	2	SPE	No reports
	Nonadecene	1	LLE	No reports
	Docosene	1	LLE	No reports
	Fluoranthene	1	LLE	1067
	Pyrene	1	LLE	502
	Naphthalene	3	LLE	1179
	2-methylnaphthalene	3	LLE	62

Type of compound	Compound	Pipeline	Extract	No. of reports in ECOTOX Knowledgebase
Hydrocarbons	Anthracene/phenanthrene	3	LLE, SPE	511/611
	Benzo(*ghi*)perylene	3	LLE	10
	Indeno(1,2,3-*cd*)pyrene	3	LLE	3
Ketones	1-methyl-2-pyrrolidinone	1	LLE	4
	2,6-di-tert-butylbenzoquinone	1	LLE	No reports
	4,6-dimethyl-2H-pyran-2-one	1	SPE	No reports
	7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	1	LLE	No reports
	Benzophenone	1, 2	LLE	No reports
	3,5-dimethyl-2-furyl methyl ketone	2	SPE	No reports
	5-hydroxy-4,5-dimethyl-2,5-dihydrofuran-2-one	2	SPE	No reports
	4,6-dimethyl-2H-pyran-2-one	2	SPE	No reports
	7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	2	SPE	No reports
	3',8,8'-trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetrone	2	SPE	No reports
	1-nitro-2-octanone	3	SPE	No reports
Miscellaneous	Tetrahydro-1,1-dioxide thiophene	1, 3	LLE	No reports
	Vinyl lauryl ether	1	LLE	No reports
	2-Ethoxyethyl ether	1	LLE	No reports
	Tetradecanamide	1	LLE	No reports
	Diethyltoluamide	2	LLE	No reports

Type of compound	Compound	Pipeline	Extract	No. of reports in ECOTOX Knowledgebase
Miscellaneous	N-butyl-benzenesulfonamide	2	LLE	No reports
	N-propylbenzamide	3	LLE	No reports
	Isocyanatobenzene	3	LLE	No reports
	Benzenethiol	3	LLE	No reports
	3-mercaptopropionitrile	3	LLE	No reports
	Triacetin (1,2,3-triacetoxypropane)	2	LLE	No reports

Moreover, the variability of RWW is another obstacle interfering with the selection of such target chemicals. Alkanes were the only compounds found consistently in all three samples, and these were expected to be present. It is noteworthy that our findings are the result of grab samples rather than composite samples collected at regular intervals, therefore providing only a snapshot of the chemical composition of effluents that might not be representative of its average composition. Previous chemometric studies of RWW have also evidenced the structural diversity of RWW by reporting aliphatic and aromatic hydrocarbons, phenols, carboxylic acids, methyl, ethyl, and propyl esters, ketones, and alcohols[78,89], but no individual compounds have been detected consistently in RWW samples other than alkanes, a number of PAHs and phenols[64,65,78,79], possibly because their extraction and detection are easier than those of other chemicals. Interestingly, neither PAHs nor alkylated PAHs were detected in pipeline 2. This finding suggests that contents of PAHs were below detection limits, or that the filtration of effluents prior extraction might have drastically reduced the concentration of PAHs in the aqueous sample, as many of these compounds are non-polar and hydrophobic and tend to adsorb onto particles[79,103]. Sample preparation for SPE usually includes filtration to avoid blocking of cartridges, but LLE does not require the removal of particles and could have been carried out without filtration.

The EC_{50} (Log of µg/L) values reported in ECOTOX Knowledgebase for compounds identified in the extracts are provided in Figure 15. The discrepancy between the number of reports presented in Table 13 and plotted in Figure 15 for each chemical stems from the exclusion of studies dealing with accumulation and enzyme activity, where concentrations are reported in g of tissue or Kg of dry body weight, respectively. The figure shows that toxicity of acids increases with chain length as a result of an increase in hydrophobicity, which is a critical factor for aquatic baseline toxicity as it drives their partitioning into lipid membranes[264]. In the case of phenols, data show that not only the type and degree of substitution are key factors for their toxic action, but also the pattern of substitution. This is observed with 2,6-dichlorophenol (*ortho*-substituted) and 2,4-dichlorophenol (*para*-substituted), the latter being more toxic. As the hydroxyl group of phenols interact with the π-electrons of the aromatic ring, phenols can generate stable phenoxy radicals that are involved in the formation of intermediate metabolites that interact with biomolecules. However, chlorines in *ortho* position form hydrogen bonds and shield the ─OH group[265], impacting the formation of such radicals. Moreover, the distribution of toxicity data shows that PAHs are the most toxic group, whose toxicity also depends on their hydrophobicity[266].

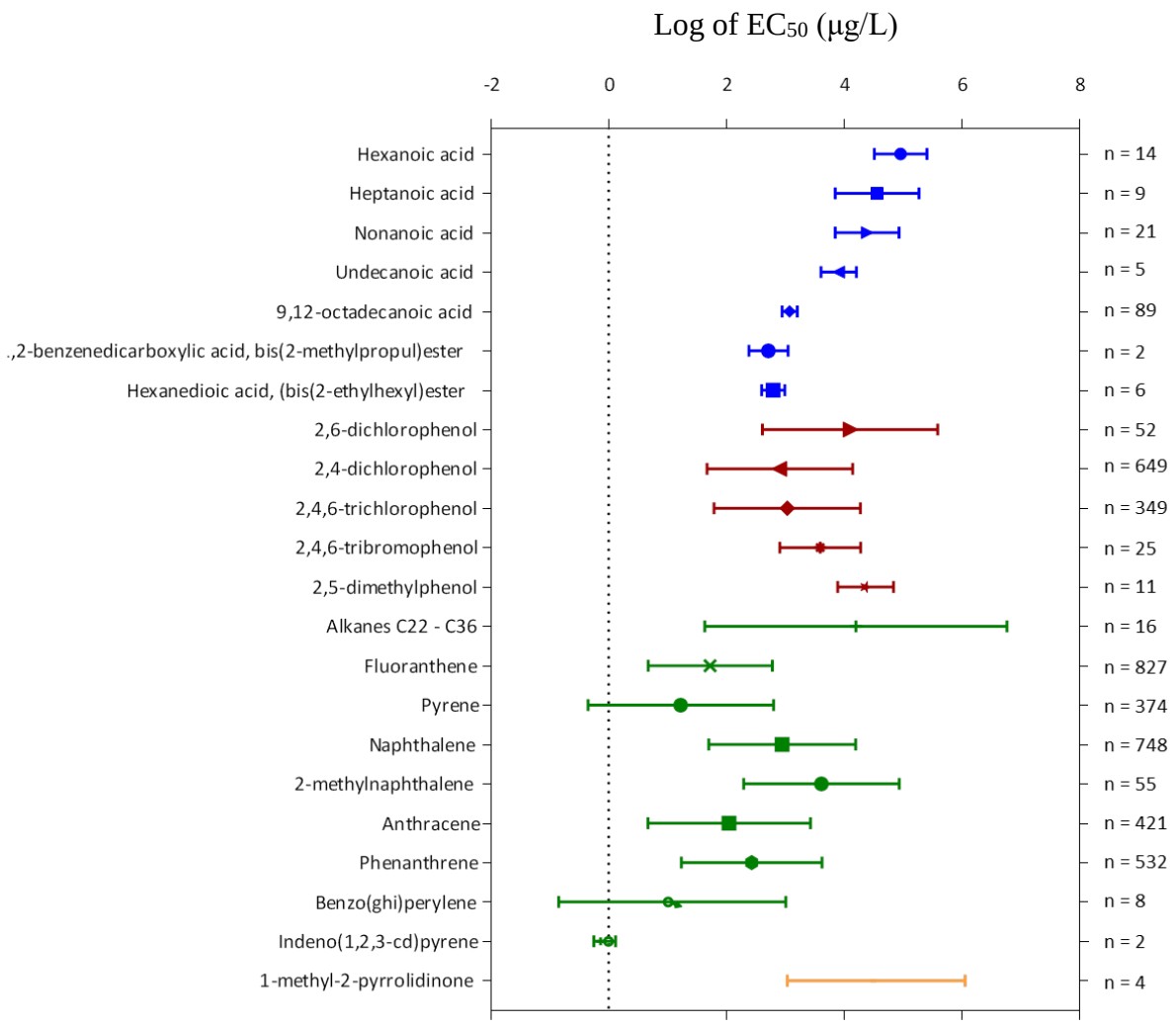


Figure 15. Aquatic toxicity (Log of EC_{50}) of compounds detected in RWW extracts, as reported in ECOTOX Knowledgebase, where n = number of reports. Acids and esters are shown in blue, phenols in red, hydrocarbons in green, and ketones in yellow.

Interestingly, petroleum refining effluent guidelines tend not to regulate specific organic toxicants, regardless their single-chemical environmental toxicity, but rather include all organic contaminants within 5-day BOD, COD, oil and grease, and phenolic compounds. In particular, the Colombian guidelines for refining effluents require the analysis and report of PAHs, BTEX, and adsorbable organic halogens, but there are no maximum discharge limits established. Within the European context, the Integrated Pollution Prevention and Control (IPCC) directive (2010/75/EU) does not set discharge limits but focuses on the application of best available technologies. The situation is the same in the US, where the concentration of PAHs, methylphenols, and other toxic organics in RWW has been found to be consistently below

treatable levels[6], so these are not considered pollutants of concern and there are no maximum discharge limits. The question remains on whether current regulations are protecting humans and wildlife from RWW with potential biological effects because effluents have a complex composition and the behaviour of chemicals in a mixture may not be as predictable as that of pure compounds. Consequently, assessing compounds separately may underestimate the biological effects of RWW because chemicals can interact and generate mixture effects, even when each chemical is present at concentrations considered safe[243].

Theoretically, concentration additivity would be expected for aliphatic hydrocarbons, PAHs, ketones, and ethers, all of which elicit toxicity by disrupting membranes as a result of their hydrophobicity[267]. However, the presence of unknowns and chemicals with different MOAs complicates the understanding of the biological effects exerted by RWW. This reinforces the advantages of effect-directed analyses when it comes to simplifying the complexity of an environmental mixture by directing chemical analysis to chemicals that actually cause toxicity, helping to determine causative effects and establish what needs to be removed during treatment.

3.3.2 Phase II of EDA

3.3.2.1 Toxicity evaluation

The increased sensitivity assay procedure was conducted to evaluate the toxicity of fractions obtained from samples and extraction blanks, for a total of 256 fractions. As opposed to the standard procedure used in Phase I, the increased sensitivity procedure challenges bacteria in a proportion 1:9 to the test sample. Due to limitations in the amount of sample, no replicates were conducted, thereby reliability of the test method was confirmed using phenol as reference standard (expected EC_{50}: 13 – 26 mg/L; experimental EC_{50} values: 17.01, 15.62, 15.18, 14.92 mg/L) and the accuracy was monitored using Cr(VI) as an intra-plate and inter-plate indicator. In all cases, RSD for Cr(VI) and phenol were < 3%.

Toxicity testing of fractions from extraction blanks revealed that many of these induced a significant reduction of luminescence, such as fractions 2, 28, 29, and 30 for the SPE blank, and fraction 30 from the LLE blank. This suggests that toxic artifacts, mainly polar, low molecular-weight plasticisers (phthalates) originating from reagents, solvents and laboratory consumables elute in these fractions. Aiming to estimate the toxicity resulting from organics in samples and not from extraction artifacts, the toxicity of blanks was subtracted from that of

samples fractions. None of the fractions induced light output from *V. fischeri* as reported in other studies[185]. Toxicity results are provided in Figure 16, revealing that fractions from pipeline 3 showed markedly higher toxicity.

Fractions with the highest toxicity values for each sample and extraction method were selected for GC-MS analysis using the 75th percentile as the cut-off value, due to the uneven distribution of toxicity data among samples. This corresponded to a total of 47 toxic fractions out of 256 total fractions, ranging from 7 to 9 fractions per sample, per extraction method.

3.3.2.2 Chemical analysis

The GC-MS analysis revealed that most of the toxic fractions contained organic acids, methyl/ethyl esters of carboxylic acids, linear and branched alkanes, and numerous unknowns, which were detected mainly in the more polar fractions. No PAHs were detected. Only a handful of compounds detected in the toxic fractions had reports for single-chemical aquatic toxicity in the US EPA ECOTOX Knowledgebase, as provided in Table 14 and Figure 17. The UCM corresponding to NAs eluted in fractions 2 and 3, both of which showed significant inhibition of luminescence (Figure 16), but the reports for NAs found in the ECOTOX Knowledgebase were not included in Figure 17 because these referred to Zn, Ca, Na, and Cu salts of NAs. In a few toxic fractions, no peaks were observed other than compounds known to correspond to column bleeding and the IS used for quality control purposes, suggesting that the compounds impacting the bioluminescence in *V. fischeri* present in these fractions were thermally labile or had low volatility and therefore these were not amenable to GC-MS analysis.

The reported (Log)EC_{50} values of the organics detected in toxic factions (Figure 17) indicate that, overall, these compounds are less toxic than those detected in extracts, with alkanes being the most toxic. This finding suggests that aliphatic hydrocarbons might have an essential contribution to the toxicity of RWW, which is in accordance with a previous report of reduction in toxicity (Microtox®) after total petroleum hydrocarbons were biodegraded[88]. Our results are comparable to other studies in the sense that chemical analysis of toxic fractions does not point at clear toxicants other than naphthenic acids as responsible for the biological effects observed. Dorris *et al.* (1974)[89] reported that none of the compounds identified in toxic fractions, which included aliphatic hydrocarbons, *m*-cresol, and dioctyl phthalate, could fully account for the

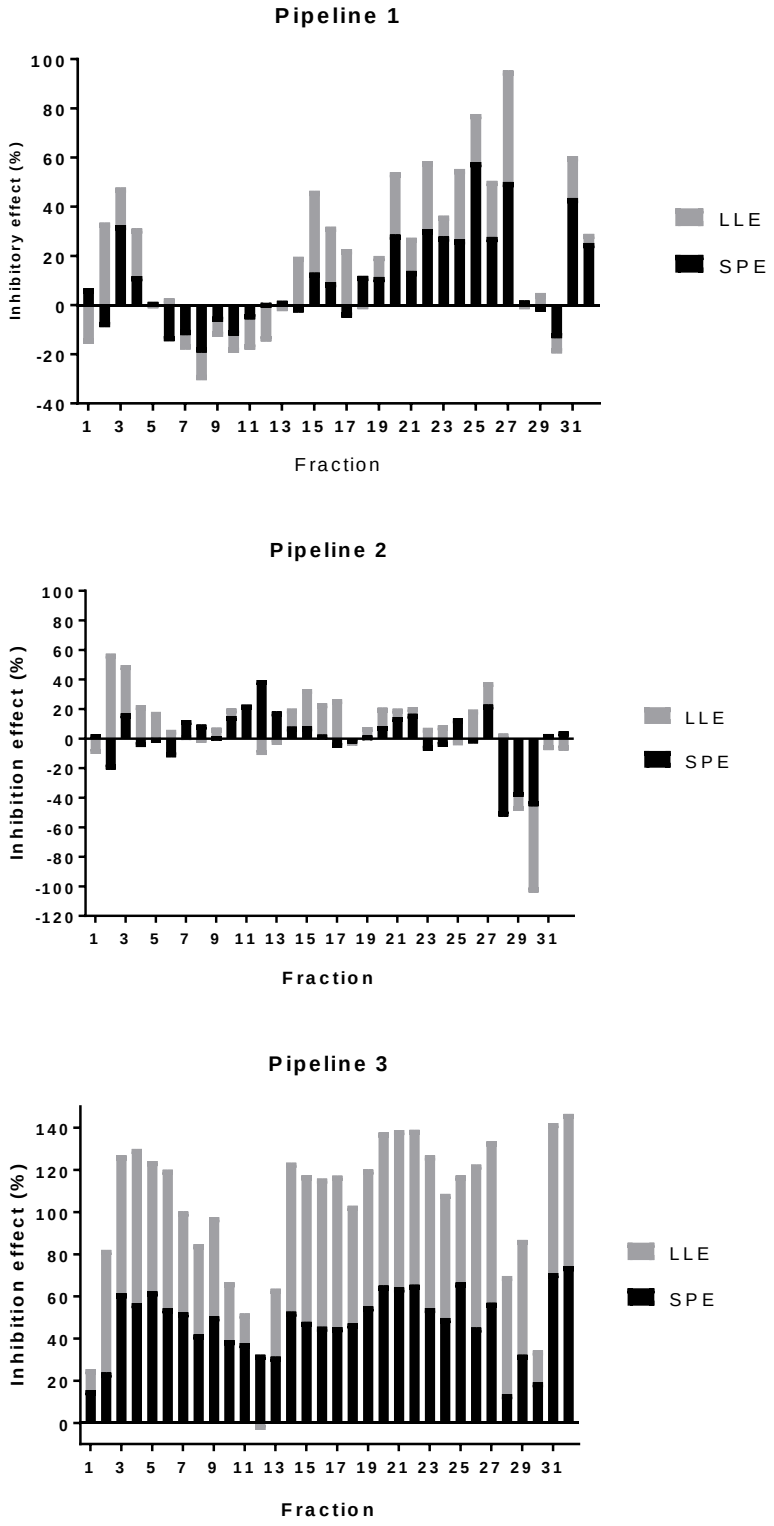


Figure 16. Toxicity of LLE and SPE fractions obtained from pipelines 1, 2, and 3 after the subtraction of toxicity from fractionation blanks

acutely lethal effects observed on *Daphnia magna*. Similarly, Ankley *et al.* (2011)[54] indicated that previous attempts to assign toxicity in RWW to single chemicals usually faced a broad distribution of toxicity among multiple fractions, complicating the establishment of a causation relationship. Leonards *et al.* (2011)[83] found that narcotic effects play an essential role in the toxicity of RWW, but these could not explain the observed toxicity for several samples, suggesting an analysis of individual organic contaminants to help to establish causative factors. These outcomes have several possible explanations. Toxicity might be related to toxicants that are not amenable for GC-MS detection or that are present in concentrations below the LoD of the instrument. Alternatively, the observed toxicity is the result of the aggregate effect of various compounds or stems from the numerous unknowns that could not be identified using the NIST library, the latter of which suggests that the range of identification could be increased using LC-MS/MS.

Table 14. Compounds with previous reports of toxicity identified in fractions selected for chemical analysis based on the LBT

Type of compound	Compound	Pipeline	Extract	Fraction	Reports in ECOTOX Knowledgebase
Organic acids	Butanoic acid	1	LLE	2	76
Phenols	2,3-dimethylphenol	3	LLE	2	14
Hydrocarbons	Tetrachloroethene	1	SPE	3	354
	1-chloropentane	1	LLE	20	2
	Alkanes $C_{22} – C_{36}$	1, 2, 3	LLE, SPE	2, 3	18
Miscellaneous	Benzenethiol	3	SPE	2	3
	Isopropyl myristate	1	LLE	2	1

As for the NAs found in toxic fractions, previous studies have reported a significant reduction in toxicity of OSPW after the removal of NAs alone[153,160,170,258], suggesting that targeting NAs during treatment of RWW could reduce their potential biological effects. Further analyses were conducted to profile the NA mixture present in the effluent sample in terms of *n* (number of carbon atoms) and *Z* families (hydrogen deficiency), as this structural information could help to select model chemicals to evaluate treatment technologies aiming to remove or degrade NAs from RWW. For this purpose, NAs were characterised using derivatisation with

MTBSTFA, low-resolution mass spectrometry, and high-resolution mass spectrometry, as described below.

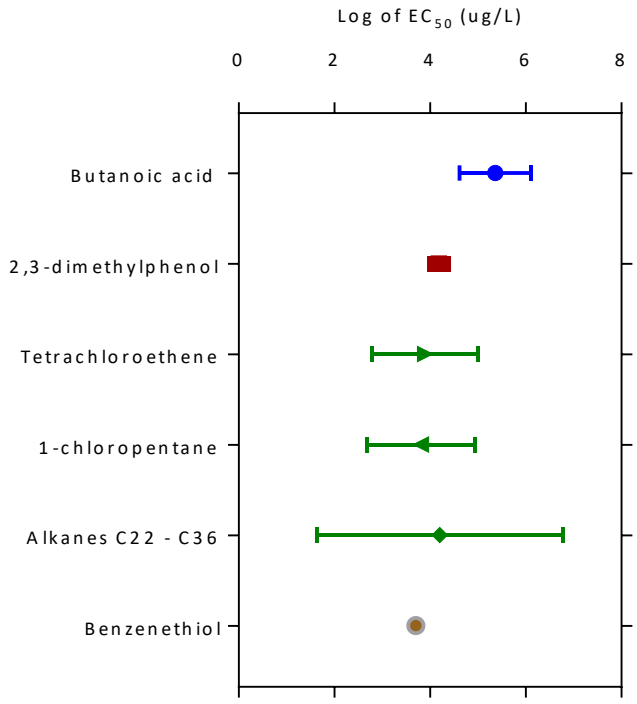


Figure 17. Aquatic toxicity (Log of EC_{50}) of compounds detected in toxic fractions, as reported in ECOTOX Knowledgebase. Acids are shown in blue, phenols in red, hydrocarbons in green, and miscellaneous in brown.

3.3.2.3 Characterisation of NAs

3.3.2.3.1 Derivatisation

The first step to confirm the presence of NAs in the UCM observed in TICs from extracts was to carry out an alkylsilylation derivatisation using MTBSTFA. During the reaction, the active hydrogen of the carboxylic acid group in NAs is replaced by the *tert*-butyldimethylsilyl (t-BDMS) group, as shown in Figure 18. The reaction results in the formation of a non-polar GC amenable derivative that is more resistant to fragmentation[268], providing a molecular ion and therefore more structural information than the non-derivatised form. The derivatisation process can produce different fragmentation patterns depending on structural characteristics of each compound, but the $[M+57]^+$ ion, where M is the molecular mass of the naphthenic acid, is typically observed for all compounds with very high relative abundance after the cleavage of the t-butyl moiety ($-C(CH_3)_3$) (Figure 18). The t-BDMS derivative is useful as it provides the molecular mass of the compounds eluting within the UCM. This method has previously been

used for the analysis of NA mixtures from commercial mixtures[257], surrogate NAs[269], and the detection of NAs in oil sands tailing waters[256], known to contain high concentrations of NAs.

4-Methyl-1-cyclohexanecarboxylic acid was used as a model compound (MW 142.20 Da) to help in the interpretation of mass spectra following derivatisation, as provided in Figure 18. The TIC and averaged mass spectra of its non-derivatised form are provided in Figure 19 (A and B, respectively), showing a RT ranging from 14.8 to 17.8 min and a molecular ion of 142 *m/z*. After derivatisation, the TIC in Figure 19 (C) revealed that the RT of the acid shifted to 19.4 – 19.9 min and showed an additional peak at 11.4 min, which corresponds to the silylation reagent. The averaged mass spectra (Figure 19, D) provides evidence of the successful formation of the *t*-BDMS derivative with a molecular ion of 199 *m/z*, which corresponds to the $[M+57]^+$ ion. The molecular mass is easily calculated by subtracting 57 from 199 *m/z*. Additionally, the ion 75 *m/z* is also observed, which corresponds to $[SiC_2H_7O]^+$ shown in Figure 18.

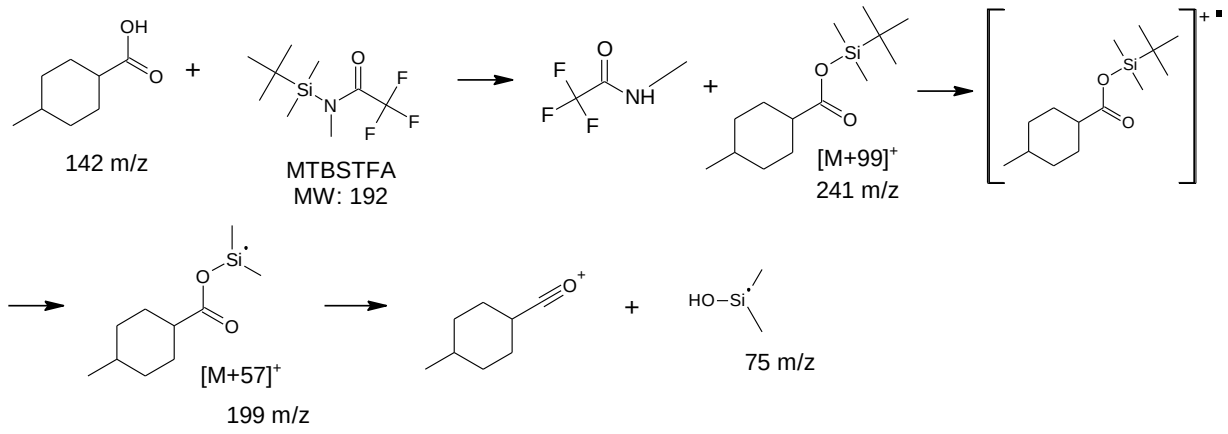


Figure 18. Silylation reaction of 4-methyl-1-cyclohexanecarboxylic acid using MTBSTFA, and fragmentation pathway leading to the predominant ions $[M+57]^+$, corresponding to *m/z* 199, and $[HO\text{-}Si(CH_3)_2]^+$, corresponding to *m/z* 75[257,268]

Figure 20 provides the TICs and averaged mass spectra for the SPE extract from pipeline 3 before (A, B) and after (C, D) derivatisation. The mass spectrum in B was the typical spectrum obtained from NAs when ionisation is achieved by electron ionisation (EI), resulting in extensive fragmentation of molecules in the mass spectrometer and total absence of molecular ions.

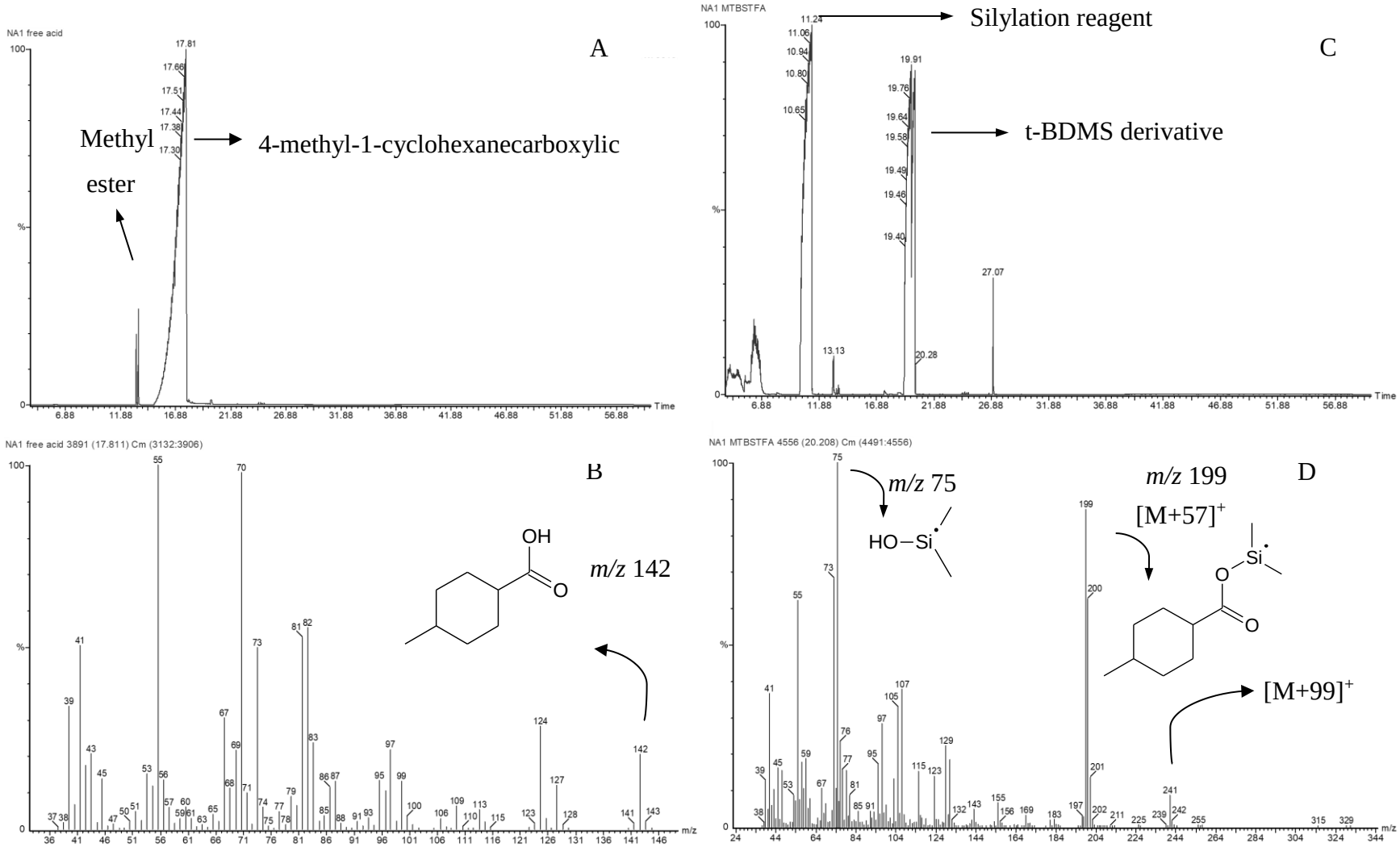


Figure 19. TICs and averaged spectra of 4-methyl-1-cyclohexanecarboxylic acid (A) (B), and its t-BDMS derivatised form (C) (D)

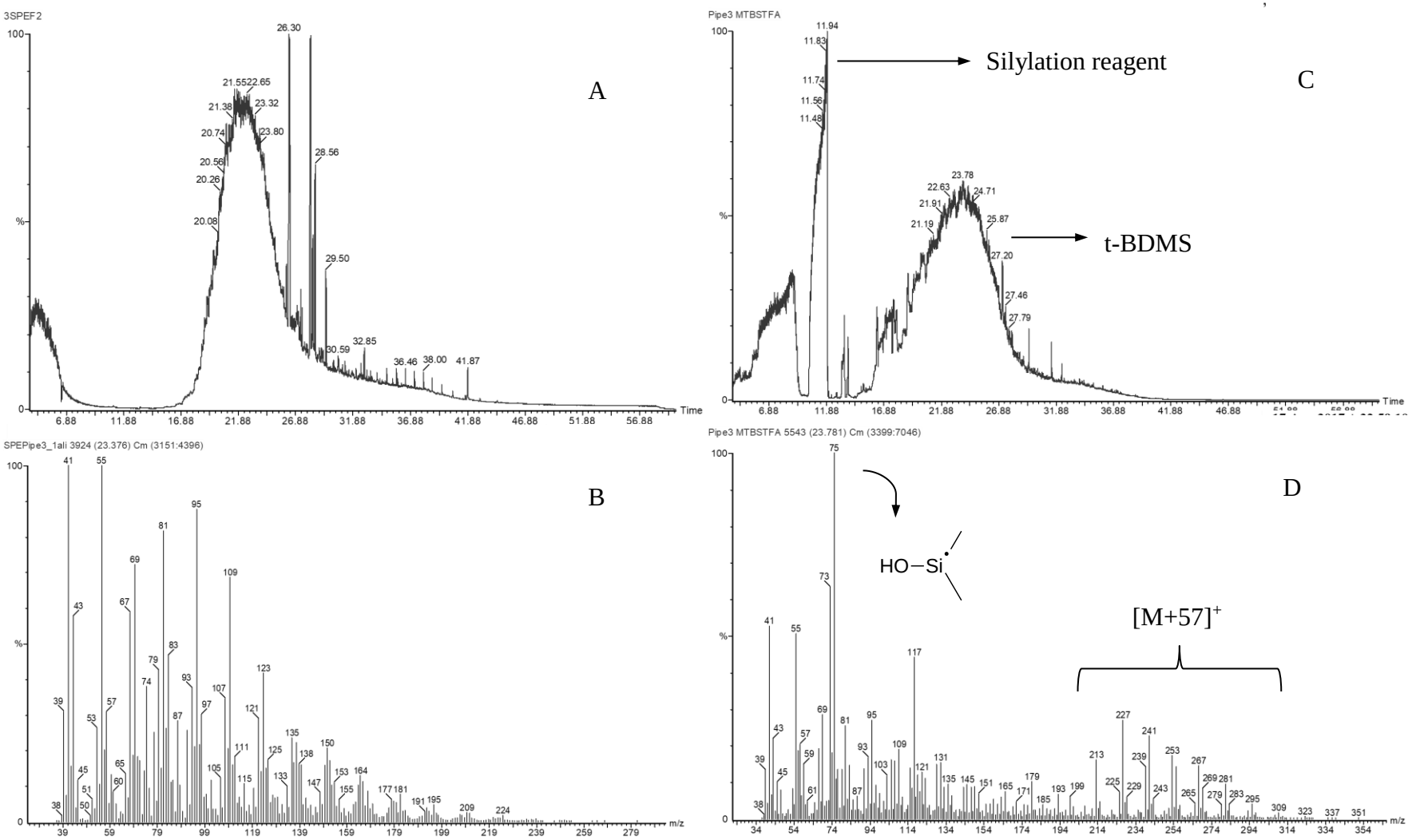


Figure 20. TICs and averaged spectra of the hump in pipeline 3(A) (B), and its t-BDMS derivatised form (C) (D)

After derivatisation, the averaged mass spectra in D showed that a significant increase in mass fragments higher than 200 *m/z*, corresponding to the t-BDMS derivatives of NAs. As set out in Table 15, it was possible to calculate the expected $[M+57]^+$ ions for any monocarboxylic NA fitting the general formula $C_nH_{2n-z}O_2$ using their molecular mass, and these predicted values were used to assign the observed mass fragments to NA congeners after subtracting 57 from the observed $[M+57]^+$ ion; the ions observed in the mass spectrum are greyed out in Table 15. As the averaged mass spectra in Figure 19(D) revealed, the ions for *t*-BDMS derivatives generated after derivatisation of the extract ranged from 213 to 295 *m/z*, indicating that the NAs present in the sample ranged from C_9 to C_{15}, and *Z* families from 0 to -8 (Table 15), all of which have been previously reported as NAs in both environmental water samples[270,271] and commercial mixtures[258,272]. The NA profile obtained is shown in Figure 21 (A).

Table 15. Predicted $[M+57]^+$ ions for a homologous series of t-BDMS derivatised NAs, ranging from C_6 – C_{20} and *Z* families between 0 and -12. $[M+57]^+$ ions observed after derivatisation of the extract from pipeline 3 are greyed-out

No. of Carbon atoms	**Z number**						
	0	**-2**	**-4**	**-6**	**-8**	**-10**	**-12**
6	173	171					
7	187	185					
8	201	199					
9	215	213					
10	229	227	225				
11	243	241	239				
12	257	255	253	251			
13	271	269	267	265			
14	285	283	281	279	277		
15	299	297	295	293	291		
16	313	311	309	307	305	303	
17	327	325	323	321	319	317	
18	341	339	337	335	333	331	329
19	355	353	351	349	347	345	343
20	369	367	365	363	361	359	357

3.3.2.3.2 HPLC/ESI-MS

Negative ion electrospray ionisation was used for further characterisation of NAs because, as a soft ionisation technique, it generates little fragmentation and provides useful structural information. Moreover, this technique has been applied in previous studies for the characterisation of NAs, so a good body of data can be found in the literature as a reference. The acidic nature of NAs was confirmed with their ionisation resulting from the addition of ammonia to the mobile phase.

The analysis of NAs in extracts from pipeline 3 using HPLC/ESI-MS gave unusually complex spectra, even though little fragmentation was achieved. Considering that the general formula of classic NAs is $C_nH_{2n+Z}O_2$, where *n* indicates the number of carbon atoms and *Z* is the hydrogen deficiency due to cyclization, the observation of significantly intense peaks at every mass number through a combination of homologues spaced every 14 *m/z* units confirmed the presence of NAs forming different *Z* families, as set out in Table 16. However, the NA profile obtained (Figure 21 - B) was very different to that obtained using GC-MS (Figure 21 – A) because it showed a wider range of ions separated in two clusters, the first ranging approximately from 127 *m/z* to 277 *m/z* and the second ranging between 283 *m/z* and 491 *m/z*. The profile indicated that the sample contained NAs ranging from C_7 to C_{33}, in contrast to the range of C_9 to C_{15} indicated by GC-MS. Moreover, the GC-MS profile showed that the *Z*=-2 family was predominant, whereas the HPLC/ESI-MS profile showed that the most intense species belonged to the *Z*=-4 family, which has been reported as the most intense family in OSPW samples[273,274]. Both profiles indicated that the most abundant *n* congeners were $C_{10\text{-}15}$, but the HPLC/ESI-MS profile showed that there was a substantial contribution from the $C_{21\text{-}28}$ region. The distribution of NAs in a first cluster with carbon numbers <21 and a second cluster with carbon numbers >21 (known as the C_{22}+ cluster) was first reported by Holowenko *et al.* (2002)[275] in water samples derived from oil sands extraction processes. Later on, Clemente *et al.* (2003)[147] reported the absence of the C_{22}+ cluster in commercial NAs preparations, whereas 6% to 26% of ions in NAs preparations from oil sands operations corresponded to the C_{22}+ cluster according to Holowenko *et al.* (2002)[275]. Other studies characterising commercial mixtures reported the presence of the $<C_{21}$ cluster only[152,258,272,273], which led some authors to suggest that commercial mixtures were not as complex as environmental mixtures in an attempt to explain why the removal/biodegradation processes developed from commercial mixtures were not fully effective in real-life samples[258].

Table 16. Predicted $[M-H]^-$ ions for a homologous series of classic NAs ranging from C_6 – C_{20}, and *Z* families between 0 and -12. NAs detected by GC-MS are greyed-out and NAs confirmed by HPLC/HRMS are shown in red

No. of Carbon atoms	Z number						
	0	-2	-4	-6	-8	-10	-12
6	115.0759						
7	129.0916	127.0759					
8	143.1076	141.0916					
9	157.1232	155.1077					
10	171.1389	169.1232	167.1099				
11	185.1545	183.1388	181.1233				
12	199.1702	197.1545	195.1389	193.1231			
13	213.1859	211.1701	209.1545	207.1388			
14	227.2016	225.1858	223.1701	221.1545	219.1389		
15	241.2173	239.2016	237.1859	235.1703	233.1545		
16	255.2328	253.2170	251.2016	249.1860	247.1702	245.1545	
17	269.2481	267.2329	265.2175	263.2016	261.1858	259.1702	
18	283.2637	281.2481	279.2327	277.2171	275.2017	273.1860	271.1703
19	297.2794	295.2637	293.2481	291.2324	289.2168	287.1862	285.1858
20	311.2950	309.2794	307.2637	305.2481	303.2324	301.2168	299.2011
21	325.3180	323.3020	321.2860	319.2700	317.2540	315.2330	313.2169
22	339.3340	337.3180	335.3020	333.2860	331.2700	329.2540	327.2380

In earlier studies, the discrepancy between the congeners observed with GC-MS and HPLC/ESI-MS was erroneously attributed to the decreased ionisation and derivatisation efficiencies in NAs with high molecular weight[273], but Bataineh *et al.* (2006) demonstrated that HPLC/ESI-MS did not discriminate against large NAs. Interestingly, previous studies of environmental samples have also detected the C_{22}+ cluster by GC-MS[275,276], which contrasts with our results because this cluster was observed only by HPLC/ESI-MS. Later on, Martin *et al.* (2008)[277] compared the ESI-MS profile with the HPLC/ESI-HRMS profile of a NA-containing extract and reported the appearance of the C_{22}+ cluster in ESI-MS analysis as false-positives stemming from unit-resolution mass spectrometry that led to misclassification of acidic components (*e.g.* high- and low-molecular-weight polyphenols and phenolic acids, phthalic acid derivatives, organic compounds containing nitrogen or sulphur) as naphthenic acids fitting the formula $C_nH_{2n+Z}O$. For this reason, further analysis of the sample was carried out using high-resolution mass spectrometry to determine whether the C_{22}+ cluster was actually

present in the sample or was a false positive.

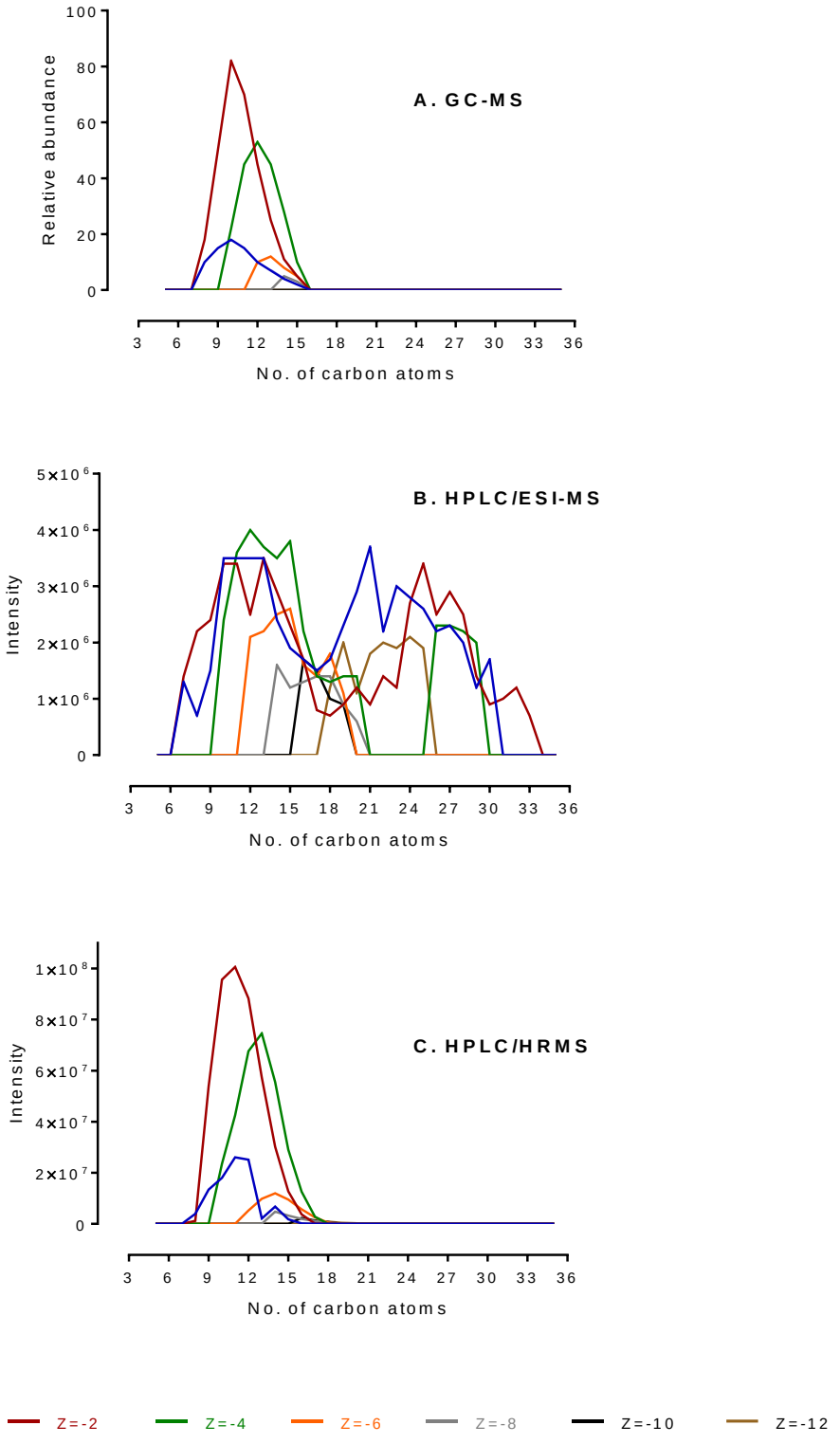


Figure 21. NA profiles for the same extract obtained from pipeline 3 analysed by (A) GC-MS after derivatisation with MTBDSTFA, (B) HPLC/ESI-MS, and (C) HPLC/HRMS

3.3.2.3.3 HPLC/HRMS

After calculating the exact masses of classic NAs fitting the formula $C_nH_{2n+Z}O_2$ for all combinations of n = 5 to 35, and Z = 0 to -12, the predicted ions were searched in the acquired mass spectra, generating the NA profile presented in Figure 21 – C. The resulting profile was similar to that obtained by GC-MS (Figure 21 – A) but differed in the low-intensity ions detected, indicating that GC is not as sensitive as HRMS, which expanded the carbon range to C_{21} and included congeners from Z = -10 and -12 (Table 16). Families Z = 0 to -6 presented the same proportional contribution (Z= -2 > -4 > 0 > -6) in both profiles. More importantly, only the $<C_{21}$ cluster was observed, corroborating the results by Martin *et al.* (2008)[277] and challenging previously published studies reporting the presence of the C_{22}+ cluster using unit-resolution ESI-MS as the only technique to characterise NAs mixtures. Some studies have now recognised that high mass accuracy is a prerequisite for a full characterisation of complex NA mixtures[141,276].

Oxidized NAs fitting the formula $C_nH_{2n+Z}O_x$ where x = 3 to 5, which result from oxidation of classic NAs[143,278], were also detected in the extract, although their intensity was much lower compared to classic NAs (Figure 22). Based on abundance, O_2 NAs corresponded to 89.8% of the NAs detected, whereas oxy-NAs corresponded to 3.5%, 6.5%, and 0.1% for O_3, O_4, and O_5, respectively. These findings corroborate the findings of previous works reporting a predominance of O_2 and O_4 in NA mixtures[143,278] but show a much higher relative abundance of O_2 NAs in relation to other studies, where classic NAs have been found to range between 15% and 72%[164,278–280]. These publications, however, did not characterise RWW but groundwater, OSPW samples, and OSPW samples from tailing ponds, where older OSPW from experimental reclamation ponds were reported to contain more oxidized NAs than parent NAs, indicating that the higher proportion of oxy-NAs results from weathering and/or biodegradation of classic NAs via hydroxylated intermediates[164]. This has important implications, as classic NAs have been regarded as the most toxic NAs[281] and could explain the significant inhibition of luminescence observed in the LBT.

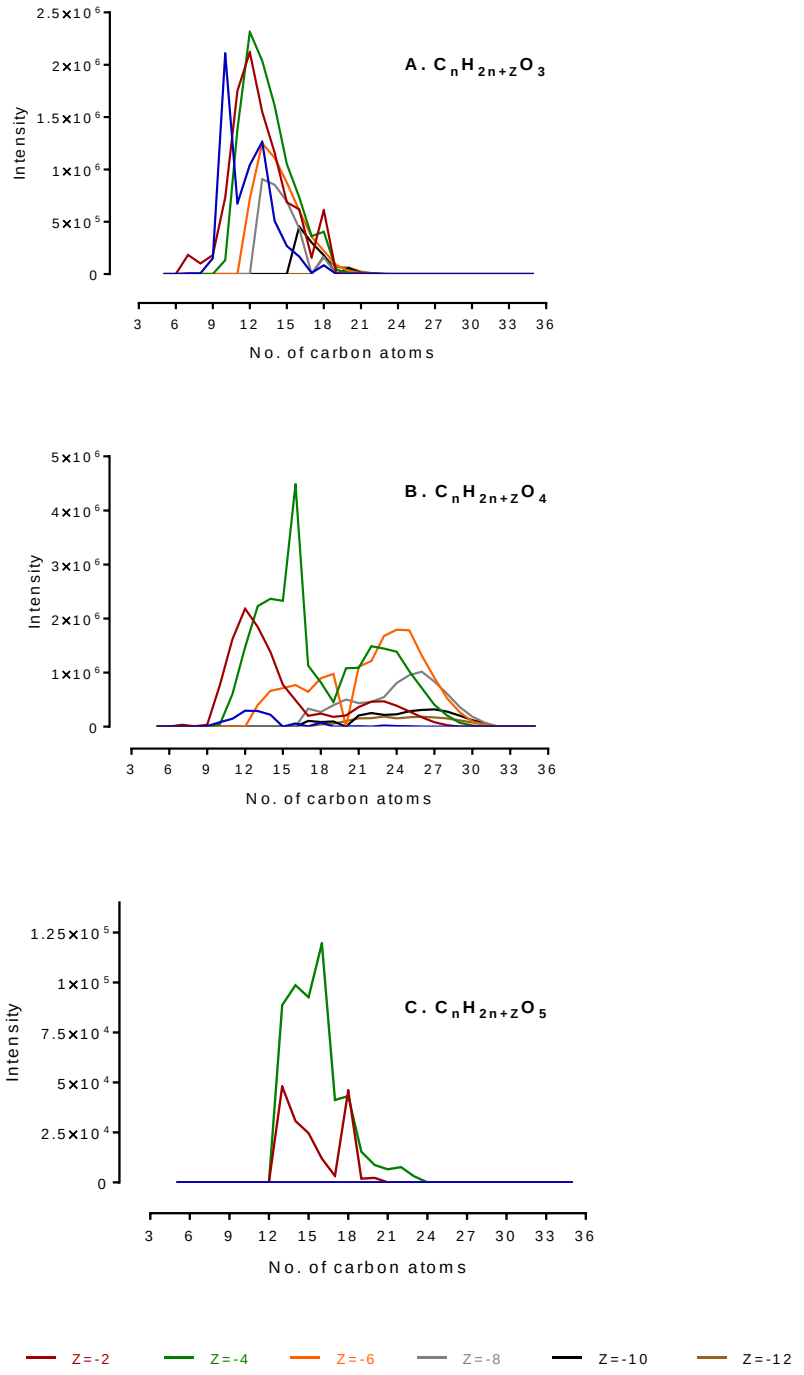


Figure 22. Profiles of oxidised NAs for pipeline 3 analysed by HPLC/HRMS. (A) $C_nH_{2n+Z}O_3$, (B) $C_nH_{2n+Z}O_4$, (B) $C_nH_{2n+Z}O_4$

3.4 Conclusions

The objective of this chapter was to detect and identify toxic organics in RWW samples following an EDA approach. The findings of this chapter confirm that the combined application of chemical and biological techniques is suitable for the analysis of complex environmental

mixtures, as it achieves a significant and selective reduction in complexity. In the same way, *Vibrio fischeri* showed to be an appropriate and convenient bio-detector for refining toxicants, as the luminescent bacteria test led to the detection of naphthenic acids in one of the samples analysed (pipeline 3). However, as previous studies following TIE approaches have suggested[79,81], most of the toxic effects observed seem to be linked to (i) organic chemicals at concentrations below detection limits of most analytical instruments and (ii) mixture effects resulting from the interaction of different organic toxicants, mainly hydrocarbons. The low number of publications involving TIE/EDA of refining effluents might be related to publication bias stemming from the "disappointing" outcome of not finding an evident chemical, or few chemicals, causing all the observed effects. In fact, one of the few references discussing toxicity-directed analyses of RWW is a book chapter[54] where unpublished TIE studies of RWW are only briefly mentioned as unsuccessful case studies due to the spread of toxicity among multiple fractions, although in all cases the assignment of toxic effects to miscellaneous organics is clear. Hence, the findings of this chapter broaden the body of evidence pointing at mixture effects and low-concentration pollutants as the cause of toxicity from RWW, in addition to NAs resulting from the processing of heavy feedstock.

Taken together, our results highlight the need of site-specific analyses to determine the cause of observed effects for RWW and to determine the appropriate treatment technologies required to achieve high-quality effluents. Characteristics of crude oil can vary drastically between geographical areas, impacting the nature of chemicals transferred to wastewater during refining. In our case study, Colombian heavy crude oil has been reported to have an acidic nature, suggesting high contents of NAs, which aligns with the results obtained in this work. It is evident that further treatment is needed in this particular wastewater treatment plant to target NAs and provide effluents that are environmentally safe. However, a limitation in this study is that the effluents analysed were grab samples due to the difficulties in establishing collaborations with refining sites, which meant that only one sampling was logistically achievable. This resulted in limited amounts of sample that restricted the extent of the research and, in the case of toxicity testing of fractions, the number of replicates conducted. Periodic samples would help to identify chemicals that are consistently present in effluents and could be used as general and site-specific indicators of wastewater treatment efficacy.

Another important finding to emerge from this chapter is that the complexity of environmental mixtures of naphthenic acids represents an analytical challenge that must be overcome in order

to develop appropriate technologies for their removal. As NAs are linked to corrosion of operative equipment in refineries and have toxic effects on a wide range of species, their removal is of great importance for recycling and discharge purposes. The variability in the composition of NA mixtures results in unpredictable behaviour, which is reflected in numerous publications aiming at degrading/removing NAs from OSPW with only limited success. Not all NAs are toxic and not all NAs are recalcitrant, but the NAs that are toxic and recalcitrant are severely impacting the sustainability of petroleum refineries processing acidic crude oil and mining sites extracting bitumen from oil sands. Therefore, there is an urgent need to develop technologies that can efficiently remove NAs and decrease the potential toxic effects of NA-containing effluents, including refining effluents generated during the processing of heavy crude oil.

CHAPTER 4

BIOPROSPECTION OF *Pseudomonas putida* FOR APPLICATION IN BIOREMEDIATION OF NAPHTHENIC ACIDS

4.1 Introduction

Microbial metabolic strategies are considered by many experts the most efficient forms of energy harvest on earth, competing over substrates and minimising energy losses in highly energy-limited conditions[282]. In natural environments, microbial degradation plays a significant role in the breakdown of environmental pollutants because of the remarkable metabolic diversity of microorganisms; these capabilities are exploited in wastewater treatment plants to recreate natural degradation processes and provide a highly cost-effective clean-up of industrial effluents. However, regardless of the numerous advancements and major breakthroughs in the optimisation of treatment plants, biological treatment of recalcitrant compounds remains poor or incomplete, leading to the release of potentially hazardous chemicals into the environment. It is, therefore, necessary to screen microorganisms capable of degrading persistent toxic chemicals as a first step in the development of efficient microbe-mediated treatment technologies that can provide high-quality effluents at low costs. The selection and identification of a suitable microorganism or consortium can ultimately lead to the identification of relevant metabolic pathways, regulation systems, enzymes, and/or genes using omics technologies that provide valuable information for biocatalysts engineering and applications[25,283].

The assessment of biodegradation potential is currently achieved using a tiered procedure that includes an initial screening step where microorganisms are exposed to the target chemicals to determine whether they can use these compounds as carbon and energy source to grow, and a subsequent characterisation of the microorganism selected as biocatalyst. The first stage in this tiered process indicates whether microorganisms possess the relevant catabolic genes and whether these genes are expressed under the test conditions, as the presence of catabolic genes does not necessarily mean that degradation will take place. This is explained by the fact that microorganisms have developed strict regulation systems — which are responsive to physiological signals and specific substrates — to control metabolic pathways and provide a highly cost-effective growth[284]. As a result of this highly efficient and tightly regulated metabolism, the rate at which chemicals are degraded is impacted by physicochemical conditions, availability of other nutrients, accessibility of such chemicals, and the presence of other competitors[285]. Therefore, not only the selection of the correct microorganisms is necessary for bioremediation purposes, but also the use of appropriate culture conditions to induce and/or improve biotransformation of chemicals[286].

Numerous variations in culture conditions have been reported to improve biodegradation rates of petrochemicals. The addition of surfactants to increase the bioavailability of non-polar compounds is common, although surfactants can exert toxicity or be used as carbon sources, ultimately inhibiting the degradation of the target chemical(s)[287–290]. The addition of complementary carbon sources is also common[23,290–293], but their influence on the biotransformation of the target chemical(s) should be examined to determine whether the added compound induces carbon catabolite repression, which is a regulation system aiming to optimise metabolism when cells are exposed to a mixture of potential carbon sources. The presence of other carbon sources can inhibit the uptake and/or expression of genes involved in the catabolism of the compound of interest[294,295]. In other cases, however, bacteria can co-metabolize different carbon sources. To avoid the carbon catabolite repression phenomenon, many studies opt to grow bacteria in minimal media and supplement it only with the target chemical (see Table 17), which might induce a strong and immediate activation, as other carbon sources can repress activation. Other carbon sources at limiting concentrations could also stimulate biotransformation of the target chemical, provided all other nutrients are in excess and cultures reach stationary phase. However, to determine how much of a carbon source is considered "limiting concentration" requires knowledge of the energy status of the cell.

Besides the presence of other potential carbon sources, the concentration of the target chemical is also key for its biotransformation because of the potential inhibitory effects on microbial growth at high concentrations, and reduced rates at very low concentrations[296,297]. It has been shown that biodegradation rates increase with increasing concentration up to a certain level but beyond this point the removal rate decreases, which indicates that metabolism is negatively impacted once the inhibitory effect is reached[298]. Similarly, lag time increases with chemical concentration[299], although such increase is equally related to the inoculum size. Cell density plays an essential role in biodegradation experiments with both free[286,300] and immobilised cells[298]. The immobilisation of bacterial biomass has shown to protects cells from very high concentrations of chemicals and facilitate removal and re-utilisation of bacteria[298]. Substrates like pumice, polyvinyl alcohol (PVA), granular silica gel, amberlite IRA-938, and exhausted perlite are used for cellular immobilisation[298,301].

As for the microorganisms to use for biodegradation, numerous species have been reported as good degraders of recalcitrant chemicals under laboratory conditions; in particular, Pseudomonads have received much attention partly because the frequent acquisition of

catabolic plasmids expands their range for biodegradation potential[302]. Some species can use more than 100 different carbon sources[28], many of which are recalcitrant and growth-inhibiting xenobiotics[25]. This genus is characterised by its ubiquitous distribution (found in most temperate, aerobic and semi-aerobic soil and water habitats, including polluted soils[28]) and versatile metabolism that makes them prolific colonisers in nature and laboratory environments[302]. Their simple nutritional requirements and fast growth in laboratory conditions are strong advantages for industrial applications[25,28]. In particular, Pseudomonads have been reported to assimilate a vast range of petrochemicals[294], including phenols and chlorophenols[303], aromatic hydrocarbons[293,304], *n*-alkanes[24], and naphthenic acids (NAs)[305,306], making this genus a great candidate for the biological treatment of refining effluents containing NAs. Removal efficiencies > 95% of commercial NA mixtures have previously been reported using a consortium of *P. putida* and *P. fluorescens*[305]. A series of culture conditions have been successfully applied to improve biotransformation of petrochemicals by *Pseudomonas* spp., such as acclimatisation (gradual increase in concentration) and pre-exposure[295,298,301,307], and the addition of supplements for the induction of natural surfactants, which increase the bioavailability of hydrophobic petrochemicals[308]. Table 17 provides some examples of biodegradation studies with *Pseudomonas* spp. and the culture conditions used to degrade crude oil and petroleum-derived contaminants, including naphthenic acids.

There is a hierarchical preference for carbon sources in *Pseudomonas*, where organic acids and aminoacids are preferred over glucose, and glucose is preferred over hydrocarbons[25,294]. This makes Pseudomonads good candidates for degradation of NAs but may represent a challenge when these co-occur with other petrochemicals, which is usually the case in RWW. In general, the presence of organic acids, such as succinate, pyruvate, and acetate, represses the expression of genes for the catabolism of glucose and other carbohydrates, including mannitol and fructose. At the same time, the presence of glucose has a repressing effect on the expression of several genes involved in the catabolism of a range of other carbon sources, such as toluene, methylphenols, and styrene. However, not all acids induce a repressive effect, and not all strains follow this preferential order; citrate, for example, induces the expression of OCT plasmid (alkane degradation pathway) in *P. putida* GPo1[294], and a study from Basu *et al.* (2006)[304] reported the preferential utilization of aromatic compounds over glucose in *P. putida* CSV86, and the co-metabolism of organic acids and aromatic compounds[304].

Table 17. Culture conditions in previous studies of biodegradation of petrochemicals by *Pseudomonas* spp.

Target chemical	Microorganism	Culture medium	Culture conditions						Reference
			Temp. (°C)	Initial concentration (mg/L)	pH	Additional C sources	Inocula	Other	
BPBA isomers (NAs)	Microbial consortia containing *Pseudomonas* spp.	MSM	20	4	-	-	2% (v/v)	Incubation for 49 days	Johnson *et al.*, 2011[306]
NA commercial mixture	*P. putida* and *P. fluorescens*	MSM	20	400	8.2	-	4% (v/v)	Incubation for up to 4 weeks	Del Rio *et al.*, 2006[305]
OSPW and commercial NA mixtures	Microbial consortia	TW + BHM 5% (v/v)	20	50, 100	-	-	Indigenous bacteria	Incubation for 28 and 98 days	Han *et al.*, 2008[309]
BPBA isomers (NAs)	*P. putida* KT2440	MSM	20	2 - 4	-	-	2% (v/v)	Incubation for 49 days	Johnson *et al.*, 2011[310]
CHCA isomers (NAs)	*P. putida*	MSM	27	2000	-	0.2% gluconate	0.01% (v/v)	-	Blakley & Papish, 1982[311]

Target chemical	Microorganism	Culture medium	Culture conditions						Reference
			Temp. (°C)	Initial concentration (mg/L)	pH	Additional C sources	Inocula	Other	
Phenol	*P. putida*	MSM	30	75	7	Glucose	-	Immobilised in PVA gel	El-Naas *et al.*, 2009 [298]
o-Cresol	*P.aeruginosa* S8	MSM	30	Up to 1250	7 - 8	-	5% (v/v) OD_{600}=0.5	-	Lassouane *et al.*, 2013 [299]
Crude oil	*P.aeruginosa*	MSM	35	700	-	3% glycerol	-	Pre-cultures for production of rhamnolipids	Zhang *et al.*, 2005[308]
Naphthalene	*P. putida* S2	MSM	28	100	-	1% citric acid	-	1% $(NH_4)_2SO_4$ 700 mg/L NaCl	Zafar *et al.*, 2010[293]
Naphthalene	*P. putida* G7	MSM	24	400	7	Sodium pyruvate	<1% (v/v) OD_{600}=0.4	24-h pre-exposure	Lee *et al.*, 2003[295]

MSM: Minimal salt medium; BPBA: butylphenyl-butanoic acid; OSPW: oil sands process water; TW: tailing water; CHCA: cyclohexanecarboxylic acid; BHM: Bushnell-Haas medium

Considering that NAs have an essential contribution to the whole effluent toxicity in some refining effluents and their degradation could help in the detoxification of such effluents, this chapter investigated the bioremediation potential of *Pseudomonas putida* for the degradation of naphthenic acids following a tiered procedure. The first step corresponded to a screening stage of 86 fully-sequenced strains of *P. putida* that aimed to (i) identify isolates capable of growing by using model NAs as carbon source, and (ii) provide data for bioinformatics analysis to detect candidate catabolic genes involved in the biodegradation of petrochemicals, which was conducted by the Centre for Systems and Synthetic Biology at Brunel University London in collaboration with Professor Nigel Saunders. The second step of the tiered procedure evaluated the strains selected during the screening stage for the biodegradation of model NAs in M9 medium following a quantitative approach. The effect of acclimatisation on the biodegradation capabilities was assessed, as it has been reported to shorten the lag phase and increase the tolerance of microorganisms to the toxicity of the target chemicals[296,297].

The model NAs used as carbon sources were 4-methyl-1-cyclohexanecarboxylic acid (C_8, *Z*=-2) (NA1) and dicyclohexylacetic acid (C_{14}, *Z*=-4) (NA2), whose structural characteristics (*i.e.* carbon content and *Z* families) match those of the NAs detected in Pipeline 3. Four additional categories of petrochemicals (phenols, aromatic hydrocarbons, aliphatic hydrocarbons, and methylated naphthalenes) were included in the screening stage because NAs co-occur with other compounds in RWW, which means that tolerance or biotransformation capabilities of other petrochemicals are highly desirable in microorganisms to be used for the treatment of RWW. The model compounds used for the screening stage and their respective 2D structures are provided in Table 18 and Figure 23. For the second stage corresponding to biodegradation experiments, it was necessary to replace NA2 with 4-(4'-*tert*-butylphenyl)-4-butanoic acid (C_{14}, *Z*=-6) (NA3) due to analytical limitations for its detection.

Table 18. Model compounds used to screen the biodegradation potential of a set of strains of *Pseudomonas putida*

Type of compound	Compound
Naphthenic acids	4-methyl-1-cyclohexanecarboxylic acid
	Dicyclohexylacetic acid
Phenols	Phenol
	o-cresol
Aromatic hydrocarbons	Benzo(a)pyrene
	Toluene
	Phenanthrene
Aliphatic hydrocarbons	Pristane
	Dotriacontane
Methylated naphthalenes	1,4,6,7-tetramethylnaphthalene

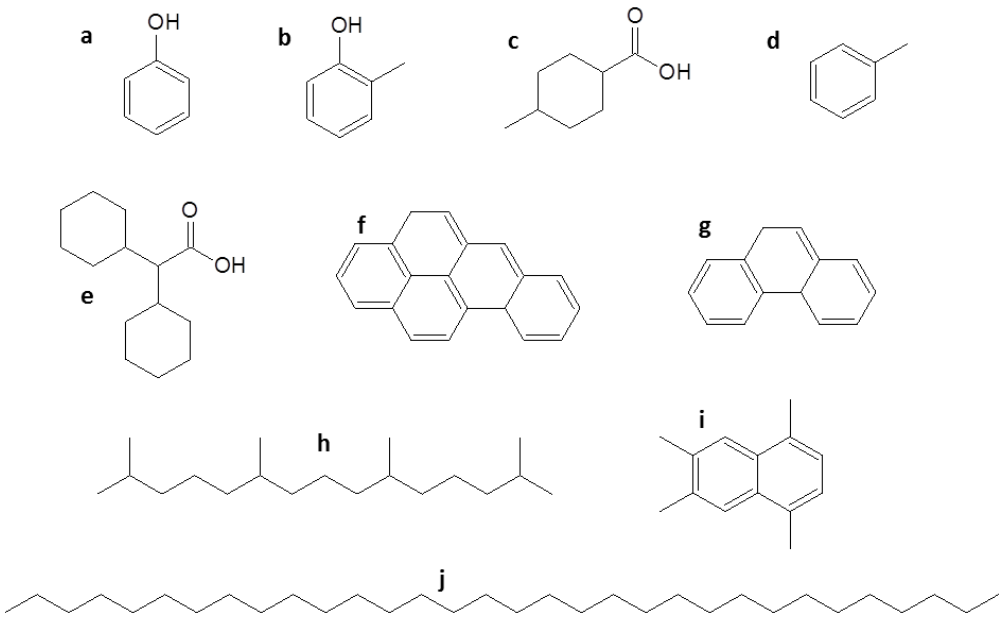


Figure 23. 2D structures of the chemicals selected for the screening for biodegradation potential due to its occurrence in petroleum refinery wastewaters and reported toxicity. (a) Phenol. (b) *o*-cresol. (c) 4-Methyl-1-cyclohexanecarboxylic acid. (d) Toluene. (e) Dicyclohexylacetic acid. (f) Benzo[*a*]pyrene. (g) Phenanthrene. (h) Pristane. (i) 1,4,6,7-Tetramethylnaphthalene. (j) Dotriacontane

4.2 Experimental section

4.2.1 Culture media

M9 minimal medium was composed of (L^{-1}): Na_2HPO_4, 33.9 g; KH_2PO_4, 15 g; NH_4Cl, 5 g; NaCl, 2.5 g; 1 M $MgSO_4 \cdot 7H_2O$, 1 mL; 1 M $CaCl_2$, 0.1 mL; 10 mM $FeSO_4 \cdot 7H_2O$, 0.1 mL; trace elements solution, 1 mL. The trace elements solutions contained (L^{-1}): H_3BO_3, 24.7 g; $CoCl_2 \cdot 6H_2O$, 7.14 g; $CuSO_4 \cdot 5H_2O$, 2.5 g; $MnCl_2 \cdot 4H_2O$, 15.8 g; $ZnSO_4 \cdot 7H_2O$, 2.88 g. Solid M9 contained 15 g L^{-1} of agar, whereas semi-solid M9 contained 5 g L^{-1}. pH was 7.0.

Nutrient agar was purchased from Sigma and used following the manufacturer's instructions.

4.2.2 Chemicals

4-methyl-1-cyclohexanecarboxylic acid, dicyclohexylacetic acid, 4-(4′-t-butylphenyl)-4-butanoic acid, dotriacontane, *o*-cresol, phenol, toluene, phenanthrene, and pristane were purchased from Sigma-Aldrich. Benzo(*a*)pyrene was purchased from Dr. Ehrenstorfer GmbH, and 1,4,6,7-tetramethylnaphthalene was obtained from Alfa Aesar. All solvents were HPLC-grade and obtained from Sigma-Aldrich or Fischer. KOH was obtained from Merck. For the derivatisation reaction, 2-nitrophenylhydrazine (2-NPH) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1-EDC·HCl) were purchased from Acros Organics.

4.2.3 Bacterial strains

A total of 86 fully-sequenced strains (JW1 – JW86) of *Pseudomonas putida* from different environmental sources were provided by Professor Nigel Saunders from the Centre for Systems and Synthetic Biology, Brunel University London. The corresponding master and work banks were established in nutrient broth using glycerol as cryoprotectant at a final concentration of 40%, using the methodology described in Appendix I. Storage was performed at -80°C. An additional strain of *P. putida* known to grow on phenol was used as positive control for the screening stage. The strain was kindly donated by Dr. Alejandro Couce from the French Institute of Health and Medical Research - Unit of Ecology and Evolution of Microorganisms.

4.2.4 Screening for biodegradation potential

The screening stage required a 2-week exposure of *P. putida* strains to the model compounds provided in Figure 23 to determine which strains were capable of utilizing NAs and other petrochemicals as a carbon source. However, some of these model petrochemicals had low solubility in water, which limited their bioavailability, biotransformation, and testing in

aqueous media. Three methods were assessed for their suitability to conduct the screening, namely the agar diffusion test, liquid microcultures, and the overlayer technique, which were evaluated in preliminary tests using 5 strains (randomly selected) and M9 for bacterial growth, as described below.

4.2.4.1 Agar diffusion test

The agar diffusion method is described elsewhere[312], and the full methodology is presented in Appendix I. Briefly, three solutions were prepared for each chemical in volatile organic solvents with concentrations of 10 mg/mL, 1 mg/mL and 0.1 mg/mL, which were later used to impregnate 6 mm filter paper discs (Whatman, no. 3). A total of 50 µL of each solution was loaded on separate discs to achieve final amounts of 500 µg, 50 µg, and 5 µg per disc. 24-h bacterial inocula previously grown at 30°C in M9 supplemented with glucose as carbon source were swabbed uniformly across M9 agar plates, and the loaded discs were then carefully placed on the surface of the agar containing the swabbed bacteria. Plates were incubated overnight at 30°C. Bacterial growth was an indication of tolerance to the chemical(s), whereas halos with no bacterial growth were an indication of growth inhibition.

4.2.4.2 Liquid microcultures

Liquid microcultures were performed using 96-well plates in M9 broth containing the test chemical as the sole carbon source, and triphenyl tetrazolium chloride (TTC) (0.01%) as redox indicator to facilitate the indirect detection of bacterial growth. Cultures were conducted with a final volume of 200 µL. Strains were exposed to 100 mg/L, 250 mg/L, 500 mg/L and 750 mg/L of the test chemicals; incubation was performed over two weeks at 30°C and 200 rpm. Determination of viability was performed every 48 hours via plating on nutrient agar.

4.2.4.3 Overlayer technique

The overlayer technique was carried out using 6-well plates following a modified version of the methodology reported by Um *et al.*, 2010[313]. A schematic representation of the procedure is shown in Figure 24. Briefly, approximately 10 mL of solid M9 medium with no carbon source were poured in each well to be used as a support medium. After solidification, 100 µL of a solvent-based 200-mg/L solution containing each test chemical were transferred to separate wells and immediately homogenised using sterile glass spreaders. After evaporation of the corresponding solvent, 2 mL of molten semi-solid M9 agar containing the bacterial suspension were transferred to each well. After solidification, plates were incubated at 30°C for two weeks, and daily checks were conducted to detect growth in the form of colonies. A

GX microscope was used at the end of the incubation time to confirm the absence/presence of bacterial growth in each well.

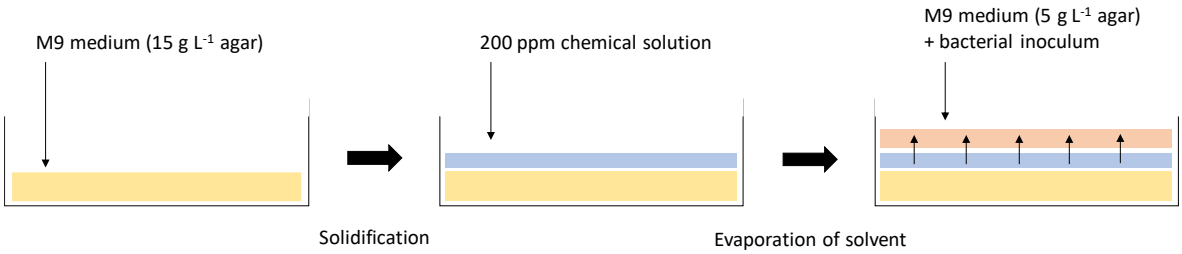


Figure 24. Schematic representation of the overlayer technique used for the screening for biodegradation potential

Bacterial inocula were prepared by reactivating the corresponding work vials onto nutrient agar plates with subsequent incubation at 30°C overnight. Then, between 3 and 5 colonies were transferred to 15-mL sterile tubes containing 10 mL of nutrient agar using a 10-µL sterile, disposable loop. The tubes were incubated overnight in a horizontal position at 30°C and 150 rpm. Grown media were then centrifuged at 4500 rpm, 4°C for 10 minutes to wash the cells and remove any trace of nutrients. The supernatant was discarded, and the pellet re-suspended in 5 mL of 0.1M of sterile phosphate buffer pH 7.2. This procedure was repeated twice, and the bacterial inoculum was finally re-suspended in 10 mL of M9 broth with no carbon source. The final bacterial suspension for the tests was prepared transferring 2.5 mL of the solution mentioned above into 22.5 mL of molten agar at 35°C.

Negative controls had no carbon source added, and positive controls contained glucose 2% (*w/v*) as a carbon source, which was added only to the upper layer of M9 medium. In addition, a reference strain of *P. putida* positive for degradation of phenol was used as a control and tested in duplicate.

4.2.5 NA-degradation experiments

The strains selected in the screening stage were combined and used as a single consortium for the biodegradation experiments, where NA2 was replaced with NA3 due to detection and quantification analytical limitations. The structures of all three model NAs are shown in Figure 25. Stock solutions of NAs were prepared in 0.1 M KOH because of their poor water solubility and acidic nature. These stock solutions were used for supplementing the M9 with the model NAs, after which the pH of culture media was adjusted to 7.0 using 0.1 M HCl.

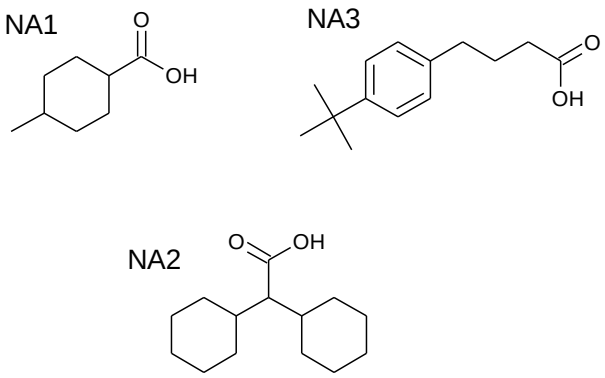


Figure 25. Model NAs used in this study as substrates for degradation by *Pseudomonas putida*. NA1: 4-methyl-1-cyclohexanecarboxylic acid; NA2: Dicyclohexylacetic acid; NA3: 4-(4′-t-butylphenyl)-4-butanoic acid

Cultures were performed in 100-mL conical flasks containing 50 mL of M9, at 30°C, 200 rpm, for 30 days, with no other carbon sources available other than NA1 or NA3. Controls included (i) biomass-free experiments to determine the abiotic loss of NAs, (ii) non-viable experiments with autoclaved cells to determine loss of NAs by adherence to cells, and (iii) glucose-based experiments to confirm the viability of inocula. Two concentrations were tested for each NA: 100 mg/L and 350 mg/L for NA1, and 30 mg/L and 70 mg/L for NA3, which were selected based on the solubility in water and analytical range of detection for each compound. Cultures were conducted in 4 separate batches, as presented in Figure 26. Biodegradation and control experiments were performed in triplicate. Acclimatisation was evaluated as pre-treatment to increase the rate of biodegradation; it was conducted by a gradual increase in the NA concentration in combination with a gradual reduction of glucose (starting concentration of 1%), as shown in Figure 27. As provided in the figure, an additional control was conducted to verify the viability of cells at each step during acclimatisation. Inocula for experiments with no pre-treatment were prepared as described above for the overlayer technique, whereas those for experiments with pre-exposure were prepared using the biomass resulting from the preceding exposure flask, washing the cells twice before transferring them into culture flasks.

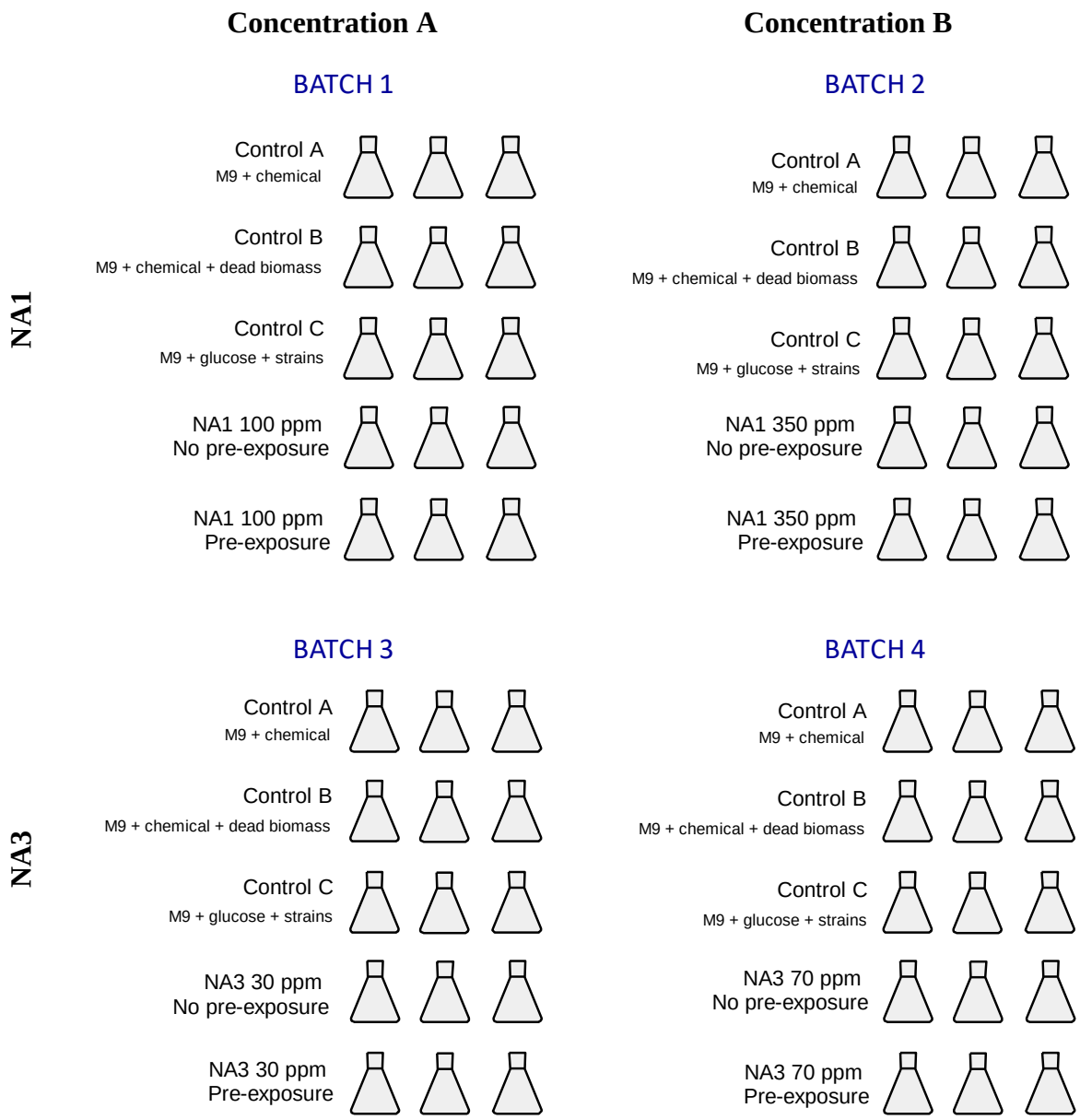


Figure 26. Experimental design for biodegradation of NAs, where Control A = Abiotic loss; Control B = Biomass adsorption; Control C = Cell viability. Two concentrations were evaluated for each NA (NA1: 100 mg/L, 350 mg/L; NA3: 30 mg/L, 70 mg/L), for a total of 4 batches

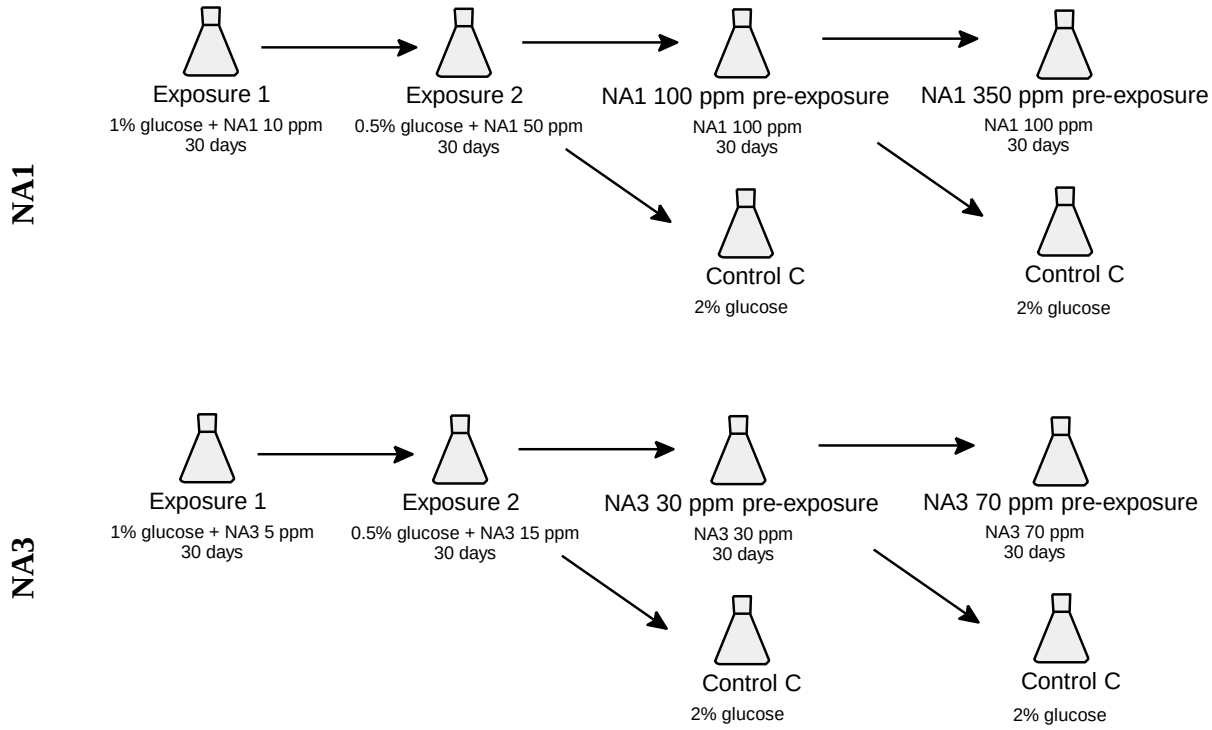


Figure 27. Acclimatisation of the bacterial consortium to increasing concentrations of NAs showing two 30-day exposure cultures (Exposure 1 and 2) followed by two 30-day biodegradation cultures, where Control C = Cell viability

Destructive sampling was conducted every third day, in which 1-mL aliquots were centrifuged at 4000 rpm for 10 minutes in 2-mL plastic microcentrifuge tubes to eliminate biomass. Pellets were discarded and supernatants were transferred to 2-mL glass vials and stored at -20°C until analysis.

For assessing biodegradation, the residual NAs in the culture medium were quantified via DAD-HPLC. To do so, the sample preparation required an extraction step and a derivatisation reaction followed by quantification, as described below and provided in Figure 28.

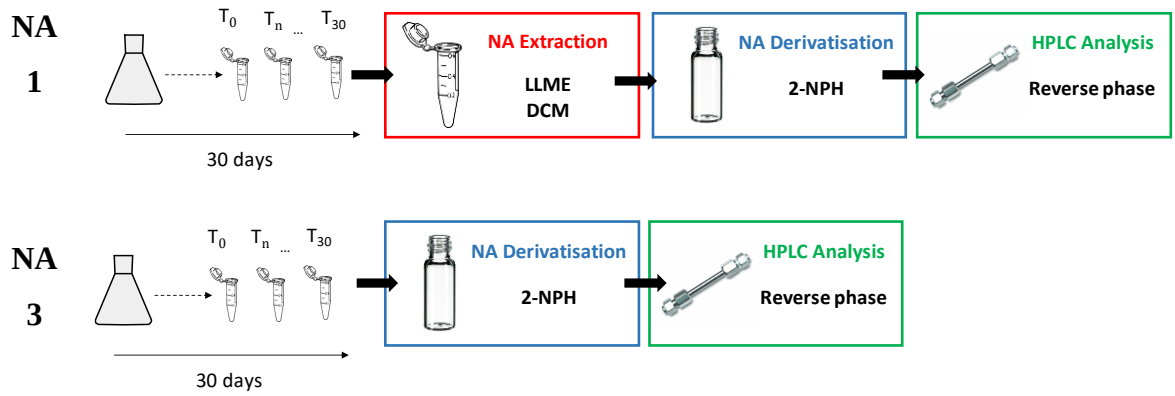


Figure 28. Sample preparation and quantification of NA1 and NA3 in culture medium after biodegradation experiments where T_n are time intervals for sampling (days)

4.2.6 Extraction of NA1 from M9

Extraction of NA1 from M9 was conducted using micro liquid-liquid extraction (MLLE) as follows. A total of 400 µL of the supernatant were transferred to 2-mL microcentrifuge plastic tubes, followed by the addition of 3 drops of 1 M HCl and 200 µL of DCM. Tubes were then vortexed for 30 seconds. Separation of the aqueous and organic phases was achieved by centrifuging at 4000 rpm for 10 minutes. Subsequently, 150 uL of the DCM in the bottom of the tube were transferred to a 2-mL glass vial containing 50 µL of deionised water used as a keeper. A second extraction was conducted as described above, and the extracts combined. Samples were then dried under a gentle nitrogen flow in a TurboVap® LV concentration evaporator workstation.

4.2.7 Derivatisation procedure for NAs

The derivatisation of carboxylic acids into acid hydrazines allows a sensitive colourimetric determination of NAs using HPLC-DAD. The assay was conducted following the procedure described by Miwa *et al.*, 1985[314]. Three solutions were used for the derivatisation reaction, as follows. Solution A was prepared by dissolving 120 mg of 2-NPH in 20 mL of ethanol and mixing the resulting solution with 20 mL of 0.1 M HCl; Solution B was prepared by dissolving 960 mg of 1-EDC·HCl in 20 mL of 95% ethanol and mixing the resulting solution with 20 mL of 3% pyridine in 95% ethanol; and Solution C was prepared by dissolving 390 mg of KOH in 100 mL of 80% methanol. Solutions were kept at 4°C at all times and used within 3 days.

The derivatisation reaction was carried out in screw-cap 2- mL glass vials by adding 200 µL of Solution A and 200 µL of Solution B to the sample. In the case of NA1, the sample corresponded to the 50 µL of water resulting from the MLLE, whereas in the case of NA3 the sample corresponded to 100 µL of the supernatant resulting from centrifuging culture medium to remove biomass. Subsequently, the vial was sealed, vortexed, and heated for 20 minutes at 65°C in an oven. The vial was then removed from heat, and 100 µL of Solution C were added. Then, the reaction mixture was heated for another 15 minutes at 65°C. The derivatised samples were vortexed once more, let to cool down, and analysed as described below.

4.2.8 Quantification of model NAs by DAD-HPLC

The depletion of NAs in M9 was calculated by measuring the residual NAs remaining in the medium after bacterial growth. Quantification was carried out by DAD-HPLC using an Agilent 1100 HPLC instrument equipped with a Luna C8 column (150 x 2 mm, 5 µm, Phenomenex) following the method reported by Clemente *et al.*, 2003[315]. The mobile phases consisted of 28 µM H_3PO_4 in deionised water (A) and methanol containing 90 µL of 0.185 M H_3PO_4 L^{-1} (B). The flow was 1.5 mL min^{-1}. The gradient elution used was 70% B 0 - 2 minutes, ramped to 100% B by minute 3 and held until minute 6, returning to 70% B by minute 7 and held for 1 minute, for a total run time of 8 min. A new calibration curve was performed every time the solutions for the derivatisation reaction were renewed. Recovery for both NAs was >90%.

Monitoring of NA1 (retention time: 2.4 min ± 0.01) and NA3 (retention time: 4.6 min ± 0.01) was carried out at 210 nm and 400 nm, respectively. Validation information for quantification methods is provided in Table 19. A number of culture positive controls (bacteria + M9 + glucose) were randomly selected and derivatised to confirm the absence of peaks in the retention times mentioned above.

4.2.9 Statistical analyses

Two-way analysis of variance (ANOVA) and the Tukey's multiple comparison test were used to determine significant differences within and between treatments using Prism version 7.03 (Graphpad software, San Diego, CA).

Table 19. Validation parameters for the quantification methods used for model NAs

Parameter	Model Compound	
	NA1	**NA3**
Wavelength of detection	210 nm	400 nm
Quantification range	5 – 400 mg/L	3 – 75 mg/L
LoD*	2 mg/L	0.5 mg/L
LoQ**	6 mg/L	2 mg/L
% RSD of retention time	< 1.0	< 1.0

* LoD = (SD_{blank}) * (3.14); n=7

** LoQ = LoD * 3.14

4.3 Results and Discussion

4.3.1 Screening: Method selection

Emerging wastewater treatment technologies targeting NAs have only had limited success because recalcitrant compounds remain[153]. The current trend is towards combinatorial techniques that improve the efficiency of the process, mostly employing biological treatment in combination with other techniques because of its low costs[30,153] but the use of an appropriate set of microorganisms is the core of treatment efficiency. Therefore, the identification/selection of a biocatalyst (*i.e.* microorganism, consortium, enzyme) suitable for degradation of NAs is the first step towards exploiting microbial metabolism for the clean-up of RWW. For this purpose, we selected *P. putida* because of its metabolic flexibility and a well-known arsenal of degradation capabilities for recalcitrant organics[25,27,28,316]. It is considered a model catalytic microorganism[26] and has been reported to degrade surrogate[306,311] and commercial NAs[305]. Different subspecies of *P. putida* vary in their genetic repertoire and phenotypic behaviour because of its tendency to acquire and express plasmids from other bacteria due to a relaxed gene expression system[28], leading to metabolic heterogeneity within the same species[25]. Therefore, a pool of 86 different strains of *P. putida* presented a significant potential for NA-degrading capabilities. More importantly, *P. putida* has great potential for industrial application because it grows rapidly, has low nutritional requirements, is non-pathogenic, and is easy to handle in laboratory settings[25,28].

However, it is necessary to consider that NAs co-occur with other petrochemicals in refining effluents and that any biocatalyst to be used for the biotransformation of NAs should either degrade such background chemicals or be active in their presence. For this reason, four additional categories of petrochemicals (phenols, aromatic hydrocarbons, aliphatic

hydrocarbons, and methylated naphthalenes; see Table 18) were used during the screening stage, as the evident differences in structural characteristics among all 5 types of petrochemicals were expected to involve different catabolic pathways. The inclusion of these additional chemicals, however, had practical implications for testing, as their structural diversity resulted in a wide range of physicochemical properties. Compounds with low water solubility, for example, can adsorb on the surface of the test vessel or distribute unevenly on a solid microbiological medium. As provided in Table 20, the partition coefficient and water solubility of the model compounds used during screening vary significantly, with phenol and dotriacontane as extremes in the spectrum. This represented a challenge for the screening process, as the method to be used had to force the chemicals and the cells into the same phase, allow safe handling of the hazardous chemicals, and avoid microbial contamination. The results obtained for the three methods evaluated are described below.

Table 20. $LogK_{ow}$ and predicted water solubility of model compounds

Model compound	$LogK_{ow}$ [a]	Water solubility (mg/L)[b]
NA1	2.27	870.30
NA2	4.71	1.52
Phenol	1.48	2.60×10^{4}
o-cresol	1.94	9066.00
Benzo(a)pyrene	6.40	0.17
Toluene	2.68	573.10
Pristane	10.11	5.30×10^{-5}
Dotriacontane	17.76	8.29×10^{-12}
Phenanthrene	4.68	0.68
1,4,6,7-tetramethylnaphthalene	5.29	1.39

[a] Chemspider, predicted data generated using the ACD/Labs Percepta Platform - PhysChem Module

[b] Chemspider, predicted data generated using the US Environmental Protection Agency's EPISuite™

4.3.1.1 Agar diffusion method

No inhibition of bacterial growth was achieved after exposing cells to amounts of chemicals ranging from 5 µg to 10000 µg per disc. Consequently, two additional agar diffusion tests were performed to determine whether chemicals were actually diffusing through the agar. As water solubility varied among test chemicals and this characteristic could have a significant impact

on diffusion, the first test was performed using dyes of different polarities (phenol red > crystal violet > sudan red) aiming to facilitate the observation of diffusion. A total of 10, 100 and 1000 µg of each dye were used to impregnate the discs. The second test evaluated the direct exposure of bacteria to toluene by placing a 50-µL drop directly on the agar surface versus the indirect exposure to the same volume of toluene but in a paper disc. Likewise, a cold pre-incubation period to enhance diffusion was evaluated (4°C, overnight). Incubation was carried out at 30°C for 24 hours.

As shown in Figure 29, no diffusion was observed with sudan red, a lipophilic dye, regardless of the amount placed in the disc. On the contrary, diffusion observed for phenol red is wide and started quickly, indicating that diffusion is directly related to the polarity/water solubility of the test chemical. Therefore, the comparison of zones of inhibition for different classes of compounds would give the impression of weak activity from non-polar compounds, demonstrating the unsuitability of this technique for the screening stage. The cold pre-incubation time enhanced diffusion slightly for phenol red and crystal violet but did not improve diffusion for sudan red. As for the second test, growth inhibition was achieved with direct exposure only, demonstrating the lack of diffusion of toluene through the agar and confirming that the agar diffusion method was not suitable for the detection of strains with tolerance/degradation capabilities. These results are in agreement with previous research that recommend not to use this technique for chemicals whose water solubility is low or unknown[312,317–319].

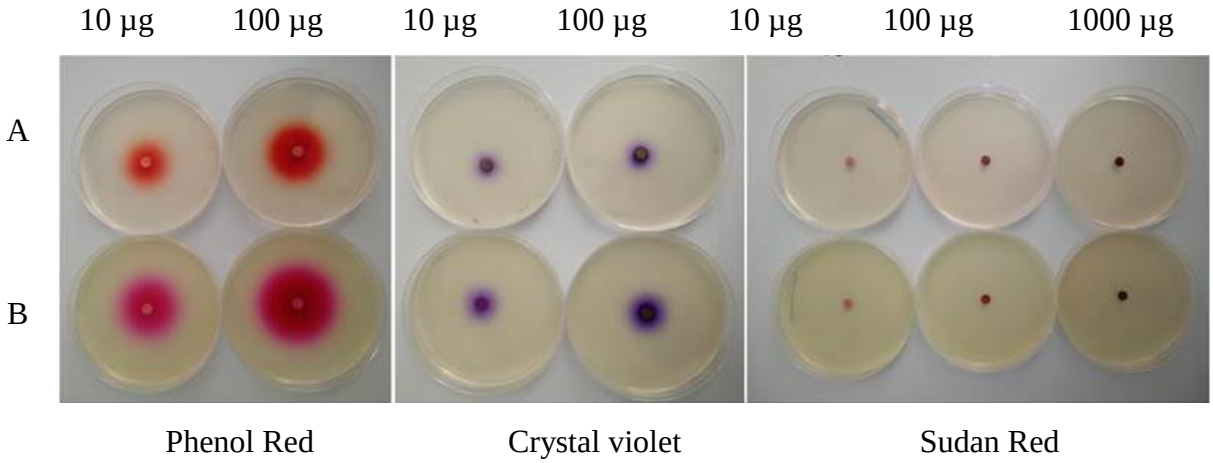


Figure 29. Diffusion of phenol red, crystal violet, and Sudan red in agar after 24 hours of incubation at 30°C with (A) no cold pre-incubation and (B) cold pre-incubation

4.3.1.2 Liquid microcultures

Experiments in liquid medium (M9 with no carbon source other than the test chemical) showed a fast and uneven evaporation rate along the plate, which affected the concentrations tested. Moreover, no detectable changes in the colour of TTC were observed, indicating the absence of bacterial growth and biodegradation. Still, streakings on nutrient agar performed every 48 hours showed viability in most of the concentrations. This finding is consistent with previous reports of survival of *P. putida* in an active non-dormant state for periods of up to one month in conditions of nutrients depletion that are followed by metabolic reactivation in suitable conditions[320]. It is possible that the adsorption of hydrophobic chemicals to the surface of the wells might have reduced the concentration available for biotransformation, hence reducing the likelihood of growth.

4.3.1.3 Overlayer technique

As opposed to the agar diffusion test and liquid microcultures, the overlayer technique allowed the semi-quantitative detection of bacterial growth resulting from the utilisation of the test chemicals as a carbon source, and was therefore used for the screening of biodegradation potential in the pool of 86 strains. Bacterial colonies embedded in the semi-solid agar containing the recalcitrant compounds were observed directly under a microscope, as provided in Figure 30. Positive and negative controls showed presence and absence of growth, respectively, and the reference strain consistently showed degradation of phenol, demonstrating the repeatability of the technique. The distribution of water-soluble compounds in the medium was even, whereas hydrophobic chemicals distributed in a irregular resulting from the formation of crystals or hydrophobic drops (Figure 31). Still, bacterial growth was observed even in the most hydrophobic compound tested, dotriacontane, in the area near the well sidewalls, where the amount of chemical seemed to be lower by visual inspection. Overall, results demonstrated that this method was suitable to screen bacterial strains for biodegradation potential of chemicals with different water solubility.

4.3.2 Screening: Strain selection

All 86 strains were exposed to the 10 model compounds in 6-well plates as shown in Figure 33, and the tests were distributed in 9 separate batches. Cell viability was confirmed with growth in glucose-supplemented positive controls, and the absence of unexpected carbon sources in the medium was confirmed with the absence of growth in negative controls, which were not supplemented with any potential carbon source. A reference strain was tested in

duplicate in each batch to determine the repeatability of results within and between batches, showing a consistent degradation of phenol and growth every time.

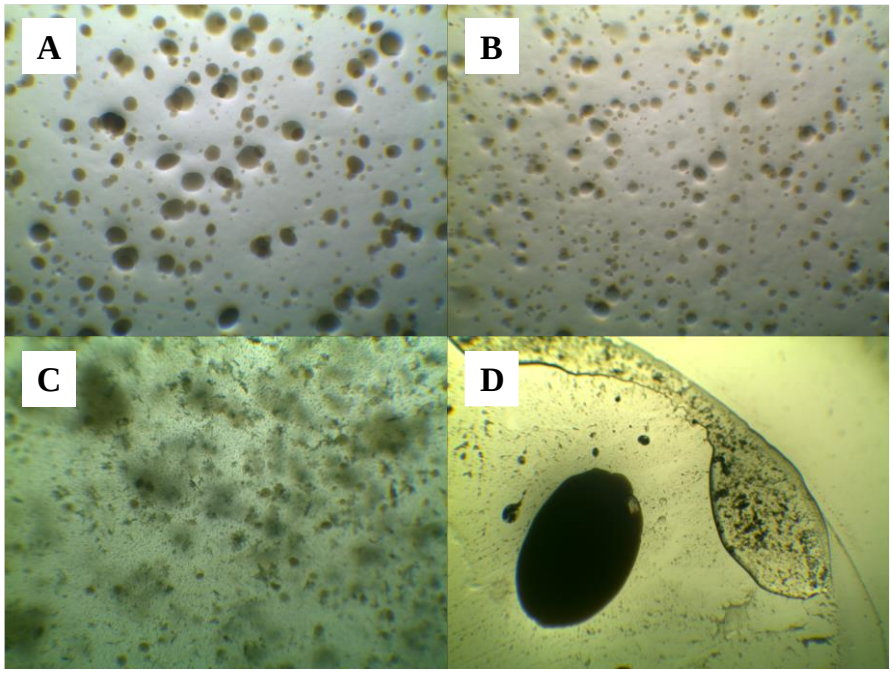


Figure 30. Microscopic view of bacterial growth resulting from the degradation of various test chemicals as carbon source, using a PL4/0.10 1X objective, in (A) glucose, (B) phenol (C) benzo(*a*)pyrene (D) Toluene

As presented in Figure 32, the pool of bacteria screened for their ability to degrade NAs and other petrochemicals did not contain degraders for *o*-cresol and phenanthrene. Likewise, a very low frequency of degraders for the methylated naphthalene, dotriacontane, pristane, and toluene was observed, with only one strain showing metabolic capabilities for the degradation of each. Only 3 strains (JW4, JW23, JW29) utilised both NAs as an energy source, suggesting that the catabolic genes for the degradation of these types of compounds are not widespread in the pool of strains tested or the expression of such genes under these conditions was achieved only in these 3 strains. A total of 10 strains utilised NA1 as carbon source compared to only 3 strains that degraded NA2, which could be associated to the higher mass transfer rate of NA1 stemming from a K_{ow} of 2.27 in contrast to that of NA2 (4.71). It is known that biotransformation depends on the rate of uptake and metabolism of a chemical, which is intrinsic to the cell, but also on the rate of mass transfer of the chemical to the cell, which is influenced by the hydrophobicity and water solubility of the chemical[321,322]. Overall, this is reflected in lower or insignificant degradation rates of hydrophobic compounds compared to those of hydrophilic compounds because such chemicals are not readily bioavailable for

microbial conversion[308]. Moreover, our findings are in agreement with those of Del Rio *et al.*, 2003, where bicyclic NAs proved to be more challenging to degrade compared to monocyclic NAs[305].

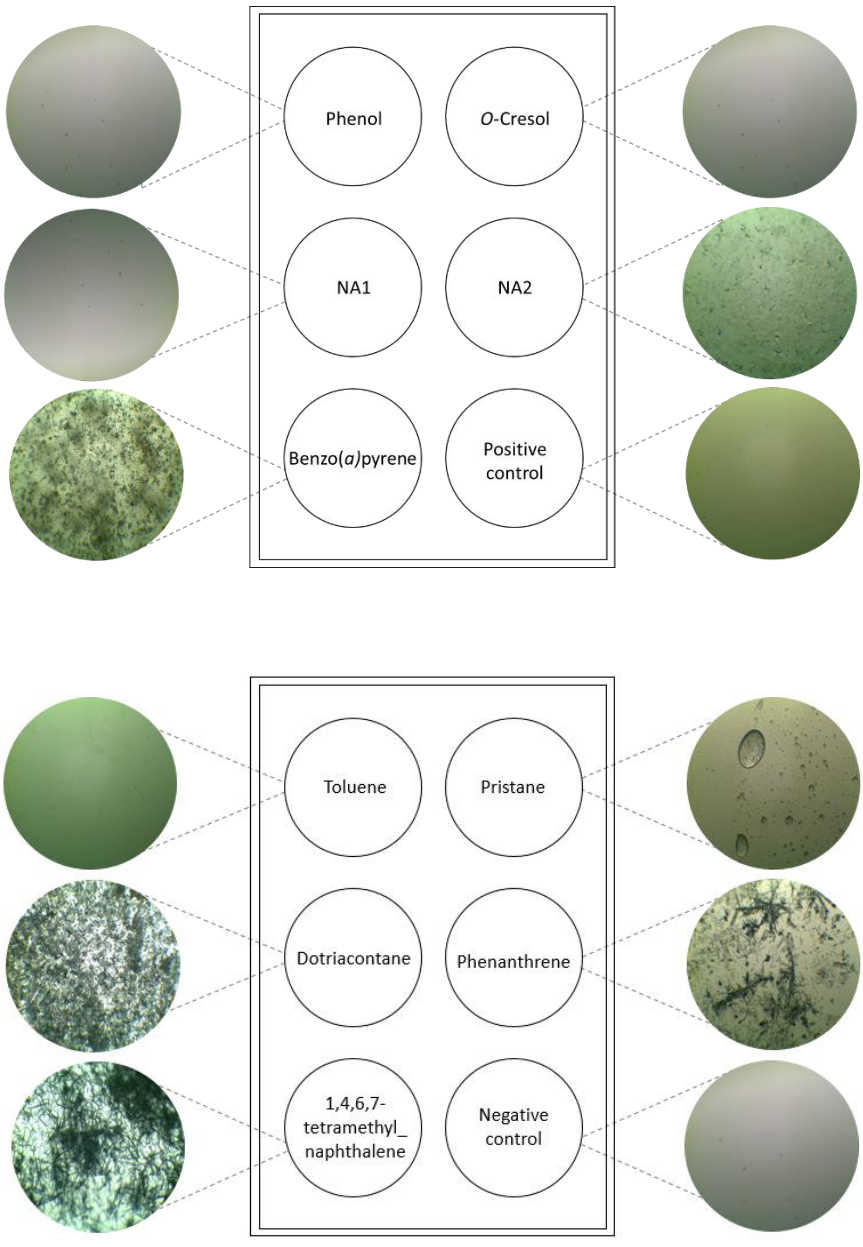


Figure 31. Distribution of model chemicals in 6-well plates for the screening stage and microscopic view of the test chemicals in the overlayer technique using a PL4/0.10 1X objective, showing that water-soluble compounds distribute evenly throughout the semi-solid medium, whereas some hydrophobic chemicals, such as pristine and dotriacontane, distribute irregularly in the form of crystals or drops

An interesting result to emerge from the data is that catabolic genes for the degradation of phenol are present and expressed in a considerable portion (50%) of the strains tested, including the 3 strains utilizing NAs. This is a desirable characteristic for bacteria to be used for treatment of RWW due to the high content of phenols usually co-occurring with NAs. However, no strains utilised *o*-cresol regardless of its structural resemblance to phenol and the fact that similar enzymatic systems are known to be involved in the hydroxylation of both compounds during biodegradation under aerobic conditions. For instance, the plasmid pVI150 is known to encode a complete pathway for the catabolism of phenols and methylated phenols, with all catabolic genes clustered on the single operon *dmp*[294]. Moreover, both compounds are considered biodegradable[323], and their high water solubility (K_{ow} 1.48 and 1.94) does not limit their mass transfer to cells. This finding may be explained by the fact that numerous pathways exist in *Pseudomonas* spp. for the degradation of phenol and methylated phenols with different patterns of substitution[323,324], so strains capable of degrading phenol and cresols can do so through different catabolic pathways[325]. This finding indicates that the degradation of phenol by a strain does not necessarily imply the metabolic capability to degrade other phenols, including cresols, regardless of their structural similarity.

As for the other petrochemicals, the low frequency of degraders is somewhat surprising because *P. putida* shows a naturally high capacity to tolerate and modify aliphatic and aromatic compounds[283], and in general to exploit a wide range of toxic organics[25]. The nutritional flexibility of *P. putida* puts it at the centre of the recycling of organic compounds in aerobic compartments in the environment, and its fast growth under laboratory conditions with unusual and/or toxic carbon sources is well known[28]. A possible explanation for this result may be that some of these chemicals have particularly high K_{ow}, such as dotriacontane and pristane with K_{ow} values of 17.76 and 10.11, respectively, indicating that they are highly hydrophobic and therefore their bioavailability is limited. Similarly, the methylated naphthalene, benzo(*a*)pyrene, and phenanthrene have relatively high K_{ow} ranging between 4.5 and 6.5, which could have impacted their mass rate transfer to the cells, which in turn decreases their bioavailability.

Further research is recommended on strains JW1, JW12, JW13, and JW72, as these were capable of degrading phenol, benzo(*a*)pyrene, toluene, pristane, dotriacontane, and 1,4,6,7-tetramethylnaphthalene, covering 4 out of 5 categories of petrochemicals. However, as the aim of the screening was to select strains with metabolic capabilities to degrade NAs, only the strains JW4, JW23, JW29 were selected for further biotransformation experiments.

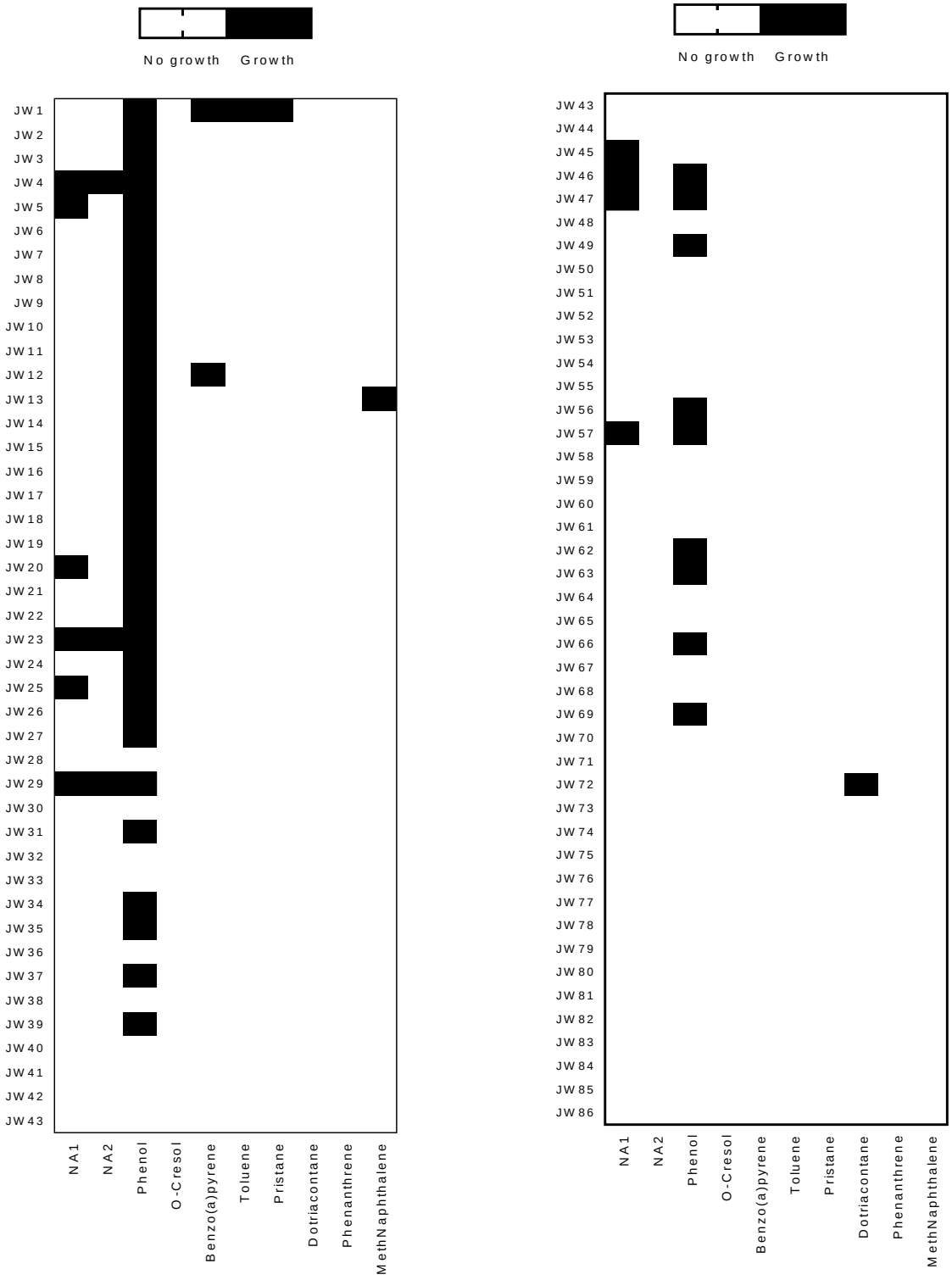


Figure 32. Heat map of the screening of *P. putida* for biodegradation potential of the model compounds showing that only three strains (JW4, JW23, JW29) degraded both model NAs

Furthermore, we attempted to identify candidate catabolic genes in the pool of 86 fully-sequenced strains by comparative genomics. Previous studies profiling metabolic and regulatory features in *P. putida* have used transcriptomics, proteomics, metabolomics, and fluxomics to identify critical cellular responses in the presence of chemicals that can be both potential nutrients and toxicants[25,316,326]; this information is highly relevant for strain improvement and systems biology. However, to our knowledge, no reports so far focus on the genes involved in the biotransformation of NAs by *P. putida*, even though this species is considered a paradigm of metabolically versatile microorganisms[28]. Unfortunately, the low frequency of degraders during the screening stage limited the bioinformatics analysis performed by the Centre for Systems and Synthetic Biology, resulting in an unsuccessful identification of candidate genes.

4.3.3 Biodegradation of model NAs

The three strains capable of growing on NAs (JW4, JW23, JW29) during the screening stage were combined in a consortium for the biodegradation experiments because mixed bacterial populations have been shown to degrade recalcitrant NAs that single strains cannot, suggesting that consortia are more suitable for bioremediation of NAs[327]. This is because microorganisms in natural environments function within large and highly diverse communities where degradation processes result from the interaction of multiple enzymes excreted by different microorganisms. Natural enzymes are highly specific, meaning that each enzyme plays one or a few unique roles in the breakdown of chemicals, and works optimally for one or a few substrates only. Therefore, the combination of degraders into a single consortium can enhance their bioremediation potential by combining their enzymatic capabilities.

The efficiency of the *P. putida* consortium for the degradation of model NAs was evaluated using DAD-HPLC to quantify the residual NAs remaining in the medium after the 30-day incubation period. Raw data is provided in Appendix IV and calibration curves for calculations in Appendix II**.** In all cases, positive controls confirmed the viability of inocula when grown in glucose-supplemented M9, and negative controls confirmed the absence of unexpected energy sources in the medium when no carbon sources were added. For batches 1, 2, and 3 (Figure 26), Tukey's multiple comparisons test showed no statistical significances within controls (A and B) over time, and between controls A and B, indicating that there is no abiotic loss of NAs by evaporation, adsorption to culture flasks, or adsorption to biomass. In contrast, significant differences were found within controls over time in batch 4 (NA3 70 mg/L), which seemed to

be the result of variations during quantification rather than to abiotic loss, as the variations were random instead of a trend showing a decrease in concentration over time (Figure 33). These variations stemming from quantification were also evident in the comparison between treatments; in batch 1 (NA1 at 100 mg/L), for example, the pre-exposure treatment showed statistically significant differences with respect to control A ($p=0.0009$), control B ($p=<0.0001$), and the no pre-exposure group ($p=0.0001$) from day 27, but data showed that the concentration at T_{30} was higher than that at T_0. These variations are likely to be related to the sample preparation procedure using extraction and derivatisation of NAs for their chromatographic analysis (see Figure 28) by increasing the sources of experimental errors. However, these steps were necessary because of the presence of salts in the matrix that impeded the direct detection of NAs in M9. After trying different extraction methods (data not shown), we opted for miniaturising the traditional method for extraction of NAs from aqueous matrixes and developed a liquid-liquid microextraction with DCM, which provided good analyte recovery (90%) and led to lower consumption of chlorinated solvents and reduced amounts of waste.

As provided in Figure 33, our consortium did not degrade NA1 in any of the batches regardless of the acclimatisation treatment. It is known that alkyl substitutions on the cycloalkane ring, such as the methyl group in *para* position with respect to the carboxyl group in NA1, confer certain resistance to biotransformation[306,309,328,329]. However, the fact that these strains showed growth on NA1 during the screening stage suggests that the catabolic genes necessary to utilise NA1 as carbon source were present and expressed under the test conditions. A possible explanation for the lack of biodegradation might be that the concentrations tested (100 and 350 mg/L) were too high and reached the threshold for inhibitory effects on microbial growth. Previous research by Headley *et al.*, 2002[330] used NA1 as a model compound to supplement river samples at 9 mg/L for biodegradation by heterotrophic populations, which successfully utilised NA1 as carbon source within approximately 20 days, but no percentage of degradation is reported. Still, the authors found that the *trans*-isomer degraded more rapidly than the *cis*-isomer, with half-lives of 4.8 ± 0.9 days and 24 ± 0.2 days, respectively, at 30°C. Another study by Paslawski *et al.*, 2009[331] used NA1 as a model compound and tested concentrations ranging from 50 to 800 mg/L, indicating that 750 mg/L was the maximum biodegradable concentration. This study used a microbial consortium (*Pseudomonas putida* + *Pseudomonas aeruginosa* + *Variovorax paradoxus*) that was isolated using the enrichment technique, which automatically selected strains that could use a commercial mixture of NAs as substrate. Also, the consortium

was grown in medium containing 100 mg/L of NA1 for months prior the biodegradation experiments, which means that a long-term acclimatisation period was conducted that led to shorter degradation times and increased resistance/metabolism of high concentrations of the compound. The authors, however, used only the *trans*-isomer to conduct the biodegradation experiments, which does not account for the isomeric mixture in environmental samples. In contrast, an isomeric mixture of NA1 was used for our biodegradation experiments. Overall, results suggest that further studies with our consortium could use a more extended acclimatisation period to increase its tolerance to the toxicity of NA1[296,297].

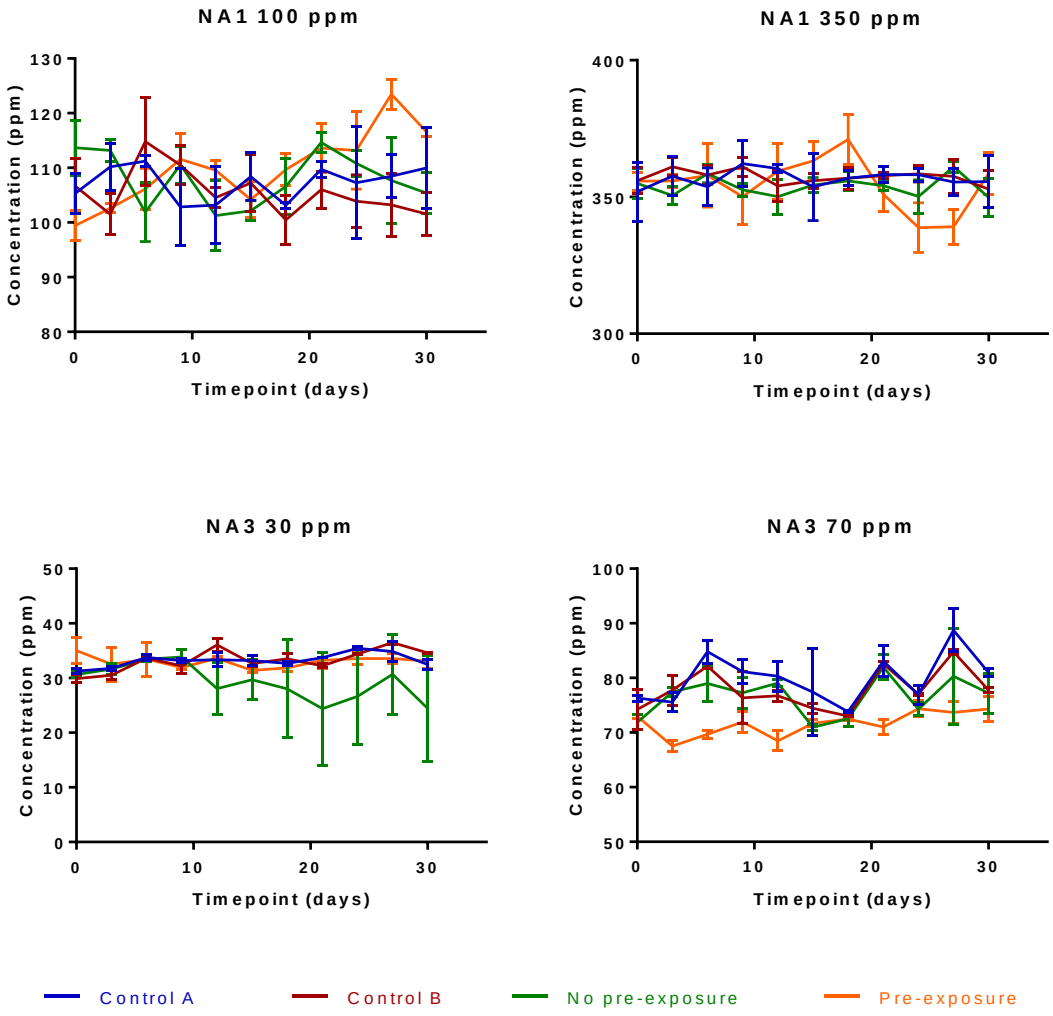


Figure 33. Concentration of NAs (mg/L) over time (days) during biodegradation experiments with the *P. putida* consortium exposed to NA1 at 100 and 350 mg/L, and to NA3 at 30 and 70 mg/L. Control A = Abiotic loss; Control B = Biomass adsorption. Degradation was observed in cultures with NA3 at 30 mg/L

As for NA3, Figure 33 shows a decrease in concentration in batch 3 (30 mg/L) in the cultures with no pre-exposure, with a final degradation of 20%. The final concentration was found to be statistically significantly different with respect to controls A and B, and the pre-exposure cultures at T_{21} (p=0.0057, p=0.0278, p=0.0097, respectively), T_{24} (p=0.0057, p=0.0278, p=0.0097, respectively), T_{27} (p=0.0057, p=0.0278, p=0.0097, respectively), and T_{30} (p=0.0057, p=0.0278, p=0.0097, respectively), indicating that degradation started after 21 days of incubation. Interestingly, the acclimatised cultures showed no degradation of the compound, which might be related to the lower cell density observed at T_0 for pre-exposed cultures, as these inocula underwent chemical stress during the acclimatisation steps and cell density decreased with increasing concentrations of NAs (data not shown). No degradation of NA3 was observed in batch 4 (70 mg/L) possibly because the concentration was too high and inhibited microbial growth.

Previous studies using NA3 as model NA used concentrations ranging from 2 to 4 mg/L and observed only a slight degradation (no percentage reported)[306] or no degradation at all[310] over 49 days. The authors suggested a possible steric hindrance for degradation stemming from the branched side chain, as NAs with branched alkyl side chains (such as NA3) have shown even more resistance to biodegradation than non-branched alkylated NAs (such as NA1)[306]. This suggests that NA3 is especially challenging to biodegrade, yet it was successfully utilised as a carbon source by our consortium within 30 days at a high concentration, resulting in a 20% degradation. Despite the promising results with NA3, it could be argued that the variability was particularly high, but processes mediated by microorganisms are typically highly variable[283]. The observed variability is likely to be related to phenotypic bifurcations resulting from alternative traits that can be expressed in genetically identical populations when exposed to environmental stress, as cells have the capacity to generate variable offspring in an attempt to facilitate adaptation and increase chances of survival[26,332]. These are not mutants but rather different phenotypes with differences in gene expression within the same population. It is possible that from the phenotypically diverse individuals, only a fraction expressed relevant catabolic genes for the biotransformation of NA3 in each one of the replicates, leading to a significant difference of residual NA. It is still unclear whether degradation of chemical pollutants by bacteria is achieved by expression of catabolic genes at the population level or through a division of metabolic labour by individual cells; this is difficult to determine because standard laboratory methods measure bacteria as populations and not as single cells[333]. However, it seems that the latter option is far more efficient for populations because it allows

accessing nutritional resources that cannot be utilised by isolated cells, whether these are single-species or multi-species populations[26].

It is noteworthy that the concentrations tested in our biodegradation experiments are certainly higher than those found in wastewater treatment plants, thus our experiments are far from reflecting realistic scenarios. However, the analytical range of detection of these model compounds by DAD-HPLC (LoQ NA1 = 6 mg/L; LoQ NA3 = 2 mg/L) was the reason to select these concentrations, so low residual concentrations could be reported with confidence. The use of LC-MS in further studies for detection and quantification purposes could help to evaluate lower concentrations that simulate realistic conditions and to identify degradation by-products, which is highly relevant to determine the efficiency of treatment but cannot be achieved using DAD-HPLC. It is critical to determine whether NAs are degraded slowly but completely mineralised, or whether they are degraded slowly, and by-products accumulate. This is because the breakdown of a chemical can occur as a result of two scenarios: for detoxification of a toxic compound or for use as a source of carbon, nitrogen, or phosphorus[334]. The detoxification of chemicals does not involve their mineralisation but rather the transformation of the parent compound into non-toxic or less toxic by-products, which often requires a single step[334]; for instance, the pesticide paraoxon is not fully biodegraded but efficiently detoxified into diethyl phosphate and *p*-nitrophenol[335]. Mineralisation of toxic chemicals, such as NAs, is desirable but also difficult because this requires a multi-step conversion of the target chemical into intermediates of a standard metabolic pathway[334] so that it can be further metabolised by the same microorganism or by other microorganisms in the proximity.

The likelihood of finding NA-degrading pathways in strains with possibly no previous exposure to NAs is low because these enzymes have never been necessary for their fitness and survival. This might the case of the pool of 86 strains of *P. putida*, which were isolated from various environmental sources unrelated to NAs but had the advantage of being fully sequenced and available for testing. Hence, the isolation of microorganisms from environments chronically exposed to NAs would increase the chances of finding strains capable of degrading these compounds. In fact, this has been the traditional approach used for strain selection in previous studies. For example, indigenous microorganisms from oil sands process-affected waters and soil repeatedly exposed to NAs have played a central role in previous research in

this field[161,258,329,336], as these microorganisms have had a long-term acclimatisation period to complex mixtures of NAs under natural conditions.

Our experiments also differ from realistic conditions in wastewater treatment plants in that NAs occur as complex mixtures rather than as single substances. It is noteworthy that there are significant differences in NA substrates used for degradation research, and the selection of substrates has a strong influence on the scope of a study. The three categories are as follow:

- Surrogate NAs: refer to any individual carboxylic acid that fits the formula $C_nH_{2n+z}O_2$[157]. Fatty acids and cyclic carboxylic acids are frequently used. NA1, NA2, and NA3 belong to this category.
- Commercially available NAs: refer to acids extracted from petroleum fractions, typically gas oils. These are usually a mixture of NAs with 0, 1, and 2 rings, ranging from 7 to 17 carbon atoms; variations occur among suppliers, which include Fluka, Kodak, Pfaltz & Bauer, Merichem, and TCI. Commercial products tend to have lower molecular weight (with just a few compounds above 300 amu), a narrower range of molecular weights, and lower structural complexity than those extracted from wastewater[258,259]. Variations are significant among batches, which also complicates the use of a mixture as reference[272].
- NAs from environmental samples: typically a very complex mixture of NAs with up to 28 carbon atoms, with the majority of compounds within the range of 150 - 350 amu; some studies have also reported NAs of up to 600 amu[259,261,278].

The difference between commercial and environmental mixtures of NAs seems to be related to the extensive biodegradation processes that NAs from environmental sources (mainly RWW and OSPW) have undergone[309]. Consequently, the NAs found in wastewater are those that resisted biodegradation by indigenous microorganisms. This means that the reporting of "NA-degrading microorganisms" can refer to very different scenarios. As expected, commercial and surrogate NAs are more readily degraded that NAs found in wastewater[309], and some commercial mixtures are more easily degraded than others, as their distribution of acids varies. A number of studies have consistently indicated that the Kodak salts and the refined Merichem naphthenic acids are more closely related to NAs found in wastewater, as high-molecular-weight and multi-ring acids are present in high concentrations[258,261,278]. Therefore, the implications of a strain (or consortium) degrading NAs from wastewater are very different to those of a strain degrading surrogate NAs partly because of their application potential in real life. In our case, the reporting of a consortium degrading a recalcitrant surrogate NA suggests

that further work is needed to improve the metabolic capabilities of the strains and to evaluate more complex substrates, such as commercial or environmental NAs. Yet, surrogate and commercial NAs provide practical advantages that make them a suitable option for investigating the microbial degradation of NAs. For instance, the use of surrogate NAs makes method development and quantification easier, as the complexity of environmental mixtures of NAs is reflected in analytical limitations to understand and characterise such mixtures. Equally, commercial and surrogate NAs have helped to understand the microbial degradation of NAs, revealing metabolic pathways and biotic and abiotic factors shown to affect NA biodegradation in the environment.

Thus, it is true that the model NAs used in this study for the selection of NA-degraders are not representative of NA mixtures found in RWW. However, our findings with NA1 and NA3 do give an indication of the metabolic capabilities of the consortium to degrade NAs with structural features known to confer resistance to degradation (*e.g.* alkyl substitution on the cycloalkane ring in NA1; high degree of alkyl branching in NA3), which are the ones that remain after treatment and have the potential to exert biological effects. Results show that our *P. putida* consortium was able to degrade 20% of NA3 at a high concentration (30 mg/L) within 30 days, which has not been achieved by any other study. This finding, while preliminary, suggests that our consortium requires optimisation of culture conditions for better biodegradation rates, yet it holds promise for the advanced treatment of NA-containing effluents. Further work is required to establish the metabolic capabilities of our consortium for degrading NAs with higher degrees of cyclicity, as these have been shown to degrade more slowly[305] than monocyclic or linear NAs or not degrade at all[309]. Moreover, cyclicity (*Z* family) is reported to have a much higher influence on persistence than *n*[309]. Similarly, the exposure of our consortium to NA mixtures, either commercial or environmental, is necessary to determine its full bioremediation potential.

Overall, these results confirm that, despite their relatively simple structure, biotransformation of model NAs is not easily achieved, recreating the scenario observed with NAs in petrochemical effluents, including RWW.

4.4 Conclusions

This chapter screened for biodegradation potential of model NAs in a fully-sequenced pool of 86 strains of *P. putida* and evaluated the selected strains as a consortium for the biodegradation of NAs, assessing the effect of acclimatisation on the biodegradation capabilities. The

screening stage was conducted using the overlayer technique, which proved to be a reliable methodology to test hydrophobic and hydrophilic chemicals in aqueous microbial medium. Findings showed that three strains (JW4, JW23, JW29) could utilise both model NAs as a carbon source, indicating that the corresponding catabolic genes are present in these strains and expressed under the test conditions. Additionally, four strains showed the ability to metabolise petrochemicals known for their toxicity and resistance to degradation, such as dotriacontane and benzo(*a*)pyrene, and further work is recommended to determine their potential for bioremediation. Unfortunately, the low frequency of degraders of NAs and other petrochemicals did not allow the identification of candidate catabolic genes by genomic profiling.

For the biodegradation experiments, method development for the quantification of residual NAs in culture media led to a sample preparation procedure that included liquid-liquid microextraction and derivatisation with 2-NPH, followed by detection and quantification by DAD-HPLC. The strains selected during the screening stage (JW4, JW23, JW29) were combined and used as a consortium because mixed bacterial populations have shown to degrade recalcitrant NAs that are not degraded using single strains[327]. Results revealed that the consortium was unable to metabolise NA1, which we believe might be related to the high concentrations tested in combination with a short acclimatisation period, thus we recommend further studies with the same consortium but at lower concentrations and/or longer pre-exposure times. Likewise, we recommend using LC-MS/MS for further studies to achieve higher sensitivity, as the analytical range of detection of NAs using DAD-HPLC did not allow to simulate realistic concentrations in refining effluents.

As for NA3, the consortium showed a successful degradation of 20% at 30 mg/L without acclimatisation period. This finding suggests that our consortium holds promise for the advanced treatment of NA-containing effluents, as the presence of the *tert*-methyl substitution in NA3 confers resistance to biotransformation and yet our findings exceed those of other studies, which used considerably lower concentrations. Future research is required to establish the metabolic capabilities of our consortium for degrading NAs with higher degrees of cyclicity, as it is reported to have a critical role on persistence[309], and environmental mixtures of NAs. Further research could also be conducted with microorganisms isolated from environmental samples chronically exposed to NAs because this would increase the chances of

obtaining highly efficient NA degraders, as the corresponding catabolic pathways would have been crucial for survival in the environment.

It is important to mention that the completion of this chapter involved numerous difficulties related to the incompatibility between practical aspects of chemistry and microbiology. For example, the method development for the screening stage required the testing of different techniques that could maintain cells and chemicals in the same phase throughout the screening (two weeks), regardless of the water solubility of the chemicals. Traditional approaches such as the agar diffusion test and liquid microcultures failed to achieve so, but the overlayer technique proved to be useful for this purpose, which was confirmed using a phenol-degrading bacterial strain as a control. This technique, however, is time-consuming, requires significant space for incubation (tests were conducted in 6-well plates), and presents a high risk of contamination, as one of the steps is conducted outside the microbiological safety cabinet. Likewise, the procedure requires the preparation of numerous solutions that need to be sterilised separately and uses semi-solid agar that needs to cool down slowly and be used at a specific temperature to maintain the viability of inocula. As the screening tested 86 strains in 10 different chemicals with their corresponding controls, it was necessary to carry out more than 1000 tests to select the strains with biodegradation potential; the workload and duration of the screening stage were therefore excessive, and alternative techniques need to be evaluated for future studies.

Moreover, during the second stage where biodegradation of model NAs was conducted, the addition of supplements (such as vegetable oils and yeast extract) to the culture medium for the induction of natural surfactants was evaluated (data not shown) because some studies have reported positive outcomes with this approach[290,293]. However, the use of these additives hampered the analytical detection of NAs because the matrix and chemical background became extremely complex. Under these circumstances and considering studies reporting good biodegradation results using minimal media, M9 was selected for the biotransformation experiments. However, regardless of the relatively simple composition of this medium in comparison to other microbiological media, the detection and quantification of NAs were still challenging because of the complex nature of the medium as a matrix in an analytical context. Therefore, method development played a crucial role again. It was necessary to extract (in triplicate), derivatise (three-step procedure), and analyse samples via HPLC for the detection and quantification of NA1, whereas derivatisation and analysis were required for NA3. The

number of samples to process (a total of 600 samples) following these multi-step procedures added a significant amount of workload and time to the overall project. Yet, the liquid-liquid microextraction conducted allowed the miniaturisation of the traditional method used for extraction of NAs from aqueous matrixes, leading to smaller culture volumes, lower consumption of chlorinated solvents, and reduced generation of waste.

CHAPTER 5

OXIDATION OF NAPHTHENIC ACIDS AND TOXICITY REDUCTION IN REFINING EFFLUENTS USING Fe-TAML/H_2O_2 SYSTEMS

5.1 Introduction

Three aspects define the sustainability of current production systems: the emission of greenhouse gases, the generation of waste, and the consumption of resources. Overcoming problems related to these three aspects will help to increase resource efficiency and move industrial sectors towards a more sustainable consumption and production, facilitating the transition of current societies into circular economy and sustainable development. However, humanity might struggle in the short term to cut off completely its dependency to one of the most questionable sectors when it comes to sustainability: the petroleum industry. It is an essential provider of the global energy requirements and numerous raw materials, yet it uses production systems that are frequently questioned in relation to the three aspects mentioned above. The refining sector, in particular, consumes large amounts of water and produce vast quantities of wastewater, which, if inadequately treated, causes serious pollution problems that impact aquatic ecosystems and public health. In response, this sector has defined best practices for waste and water management aiming at recovering and recycling wastewater to minimize environmental risk and alleviate the pressure on ecosystems resulting from water withdrawal[337]. However, the implementation of such practices is not compulsory, so local environmental legislation are strong technical drivers with a significant contribution on the way refineries manage their waste and water resources. In other words, refining sites at different locations are likely to treat, discharge, and manage their wastewater differently because environmental regulations vary considerably from one place to another. From the technical point of view, the provision of high-quality effluents suitable for recycling is not easy for the refining sector because of the large volumes of wastewater containing recalcitrant compounds, frequently resulting in low-quality effluents with little recycling potential.

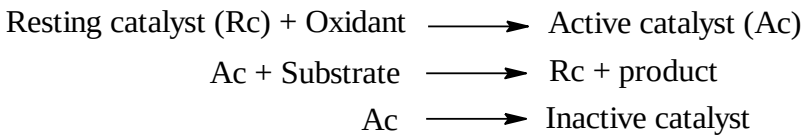
NAs are a great example to illustrate this situation. These chemicals have been reported in high concentrations in process water and have been linked to corrosion of equipment and operation pipelines, leading to outages and repairs[338]. Consequently, water contaminated with NAs cannot be recycled, contributing to high water consumption and operational costs. Furthermore, evidence suggests that the discharge of these acids to waterways pose a significant health risk to humans and wildlife[147,155,156,171,339,340], and therefore treatment technologies are pivotal for the appropriate management of wastewater containing NAs. Emerging technologies for the treatment of NAs include biodegradation, membrane filtration, coagulation/flocculation, advanced oxidation processes, and adsorption[153]. Overall, it is evident that only limited success

has been achieved with the technologies developed over the last decade, as NAs with low molecular weight are often resistant to degradation.

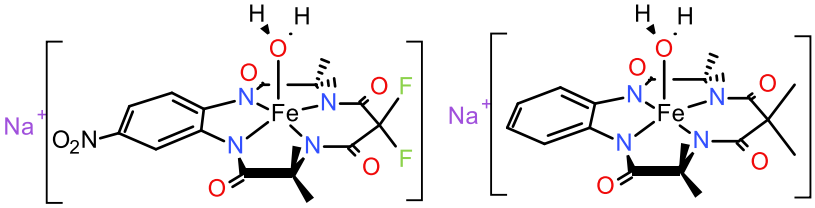
Moreover, the upscaling of many of the emerging technologies developed under laboratory conditions is cost-prohibitive, thus there is an urgent need for developing new technologies targeting NAs that align with the ideal features of treatment technologies, namely low costs, mild conditions, and high efficiency. Biological processes are an excellent example of what is highly desired in treatment technologies, as these are based on highly efficient and energy-saving chemical reactions achieved by the action of enzymes that increase the rate of virtually all the chemical reactions within cells. Consequently, enzyme-based wastewater treatment approaches were developed decades ago with several enzymes that have the potential to reduce environmental pollution by degrading a wide range of organic and inorganic compounds. For instance, peroxidases catalyse the oxidation of a variety of pollutants in the presence of peroxides, such as H_2O_2[341]. The use of filamentous fungi (e.g. *Phanaerochaete chrysosporium*, *Trametes versicolor*) for bioremediation of industrial wastewater derives from their ability to produce a set of powerful extracellular enzymes, including peroxidases, that can degrade highly recalcitrant chemicals, including lignocellulosic materials[342]. However, even though peroxidases exhibit excellent catalytic activity and use a cheap, readily available substrate like H_2O_2, their technological applicability is limited by their oxidative and hydrolytic fragility[343]. The approach over the last few decades has been to develop synthetic enzyme-like molecules that compete functionally with natural enzymes but exhibit greater resistance to oxidative destruction, deriving in catalyst-mediated technologies that are especially attractive for sustainability purposes, as catalysts improve operating times, capital costs, and overall economic and environmental features of treatment technologies[33].

The family of Fe-TAML® (TetraAmido Macrocyclic Ligands) activators, which mimic peroxidase enzymes, has resulted from the approach of developing small-molecule synthetic enzymes. These homogeneous catalysts resulted from iterative ligand design initiated in the 1980s within the frame of green chemistry, achieving practical, efficient, and selective catalysts with great potential in numerous fields of use, especially where the removal of highly recalcitrant organic pollutants is needed[344]. Fe-TAML catalysts can resist high turnover frequency numbers and this provides more resistance to suicidal inactivation than natural peroxidases[345]. Fe-TAML activators start as resting catalysts (Rc) that activate in the presence of an oxidant to form the active catalyst (Ac), which oxidises the corresponding substrates in a peroxidase-like manner and also decomposes peroxide in a catalase-like way[33]. Ac ultimately

undergoes suicidal inactivation along the oxidation process, which limits the overall catalytic rate but is highly desired as it reduces the risk associated with the discharge of TAML catalysts to the environment after water treatment. The catalytic performance of Fe-TAML activators is typically a function of the processes in the general catalytic cycle shown below[345], which means that any structural modification that alters any of the processes below (*e.g.* by increasing oxidative aggression or improving hydrolytic stability) will have an impact on the catalytic performance.



Consequently, the design of TAML activators has produced 5 generations of catalysts that differ on the ligand structure, and within each generation various catalysts exist that differ in the substitution groups of the ligand. As expected, these structural features within and between generations provide differential reactivities for the processes occurring during oxidation[346]. For example, the TAML activators used in this study and provided in Figure 34 belong to the first generation, which means that both present the same basic ligand structure but differ in its substitutions (F, F, H, NO_2 for **1a**; CH_3, CH_3, H, H for **1b**), and therefore their catalytic performance differs. For instance, the substitution of the germinal methyl groups in **1b** for the fluorine atoms in **1a** has shown to provide more hydrolytic stability by protecting from proton-induced demetalation, and the reactivity of **1b** towards H_2O_2 is highest at pH values near 10[40,346]. However, there are practical reasons that make **1b** an attractive catalyst; as it does not contain halogens, it is likely that degradation fragments are readily degraded once discharged into the environment. Likewise, the difluoro group in **1a** is expected to increase the production costs of the catalysts[347].



1a **1b**

Figure 34. Structure of the activated Fe-TAML catalysts used in this study

Previous studies with Fe-TAML/H_2O_2-based treatment processes have reported successful removal of a wide variety of organics in wastewater, including estrogenic compounds[38,39,348], dyes[42–45], dibenzothiophene-derivatives[349], pharmaceuticals[35], molluscicides[40,41], halogenated phenols[36,37], and nitrophenols[350]. More importantly, these studies report successful removal of contaminants with very low concentrations of TAML activators, ranging from nM to µM, which makes them very attractive for upscaling purposes, especially for the refining sector because of the vast amount of wastewater generated on a daily basis. Furthermore, their efficiency in degrading highly recalcitrant compounds like metaldehyde in a few days[40,41] suggests that the catalytic activity of Fe-TAML catalysts might be aggressive enough to oxidise recalcitrant NAs, helping to clean-up and detoxify refining effluents for recycling and discharge purposes. Hence, the purpose of this chapter was to investigate the catalytic performance of Fe-TAML/H_2O_2 oxidation systems for the degradation of NAs. The study was conducted in a tiered process as follows: (i) evaluation of Fe-TAML/H_2O_2 systems for the degradation of NAs using model compounds NA1 and NA3 (Figure 35), (ii) investigation of optimal conditions of Fe-TAML/H_2O_2 using batch and semi-batch processes, (iii) application of optimized Fe-TAML/H_2O_2 conditions to a real sample of refining wastewater sample, and (iv) toxicity testing of Fe-TAML treated effluents using the luminescent bacteria test to assess toxicity reduction.

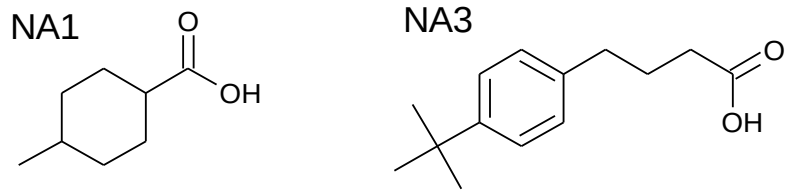


Figure 35. 2D structures of the model naphthenic acids used in this study

5.2 Experimental section

5.2.1 Chemicals

Model NAs (4-methyl-1-cyclohexanecarboxylic acid and 4-(4-*tert*-butylphenyl)butanoic acid; see Figure 35) were purchased from Sigma-Aldrich. HPLC-grade acetonitrile was obtained from Fisher Chemical. Both TAML activators (**1a** and **1b**) were provided by Prof. Terry Collins from the Institute for Green Science, Carnegie Mellon University. H_2O_2 (30% w/w) was purchased from Sigma Aldrich and diluted to 0.3% using Milli-Q® water before use. The H_2O_2 stock solution (30% w/w) was standardised every 48 hours by redox titration with potassium permanganate ($KMnO_4$), which was obtained from Sigma Aldrich. Sodium oxalate ($Na_2C_2O_4$)

for standardisation of $KMnO_4$ was purchased from Fisher Chemical. Unbuffered Milli-Q® water was used as the reaction medium. The pH of reaction mixtures was adjusted by addition of 0.01 M KOH or 0.01 M HCl. Catalase from bovine liver was used for the quenching of H_2O_2 and obtained from Sigma Aldrich. The Internal standard (IS) solution (1-chlorooctadecane and *o*-terphenyl) for extraction purposes was obtained from Restek UK. Phenol and potassium dichromate ($K_2Cr_2O_7$) for toxicity testing were purchased from Sigma Aldrich.

Stock solutions of 17α-ethinylestradiol (EE2) (10 mg/L) and TAML catalysts (40 mg/L) were prepared in water and stored at 4°C. Stock solutions of catalase (500 mg/L) were prepared in water and stored at 4°C for a maximum of one week before disposal.

5.2.2 Fe-TAML/H_2O_2 oxidation of model NAs

Reactions were performed in triplicate in 15-mL glass vials containing 10 mL of the reaction medium and initiated by the addition of one aliquot of H_2O_2 to a solution of a model NA (50 mg/L) and a TAML catalyst in Milli-Q® water. Reactions were conducted at pH 7, 8, and 9. Batch and semi-batch reactions were conducted, the latter of which had additional aliquots of both H_2O_2 and TAML activators every 8 hours. Aqueous solutions of the model NAs and uncatalysed reactions were used as controls; quality control of reagents and Fe-TAML activators was performed by following the procedure described by Mills *et al.* (2015)[348] to degrade EE2 at pH 7 with 20 mg/L of H_2O_2 and 40 ppb of catalyst (**1a** or **1b**). Figure 36 shows the design for each batch containing two controls, two catalysts (**1a** and **1b**), and two treatments (batch and semi-batch) for each model NA at each pH value, for a total of 6 batches and 18 reaction vessels per batch.

Oxidation reactions were carried out for 72 hours, and 500-µL aliquots were sampled every 8 hours in 2-mL glass vials. A total of 50 µL of the catalase solution was transferred to each 2-mL glass vial and left for 5 minutes to quench the H_2O_2 and stop the reaction, and 50 µL of 1M HCl were added after the quenching time to lower the pH and allow detection via HPLC-DAD.

5.2.3 Fe-TAML/H_2O_2 oxidation of NAs in RWW

Reactions were performed for 72 hours in duplicate (due to limitations in the amount of sample) in 500-mL conical flasks containing 200 mL of RWW (pipeline 3) and initiated by the addition of one aliquot of H_2O_2 to samples containing TAML catalyst. MilliQ® water, RWW alone, and an uncatalysed reaction of RWW were used as controls. Oxidation reactions were stopped by transferring 5 mL of a 500-mg/L catalase solution and left for 15 minutes to make sure the

H_2O_2 was fully quenched. A 5-mL aliquot was taken from each flask for toxicity tests, and samples were then extracted for analysis, as described below.

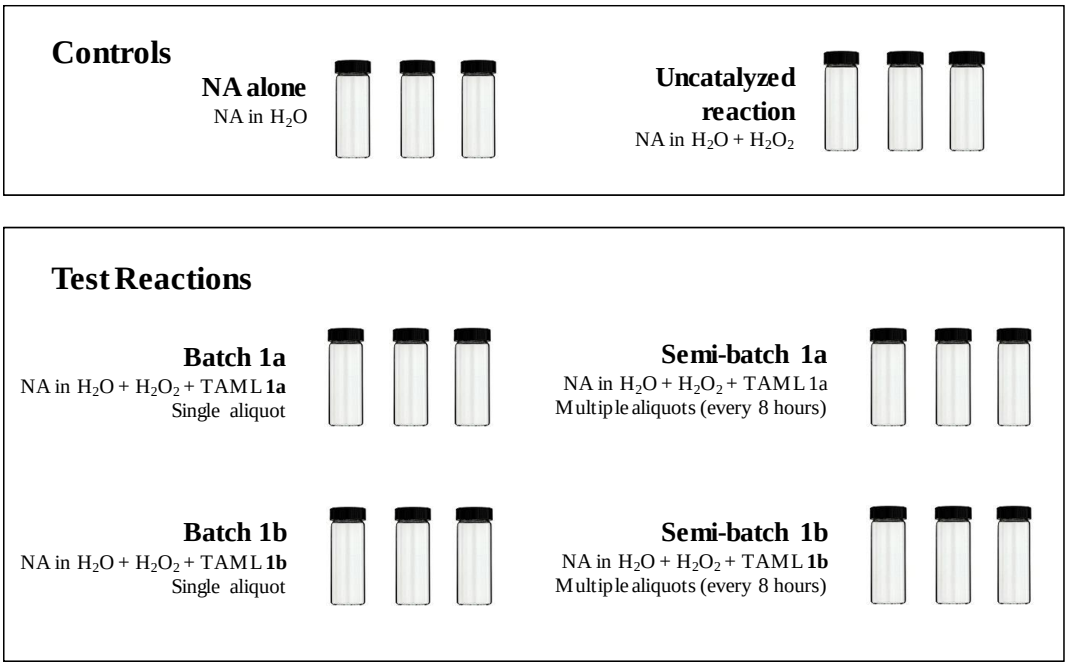


Figure 36. Control and test reactions conducted with Fe-TAML/H_2O_2 systems for the degradation of model NAs in batch and semi-batch processes

5.2.4 Liquid-liquid extraction

An aliquot of the IS solution was added to controls and treated RWW samples to a final concentration of 100 µg/L, and the pH was then adjusted to 2 using 1 M HCl. Samples were then transferred to separatory funnels and extracted three times with 20 mL of DCM, and the resulting organic extracts were reduced to incipient dryness in a TurboVap® LV concentration evaporator workstation. The extracts were then split in half and reconstituted into 100 µL in DCM for GC-MS analysis and 100 µL in methanol for HPLC/HRMS analysis.

5.2.5 Quantification of model NAs by DAD-HPLC

The depletion of model NAs in water was calculated by measuring the residual NAs remaining in the medium after oxidation by H_2O_2 + Fe-TAML catalysts. Quantification was performed using high-performance liquid chromatography (HPLC) coupled to a diode array detector (DAD) in an Agilent 1100 HPLC instrument on a Hyperclone® C8 (150 x 2 mm, 5 µm, Phenomenex). The mobile phases consisted of HPLC-grade water (A) and acetonitrile (ACN) (B), which were pumped at 0.7 mL min^{-1}. The gradient elution used was 1% B 0 - 1 min,

ramped to 100% B by 2 min and held until 3 min, returning to 1% B by 4 min and held for 2 minutes, for a total run of 6 minutes. Monitoring of NA1 was carried out at 210 nm (retention time: 3.95 min ± 0.01), and that of NA3 at 254 nm (retention time: 4.11 min ± 0.01) using the calibration curves provided in Appendix II. Calibration solutions were prepared in Milli-Q® water.

5.2.6 GC-MS

GC-MS analysis of LLE extracts was performed to monitor NAs present in RWW samples after treatment with Fe-TAML/H_2O_2 systems. Analyses were conducted using a Perkin Elmer Clarus® 500 instrument equipped with a DB-5 capillary column (30 m x 0.25 mm I.D) coated with 0.25 µm film of 5% phenyl polysilphenylene siloxane. High purity helium was used as carrier gas with flow-rate at 1.0 ml/min. The inlet was held at 250°C, and the injection volume was 1 µL. The column was held at 35°C for 4 minutes, ramped at 8 °C/min to 310°C, and held for 10 minutes, for a total run time of 48 minutes. The mass spectrometer was operated in electron ionisation (EI) mode with ionisation energy of 70 eV, and the scan range was 50 to 600 amu. The instrument was mass calibrated using perfluorotributyl-amine (PFTBA) using ions m/z 69, 131, 219 and 502. The filament emission current was set at 0.06 pA.

5.2.7 HPLC/HRMS

LLE extracts from controls and treated RWW samples were analysed using high-resolution mass spectrometry, which was carried out using a Thermo Accela LC pump and a CTC autosampler interfaced directly to a Thermo Exactive® mass spectrometer. Chromatographic separation was conducted using a Varian Pursuit XRs C18 (100 x 3.0 mm, 3 µm, 100 Å) column. The mobile phase consisted of 0.1% NH4OH in HPLC water (A) and 0.1% NH4OH in methanol (B), which was pumped at a flow rate of 600 µL min^{-1}. The gradient elution used was 10% B from 0 min to 2 min, ramped to 70% B by 2.5 min and held until 6 min, ramped to 100% B by 6.5 min and held until 7.5 min, returning to 10% B by 9 min and held for 3 minutes, for a total run of 12 minutes. Detection was performed in negative ion mode with a scan range of 80 – 500 m/z, and the following settings were applied for the heated-electrospray ionisation (H-ESI) source: sheath gas flow rate 50 units; spray voltage 4000 V; capillary temperature 350 °C; capillary voltage 55 V; tube lens voltage 105 V; skimmer voltage 26 V; heater temperature 300 °C.

5.2.8 Toxicity evaluation

Toxicity of aqueous RWW after treatment with Fe-TAML/H_2O_2 was measured using a modified version of the luminescent bacteria test (LBT) described in BS EN ISO 11348-3:2008 "Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria) – Part 3: Method using freeze-dried bacteria". The procedure was adapted to 96-well plates. Samples were analysed in duplicate using phenol (400 mg/L in saline solution) as reference substance (expected EC_{50} = 13 – 26 mg/L), saline solution (20% NaCl solution) as the negative control, and Cr(VI) (105.8 mg/L of $K_2Cr_2O_7$ in saline solution) as the positive control. Standard transparent microplates were used for the preparation of sample dilutions, and white opaque microplates were used for the measurement of luminescence output. A Promega GloMaxTM luminometer was used for light readings, and incubation during contact time was performed in an Aqualytic thermostatic cabinet at 25°C ±0.3. All samples were analysed in dilution series to determine EC_{50} values, which were determined using a linear regression analysis of the logarithm of the gamma value (ratio of light lost to the amount of light remaining at time *t*) and the logarithm of the concentration of the sample, considering only inhibition values between 10% and 90%. Calculations were carried out in accordance to BS EN ISO 11348-3:2008.

5.2.9 Statistical analyses

Two-way analysis of variance (ANOVA) and the Tukey's multiple comparison test were used to determine significant differences within and between treatments using Prism version 7.03 (Graphpad software, San Diego, CA).

5.3 Results and discussion

5.3.1 Method development

Preliminary oxidation reactions of model NAs were conducted with Fe-TAML catalysts **1a** and **1b** ranging from 40 to 100 ppb and H_2O_2 ranging from 20 to 100 mg/L, at a concentration of 50 mg/L of model NAs. Decomposition of NAs was observed at 100 ppb of Fe-TAML activators in combination with 40 mg/L of H_2O_2, and these conditions were used for the degradation reactions conducted subsequently. No decomposition was observed with Fe-TAML concentrations below 100 ppb. The activity of aqueous solutions of **1a** and **1b** was confirmed before conducting degradation experiments because when TAML activators are dissolved in water, several decomposition pathways can occur and result in suicide inactivation [345]. Confirmation of catalytic activity was conducted by degrading EE2 under the conditions

described by Mills *et al.* (2015)[348], which showed the higher oxidation rate of EE2 of **1a** (Figure 37).

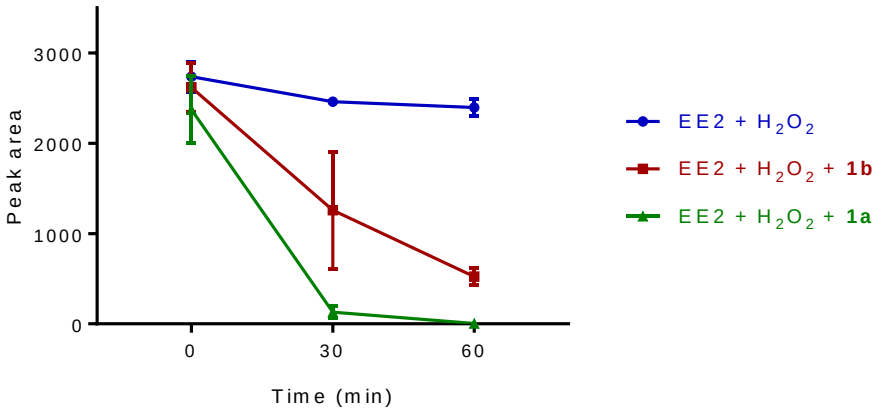


Figure 37. Degradation of EE2 by Fe-TAML/H_2O_2 used to confirm the activity of aqueous solutions of 1a and 1b before oxidation experiments

5.3.2 Performance of 1a and 1b for the oxidation of model NAs

The overall performance of both catalysts is provided in Figure 38; raw data are set out in Appendix IV and calibration curves for calculations in Apendix II. Performance of **1a** and **1b** was comparable in batch experiments, even thought **1a** was expected to have superior catalytic activity due to higher hydrolytic stability stemming from the inhibition of proton-induced demetalation by fluoride atoms, and faster rate near neutral pH[346,351]. The final concentrations were not significantly different at any pH when treated with **1a** or **1b** for NA1 ($\alpha = 0.05$; pH 7 $p = 0.9618$; pH 8 $p = 0.9315$; pH 9 $p = 0.9999$) or NA3 ($\alpha = 0.05$; pH 7 $p = 0.9895$; pH 8 $p = >0.9999$; pH 9 $p = >0.9999$), although the catalysed batch reactions for NA1 presented final concentrations that significantly differed with respect to controls at all pH values ($p = <0.0001 – 0.0004$). On the contrary, catalysed reactions for NA3 did not show statistically significant differences with respect to controls at any pH value ($p = 0.9996$ - >0.9999), which indicates that NA3 is more resistant to oxidation than NA1. This is also suggested by the fact that NA1 was slightly degraded in the uncatalysed reactions, as observed in Figure 38, although the differences with respect to NA1 alone were not statistically significant ($p = >0.9999$).

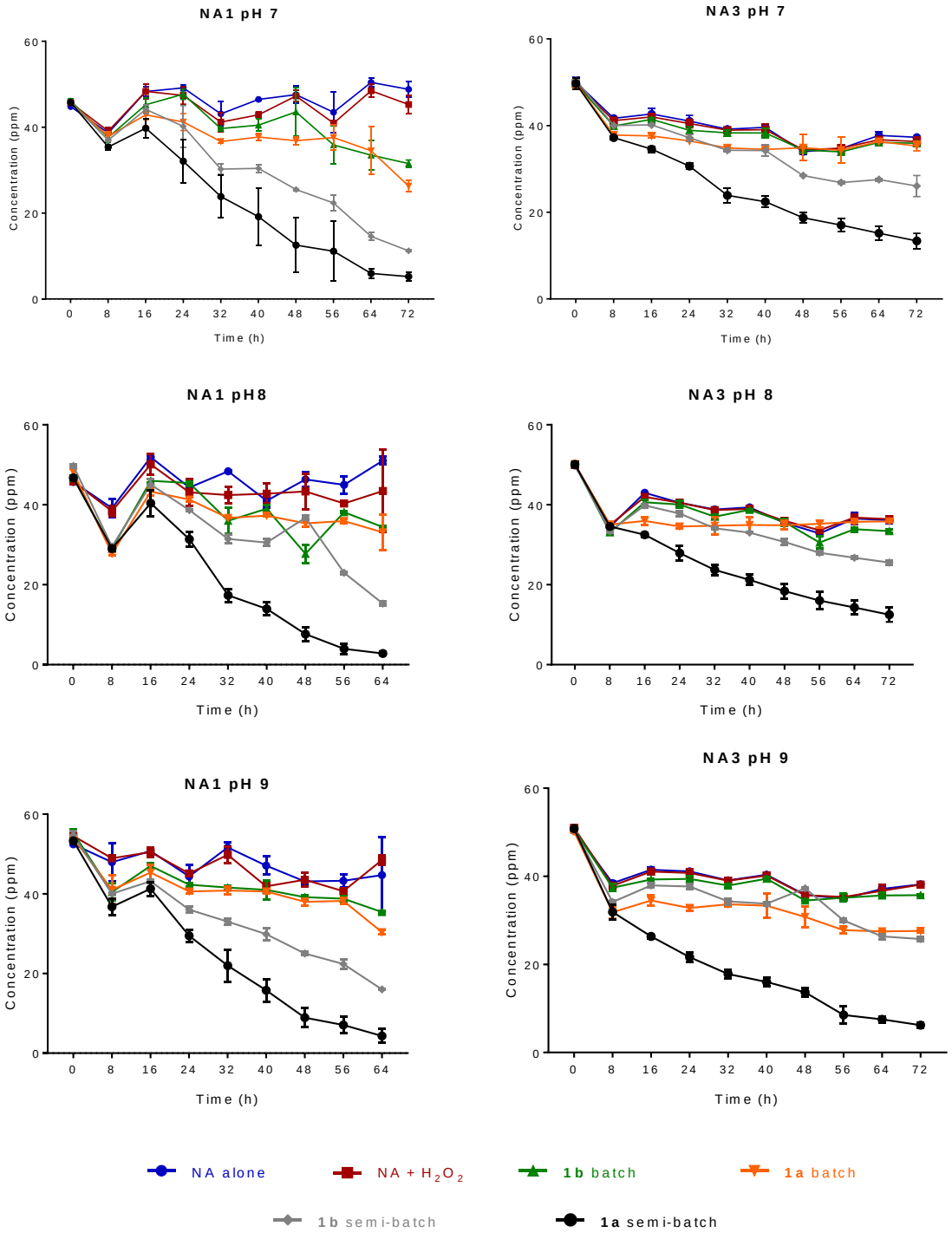


Figure 38. Residual concentration of model NAs (*n*=3) after oxidation reactions using TAML activators (**1a** and **1b**) and H_2O_2 at different pH values. Conditions: MilliQ® water, 100 ppb of Fe-TAML activators, 40 mg/L of H_2O_2, 72 hours of reaction

The lack of decomposition observed in batch reactions suggested that the catalysts underwent inactivation during the oxidation process, as proposed previously by Tang *et al.* (2016)[40]. It is known that Fe-TAML catalysts in solution undergo degradation; the resting catalyst undergoes inactivation induced by the reaction medium, whereas the active catalyst can be destroyed by intra- and intermolecular inactivation[33]. Previous studies have reported proton-induced demetalation of resting TAML catalysts in the presence of acidic substrates, as these are an important source of protons in the solution and might be partially responsible for the cleavage of Fe-N bonds by enhancing the rate of acid catalysis[347]. In TAML activators, the cleavage of only one of the four Fe-N bonds leads to rapid hydrolysis of the other three bonds and liberation of Fe^{III}, resulting in irreversible inactivation. However, our oxidation reactions were carried out under neutral/alkaline conditions above the pKa of NAs (5 – 6), meaning that the protonated forms predominated and thus proton-induced demetalation is unlikely. Rather, the fast inactivation of **1a** and **1b** in the batch experiments might be linked to intra- and intermolecular oxidative degradation of the active catalyst. Furthermore, it has been reported that when the active catalyst is unable to attack substrates that are resistant or difficult to oxidise, the catalase-like activity prevails over the peroxidase-like activity of TAML activators[353]. In contrast to peroxidases, which transfer oxidation equivalents from H_2O_2 to reducing substrates, catalases catalyse the decomposition of H_2O_2 to water and oxygen. As these two reactivities act on the same substrate (H_2O_2), competition occurs, which means that the H_2O_2 is wasted if the catalase-like activity predominates, with insufficient oxidant left for the oxidation of NAs[354]. The efficacy of multiple aliquots of catalysts and H_2O_2 (every 8 hours) was evaluated to address inactivation by renewing the resting catalyst and reducing the impact of catalyst inactivation on the overall catalytic performance. These semi-batch oxidation reactions revealed that lower final concentrations of model NAs were consistently achieved at all pH values using **1a**/H_2O_2 (Figure 38), which was anticipated due to the increased oxidative aggression provided by the electron-withdrawing capacity of the NO_2 group. Moreover, the superiority of **1a** over **1b** was statistically significant at all pH values for both NAs ($p = <0.0001$).

Even though the self-degradation of Fe-TAML activators is highly desired as this reduces the risks associated with the discharge of TAML-containing effluents into the environment after treatment, a problem arises when complete deactivation occurs before a large number of catalytic turnovers is achieved[33], as the need of additional aliquots of reactants increases overall economic costs. Nevertheless, as set out in Figure 38, semi-batch reactions with **1a** achieved degradation of approximately 95% for NA1 and 90% for NA3 within 72 hours, demonstrating the suitability of Fe-TAML/H_2O_2 systems to decompose model NAs under laboratory

conditions. These findings contrast with results from Chapter 3 that indicated no degradation of NA1 and only 20% degradation of NA3 by the consortium of *P. putida* after 28 days, demonstrating the superiority of Fe-TAML/H_2O_2 systems for the degradation of these model NAs.

5.3.3 Effect of initial pH on the oxidation of model NAs by 1a and 1b

Oxidation reactions were conducted in unbuffered water as this is a better approximation to realistic conditions during wastewater treatment than buffered reactions, which constitute most of the studies published with Fe-TAML/H_2O_2 systems[43,344,348,355–357] because the reactivity of some of these catalysts is highly dependent on pH, with the highest rates observed near pH 10 in previous studies on various substrates[351,356,358]. Reactions took place in MilliQ® water and pH was adjusted to 7, 8, or 9 at the beginning of the oxidation processes. These pH values were selected based on the pH of our RWW samples (~ 7) and the pH values reported for OSPW (typically between 8 and 9[141] due to the presence of NaOH used in the extraction of bitumen[359]) because these effluents are important sources of NAs into the environment.

Performance of **1a** and **1b** for the degradation of NA1 and NA3 at different pH values is set out in Figure 39. There was not a marked effect of pH on the oxidation of NA1 in semi-batch experiments with **1a** ($\alpha = 0.05$; $p = 0.8253, 0.7944, 0.4356$) or **1b** ($\alpha = 0.05$; $p = 0.7645, 0.3481, 0.7645$), nor batch experiments with **1b** ($\alpha = 0.05$; $p = 0.7917, 0.4447, 0.8361$). On the contrary, there were statistically significant differences between the final concentration of NA1 in the batch experiments with **1a** at pH 7 and pH 9 ($\alpha = 0.05$; $p = 0.0203$), with better performance at pH 7. Regarding NA3, semi-batch reactions at pH 9 were significantly superior to those at pH 7 and 8 with **1a** ($\alpha = 0.05$; $p = <0.0001, <0.0001$) but no differences were observed with **1b** at any pH ($\alpha = 0.05$; $p = 0.8914, 0.8717, 0.6067$). Batch experiments showed no significant differences between pH values with **1a** ($\alpha = 0.05$; $p = 0.9875, <0.0001, <0.0001$) or **1b** ($\alpha = 0.05$; $p = 0.1521$; $p = >0.9999$; $p = 0.0730$).

Previous studies using ozone to oxidise NAs have also reported higher efficiency at basic pH[357,360], which has been linked to the decomposition of ozone at high pH values and the resulting formation of hydroxyl radicals[228,360]. Contrary to ozone, which is quite selective in its oxidation reactions and useful for aromatics and C-C multiple bonds only, hydroxyl radicals can react with aliphatic carbon chains by hydrogen abstraction[361], resulting in higher oxidation efficiency of NAs. However, hydroxyl radicals seem not to be involved in Fe-TAML oxidation reactions, and previous research suggests that the pH-dependant efficiency is more likely to be

related to the interaction between Fe-TAML activators and H_2O_2[343,352], which appears to be a redox reaction where H_2O_2 binds to the Fe-TAMLs and decomposes to an oxo ligand and water, giving an oxidized Fe-TAML[352].

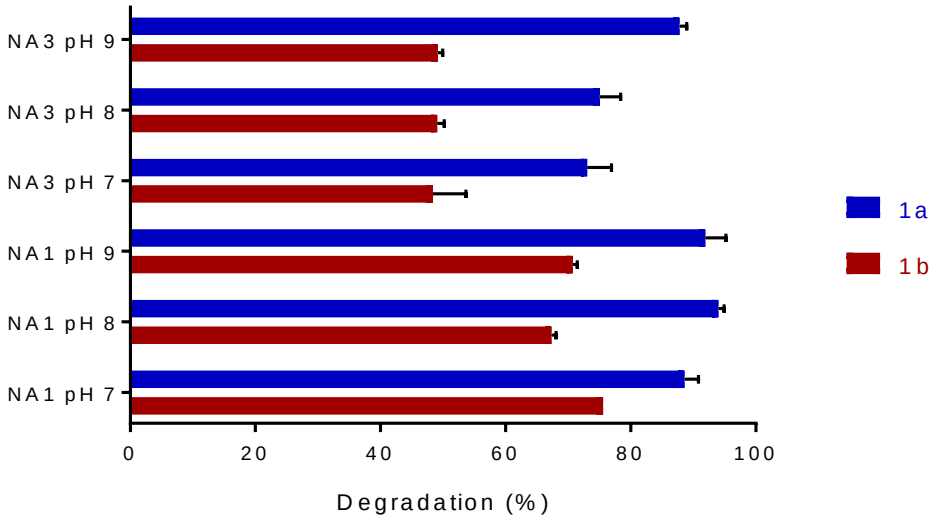


Figure 39. Overall performance (*n*=3) of Fe-TAML catalysts **1a** and **1b** for the degradation of model NA1 and NA3 at different pH, showing higher efficacy of **1a** over **1b**

5.3.4 By-product formation from degradation of NA3

HPLC-DAD analysis of the reaction medium after degradation of NA3 by **1a** showed that as NA3 degraded, a by-product was formed with increasing peak area over time, as set out in Figure 40. HRMS results confirmed the presence of an additional chemical that was absent in blanks and reaction media at T_0, and that co-eluted with NA3 in an unresolved peak, as observed in Figure 41. The peak was composed of 3 unresolved peaks corresponding to NA3 (RT: 3.78 and 3.72 min; two isomeric forms) and the by-product (RT: 3.64 min), which presented an accurate mass of 233.1181, providing a chemical formula of $C_{14}H_{17}O_3$ (4 mg/L error). ESI fragmentation showed product ions of *m/z* 188.7 and *m/z* 55.0, corresponding to $[M\text{-}COOH]^-$ and $[C_4H^7]^-$, leading to the proposed structure provided in Figure 42.

This finding is in agreement with previous studies degrading NAs by other oxidation processes, where the proportion of classic NAs fitting the formula $C_nH_{2n+z}O_2$ decreases and that of oxidised species with O_3, O_4, O_5, and O_6 increases[279], indicating oxidation of classic NAs. As a classic NA, NA3 underwent oxidation by **1a**/H_2O_2 and transitioned from $C_{14}H_{20}O_2$ to $C_{14}H_{17}O_3$.

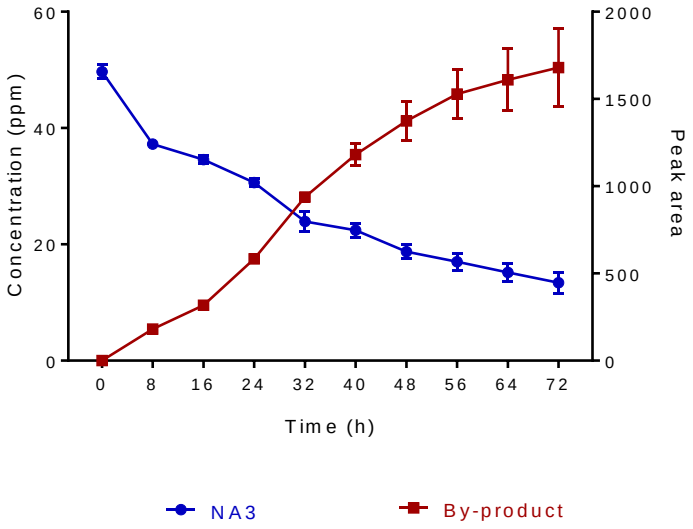


Figure 40. Concentration of NA3 (*n*=3) and peak area (*n*=3) of a degradation by-product over time in semi-batch experiments with **1a**

5.3.5 Degradation of NAs in RWW

After establishing the rapid inactivation of Fe-TAML catalysts in batch experiments resulting in little degradation of model NAs, the superiority of **1a** over **1b** in semi-batch experiments, and the higher catalytic performance of **1a** in semi-batch reactions at pH 9 to degrade NA3, the oxidation experiments of RWW (pipeline 3) were conducted for 72 hours in a semi-batch process with **1a** at 100 ppb, H_2O_2 at 40 mg/L, and pH 9. To determine the performance of the Fe-TAML/H_2O_2 treatment, 4 endpoints were monitored: (i) toxicity measured by the LBT, (ii) concentration of NAs determined by the single point external standard method using total ion chromatograms from GC-MS, (iii) relative abundance of classic and oxy-NAs identified using HRMS, and (iv) NA profiles based on *n* values and *Z* families obtained from HRMS data.

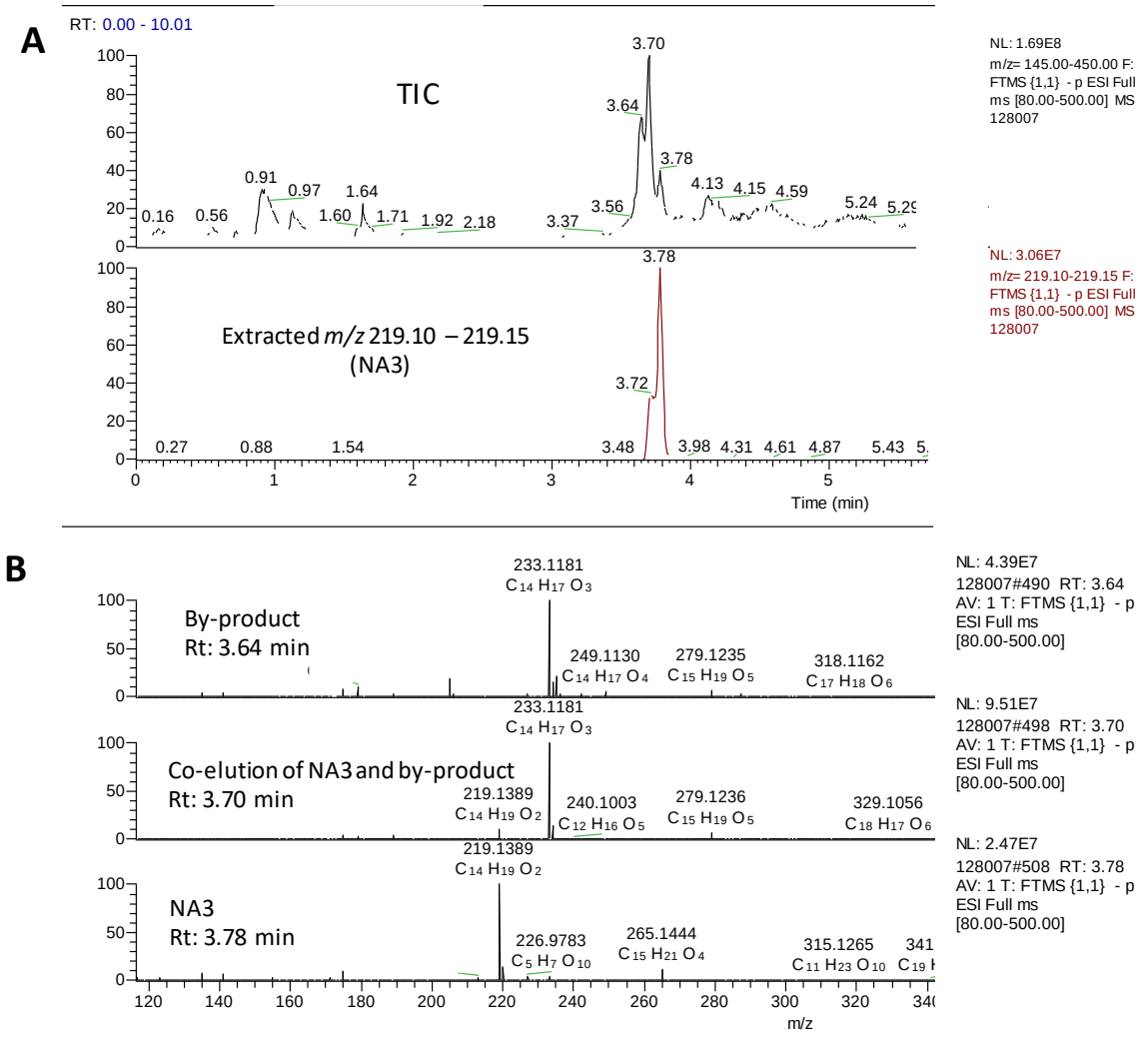


Figure 41. Detection of a by-product generated from the degradation of NA3. (A) Total ion chromatogram of the reaction medium in semi-batch reactions of NA3 with 1a/H_2O_2 and (B) high-resolution mass spectra for NA3 and the degradation by-product

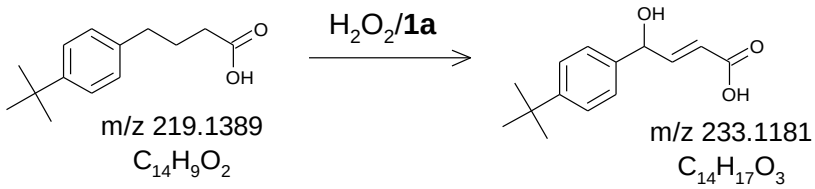


Figure 42. NA3 and the proposed degradation by-product generated during oxidation reactions with 1a/H_2O_2

Toxicity results (raw data in Appendix III; calibration curves in Appendix II) confirmed that the catalase effectively quenched all the H_2O_2 remaining at T_{72} and that no toxicity was exerted by the reaction matrix composed of TAML activators, catalase, and, potentially, H_2O_2. Moreover, as presented in Figure 43, treatment with **1a**/H_2O_2 reduced the toxicity of RWW by 4-fold; such reduction in toxicity can be unequivocally linked to **1a** in the reaction medium because the uncatalysed reactions showed no statistically significant difference with respect to T_0 and RWW alone at T_{72}. In comparison, ozonation has been reported to reduce toxicity of OSPW by 3-fold, but it requires the continuous supply of ozone throughout reactions (60 min; constant ozone concentration of 25 - 35 mg/L in the gas-phase)[142], which would derive in high operational costs for scaling up.

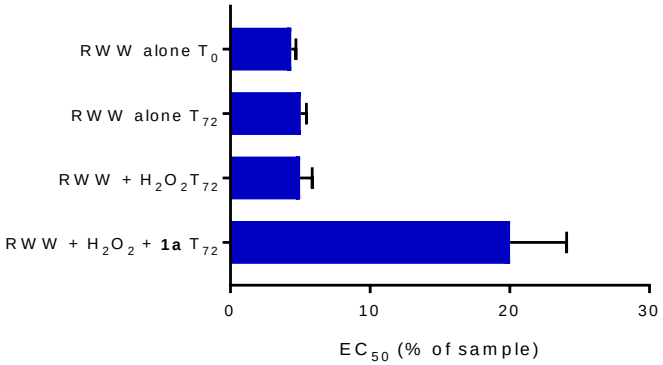


Figure 43. EC_{50} (% of sample; *n*=3) of treated and untreated RWW at T_0 and T_{72} towards *Vibrio fischeri*

Semi-quantification of NAs from the TIC observed in GC-MS was conducted based on the peak area of the unresolved hump assigned previously to NAs based on EI-MS, ESI-MS, and HRMS. The single point external standard method was followed, using the formula below:

$$Concentration\ of\ NAs = \frac{Peak\ area\ of\ NAs}{Peak\ area\ of\ IS}\ x\ Concentration\ of\ IS$$

From the two IS used, 1-chlorooctadecane was used for the calculation because its %RSD was lower compared to that of *o*-terphenyl (18.3 % and 41.6% respectively; spiking concentration 100 µg/L). The concentration of NAs was estimated to be roughly 90.4 ± 25.6 mg/L, 86.9 ± 48.2 mg/L, and 133.3 ± 3.7 mg/L for untreated, uncatalysed, and catalysed RWW respectively, indicating that the total NA content did not decrease after treatment. Variability, however, was significant in both controls.

HRMS profiles showed that the distribution and abundance of NA species in the mixture changed after treatment, explaining the transformation of the toxicological properties of the treated sample even though total NA content remained constant. Chemical analysis revealed that the relative abundance of O_2 NAs decreased after treatment with **1a**/H_2O_2, with significant differences with respect to untreated and uncatalysed RWW ($\alpha = 0.05$; $p = <0.0001$), whereas relative abundance of O_3 and O_4 NAs increased with statistical significance ($\alpha = 0.05$; $p = <0.0001$) (Figure 44), suggesting oxidation of O_2 NAs into oxy-NAs. This finding corroborates the result obtained in our degradation experiments with model NAs where NA3, an O_2 species, oxidised into an O_3 species, and is in accordance with previous research reporting increased percentage of oxidised NA species after ozone-based oxidation treatments[279,362].

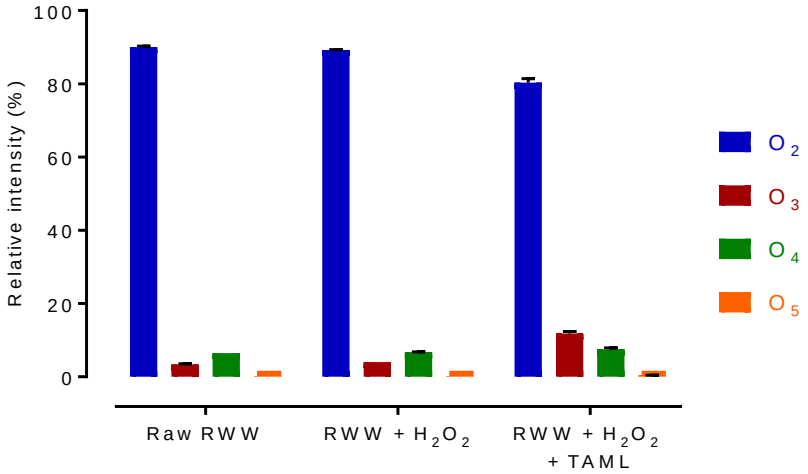


Figure 44. Relative abundance (%; *n*=2) of classic and oxy-NAs in raw, uncatalysed, and catalysed (**1a**) RWW

O_2 NAs have been regarded as the most toxic NAs[281,363], so their decreased relative abundance could explain the different outcomes observed in the toxicity tests of treated and untreated samples. Previous research has suggested that the most probable mode of action for acute toxicity of NAs is narcosis because of their surfactant properties and the lack of functional groups that could target specific receptors[339]. But, interestingly, studies have shown that low-molecular-weight NAs were key in the acute toxicity exerted by NA mixtures in OSPW[275,364]. As narcosis depends on hydrophobicity and size of a molecule, high-molecular-weight NAs would be expected to cause greater disruption to cell membranes[267], which is precisely what is observed with surrogate NAs, whose toxicity is proportional to carbon number up to the point where insolubility inhibits biological effects[365]. However, a study by Frank *et al.*, 2009[339], demonstrated that high-molecular-weight NAs in OSPW tended to have more carboxylic acid

moieties, resulting in structures that are less hydrophobic than low-molecular-weight monocarboxylic NAs, explaining the toxicity data. In our case, the oxidation of O_2 into O_3 species might have the same effect by lowering the hydrophobicity of alkyl groups, resulting in lower toxicity towards *V. fischeri*. Nevertheless, O_3 NAs have been implicated in estrogenicity[366], and further biological tests could help to confirm this activity for our sample. It is also possible, however, that other organics present in RWW were degraded during treatment with **1a**/H_2O_2, such as aliphatic hydrocarbons, which have been linked to narcosis in previous research[267].

NA profiles of untreated RWW obtained from HRMS data are provided in Figure 45, showing the distribution of O_2, O_3, O_4 and O_5 NAs based on *n* values and *Z* families. It can be seen that families *Z* = -2 and -4 were predominant in all cases, and that *Z* = 0 NAs were also abundant in O_3 NAs; these findings contrast with a previous study of RWW[163] reporting abundance of families Z = -6 > -8 > -12 > -14 > -10 > -4 > 0 in O_2 NAs, but are consistent with other reports from OSPW[142,273,274]. Regarding *n*, the distribution of O_2 and O_3 NAs was somewhat similar, with the most abundant *n* congeners within O_2 NAs centred around $C_{9\text{-}13}$ and those within O_3 species between $C_{9\text{-}15}$. O_4 species peaked around $C_{12\text{-}16}$ and $C_{22\text{-}26}$, whereas O_5 NAs peaked around $C_{13\text{-}18}$.

The NA profiles for classic and oxy-NAs after catalysed and uncatalysed treatments, together with untreated RWW, are provided in Figure 46, revealing that the profiles from the uncatalysed treatment were not different from the untreated sample. The biggest impact of treatment with **1a**/H_2O_2 was observed on the overall profiles of O_3, O_4, and O_5 NAs, with an evident increase in the intensity of O_4 NAs with $C_{11\text{-}13}$ and *Z* = -2, and of *Z* = -2 and -4 in O_5 NAs. The statistical analysis (Figure 47) revealed significant differences in *Z* = 0, -2, -4, and -8 for O_3, and *Z* = 0, -2, -4, -6, and -8 for O_4 in treated samples with respect to controls. However, the differences of O_5 NAs after treatment did not have statistical significance. For both O_3 and O_4 congeners, the relative abundance of *Z* = -8 decreased after treatment, whereas *Z* = -6 decreased in O_4 species only. Family Z = -4 increased for O_3 NAs but decreased for O_4 NAs, *Z* = -2 increased in both groups, and Z = 0 decreased for O_3 but increased for O_4. These findings corroborate the results from previous studies applying other oxidation processes to NAs, where species with low *Z* number (0 or -2) were formed during treatment possibly due to ring opening[142,279,357,367]. However, the relative abundance of O_2 and O_5 congeners with lower cyclicity did not change, and the reason behind this is not yet clear. Nevertheless, results show that the catalysed treatment decreased the overall degree of cyclization of the NA mixture,

which has important practical implications because cyclization remains a major factor contributing to persistence, with faster biodegradation rates in NA mixtures with lower degrees of cyclization[309]. The higher reactivity of **1a**/H_2O_2 and other AOPs to NAs with increasing number of rings might be related to the higher number of tertiary carbons, which are more reactive than secondary or primary carbons[360].

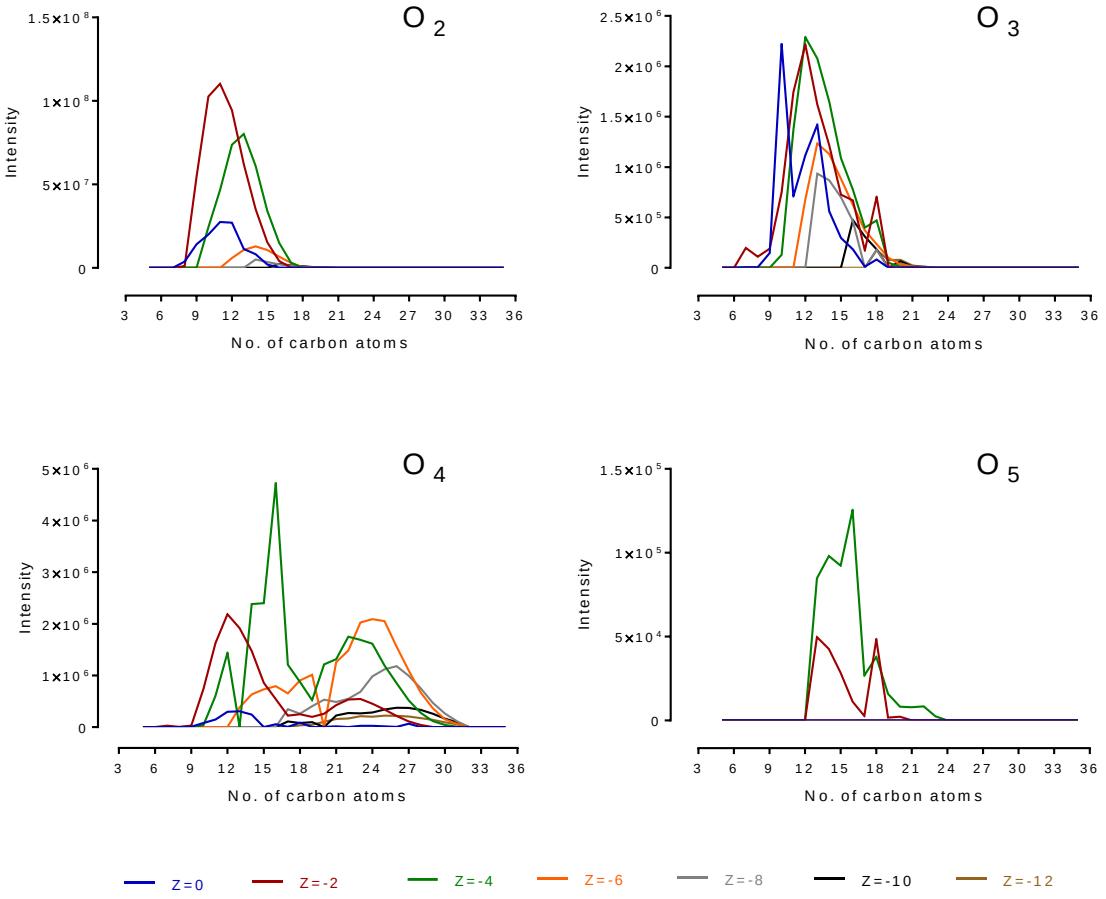


Figure 45. NA profiles for O_2, O_3, O_4, and O_5 NAs from untreated RWW based on *n* values and *Z* families obtained from HRMS data

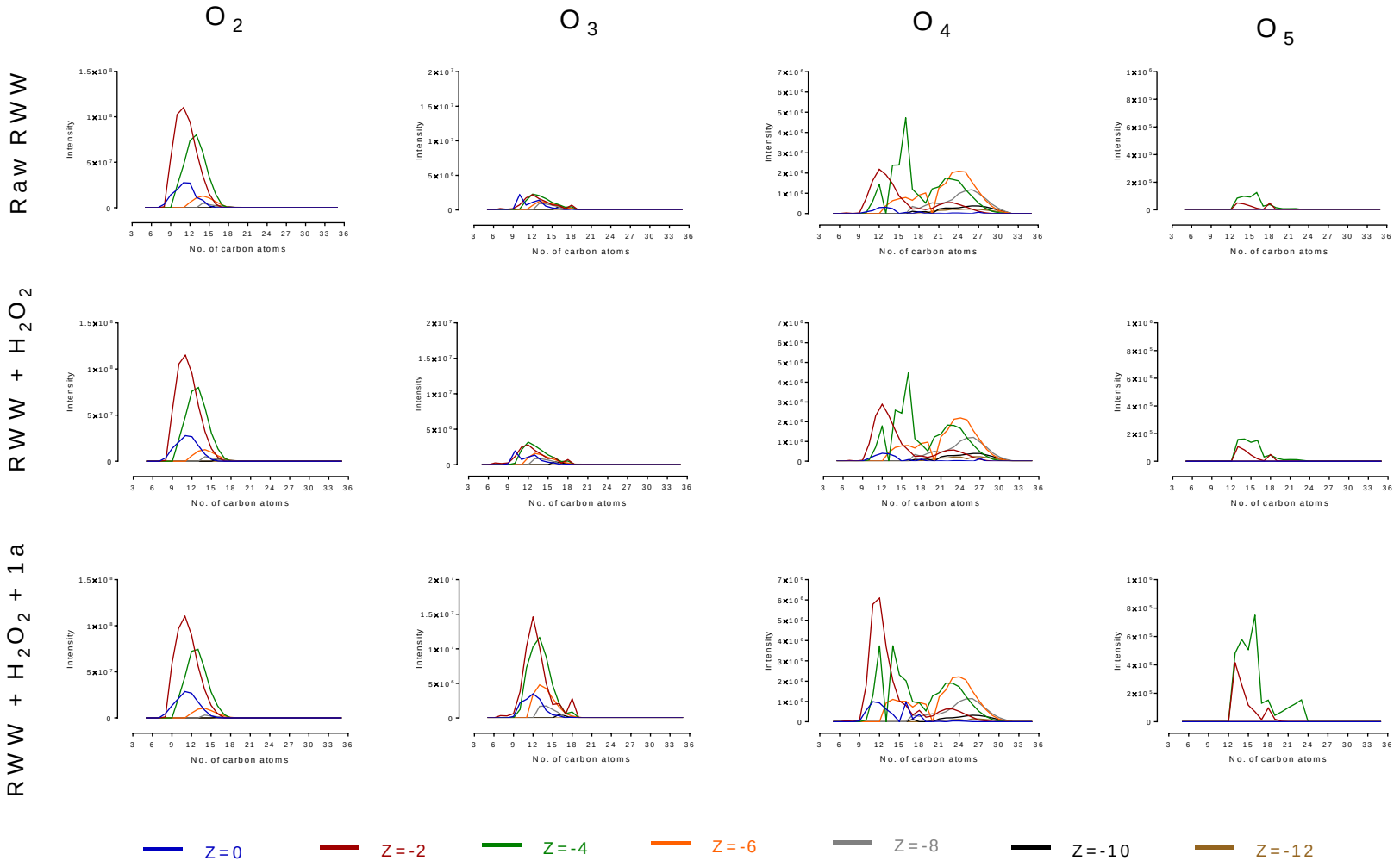


Figure 46. NA profiles of treated and untreated RWW based on *Z* families and *n* carbon atoms

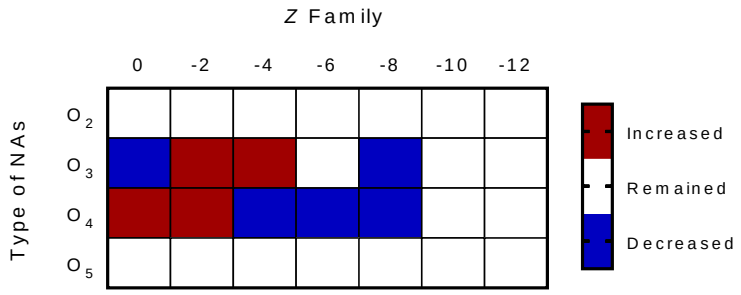


Figure 47. Heat map showing statistically significant changes in relative abundance of *Z* families for O_2 to O_5 NAs in treated RWW with respect to controls

Regarding the carbon content of controls and catalysed samples, profiles provided in Figure 46 show that the overall distribution of O_2 NAs looks unchanged, whereas O_3 congeners peaked around $C_{12\text{-}13}$, O_4 species centred on $C_{11\text{-}14}$ and $C_{22\text{-}25}$, and O_5 between $C_{13\text{-}17}$. Previous studies have used n = 15 as margin to classify NAs into low and high molecular weight (MW)[279,368], and we used the same approach to determine whether treatment with **1a**/H_2O_2 had any effect on high MW species, which are reported to be less biodegradable. As set out in Figure 48, both uncatalysed and catalysed treatments reduced high MW O_2 NAs from 11% to 4%, which can be linked to oxidation by hydroxyl radicals from H_2O_2. In contrast, low MW NAs in O_3 and O_4 species increased with respect to controls after treatment with Fe-TAML only, reaching 90% and 43% respectively. Interestingly, low MW congeners in O_5 NAs increased in the uncatalysed treatment only, even though the catalysed reaction had the same concentration of H_2O_2. The explanation behind this finding is not yet known. In alignment with previous research of other oxidation processes[279,357,360], **1a**/H_2O_2 showed preferential oxidation towards high MW NAs, which can be attributed to the increasing number of H atoms vulnerable to abstraction by hydroxyl radicals[360].

5.3.6 Comparison of Fe-TAML/H_2O_2 with other treatment options

The toxicity and persistence of NAs in OSPW (and to a lesser extent in RWW) have necessitated the development of numerous treatment methods for their removal. Biodegradation is considered the most cost-effective treatment for degrading NAs[147] and numerous microorganisms isolated from environmental samples chronically exposed to NAs have demonstrated their metabolic potential to use NAs as carbon sources. However, biodegradation fails to degrade species with high molecular weight and high degrees of cyclicity, and is usually slow, with an estimated half-life of NAs in nature of 12.8 – 13.6

years[164]. Hence, other treatment technologies have been investigated, including ozonation and adsorption-based technologies.

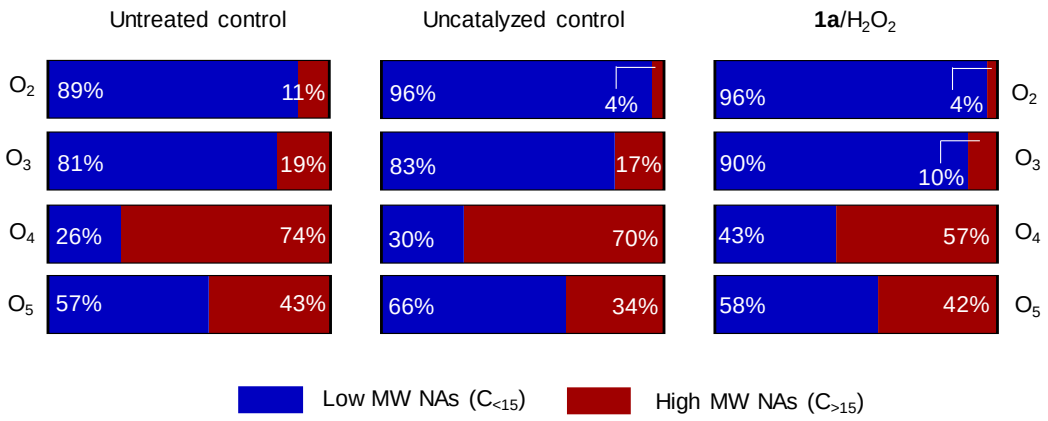


Figure 48. Variations in the carbon number of NAs in untreated, uncatalysed, and catalysed RWW using C_{15} as margin to separate NA congeners into low and high MW species

Ozone has been reported to be an effective advanced oxidation technology to oxidise model NAs and NAs from OSPW, increasing their biodegradability and reducing toxicity towards *V. fischeri*[142,357,359,369]. For instance, complete detoxification of OSPW (based on the Microtox® assay) has been achieved after only 50 minutes of ozonation[367]. However, when used alone, ozone is frequently applied in a semi-batch mode with the gas running continuously[142,370], thereby providing excess ozone for the duration of the reaction[367]. The high cost of ozonation (due to the high consumption of energy and ozone) for the vast quantities of NA-containing wastewater typically produced in the petroleum sector has been acknowledged by previous studies, questioning the economic feasibility of ozone-based technologies for removal of NAs in realistic scenarios[359,367]. In fact, effective concentrations of ozone reported in previous research can go up to 150 mg/L[357,359,362]. Dosing has been referred to as intensive at concentrations equal or above 80 mg O_3/L, mild at 30 – 50 mg O_3/L[371], and light at approximately 25 mg/L[142], but these concentrations are still cost-prohibitive taking into account the millions of m^3 of wastewater produced on a daily basis in a single refining site[67]. It has been estimated that implementing ozonation at 7.7 mg O_3/L with a retention time of 25 minutes (10 kWh/kg of produced ozone per hour) would increase treatment cost by 0.18 - 0.22 €/m^3 in the Netherlands and from 0.10 – 0.18 €/m^3 in Germany for municipal wastewater[372], but these costs do not account for the recalcitrant compounds commonly found in RWW, such as NAs. These costs might result cost-prohibitive for refining sites in low- and middle-income

countries, considering that a total of 3.5 – 5 m^3 of wastewater are produced per ton of crude refined when cooling water is recycled[69]. Consequently, ozone alone is hardly applicable for the treatment of RWW. Instead, it is lately seen as a potential pre-treatment for biological degradation[30,371] because it targets NAs with greater MW (higher possibility for hydrogen abstraction) and number of rings (more tertiary carbons, and therefore higher reactivity), which tend to be more resistant to biodegradation[15,357,360]. Likewise, adsorption has been proposed as a pre-treatment step of ozonation to reduce the levels of applied and utilised ozone[359].

Our results showed the effectiveness of the Fe-TAML to reduce the toxicity of RWW by a factor of 4 using a semi-batch process in which a total of 900 µg/L of **1a** were added throughout the reaction, thereby comparing favourably with a similar approach undertaken previously by Vaiopoulou *et al.*, 2015[142], where ozone at 25-35 mg O_3/L resulted in a 3.3-fold reduction in toxicity. However, the same study achieved a 15-fold decrease in toxicity towards *V. fischeri* when ozone was used in combination with biodegradation because this approach targets a wide range of NAs, and therefore further work is required to determine if the same benefit can be obtained by using Fe-TAML/H_2O_2 and biodegradation combined. New generations of TAML activators have been developed recently, which may have greater efficacy for NA degradation and detoxification of RWW at lower concentrations and with lower requirements of H_2O_2.

5.4 Conclusions

In this study, we provided an insight into the efficacy of Fe-TAML/H_2O_2 systems to oxidise surrogate NAs and detoxify refining effluents containing naphthenic acids. It was demonstrated that only very low concentrations of Fe-TAML activators (100 ppb) were required to oxidise aqueous solutions of NAs at high concentration (50 mg/L), although the catalysts underwent inactivation during oxidation reactions, which might be linked to the acidic nature of NAs, contributing to the irreversible cleavage of Fe-N bonds in the structure of the catalyst. A semi-batch process (multiple aliquots of catalysts and H_2O_2 every 8 hours) proved to address such inactivation, achieving up to 95% degradation of surrogate NAs within 72 hours. An oxidation by-product was identified, revealing hydroxylation of the parent NA by means of HRMS. Catalyst **1a** proved to be superior to **1b** in semi-batch reactions, which is explained by the increased oxidative aggression provided by the electron-withdrawing capacity of the NO_2 group[346,351] in its ligand structure.

Treatment of real refining effluents with **1a**/H_2O_2 revealed that there is no need of complete mineralisation of NAs to reduce their biological effects. Even though the total NA content

remained unchanged after treatment, changes in the distribution and abundance of classic and oxy-NAs resulted in effluents that were less toxic towards *V. fischeri*, with a reduction in toxicity of 400%. The relative abundance of O_2 NAs decreased after treatment, and that of O_3 and O_4 increased. Oxidation of O_2 NAs might lower hydrophobicity of alkyl groups, resulting in a weaker narcotic effect. Likewise, **1a**/H_2O_2 reduced the degree of cyclization on the NA mixture and showed preferential oxidation towards high molecular weight NAs, showing comparable results with ozonation, which is one of the most effective oxidation technologies for the removal of NAs from oil sands process water[142,153,279,357]. However, concentrations of Fe-TAML activators required for treatment are significantly lower than those of ozone, which is highly relevant for up-scaling purposes.

Further work is required to evaluate Fe-TAML/H_2O_2 systems in combination with biodegradation to achieve lower residual NAs concentrations in refinery effluents and thus lower toxicity. Likewise, further research is needed to evaluate new generations of Fe-TAML activators in an attempt to reduce H_2O_2 concentrations and reaction times. Overall, our findings suggest that Fe-TAML/H_2O_2 systems are good candidates for achieving a high degree of NAs removal in refining effluents and other petrochemical effluents containing these pollutants, moving a step forward in water sustainability.

CHAPTER 6
CONCLUSIONS AND RECOMMENDATIONS

6.1 Background

This study was framed within the wastewater quality triad linking chemistry, toxicity, and wastewater treatment for the petroleum refining sector. The provision of refining effluents that are safe for disposal or recycling requires the understanding of their composition, their potential environmental toxicity, and the fate and behaviour of their key toxicants in wastewater treatment plants. And the relationship between all these elements is equally important. Toxic chemicals, for example, are less likely to be biodegraded during secondary treatment, as these can decrease the oxidation potential of the microbial communities used in this stage[30,373–375], thus the implementation of advanced treatment can significantly improve removal of such compounds. In other words, the development of adequate wastewater treatment requires a deep understanding of what is present in wastewater and what needs to be removed.

As discussed in Chapter 2, the identification of target contaminants for treatment is dependent on the successful linking of biological effects with causative agents, which is not straightforward. Such link is not well established for petroleum refining effluents. Previous studies have reported various toxic chemicals in oil refining effluents, including alkanes[80,88], PAHs[65,78–81], BTEX[81,93,376], phenols[64,65,78,80], metals, and carboxylic acids[78,80], but the exact nature of organic toxicants remains unclear, especially polar organics. Therefore, addressing this gap in knowledge might help establish the missing link between toxicity and chemistry in refining effluents. Based on this premise, two key questions were the core of this research: (1) what is the identity of the organic chemicals causing toxic effects? and (2) how can these toxic organics be degraded cost-effectively? To answer these questions, a petroleum refinery processing heavy crude oil located in Barrancabermeja, Colombia, was used as a case study. Three end-of-pipe effluent grab samples were collected for further study.

6.2 Effluent Characterisation & Effect Directed analysis

The first question was addressed in Chapter 3; the objective was to identify toxic organics in refining effluents in order to determine what chemicals are persistent and resist wastewater treatment and thus reach aquatic ecosystems. As the existing research on refining effluents set a consistent, yet incomplete platform of data resulting from characterisation studies using exclusively the TIE approach, this chapter followed an EDA approach which, to our knowledge, has not been reported before in refining effluents and was conducted herein.

The EDA approach used in this chapter used a sequential SPE extraction method to extend the range of chemicals extracted to include polar compounds and LLE as the traditional extraction

method for effluents. The LBT revealed that the toxicity of two aqueous samples (pipelines 1 and 2) could not be determined due to their low toxicity to *V. fischeri*, but the third sample (pipeline 3) presented a high TOC value (nearly 130 mg/L) and 5.0 TU (EC_{50} = 20%). Likewise, extracts from pipeline 3 were significantly more toxic than those of pipelines 1 and 2, with TU values that were up to 350-fold more toxic. GC-MS analyses of SPE and LLE extracts from all three samples revealed the presence of organic acids, phenols, aliphatic and aromatic hydrocarbons, ketones, and miscellaneous organics. In particular, pipeline 3 was shown to contain NAs, which were semi-quantitatively estimated to be present at approximately 85 - 135 mg/L. After fractionation, consistent with the literature[54], we found that toxicity spread in many fractions, each presenting a large number of compounds, suggesting that the toxicity of refinery effluents stems from the interaction of organic compounds, including alkanes. Moreover, our results showed that NAs were present alone in two fractions that impacted significantly the bioluminescence output in the LBT, indicating that they have an important contribution to the biological effects of RWW. The high percentages of inhibition of bacterial luminescence in the toxicity assessment showed that further treatment is needed to protect receiving environments, which could be achieved by implementing a tertiary stage that targets NAs.

Characterisation of the NA mixture present in pipeline 3 required different analytical techniques (GC-MS, HPLC/ESI-MS, and HPLC/HRMS) to confirm the identity of NAs and profile NA mixtures based on families and carbon content. Results showed that HPLC/ESI-MS is not a reliable technique to profile NA mixtures due to the observation of numerous false positives, making HPLC/HRMS necessary for accurate profiling. The application of this technique in industrial settings might be cost-prohibitive for routine analyses, but it sets a baseline for the characterisation of site-specific samples and could be used as a gold standard for the development of cheaper and more practical alternatives.

From a regulatory point of view, the Colombian case study is an excellent example of how environmental regulations function as technical drivers. Our study site discharged effluents with significant concentrations of NAs into the environment because treatment technologies on-site do not target such chemicals, which stems from legislation and effluent guidelines that do not require refineries to report/limit the concentration of these contaminants in effluents. In contrast, mining sites that extract bitumen from the oil sands in northern Alberta, Canada, and produce wastewater with similar concentrations of naphthenic acids[160,164,278] have very

different wastewater management practices. Canada has a zero-discharge policy for wastewater containing NAs, so these effluents must be stored in settling ponds until the concentration of naphthenic acids reaches non-hazardous levels[157,160]. NAs play a central role in the wastewater management practices of this productive sector, and significant funding from public sources has been granted for research on treatment technologies to remove these chemicals. Within this context, Colombia and Canada have similar scenarios when it comes to NAs but evident differences in their regulatory approaches. Our findings show that NAs are also relevant within the refining sector thus we anticipate our results to be a starting point for better environmental legislation related to refining effluents from the processing of heavy crude oil, as these do not consider naphthenic acids at the moment.

Overall, our findings stress the importance of site-specific analyses for wastewater treatment purposes, as many factors contribute to the characteristics of refining effluents, including the characteristics of the feedstock, water management practices, and local environmental regulations. Simply said, effective treatment cannot be achieved without an accurate diagnosis.

6.3 Evaluation of *Pseudomonas putida* for degradation of naphthenic acids

Once naphthenic acids were identified as crucial toxicants in refining effluents, two lab-scale methods were evaluated for the degradation of naphthenic acids, aiming at detoxifying refining effluents. Chapter 4 presented the bioprospection of *Pseudomonas putida* for application in bioremediation of naphthenic acids, as biodegradation has been shown to be the most cost-effective treatments for the removal of dissolved organics and has good potential for scaling-up[377,378]. Surrogate naphthenic acids were used in a tiered procedure that included (i) a screening step to identify isolates capable of growing using model NAs as carbon source and provide data for bioinformatics analysis to detect candidate catabolic genes involved in the biodegradation of petrochemicals; and (ii) an evaluation of the strains selected during the screening stage for the biodegradation of model NAs in M9 medium following a quantitative approach, and assessing the effect of acclimatization on biodegradation capabilities.

Despite the low frequency of NA degraders within the set of 86 fully-sequenced strains of *P. putida*, a three-strain consortium was established, which showed 20% degradation of NA3 at 30 mg/L after 30 days but no degradation of NA1 at any concentration (100 mg/L, 350 mg/L). Our findings indicated that the acclimatisation of cells to increasing concentrations of NAs did not improve the biodegradation rate, which might be partly linked to the decreased number of viable cells at T_0 as a result of the chemical stress induced during the pre-exposure stage.

Nevertheless, the degradation of NA3 at the concentration tested suggests that our consortium holds promise for the biodegradation of NAs, as NAs with branched alkyl side chains (like NA3) are particularly difficult to break down, and other studies have reported negligible or no degradation using NA3[306,310].

6.4 Evaluation of Fe-TAML/H_2O_2 systems for degradation of naphthenic acids

Model compounds were used for the evaluation and optimisation of Fe-TAML/H_2O_2 systems for the oxidation of naphthenic acids and the subsequent treatment of a refining effluent sample. Oxidation experiments of model NAs demonstrated that both TAML activators underwent inactivation during the oxidation reaction, which we believe is linked to the acidic nature of NAs, as protons might be partially responsible for the cleavage of Fe-N bonds in the TAML structure, leading to irreversible inactivation[347]. Under these circumstances, the addition of repeated aliquots of TAML/H_2O_2 was evaluated in semi-batch experiments, demonstrating that the renewal of resting catalyst reduced the impact of catalyst inactivation and improved the catalytic performance of both TAML activators significantly, although **1a** was consistently superior to **1b**. The electron-withdrawing capacity of the NO_2 group present in **1a** is known to increase the oxidative aggression of this activator when compared to **1b**, which presents an H in the same position of the structure[346]. As for the catalytic performance of TAML activators in semi-batch reactions at different pH values, **1b** had similar performance (approximately 50% removal for NA3 and 70% removal for NA1) with no significant differences between pH values, whereas **1a** showed better performance at pH 9. Under these conditions, **1a** achieved nearly 95% degradation of NA1 and 90% of NA3 within 72 hours, which contrasts with the low degradation results ranging from 0 to 20% within 30 days using the bacterial consortium.

These results in semi-batch conditions led us to conduct oxidation reactions in the refining effluent sample containing NAs (pipeline 3) with **1a** (100 ppb) at pH 9 for 72 hours in a semi-batch process. A total of 4 endpoints were monitored: (i) toxicity measured by the luminescent bacteria test, (ii) concentration of NAs determined by the single point external standard method using total ion chromatograms from GC-MS, (iii) relative abundance of classic and oxy-NAs identified using HRMS, and (iv) NA profiles based on n values and Z families obtained from HRMS data.

Chemical analyses revealed that treatment with **1a**/H_2O_2 reduced the relative abundance of O_2 NAs and increased that of O_3 and O_4 NAs, indicating oxidation of classic NAs into oxy-NAs. Moreover, the treatment decreased the degree of cyclization of the NA mixture and showed

preferential oxidation towards high molecular weight NAs. These findings have important implications because classic NAs have been reported as the most toxic[281,329], and cyclization remains a major factor contributing to persistence, with faster biodegradation rates in NA mixtures with lower degrees of cyclization[309]. Furthermore, the toxicity test revealed that there was a **4-fold reduction in toxicity** as determined using the luminescent bacteria test after treating effluents with **1a**/H_2O_2, which could be explained by the decreased relative abundance of O_2 NAs. These findings demonstrate that detoxification of effluents containing naphthenic acids do not require their complete break-down, as the oxidation of O_2 NAs suffice for the provision of safer effluents.

6.5 Future perspectives and challenges

- Further research is needed to test more extended acclimatisation periods and/or lower concentrations with the three-strain consortium of *P. putida* used in this study, as previous research has successfully conditioned bacterial strains to higher concentrations of target chemicals after months of exposure to low concentrations[331]. Likewise, further research could be conducted to isolate microorganisms from environmental samples chronically exposed to NAs, such as soil or wastewater samples. Tailoring enrichment/isolation methodologies would increase the probability of obtaining strains with high potential for degradation and high tolerance to NAs.
- Treatment with Fe-TAML/H_2O_2 achieved a reduction in toxicity of 400% in a real RWW sample, but the complete elimination of toxic effects towards *Vibrio fischeri* was not achieved. Additional experiments with other TAML activators and/or longer reaction times with **1a**/H_2O_2 could determine whether Fe-TAML/H_2O_2 systems are aggressive and stable enough to provide a cost-effective solution suitable for scaling-up in real petroleum refineries.
- Other studies have successfully treated NAs in OSPW by a combination of ozone and biological treatment. Just like Fe-TAML/H_2O_2, ozone decreases the degree of cyclicity of NA mixtures and shows preferential oxidation towards high molecular weight NAs[142,153,279,357], increasing the biodegradability of NAs. A further study could assess a similar combinatorial approach of Fe-TAML/H_2O_2 followed by biodegradation to determine whether similar or better results could be obtained in terms of toxicity reduction and operating costs.

- Studies undertaken in this project were conducted in static laboratory conditions, and

further work is required with laboratory flow-through experiments or at pilot scale.

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APPENDIX I: PROTOCOLS

PROTOCOL FOR LIQUID-LIQUID EXTRACTION

Reagents and materials

- 800 mL of refining effluent samples.
- Universal pH paper.
- 100 mL of H_2SO_4:water 1:1 (v/v)
- 100 mL of 10N NaOH.
- Dichloromethane (DCM) (approximately 400 mL per sample).
- Sodium sulphate anhydrous.
- Measuring cylinder 1L.
- Separatory funnel 2L (one per sample).
- Beakers 500 mL (one per sample).
- Pasteur pipettes.
- Solvent concentrator.

Procedure

Extraction at neutral pH

1) Take an initial pH reading of the sample using pH paper and record. Adjust pH to be between 6-8 using H_2SO_4:water 1:1 (v/v) or 10N NaOH as required.
2) Use a clean glass graduated cylinder to measure 800 mL of sample and transfer into a 2L separatory funnel (in duplicate).
3) Add 60mL of DCM.
4) Shake sample sufficiently; vent pressure periodically about every 1-2 minutes; let rest until layers separate.
5) Collect the DCM in a pre-labeled beaker.
6) Repeat steps 3 to 5.

Extraction at acidic pH

7) Adjust pH ≤ 2 using H_2SO_4:water 1:1 (v/v). This should require ½ pipette full of acid. Do not add more acid than is necessary. Check pH with pH paper.
8) Add 60mL of DCM and shake 1-2 minutes. Vent pressure periodically about every 1-2 minutes; let rest until layers separate.
9) Repeat steps 3 to 5 using the same beaker employed during neutral extraction.

Extraction at alkaline pH

10) Adjust pH to 12 with 10N NaOH. This should take 2-3 pipettes full of 10N NaOH. Then add an additional 2 pipettes full of 10N NaOH.
11) Add 60mL of DCM and shake 1-2 minutes. Vent pressure periodically about every 1-2 minutes; let rest until layers separate.
12) Repeat steps 3 to 5 using the same beaker employed during neutral and acidic extraction. A total of 6 extractions per sample should have been made: 2 at neutral pH, 2 at acidic pH, and 2 at basic pH.
13) Dry over the DCM adding sodium sulphate. Stir and filter.
14) Take the sample to incipient dryness and reconstitute to 1 mL using DCM.

PROTOCOL FOR SOLID PHASE EXTRACTION

Reagents and materials

- 800 mL of refining effluent samples.
- 100 mL of H_2SO_4:water 1:1 (v/v)
- 100 mL of 10N NaOH.
- Deionized water.
- Methanol.
- 100 mL of 5% methanol in water.
- 100 mL of 5% NH_4OH in methanol.
- 100 mL of 2M HCl:ethyl acetate 1:10 (v/v).
- Hexane.
- HLB (6 mL, 200 mg; three per sample) and WAX cartridges (6 mL, 200 mg; one of each per sample).
- Beakers 100mL (one per sample).
- 10-mL test tubes (two per sample).
- Vacuum manifold and vacuum pump.
- Solvent concentrator.

Procedure

Extraction with HLB cartridges at neutral pH

1) Take an initial pH reading of the sample using pH paper and record. Adjust pH to be between 6-8 using H_2SO_4:water 1:1 (v/v) or 10N NaOH as required.
2) Place an Oasis HLB cartridge on the vacuum manifold and set the vacuum to 5” Hg.
3) Condition the cartridge by adding methanol and switching on the vacuum pump.
4) Switch off the vacuum pump after reducing the vacuum to the lowest possible setting.
5) Equilibrate the cartridge by adding water and switching on the vacuum pump.
6) Switch off the vacuum pump after reducing the vacuum to the lowest possible setting.
7) Load the sample.
8) Switch on the vacuum pump and gradually increase as needed until all sample has passed through the sorbent bed.
9) Switch off the vacuum pump.
10) Collect the extracted sample for further extraction at alkaline pH.

11) Apply 10 mL of 5% methanol in water as wash solvent.
12) Switch on vacuum to 5” Hg (adjust/increase as needed).
13) Pull vacuum for another 30 seconds - 1 minute to eliminate residual wash solvent.
14) Switch off the vacuum pump after reducing the vacuum to the lowest possible setting.
15) Release vacuum and insert test tubes to collect elution solvent.
16) Apply 20 mL of 100% methanol and let it flow through by gravity before switching on the vacuum pump.
17) Switch on the vacuum pump and gradually increase as needed.
18) Pull vacuum for another 30 seconds - 1 minute to collect all elution solvent.
19) Remove test tubes with the elution solvent.
20) Transfer methanol to separate beakers for different samples.

Extraction with HLB cartridges at alkaline pH

21) Adjust pH to 12 with 10N NaOH. This should take 2-3 pipettes full of 10N NaOH. Then add an additional 2 pipettes full of 10N NaOH.
22) Repeat steps 2 to 9.
23) Collect the extracted sample for further extraction at acidic pH.
24) Apply 10 mL of 5% methanol in water as wash solvent.
25) Switch on vacuum to 5” Hg (adjust/increase as needed).
26) Apply 10 mL of 5% NH_4OH in methanol as a second wash solvent.
27) Switch on vacuum to 5” Hg (adjust/increase as needed).
28) Pull vacuum for another 30 seconds - 1 minute to eliminate residual wash solvent.
29) Switch off the vacuum pump after reducing the vacuum to the lowest possible setting.
30) Release vacuum and insert test tubes to collect elution solvent.
31) Apply 20 mL of 2% formic acid in methanol and let it flow through by gravity before switching on the vacuum pump.
32) Repeat steps 17 to 19.
33) Transfer the elution solvent of each sample to the corresponding beaker used during the neutral extraction for a composite extract.

Extraction with HLB cartridges at acidic pH

34) Adjust pH ≤ 2 using H_2SO_4:water 1:1 (v/v). This should require ½ pipette full of acid. Do not add more acid than is necessary. Check pH with pH paper.

35) Repeat steps 2 to 9.
36) Collect the extracted sample for further extraction using WAX cartridges.
37) Apply 10 mL of 5% methanol in water as wash solvent.
38) Switch on vacuum to 5” Hg (adjust/increase as needed).
39) Apply 10 mL of 2% formic acid in methanol as a second wash solvent.
40) Switch on vacuum to 5” Hg (adjust/increase as needed).
41) Pull vacuum for another 30 seconds - 1 minute to eliminate residual wash solvent.
42) Switch off the vacuum pump after reducing the vacuum to the lowest possible setting.
43) Release vacuum and insert test tubes to collect elution solvent.
44) Apply 20 mL of 5% NH_4OH in methanol and let it flow through by gravity before switching on the vacuum pump.
45) Repeat steps 17 to 19.
46) Transfer the elution solvent of each sample to the corresponding beaker used during the neutral and alkaline extraction for a composite extract.

47) Extraction with WAX Cartridges
48) Place an Oasis WAX cartridge on the vacuum manifold and set the vacuum to 5” Hg.
49) Repeat steps 3 to 9.
50) Discard extracted sample.
51) Apply 10 mL of 5% ammonia in water as first wash solvent.
52) Switch on vacuum to 5” Hg (adjust/increase as needed).
53) Apply 100% methanol as second wash solvent.
54) Switch on vacuum to 5” Hg (adjust/increase as needed).
55) Pull vacuum for another 30 seconds - 1 minute to eliminate residual wash solvent.
56) Switch off the vacuum pump after reducing the vacuum to the lowest possible setting.
57) Release vacuum and insert test tubes to collect elution solvent.
58) Apply ethyl acetate saturated with hydrochloric acid (2 M HCl:ethyl acetate (1:10) v/v) and let it flow through by gravity before switching on the vacuum pump.
59) Repeat steps 17 to 19.
60) Transfer the elution solvent of each sample to the corresponding beaker used during the neutral, acidic, and alkaline extraction for a composite extract.
61) Take each sample to incipient dryness and reconstitute to 1 mL using DCM.

PROTOCOL FOR TOXICITY EVALUATION USING THE LUMINISCENT BACTERIA TEST

(Method using freeze-dried bacteria)

Reagents and materials

Use deionized water for the preparation of all solutions described below.

- Test bacteria: Microtox® acute reagent containing *Vibrio fischeri* NRRL B-11177.
- 2% sodium chloride solution (diluent).
- 1M Sodium hydroxide solution.
- 1M hydrochloric acid solution.
- 105.8 mg/L solution of potassium dichromate in saline solution 2% (w/v).
- 400 mg/L solution of phenol.
- Opaque, white plastic 96-well plates (for luminescence readings).
- Standard polystyrene transparent 96-well plates (for sample preparation)

Procedure

Preparation of stock solutions of standard substances (phenol and potassium dichromate)

1) Prepare a 105.8 mg/L solution of potassium dichromate in 2% sodium chloride solution. This solution is used as a reference substance, in triplicate, to fall within 40 – 60% of light inhibition after 15 minutes of contact time.
2) Prepare a 400-mg/L solution of phenol in 2% sodium chloride solution. Use an amber volumetric flask.

Preparation of sample prior testing

3) Measure the pH of each sample; values should range between 6 and 8.5. If necessary, use 1M NaOH or 1M HCl for pH adjustments.
4) Add 20g of NaCl per litre of sample.
5) Stir for 30 minutes for oxygenation. Deficiencies in oxygen may lead to luminescence inhibition.
6) In case or turbid samples, allow sample to settle for 1 hour or filtrate.

Preparation of dilutions of samples, controls, and standards in transparent 96-well plate(s)

7) For phenol, follow the table below to prepare the corresponding test solutions.

Dilution	Volume of stock solution (µL)	Volume of previous dilution (µL)	Volume of diluent (µL)	Resulting concentration (mg/L)	Final test concentration (mg/L)
2	300		0	400	200
4		150	150	200	100
3	200		100	266.66	133.33
6		200	100	133.33	66.66
12		150	150	66.66	33.33
24		150	150	33.33	16.66
48		150	150	16.66	8.35
96		150	150	8.35	4.20
192		150	150	4.20	2.08

8) For samples, follow the table below to prepare the corresponding test solutions.

Dilution	Volume of sample (µL)	Volume of previous dilution (µL)	Volume of diluent (µL)	Resulting concentration (%)	Final test concentration (%)
2	300		0	100	50
4		150	150	50	25
8		150	150	25	12.5
16		150	150	12.5	6.25
32		150	150	6.25	3.12
64		150	150	3.12	1.56
128		150	150	1.56	0.78
256		150	150	0.78	0.39
3	200		100	66.66	33.33
6		150	150	33.33	16.66
12		150	150	16.67	8.35
24		150	150	8.33	4.2
48		150	150	4.17	2.1
96		150	150	2.08	1.05
192		150	150	1.05	0.52
384		150	150	0.52	0.26

Preparation of bacterial stock and test suspensions

9) Prepare the bacterial stock suspension by removing one vial from the freezer and quickly adding 1 mL of previously cooled water (4°C ± 3°C). Add the cooled water quickly in order to minimize clumping and loss of light emission. Wait at least 10 minutes before using this suspension, keeping it at 4 °C at all times.
10) Prepare the bacterial test suspension by transferring the total volume of the bacterial stock suspension into a 10-mL test tube containing 9 mL of diluent. Mix thoroughly.

Transference of bacterial test solution, diluent, and samples to white plate(s) for light readings

11) Transfer 20 µL of the bacterial test suspension and 80 µL of diluent to each well of the opaque, white microplate(s).
12) Allow the microplates to equilibrate to 15°C during 15 – 20 minutes.
13) When stable, read luminescence as I_0.
14) Add 100 µL of sample (in the case of controls, add diluent) in each well for a final volume of 200 µL.
15) Incubate for 15 minutes at 15°C.
16) Read luminescence as I_{15}.

PROTOCOL FOR THE ESTABLISHMENT OF BACTERIAL MASTER AND WORK BANKS

Reagents and materials

- Strains to be preserved.
- Nutrient agar
- Nutrient broth with glycerol (40%)
- Disposable sterile 1-µL and 10-µL loops
- Incubator
- Microbiological safety cabinet level II
- Autoclave
- 2-mL cryovials

Procedure

Preparation of culture media

1) Prepare 2 plates of nutrient agar per strain following the manufacturer's instructions.
2) Prepare 4 mL of nutrient broth containing glycerol at a final concentration of 40% per strain, and transfer aliquots of 1 mL into 2-mL cryovials.

Establishment of master bank

3) Streak the strain to be preserved onto nutrient agar (plate ①) and incubate at 30°C overnight.
4) Take an isolated colony from ① and streak it on nutrient agar (plate ②). Incubate at 30°C overnight.
5) Scrap 50% of the biomass in ① using a 10-µL disposable loop and suspend it in a 2-mL cryovial containing 1 mL of nutrient broth and glycerol (40%).
6) Scrap the remaining biomass in ① and suspend it in a second cryovial with nutrient broth and glycerol.
7) Vortex the cryovials for 30 seconds.
8) Store in an appropriate rak and freeze at -80°C.

Establishment of work bank

9) Scrap 50% of the biomass in ② using a 10-µL disposable loop and suspend it in a 2-mL cryovial containing 1 mL of nutrient broth and glycerol (40%).
10) Scrap the remaining biomass in ② and suspend it in a second cryovial with nutrient broth and glycerol.
11) Vortex the cryovials for 30 seconds.
12) Store in an appropriate rak and freeze at -80°C.

Quality control of master and work banks

13) After 24 hours at -80°C, randomly select a number of vials corresponding to the 10% of the total of vials.
14) Determine viability by scraping frozen biomass from the surface of the frozen suspension using a 1-µL disposable loop. Streak the biomass on nutrient agar and incubate at 30°C overnight.
15) Determine purity of strains performing a gram stain.

PROTOCOL FOR THE AGAR DIFFUSION TEST

Reagents and materials

- Test chemicals (4-methyl-1-cyclohexanecarboxylic acid, dicyclohexylacetic acid, phenol, *o*-cresol, benzo(*a*)pyrene, toluene, phenanthrene, pristane, dotriacontane, 1,4,6,7-tetramethylnaphthalene).
- Test strains (vials from work bank).
- 6-mm diameter filter paper discs.
- Methanol, hexane, pentane, dichloromethane.
- 10-mL volumetric flasks.
- Dark 20-mL vials with screw lid.
- Pasteur pipettes.
- Freezer at -20°C.

Procedure

Preparation of stock and intermediate solutions of test chemicals

1) For stock solutions (10000 µg/mL), transfer 50 mg of each chemical to separate 10-mL volumetric flasks and add the corresponding solvent up to the 10-mL mark, making sure that complete dissolution is achieved.
2) Transfer to dark 20-mL vials with screw lid and store at -20°C.
3) For intermediate solutions, transfer 1 and 0.1 mL of the stock solution to separate 10-mL volumetric flasks for 1000 and 100 µg/mL solutions, respectively. Add the corresponding solvent up to the 10-mL mark, making sure that complete dissolution is achieved.
4) Transfer to dark 20-mL vials with screw lid and store at -20°C.

Preparation of discs containing test chemicals

5) Apply 50 µL of each chemical solution on separate sterile 6 mm diameter filter paper discs to obtain final amounts of 500, 50, and 5 µg of chemical per disc.
6) Dry discs in a sterile biological hood for 24 hours at room temperature. Prepare control discs with solvents only.

Preparation of bacterial inoculum

7) Scrap biomass from the surface of a cryovial from the working bank using a 1-µL

disposable loop.

8) Inoculate a test tube containing 10 mL of nutrient broth and incubate overnight at 250 rpm and 30°C.

Preparation of nutrient agar plates for the test

9) Prepare the nutrient agar following the manufacturer's instructions in the same day of the test.
10) After autoclaving, dispense exactly 20 mL of agar in each petri dish, measuring the volume with a sterile graduated 20-mL pipette.
11) Wait until solid and do not place the plates in the fridge.

Swabbing of bacteria on nutrient agar plates

12) Dip a sterile cotton swab into the 10-mL bacterial culture and uniformly inoculate the surface of nutrient agar plates.
13) Allow the plate to dry for approximately 5 minutes.

Placing of chemical-containing discs on the nutrient agar plates

14) Using sterile forceps, place 3 discs per plate, evenly distributed on the agar.
15) Gently press each disc to the agar to ensure that the disc is attached to the agar.
16) Incubate overnight at 30°C.

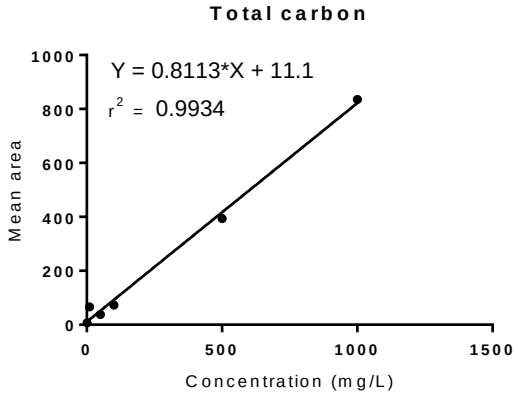
Detection and measurement of inhibition areas

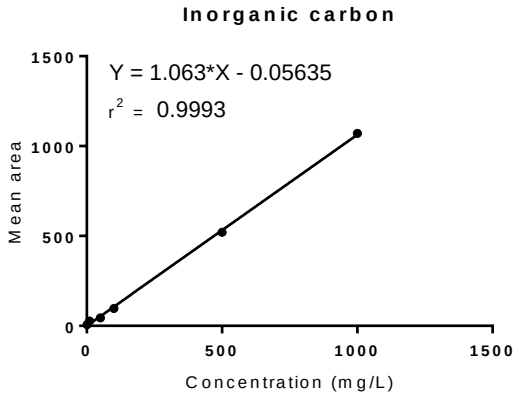
17) Measure the diameter of inhibition areas in mm for each strain when exposed to each test chemical.
18) Report as tolerant or sensitive, the latter including the corresponding diameter of the inhibition area. Inhibition is considered positive when the diameter of the inhibition zone is more than 2 mm larger than the diameter of the paper disc.

APPENDIX II: CALIBRATION CURVES

TOC DETERMINATION IN EFFLUENT SAMPLES

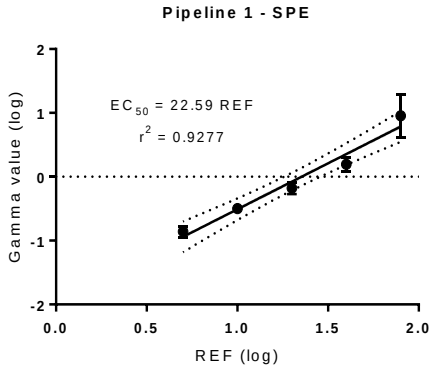
Parameter	Total Carbon	Inorganic Carbon
Furnace Temp. (°C)	680	680
No. of injections	2/3	2/3
No. of wash	2	2
Vol. injected (µL)	27	20
Maximum SD	0.1	0.1

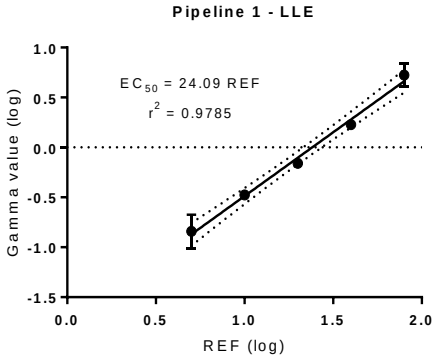


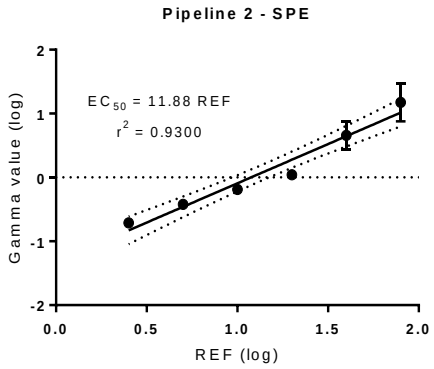


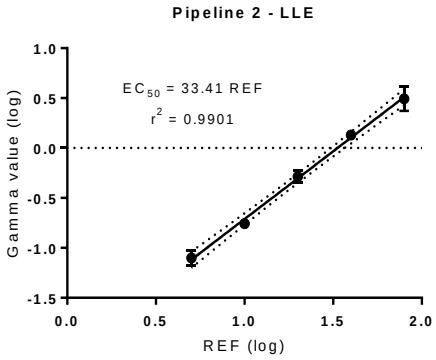
DETERMINATION OF EC_{50} VALUES FOR PHENOL AND EFFLUENT SAMPLES USING THE LUMINESCENT BACTERIA TEST

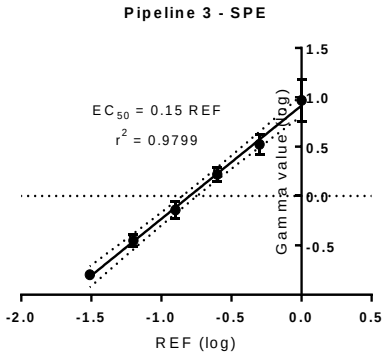
Log-log linear regression of gamma value vs. concentration of sample (relative enrichment factor - REF) for effluent samples (pipeline 1, 2, and 3):

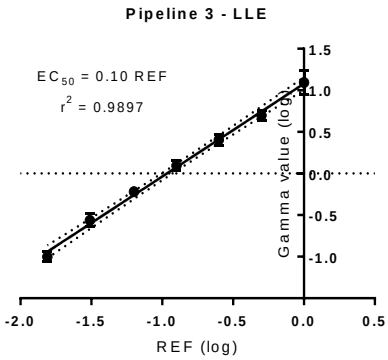




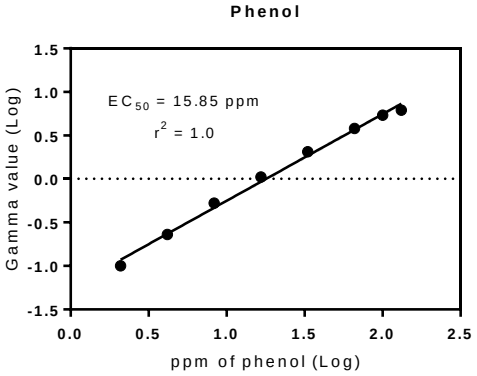






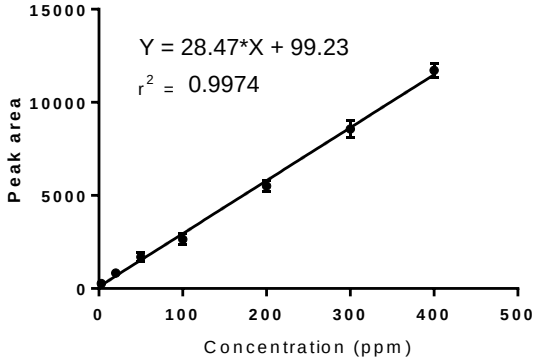


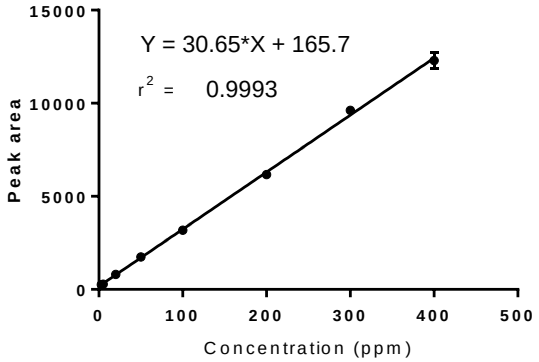
Log-log linear regression of gamma value vs. concentration of phenol (mg/L):

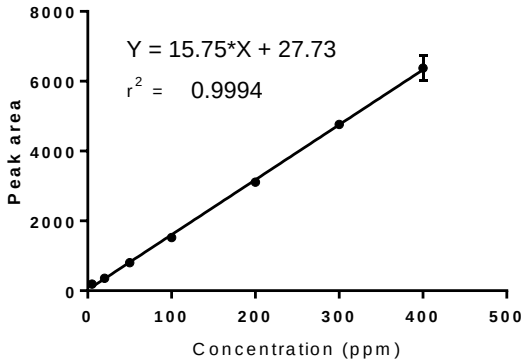


QUANTIFICATION OF MODEL NAS IN BIODEGRADATION EXPERIMENTS

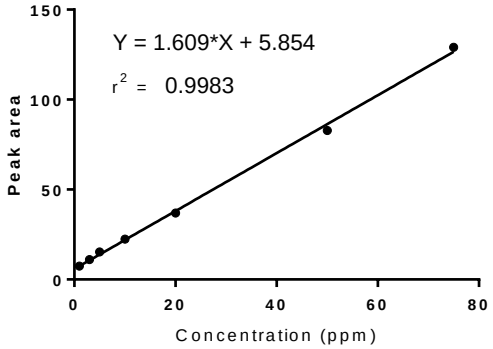
Calibration curves for quantification of NA1:

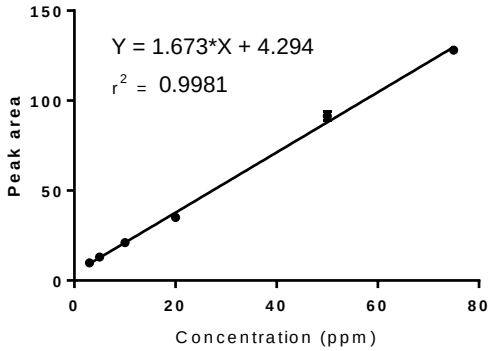




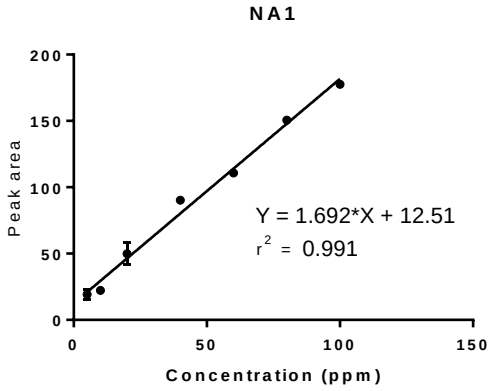


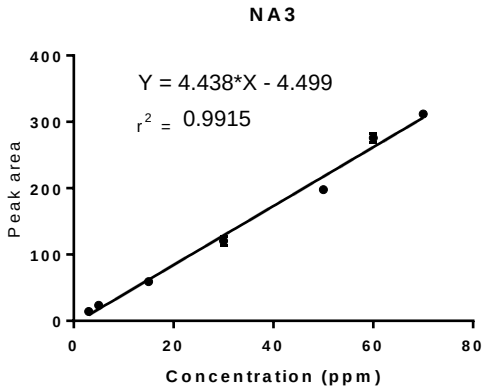
Calibration curves for quantification of NA3:





QUANTIFICATION OF MODEL NAS IN FE-TAML/H_2O_2 EXPERIMENTS





APPENDIX III: SUPPLEMENTARY DATA FOR TOXICITY ASSESSMENTS

INHIBITION EFFECT OF SPE AND BNA EXTRACTS ON *Vibrio fischeri* IN THE LUMINESCENT BACTERIA TEST

For all tables below, REF refers to relative enrichment factor, H_{15} is the inhibitory effect of the test sample after a contact time of 15 minutes, RSD refers to relative standard deviation (%), and Γ_{15} is the gamma value of the test sample after 15 minutes. H_{15} and Γ_{15} were used for the determination of EC_{50} values.

Data for pipeline 1 are shown in the table below; data used to calculate EC_{50} values are greyed out:

REF	Replicate	SPE H_{15}	SPE Average H_{15}	SPE RSD (n=2)	SPE Γ_{15}	LLE H_{15}	LLE Average H_{15}	BNA RSD (n=2)	LLE Γ_{15}
80	1	91.75	93.96	2.29	15.56	86.82	86.41	15.06	6.36
		94.10				86.01			
	2	96.04	83.88	3.84	5.20	84.43	81.53	11.66	4.41
		91.49				78.63			
40	1	64.11	65.27	0.88	1.88	63.88	63.04	1.89	1.71
		64.93				62.20			
	2	66.78	56.55	8.60	1.30	65.85	62.71	7.07	1.68
		59.90				59.58			
20	1	42.96	43.43	1.08	0.77	42.99	42.24	2.53	0.73
		43.63				41.48			
	2	43.70	36.30	27.69	0.57	40.25	39.94	1.10	0.66
		40.33				39.63			
10	1	24.86	26.38	1.20	0.36	23.66	23.41	1.54	0.31
		25.31				23.15			
	2	28.97	21.82	16.53	0.28	25.85	26.55	3.72	0.36
		23.87				27.25			
5	1	12.63	13.80	3.66	0.16	10.67	9.90	34.51	0.11
		13.34				9.13			

REF	Replicate	SPE H_{15}	SPE Average H_{15}	SPE RSD (n=2)	SPE Γ_{15}	LLE H_{15}	LLE Average H_{15}	BNA RSD (n=2)	LLE Γ_{15}
	2	15.44	10.69	24.40	0.12	15.83	15.71	39.70	0.19
		11.75				15.59			
2.5	1	4.86	5.56	21.30	0.06	4.13	3.84	10.69	0.04
		4.89				3.55			
	2	6.93	5.00	19.85	0.05	4.91	2.80	106.32	0.03
		5.52				0.70			
1.25	1	2.56	3.41	12.99	0.04	1.24	1.33	9.29	0.01
		3.19				1.42			
	2	4.48	5.39	7.75	0.06	4.14	3.49	26.35	0.04
		3.89				2.84			
0.625	1	7.79	7.53	4.49	0.08	6.27	5.82	10.98	0.06
		7.31				5.37			
	2	7.48	5.90	59.71	0.06	0.35	-3.67	155.08	-0.04
		8.34				-7.70			
0.31	1	1.86	1.63	30.25	0.02	0.30	-0.52	179.72	-0.01
		1.16				-1.35			
	2	1.88	1.11	59.68	0.01	-4.52	-4.28	86.67	-0.04
		0.94				-4.04			
0.15	1	0.51	0.81	38.05	0.01	0.25	-0.17	347.54	0.00
		0.94				-0.60			
	2	0.98	7.36	8.05	0.08	2.13	0.90	193.23	0.01
		0.14				-0.33			
0.07	1	-0.03	-0.74	84.24	-0.01	-0.72	-0.95	35.13	-0.01
		-0.91				-1.19			
	2	-1.28	-0.98	0.64	-0.01	0.90	-2.00	204.94	-0.02
		-1.27				-4.90			
0.03	1	-0.39	36.69	2.19	0.58	-1.56	-1.51	4.77	-0.01

REF	Replicate	SPE H_{15}	SPE Average H_{15}	SPE RSD (n=2)	SPE Γ_{15}	LLE H_{15}	LLE Average H_{15}	BNA RSD (n=2)	LLE Γ_{15}
		-1.53				-1.46			
	2	-0.70	28.43	0.33	0.40	-8.54	-4.13	151.27	-0.04
		-0.84				0.29			

Data for pipeline 2 are shown in the table below; data used to calculate EC_{50} values are shown in grey:

REF	Replicate	SPE H_{15}	SPE Average H_{15}	SPE RSD (n=2)	SPE Γ_{15}	LLE H_{15}	LLE Average H_{15}	LLE RSD (n=2)	LLE Γ_{15}
80	1	99.99	96.03	6.38	24.22	81.27	79.08	4.88	3.78
		99.14				81.34			
	2	88.97	90.22	0.98	9.22	74.62	71.86	10.98	2.55
		90.22				85.77			
40	1	91.45	86.77	3.30	6.56	55.19	58.77	0.43	1.43
		87.40				54.83			
	2	81.47	76.06	5.16	3.18	66.30	55.89	1.37	1.27
		87.02				67.38			
20	1	59.68	53.85	5.21	1.17	33.99	36.18	0.50	0.57
		55.71				33.73			
	2	46.14	50.88	15.30	1.04	40.81	31.96	48.69	0.47
		59.86				41.08			
10	1	46.63	41.83	12.80	0.72	13.99	15.16	9.45	0.18
		39.06				11.97			
	2	39.79	36.75	2.04	0.58	19.53	14.83	3.11	0.17
		40.85				20.19			
5	1	29.61	28.47	9.62	0.40	4.77	6.30	7.85	0.07
		25.74				5.47			
	2	30.05	26.65	10.85	0.36	8.66	8.30	10.97	0.09
		34.14				7.37			

REF	Replicate	SPE H_{15}	SPE Average H_{15}	SPE RSD (n=2)	SPE Γ_{15}	LLE H_{15}	LLE Average H_{15}	LLE RSD (n=2)	LLE Γ_{15}
2.5	1	15.77	15.31	16.66	0.18	8.86	12.83	27.33	0.15
		12.56				14.16			
	2	17.60	17.17	28.85	0.21	15.49	7.75	60.54	0.08
		10.60				8.85			
1.25	1	23.31	13.31	53.94	0.15	-1.10	-1.22	41.53	-0.01
		13.16				-1.82			
	2	3.46	3.89	76.44	0.04	-0.73	2.31	380.95	0.02
		7.66				11.69			
0.625	1	0.54	-0.27	884.42	0.00	-4.04	-4.11	7.85	-0.04
		-2.88				-4.49			
	2	1.52	0.64	159.58	0.01	-3.79	-4.32	15.15	-0.04
		0.89				-5.05			
0.31	1	-0.48	-1.78	56.51	-0.02	-4.13	-4.41	8.42	-0.04
		0.94				-4.65			
	2	-5.79	-6.64	25.48	-0.06	-4.46	-4.67	20.94	-0.04
		-8.19				-5.84			
0.15	1	-5.94	-5.39	3.97	-0.05	-3.71	-4.17	10.77	-0.04
		-5.64				-4.35			
	2	-4.60	5.33	53.83	0.06	-4.47	-3.21	6.73	-0.03
		-8.66				-4.77			
0.07	1	-2.38	-0.95	4.17	-0.01	-3.34	-0.31	1828.07	0.00
		-2.44				4.68			
	2	1.97	-0.11	3099.94	0.00	-2.28	-3.24	12.24	-0.03
		-2.68				-2.84			
0.03	1	0.40	35.20	5.18	0.54	-4.62	-4.77	30.21	-0.05
		-2.18				-6.65			
	2	-2.36	26.41	1.27	0.36	-5.36	-3.90	53.04	-0.04

REF	Replicate	SPE H_{15}	SPE Average H_{15}	SPE RSD (n=2)	SPE Γ_{15}	LLE H_{15}	LLE Average H_{15}	LLE RSD (n=2)	LLE Γ_{15}
		-2.84				-2.43			

Data for pipeline 3 are shown in the table below; data used to calculate EC_{50} values are greyed out:

REF	Replicate	SPE H_{15}	SPE Average H_{15}	SPE RSD (n=2)	SPE Γ_{15}	LLE H_{15}	LLE Average H_{15}	LLE RSD (n=2)	LLE Γ_{15}
1	1	94.96	92.96	2.67%	13.21	93.40	94.02	1.14	15.72
		93.74				95.26			
	2	90.18	86.78	1.08%	6.57	93.39	90.73	1.78	9.78
		88.86				95.67			
0.5	1	81.30	79.71	2.83%	3.93	83.11	84.66	2.31	5.52
		78.11				85.88			
	2	79.70	73.99	3.74%	2.84	84.98	81.86	2.34	4.51
		75.79				87.70			
0.25	1	66.47	65.19	5.43%	1.87	72.90	73.95	1.95	2.84
		61.47				74.94			
	2	67.64	59.46	18.46%	1.47	74.02	69.25	17.37	2.25
		63.76				78.17			
0.13	1	46.99	45.44	7.20%	0.83	55.57	57.83	5.32	1.37
		42.36				59.92			
	2	46.97	38.63	11.59%	0.63	57.99	52.92	7.13	1.12
		40.64				63.33			
0.06	1	28.29	28.06	6.37%	0.39	37.43	39.53	7.24	0.65
		25.76				41.47			
	2	30.13	24.06	7.34%	0.32	39.69	35.71	12.11	0.56
		27.63				45.80			
0.03	1	14.40	14.29	9.21%	0.17	21.63	23.72	8.05	0.31

		12.92				25.37			
	2	15.54	12.74	3.06%	0.15	24.17	19.35	2.19	0.24
		14.99				24.77			
0.02	1	7.70	9.18	34.37%	0.10	9.10	9.99	16.99	0.11
		12.16				11.50			
	2	7.67	5.97	9.23	0.06	9.37	7.91	33.86	0.09
		6.89				13.16			
0.008	1	3.34	3.21	19.13	0.03	1.19	1.84	49.41	0.02
		2.47				2.47			
	2	3.81	2.39	56.22	0.02	1.85	1.35	284.34	0.01
		2.25				4.93			
0.004	1	1.13	1.65	31.00	0.02	-2.72	-2.86	12.15	-0.03
		1.85				-2.23			
	2	1.97	1.52	6.87	0.02	-3.63	-3.91	13.50	-0.04
		2.11				-2.89			
0.002	1	0.49	0.79	75.38	0.01	-5.22	-4.82	15.05	-0.05
		1.34				-4.20			
	2	0.55	0.43	21.56	0.00	-5.04	-5.67	5.90	-0.05
		0.42				-5.52			
0.001	1	0.32	0.49	56.34	0.00	-6.44	-5.83	10.25	-0.06
		0.33				-5.25			
	2	0.80	0.15	235.29	0.00	-5.79	-5.98	5.54	-0.06
		0.29				-6.26			
0.0001	1	-0.64	-0.88	24.81	-0.01	-5.89	-5.04	11.66	-0.05
		-0.33				-5.06			
	2	-1.69	-1.88	13.71	-0.02	-4.18	-6.16	45.48	-0.06
		-2.06				-8.14			

INHIBITION EFFECT OF RWW ON *Vibrio fischeri* IN THE LUMINESCENT BACTERIA TEST AFTER Fe-TAML/H_2O_2 EXPERIMENTS

For all tables below, H_{15} is the inhibitory effect of the test sample after a contact time of 15 minutes, RSD refers to relative standard deviation (%), and Γ_{15} is the gamma value of the test sample after 15 minutes. H_{15} and Γ_{15} were used for the determination of EC_{50} values.

Water, T_{72}, control:

Concentration (%)	Replicate	H_{15}	Average H_{15}	RSD (n=2)	Γ_{15}
50	1	11.60	11.87	6.58	0.13
		12.14			
	2	3.84	3.07	102.89	0.03
		2.29			
33.3	1	10.60	12.16	18.17	0.14
		13.73			
	2	-2.23	-2.48	14.25	-0.02
		-2.73			
25	1	3.01	5.19	59.43	0.05
		7.36			
	2	-3.25	-3.28	1.39	-0.03
		-3.31			
16.7	1	4.59	7.07	49.62	0.08
		9.55			
	2	-6.38	-7.13	14.78	-0.07
		-7.87			
12.5	1	0.79	1.67	74.71	0.02
		2.55			
	2	-5.95	-3.75	83.19	-0.04
		-1.54			
8.3	1	3.75	5.83	77.32	0.06
		7.91			
	2	-9.83	-9.93	7.64	-0.09
		-10.04			
6.3	1	-1.10	2.92	194.64	0.03
		6.93			
	2	-8.63	-8.83	3.14	-0.08
		-9.02			
4.2	1	2.31	4.08	61.14	0.04
		5.84			
	2	-11.90	-12.80	9.93	-0.11
		-13.70			

Concentration (%)	Replicate	H_{15}	Average H_{15}	RSD (n=2)	Γ_{15}
3.12	1	-12.46	-15.83	30.11	-0.14
		-19.20			
	2	-12.12	-11.09	13.15	-0.10
		-10.06			
2.1	1	6.21	5.97	5.60	0.06
		5.73			
	2	-14.22	-13.63	6.16	-0.12
		-13.04			
1.56	1	-1.35	0.67	367.06	0.01
		2.70			
	2	-17.32	-16.94	2.69	-0.14
		-16.55			
1.05	1	3.14	3.92	28.11	0.04
		4.70			
	2	-17.35	-16.60	6.33	-0.14
		-15.86			
0.78	1	2.81	0.70	423.43	0.01
		-1.40			
	2	-14.55	-15.13	5.39	-0.13
		-15.71			
0.52	1	2.08	4.10	69.46	0.04
		6.11			
	2	-17.72	-17.63	0.76	-0.15
		-17.53			
0.39	1	4.08	3.76	12.12	0.04
		3.44			
	2	-18.17	-18.18	0.06	-0.15
		-18.18			
0.26	1	3.99	6.24	51.06	0.07
		8.49			
	2	-18.08	-17.00	8.99	-0.15
		-15.92			

Pipeline 3, T_{72}, Control:

Concentration (%)	Replicate	H_{15}	Average H_{15}	RSD (n=2)	Γ_{15}
50	1	94.21	94.20	2.59	16.25
		94.20			
	2	93.84	93.08	2.17	13.46
		94.62			
33.3	1	89.97	89.94	0.05	8.94

Concentration (%)	Replicate	H_{15}	Average H_{15}	RSD (n=2)	Γ_{15}
		89.91			
	2	90.79	88.94	1.28	8.04
		89.19			
25	1	83.27	84.51	2.06	5.45
		85.74			
	2	86.83	85.21	0.37	5.76
		87.27			
16.7	1	79.94	78.82	2.02	3.72
		77.69			
	2	81.54	80.27	0.37	4.07
		81.96			
12.5	1	72.96	70.85	4.20	2.43
		68.75			
	2	77.33	73.90	0.92	2.83
		76.37			
8.3	1	59.81	62.90	16.76	1.70
		65.99			
	2	68.01	67.20	5.92	2.05
		70.71			
6.3	1	45.45	46.56	3.37	0.87
		47.67			
	2	62.88	57.07	1.28	1.33
		61.84			
4.2	1	40.09	45.36	16.44	0.83
		50.64			
	2	46.49	48.38	14.60	0.94
		56.48			
3.12	1	31.59	38.54	25.50	0.63
		45.49			
	2	42.18	40.39	4.31	0.68
		44.65			
2.1	1	36.48	32.27	18.46	0.48
		28.05			
	2	34.33	34.34	7.15	0.52
		37.80			
1.56	1	27.87	28.20	11.94	0.39
		28.54			
	2	30.89	27.09	24.27	0.37
		30.88			
1.05	1	22.40	22.45	0.33	0.29
		22.50			
	2	19.50	20.51	14.76	0.26
		23.78			

Concentration (%)	Replicate	H_{15}	Average H_{15}	RSD (n=2)	Γ_{15}
0.78	1	15.76	14.76	9.60	0.17
		13.76			
	2	18.25	16.71	3.98	0.20
		19.19			
0.52	1	13.27	11.92	16.00	0.14
		10.57			
	2	12.69	14.05	21.45	0.16
		16.96			
0.39	1	11.16	11.01	1.95	0.12
		10.86			
	2	12.51	10.46	2.57	0.12
		12.12			
0.26	1	7.88	7.48	7.69	0.08
		7.07			
	2	6.74	10.32	48.98	0.12
		13.89			

Pipeline 3, T_{72}, Uncatalysed control (H_2O_2 only):

Concentration (%)	Replicate	H_{15}	Average H_{15}	RSD (n=2)	Γ_{15}
50	1	90.69	91.40	2.99	10.63
		92.11			
	2	88.99	89.42	1.77	8.45
		89.85			
33.3	1	86.82	86.63	0.32	6.48
		86.43			
	2	86.79	81.34	9.47	4.36
		75.90			
25	1	75.54	78.37	5.10	3.62
		81.19			
	2	84.93	84.59	0.56	5.49
		84.25			
16.7	1	79.18	79.12	0.12	3.79
		79.05			
	2	78.35	75.34	5.65	3.06
		72.33			
12.5	1	75.14	74.77	0.71	2.96
		74.39			
	2	70.66	68.04	5.45	2.13
		65.42			
8.3	1	65.30	66.23	5.15	1.96
		67.15			
	2	59.50	61.13	10.40	1.57

Concentration (%)	Replicate	H_{15}	Average H_{15}	RSD (n=2)	Γ_{15}
		62.77			
6.3	1	60.54	61.00	1.08	1.56
		61.47			
	2	50.50	49.48	2.90	0.98
		48.47			
4.2	1	45.68	48.87	9.24	0.96
		52.06			
	2	23.95	33.95	41.66	0.51
		43.95			
3.12	1	43.59	43.37	0.69	0.77
		43.16			
	2	29.47	37.11	29.12	0.59
		44.75			
2.1	1	33.82	33.07	3.18	0.49
		32.33			
	2	32.94	28.34	22.94	0.40
		23.75			
1.56	1	4.65	13.89	66.62	0.16
		23.14			
	2	31.14	24.80	69.95	0.33
		18.45			
1.05	1	13.07	14.49	13.79	0.17
		15.90			
	2	-3.17	-1.50	157.66	-0.01
		0.17			
0.78	1	18.69	19.00	2.26	0.23
		19.30			
	2	2.87	3.60	28.62	0.04
		4.32			
0.52	1	5.62	10.78	67.70	0.12
		15.94			
	2	4.97	10.50	74.56	0.12
		16.04			
0.39	1	8.35	8.22	2.14	0.09
		8.10			
	2	11.69	10.17	21.21	0.11
		8.64			
0.26	1	5.95	8.84	46.24	0.10
		11.74			
	2	7.49	9.04	24.32	0.10
		10.60			

Pipeline 3, T_{72}, Catalysed reaction (**1a** + H_2O_2):

Concentration (%)	Replicate	H_{15}	Average H_{15}	RSD (n=2)	Γ_{15}
50	1	69.98	69.71	6.42	2.30
		69.44			
	2	74.33	76.39	7.00	3.24
		78.45			
33.3	1	61.98	62.07	0.21	1.64
		62.16			
	2	67.84	67.16	1.44	2.04
		66.47			
25	1	48.25	50.60	6.56	1.02
		52.94			
	2	59.59	60.39	1.87	1.52
		61.19			
16.7	1	43.86	44.03	0.56	0.79
		44.20			
	2	49.04	49.97	2.62	1.00
		50.89			
12.5	1	33.52	33.14	1.62	0.50
		32.76			
	2	39.64	40.94	4.50	0.69
		42.25			
8.3	1	26.21	26.57	25.80	0.36
		26.93			
	2	30.65	32.19	17.67	0.47
		33.72			
6.3	1	14.71	15.36	5.98	0.18
		16.01			
	2	22.70	22.99	1.77	0.30
		23.28			
4.2	1	10.60	12.19	18.48	0.14
		13.79			
	2	18.71	19.18	3.47	0.24
		19.65			
3.12	1	8.09	8.00	1.60	0.09
		7.91			
	2	10.15	11.55	17.04	0.13
		12.94			
2.1	1	2.99	4.31	43.24	0.05
		5.62			
	2	9.38	10.12	10.34	0.11
		10.86			
1.56	1	2.07	2.65	58.70	0.03
		3.23			

Concentration (%)	Replicate	H_{15}	Average H_{15}	RSD (n=2)	Γ_{15}
	2	1.48	2.47	40.04	0.03
		3.46			
1.05	1	0.15	0.69	111.14	0.01
		1.23			
	2	2.39	3.54	46.18	0.04
		4.70			
0.78	1	-0.04	0.83	147.91	0.01
		1.69			
	2	-1.48	-4.36	93.29	-0.04
		-7.23			
0.52	1	0.11	0.63	116.32	0.01
		1.15			
	2	-12.76	-4.95	222.79	-0.05
		2.85			
0.39	1	-0.66	-0.03	3131.96	0.00
		0.60			
	2	-0.23	0.14	379.20	0.00
		0.51			
0.26	1	-0.11	-0.02	843.18	0.00
		0.08			
	2	1.63	3.67	78.75	0.04
		5.71			

APPENDIX IV: SUPPLEMENTARY DATA FOR QUANTIFICATION OF NAs

QUANTIFICATION OF RESIDUAL NAs IN M9 AFTER BIODEGRADATION BY CONSORTIUM OF *Pseudomonas putida*

For all tables below, RT refers to retention time, PA refers to peak area, and RSD refers to relative standard deviation (%). Control A = Abiotic loss; Control B = Biomass adsorption. Treatments = Pre-exposure, No pre-exposure. Concentrations = 100 and 350 mg/L for NA1; 30 and 70 mg/L for NA3.

NA1 at 100 mg/L after 30 days of biodegradation:

Control A:

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	2.438	3210.2	108.90	105.32	3.47
	2	2.459	3002.8	101.59		
	3	2.397	3112.3	105.45		
3	1	2.410	3125.6	105.92	110.20	3.90
	2	2.400	3245.9	110.16		
	3	2.402	3369.7	114.53		
6	1	2.359	3287.9	111.64	111.21	0.94
	2	2.422	3297.1	111.97		
	3	2.465	3241.9	110.02		
9	1	2.369	2989.3	101.12	102.84	6.91
	2	2.489	2865.7	96.76		
	3	2.587	3259.8	110.65		
12	1	2.400	3451	111.26	103.16	6.81
	2	2.396	3089.5	98.68		
	3	2.456	3114.9	99.56		
15	1	2.419	3248.7	104.22	108.39	4.02
	2	2.389	3358.1	108.02		
	3	2.998	3498.8	112.92		
18	1	2.402	3198.5	102.47	103.14	0.57
	2	2.418	3226.5	103.44		
	3	2.391	3228.7	103.52		
21	1	2.375	3368.1	108.37	109.75	1.33
	2	2.405	3402.9	109.58		
	3	2.412	3451.9	111.29		
24	1	2.398	3523.8	113.79	107.26	9.54
	2	2.406	3487.5	112.53		
	3	2.412	2997.5	95.48		

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
27	1	2.402	3258.4	104.55	108.48	3.67
	2	2.413	3368.2	108.37		
	3	2.411	3487.1	112.51		
30	1	2.423	1646.2	102.75	109.98	6.74
	2	2.418	1879.6	117.58		
	3	2.583	1754.2	109.61		

Control B:

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	2.420	3256.2	110.53	106.68	4.67
	2	2.435	3197.6	108.46		
	3	2.423	2987.4	101.05		
3	1	2.429	2876.5	97.14	101.51	3.76
	2	2.437	3045.8	103.11		
	3	2.420	3078.5	104.26		
6	1	2.419	3521.6	119.88	114.82	7.02
	2	2.425	3498.5	119.07		
	3	2.412	3114.3	105.52		
9	1	2.411	3256.9	110.55	110.50	3.19
	2	2.419	3154.7	106.95		
	3	2.415	3354.9	114.01		
12	1	2.420	3215.2	103.05	104.57	1.73
	2	2.405	3316.4	106.57		
	3	2.416	3245.1	104.09		
15	1	2.411	3169.5	101.46	107.24	4.94
	2	2.409	3468.5	111.86		
	3	2.421	3368.9	108.40		
18	1	2.398	3178.2	101.76	100.48	4.49
	2	2.400	2997.1	95.46		
	3	2.498	3248.6	104.21		
21	1	2.415	3156.2	101.00	99.04	3.42
	2	2.430	2987.6	95.13		
	3	2.409	3156.4	101.00		
24	1	2.415	3388.6	109.08	103.88	4.67
	2	2.411	3215.6	103.06		
	3	2.412	3112.8	99.49		
27	1	2.422	1569.3	97.87	105.93	4.46

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	2	2.623	1748.7	109.26		
	3	2.420	1643.6	102.59		
30	1	2.403	1698.5	106.07	101.58	3.85
	2	2.408	1586.3	98.95		
	3	2.402	1598.4	99.72		

No pre-exposure:

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	2.412	3498.6	119.07	113.67	4.43
	2	2.421	3321.3	112.82		
	3	2.420	3215.9	109.11		
3	1	2.411	3345.7	113.68	113.17	1.74
	2	2.423	3378.5	114.84		
	3	2.409	3269.8	111.01		
6	1	2.408	3169.4	107.47	101.88	5.27
	2	2.406	2997.5	101.41		
	3	2.405	2865.4	96.75		
9	1	2.416	3347.9	113.76	110.55	3.05
	2	2.417	3265.8	110.86		
	3	2.405	3156.9	107.03		
12	1	2.419	3154.8	100.95	101.29	6.37
	2	2.422	2984.2	95.01		
	3	2.406	3354.8	107.91		
15	1	2.405	3169.4	101.46	102.15	1.81
	2	2.413	3148.9	100.74		
	3	2.420	3249.5	104.24		
18	1	2.411	3165.9	101.34	106.59	4.79
	2	2.416	3326.5	106.92		
	3	2.407	3458.7	111.52		
21	1	2.415	3568.1	115.33	114.66	1.61
	2	2.403	3489	112.58		
	3	2.410	3589.6	116.08		
24	1	2.408	3465.2	111.75	110.81	2.17
	2	2.403	3359.8	108.08		
	3	2.402	3489.7	112.60		
27	1	2.423	1659.2	103.58	107.69	7.23
	2	2.420	1865.3	116.67		

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	3	2.421	1647.2	102.82		
30	1	2.403	1756.3	109.75	105.41	3.58
	2	2.402	1658.9	103.56		
	3	2.403	1648.7	102.91		

Pre-exposure:

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	2.406	1547.35	96.48	99.45	2.72
	2	2.398	1630.60	101.76		
	3	2.398	1604.55	100.11		
3	1	2.398	1639.55	102.33	102.59	0.81
	2	2.403	1633.10	101.92		
	3	2.401	1658.25	103.52		
6	1	2.397	1637.35	102.19	106.15	3.53
	2	2.399	1707.25	106.63		
	3	2.398	1754.55	109.63		
9	1	2.395	1749.95	109.34	111.55	4.20
	2	2.400	1734.90	108.39		
	3	2.399	1869.45	116.93		
12	1	2.396	1753.00	109.54	109.56	1.57
	2	2.399	1726.40	107.85		
	3	2.401	1780.7	111.29		
15	1	2.404	1627.2	101.55	104.19	3.17
	2	2.405	1651.95	103.12		
	3	2.405	1727.2	107.90		
18	1	2.405	1784.6	111.54	109.68	2.73
	2	2.402	1701.05	106.24		
	3	2.401	1780.4	111.28		
21	1	2.402	1809.45	113.12	113.61	3.96
	2	2.405	1750.35	109.37		
	3	2.404	1891.45	118.33		
24	1	2.400	1822.05	113.92	113.20	6.27
	2	2.400	1693.8	105.78		
	3	2.404	1916.4	119.91		
27	1	2.403	1941.25	121.49	123.47	2.22
	2	2.406	2021.8	126.61		
	3	2.401	1954.25	122.32		

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
30	1	2.401	1872.65	117.13	116.57	0.69
	2	2.402	1849.15	115.64		
	3	2.402	1869.5	116.93		

NA1 at 350 mg/L after 30 days of biodegradation:

Control A:

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	2.412	10456.0	364.29	351.96	3.08
	2	2.402	9986.3	347.74		
	3	2.418	9876.2	343.85		
3	1	2.403	10265.3	357.57	357.62	1.99
	2	2.405	10469.8	364.78		
	3	2.404	10065.2	350.52		
6	1	2.411	9978.6	347.46	353.71	1.95
	2	2.41	10123.3	352.56		
	3	2.409	10365.8	361.11		
9	1	2.416	10456.9	364.32	362.23	2.30
	2	2.417	10598.7	369.32		
	3	2.405	10136.9	353.04		
12	1	2.418	10569.5	358.94	360.35	0.43
	2	2.403	10657.2	361.99		
	3	2.401	10603.1	360.11		
15	1	2.400	10012.9	339.57	353.73	3.48
	2	2.413	10587.4	359.56		
	3	2.411	10659.2	362.06		
18	1	2.406	10469.3	355.45	356.87	0.74
	2	2.408	10597.8	359.93		
	3	2.409	10463.2	355.24		
21	1	2.415	10639.6	361.38	358.24	0.88
	2	2.411	10459.3	355.11		
	3	2.407	10549.2	358.23		
24	1	2.408	10469.3	355.45	358.10	0.66

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	2	2.411	10598.8	359.96		
	3	2.416	10567.6	358.88		
27	1	2.421	5639.2	356.33	353.17	1.26
	2	2.422	5539.9	350.02		
	3	2.419	5697.4	360.02		
30	1	2.411	5798.6	366.45	358.97	2.94
	2	2.409	5563.2	351.50		
	3	2.421	5521.1	348.83		

Control B:

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	2.405	10265.3	357.57	355.93	1.35
	2	2.421	10065.3	350.52		
	3	2.402	10325.6	359.69		
3	1	2.406	10369.8	361.25	361.05	0.94
	2	2.405	10456.9	364.32		
	3	2.412	10265.4	357.57		
6	1	2.411	10169.3	354.19	358.01	0.98
	2	2.416	10365.5	361.10		
	3	2.407	10298.8	358.75		
9	1	2.418	10458.9	364.39	361.02	0.95
	2	2.407	10365.2	361.09		
	3	2.406	10265.3	357.57		
12	1	2.411	10254.3	347.97	354.08	1.58
	2	2.41	10465.4	355.32		
	3	2.400	10569.7	358.95		
15	1	2.406	10456.6	355.01	356.00	0.78
	2	2.409	10423.1	353.85		
	3	2.41	10574.8	359.13		
18	1	2.407	10499.6	356.51	356.88	1.14
	2	2.405	10632.1	361.12		
	3	2.421	10398.7	353.00		
21	1	2.403	10545.1	358.09	357.75	0.33
	2	2.421	10497.6	356.44		
	3	2.42	10563.3	358.73		
24	1	2.422	10631.7	361.11	358.50	0.85
	2	2.416	10459.8	355.12		

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	3	2.418	10578.8	359.26		
27	1	2.423	5561.5	351.39	357.51	1.77
	2	2.424	5760.9	364.05		
	3	2.426	5651.1	357.08		
30	1	2.405	5521.3	348.84	354.90	2.29
		2.414	5707.3	360.65		
		2.416	5526.3	349.16		

No pre-exposure:

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	2.416	10363.2	361.02	354.93	1.54
	2	2.417	10062.1	350.41		
	3	2.407	10145.9	353.36		
3	1	2.412	9987.3	347.77	350.56	0.90
	2	2.413	10048.7	349.93		
	3	2.406	10163.3	353.97		
6	1	2.405	10265.3	357.57	358.51	0.95
	2	2.404	10398.9	362.28		
	3	2.415	10211.4	355.67		
9	1	2.423	10159.9	353.85	352.66	0.67
	2	2.412	10048.7	349.93		
	3	2.42	10169.6	354.20		
12	1	2.405	10158.8	344.65	350.14	1.85
	2	2.403	10269.3	348.50		
	3	2.408	10521.5	357.27		
15	1	2.422	10526.5	357.45	354.36	0.78
	2	2.418	10374.6	352.16		
	3	2.401	10412.2	353.47		
18	1	2.407	10389.9	352.69	355.91	0.97
	2	2.403	10469.9	355.48		
	3	2.413	10587.1	359.55		
21	1	2.416	10458.8	355.09	354.15	0.55
	2	2.422	10367.1	351.90		
	3	2.421	10469.3	355.45		
24	1	2.407	10125.6	343.50	350.20	1.74
	2	2.406	10359.8	351.64		
	3	2.403	10469.2	355.45		

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
27	1	2.429	5691.9	359.67	361.05	0.59
	2	2.432	5752.4	363.51		
	3	2.428	5696.7	359.98		
30	1	2.412	5548.1	350.54	349.75	1.98
	2	2.414	5421.2	342.48		
	3	2.408	5637.9	356.24		

Pre-exposure:

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	2.411	5180.6	327.20	326.22	0.26
	2	2.407	5158.0	325.77		
	3	2.406	5156.8	325.69		
3	1	2.409	5319.2	336.00	331.26	1.96
	2	2.410	5128.1	323.87		
	3	2.409	5286.4	333.92		
6	1	2.418	5463.9	345.19	357.91	3.24
	2	2.419	5708.0	360.69		
	3	2.418	5820.8	367.85		
9	1	2.420	5577.3	352.39	350.12	2.94
	2	2.419	5364.5	338.88		
	3	2.419	5682.7	359.08		
12	1	2.415	5517.1	348.57	359.46	2.86
	2	2.415	5838.8	369.00		
	3	2.416	5709.7	360.80		
15	1	2.415	6117.4	386.69	385.83	1.08
	2	2.416	6161.5	389.49		
	3	2.419	6032.7	381.31		
18	1	2.419	5887.5	372.09	371.07	2.44
	2	2.420	6005.2	379.56		
	3	2.416	5721.7	361.56		
21	1	2.407	4528.9	285.81	346.22	17.74
	2	2.425	6462.9	408.63		
	3	2.419	5448.6	344.22		
24	1	2.417	5239.9	330.96	338.75	2.68
	2	2.415	5519.6	348.73		
	3	2.414	5328.1	336.57		
27	1	2.416	5358.6	338.51	339.11	1.90

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	2	2.414	5474.1	345.84		
	3	2.414	5271.9	333.00		
30	1	2.405	5605.8	354.20	358.70	2.15
		2.419	5817.0	367.61		
		2.405	5607.0	354.28		

NA3 at 30 mg/L after 30 days of biodegradation:

Control A:

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.63	55.8	30.79	31.29	1.41
	2	4.632	57.2	31.63		
	3	4.633	56.9	31.45		
3	1	4.633	56.6	31.27	31.79	1.42
	2	4.634	57.8	31.99		
	3	4.634	58	32.11		
6	1	4.632	60.6	33.66	33.76	1.53
	2	4.632	60	33.30		
	3	4.632	61.7	34.32		
9	1	4.632	60.6	33.66	33.19	1.25
	2	4.658	59.4	32.95		
	3	4.634	59.4	32.95		
12	1	4.64	62.1	34.56	33.36	3.81
	2	4.639	60.3	33.48		
	3	4.657	57.4	32.03		
15	1	4.633	58.6	32.78	33.17	2.73
	2	4.632	58.2	32.53		
	3	4.632	60.9	34.20		
18	1	4.632	58.5	32.71	32.69	1.24
	2	4.631	57.8	32.28		
	3	4.632	59.1	33.09		
21	1	4.648	60	33.65	33.71	0.18
	2	4.651	60.1	33.71		
	3	4.652	60.2	33.77		
24	1	4.647	62.3	35.07	35.43	0.90
	2	4.643	63	35.51		
	3	4.639	63.3	35.70		
27	1	4.632	58.5	32.74	34.85	5.43

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	2	4.633	62.8	35.39		
	3	4.656	63.6	36.41		
30	1	4.629	58.4	32.65	32.47	3.01
	2	4.629	56.4	31.41		
	3	4.628	59.5	33.34		

Control B:

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.633	55.2	30.44	29.90	2.31
	2	4.632	53.0	29.12		
	3	4.633	54.7	30.14		
3	1	4.633	56.5	31.21	30.51	2.59
	2	4.634	55.6	30.67		
	3	4.633	53.9	29.66		
6	1	4.632	60.0	33.30	33.66	0.99
	2	4.631	60.7	33.72		
	3	4.632	61.1	33.96		
9	1	4.634	55.5	30.61	32.23	4.39
	2	4.634	59.2	32.83		
	3	4.634	59.9	33.25		
12	1	4.633	61.7	34.70	36.05	3.24
	2	4.632	65.0	36.75		
	3	4.632	64.9	36.69		
15	1	4.631	58.8	32.90	32.66	1.62
	2	4.632	59.0	33.02		
	3	4.633	57.9	32.05		
18	1	4.631	58.0	32.40	33.52	2.91
	2	4.633	60.5	33.96		
	3	4.633	60.9	34.20		
21	1	4.657	57.3	31.97	32.22	1.17
	2	4.658	57.4	32.03		
	3	4.659	58.4	32.65		
24	1	4.64	61.4	34.52	34.39	0.36
	2	4.641	61.0	34.27		
	3	4.64	61.2	34.39		
27	1	4.632	64.3	36.44	36.48	0.77
	2	4.632	65.0	36.22		

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	3	4.632	64.9	36.78		
30	1	4.63	61.3	34.45	34.62	0.45
	2	4.629	61.6	34.64		
	3	4.63	61.8	34.76		

No pre-exposure:

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.632	54.7	30.14	30.65	1.87
	2	4.632	55.4	30.55		
	3	4.632	56.6	31.27		
3	1	4.634	58.3	32.29	31.63	3.44
	2	4.633	55.1	30.38		
	3	4.634	58.2	32.23		
6	1	4.632	60.5	33.60	33.44	0.98
	2	4.631	59.6	33.07		
	3	4.632	60.6	33.66		
9	1	4.633	62.9	35.04	33.84	3.93
	2	4.633	58.5	32.41		
	3	4.635	61.3	34.08		
12	1	4.633	42.5	22.77	28.07	16.84
	2	4.633	57.1	31.84		
	3	4.633	53.5	29.61		
15	1	4.633	47	25.53	29.70	12.40
	2	4.633	58.7	32.53		
	3	4.633	56.2	31.03		
18	1	4.631	34.3	17.68	28.01	32.01
	2	4.633	58.4	32.65		
	3	4.633	60.1	33.71		
21	1	4.661	26	12.52	24.39	42.41
	2	4.664	56.5	31.47		
	3	4.665	52.8	29.17		
24	1	4.631	32.4	16.50	26.66	33.19
	2	4.633	58.4	32.65		
	3	4.651	54.6	30.82		
27	1	4.629	41.6	22.31	30.70	23.88
	2	4.632	63.3	35.82		
	3	4.631	60.5	33.99		

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
30	1	4.627	28.3	13.95	24.43	39.98
	2	4.629	59.4	33.27		
	3	4.629	47.8	26.06		

Pre-exposure:

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.653	58.5	32.41	35.04	6.82
	2	4.629	66.3	37.07		
	3	4.627	63.9	35.64		
3	1	4.628	64.7	36.11	32.51	9.62
	2	4.630	55.9	30.85		
	3	4.631	55.4	30.55		
6	1	4.623	66.1	36.95	33.42	9.21
	2	4.625	56.6	31.27		
	3	4.625	57.9	32.05		
9	1	4.629	57.1	31.57	31.95	1.22
	2	4.631	57.7	31.93		
	3	4.631	58.4	32.35		
12	1	4.644	61.0	33.90	33.62	0.89
	2	4.641	60.6	33.66		
	3	4.639	60.0	33.30		
15	1	4.633	56.9	31.72	31.39	1.09
	2	4.632	55.8	31.04		
	3	4.633	56.4	31.41		
18	1	4.631	58.2	32.53	31.86	1.80
	2	4.632	56.6	31.53		
	3	4.632	56.6	31.53		
21	1	4.633	61.5	34.58	33.23	4.22
	2	4.633	59.5	33.34		
	3	4.660	57.0	31.78		
24	1	4.649	58.2	32.53	33.56	3.36
	2	4.648	59.6	33.40		
	3	4.651	61.8	34.76		
27	1	4.632	58.9	33.02	33.57	2.91
	2	4.632	58.8	32.99		
	3	4.632	61.6	34.70		
30	1	4.627	56.9	31.72	33.11	4.22

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	2	4.627	59.1	33.09		
	3	4.628	61.4	34.52		

NA3 at 70 mg/L after 30 days of biodegradation:

Control A:

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.630	130.9	75.69	76.31	0.73
	2	4.632	132.2	76.47		
	3	4.633	132.7	76.77		
3	1	4.630	127.7	73.78	75.57	2.33
	2	4.629	130.8	75.63		
	3	4.630	133.6	77.31		
6	1	4.633	150.0	87.11	84.80	2.48
	2	4.632	145.3	84.30		
	3	4.634	143.1	82.99		
9	1	4.637	141.4	81.97	81.13	2.66
	2	4.638	135.9	78.68		
	3	4.638	142.7	82.75		
12	1	4.634	132.7	78.82	80.33	3.39
	2	4.632	132.5	78.70		
	3	4.634	140.2	83.48		
15	1	4.634	142.3	82.51	77.40	10.25
	2	4.634	140.5	81.43		
	3	4.650	115.7	68.26		
18	1	4.634	124.4	73.66	73.81	0.27
	2	4.634	124.5	73.73		
	3	4.634	125.0	74.04		
21	1	4.659	134.5	79.94	83.07	3.37
	2	4.661	143.2	85.35		
	3	4.664	140.9	83.92		
24	1	4.630	127.4	75.62	76.95	2.29
	2	4.631	128.3	76.27		
	3	4.631	132.6	78.94		
27	1	4.632	155.3	93.02	88.71	4.41
	2	4.633	143.0	85.38		
	3	4.633	146.9	87.74		
30	1	4.630	137.1	81.55	80.97	0.99

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	2	4.631	136.7	81.31		
	3	4.631	134.7	80.06		

Control B:

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.632	132.7	76.77	74.22	4.88
	2	4.632	131.1	75.81		
	3	4.659	121.5	70.07		
3	1	4.632	130.9	75.69	77.79	3.53
	2	4.631	139.6	80.90		
	3	4.632	132.7	76.77		
6	1	4.633	140.9	81.67	82.13	0.58
	2	4.633	141.6	82.09		
	3	4.634	142.5	82.63		
9	1	4.639	137.9	79.88	76.37	6.21
	2	4.641	123.0	70.97		
	3	4.640	135.2	78.26		
12	1	4.633	127.4	75.53	76.71	1.36
	2	4.633	130.6	77.52		
	3	4.633	129.9	77.08		
15	1	4.625	123.9	73.35	74.45	1.28
	2	4.628	126.6	75.03		
	3	4.629	126.5	74.97		
18	1	4.634	123.4	73.04	73.02	0.13
	2	4.633	123.5	73.10		
	3	4.633	123.2	72.92		
21	1	4.661	137.9	82.05	82.55	0.53
	2	4.658	139.0	82.74		
	3	4.651	139.2	82.86		
24	1	4.632	129.5	77.05	77.04	0.30
	2	4.631	129.1	76.80		
	3	4.632	130.0	77.27		
27	1	4.632	141.3	85.00	84.77	0.49
	2	4.632	141.3	85.00		
	3	4.634	141.3	84.29		
30	1	4.630	130.6	77.52	77.76	0.55
	2	4.631	130.6	77.52		

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	3	4.631	131.8	78.26		

No pre-exposure:

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.633	124.0	71.57	71.87	1.91
	2	4.634	127.0	73.36		
	3	4.634	122.5	70.67		
3	1	4.632	134.5	77.85	77.45	1.03
	2	4.632	132.3	76.53		
	3	4.633	134.7	77.97		
6	1	4.633	142.2	82.45	78.96	4.28
	2	4.633	136.0	78.74		
	3	4.648	130.9	75.69		
9	1	4.641	128.5	74.26	77.27	3.61
	2	4.644	134.4	77.79		
	3	4.647	137.7	79.76		
12	1	4.632	132.0	78.39	79.05	0.83
	2	4.633	133.1	79.07		
	3	4.633	134.1	79.69		
15	1	4.630	121.1	71.61	70.99	0.76
	2	4.630	119.7	70.74		
	3	4.631	119.5	70.62		
18	1	4.633	121.6	71.92	72.52	1.82
	2	4.633	125.0	74.04		
	3	4.633	121.1	71.61		
21	1	4.651	139.3	82.92	81.97	2.77
	2	4.645	140.4	83.61		
	3	4.650	133.6	79.38		
24	1	4.632	124.9	73.94	74.27	1.55
	2	4.632	131.8	75.56		
	3	4.633	124.2	73.32		
27	1	4.632	139.8	83.23	80.32	10.96
	2	4.632	146.7	87.30		
	3	4.651	119.2	70.43		
30	1	4.630	135.2	80.37	77.25	4.79
	2	4.631	123.6	73.17		
	2	4.631	131.7	78.20		

Pre-exposure:

Timepoint (days)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.631	123.8	73.29	72.98	0.47
	2	4.634	122.7	72.61		
	3	4.631	123.4	73.04		
3	1	4.635	112.6	66.33	67.51	1.57
	2	4.614	115.0	67.82		
	3	4.620	115.9	68.38		
6	1	4.621	117.0	69.06	69.71	1.10
	2	4.624	119.4	70.56		
	3	4.625	117.7	69.50		
9	1	4.626	120.7	71.36	71.94	2.60
	2	4.628	125.0	74.04		
	3	4.628	119.2	70.43		
12	1	4.630	112.8	66.46	68.48	2.72
	2	4.630	116.7	68.88		
	3	4.630	118.7	70.12		
15	1	4.630	122.2	72.30	71.67	0.87
	2	4.630	121.2	71.67		
	3	4.630	120.2	71.05		
18	1	4.631	122.1	72.23	72.46	0.30
	2	4.630	122.5	72.48		
	3	4.630	122.8	72.67		
21	1	4.629	118.6	70.06	71.03	2.08
	2	4.631	119.0	70.31		
	3	4.631	122.9	72.73		
24	1	4.631	122.9	72.73	74.37	2.03
	2	4.631	126.0	74.66		
	3	4.631	127.7	75.71		
27	1	4.631	125.4	74.28	73.68	2.83
	2	4.632	127.2	75.40		
	3	4.632	120.7	71.36		
30	1	4.631	126.5	74.97	74.33	3.06
	2	4.632	128.5	76.21		
	3	4.632	121.4	71.80		

QUANTIFICATION OF RESIDUAL MODEL NAs IN WATER AFTER DEGRADATION WITH 1a/H_2O_2

For all tables below, RT refers to retention time, PA refers to peak area, and RSD refers to relative standard deviation (%).

NA1 at pH 7:

NA1 alone in water

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	3.959	81.1	45.23	44.80	1.02
	2	3.961	80.5	44.84		
	3	3.963	79.7	44.32		
8	1	3.971	70.8	38.54	38.63	0.39
	2	3.969	70.8	38.54		
	3	3.969	71.2	38.8		
16	1	3.963	87.8	49.58	48.30	2.45
	2	3.962	84.2	47.24		
	3	3.963	85.5	48.09		
24	1	3.957	88.3	49.9	49.17	1.40
	2	3.950	87.0	49.06		
	3	3.950	86.2	48.54		
32	1	3.947	82.8	46.33	43.10	6.48
	2	3.942	75.3	41.46		
	3	3.942	75.4	41.52		
40	1	3.947	83.4	46.72	46.48	0.45
	2	3.952	82.9	46.40		
	3	3.947	82.8	46.33		
48	1	3.965	85.1	47.83	47.59	4.32
	2	3.964	81.4	45.42		
	3	3.966	87.7	49.51		
56	1	3.965	86.0	48.41	43.47	10.93
	2	3.963	71.4	38.93		
	3	3.967	77.8	43.08		
64	1	3.961	88.4	49.97	50.42	2.02
	2	3.973	90.9	51.59		
	3	3.977	88.0	49.71		
72	1	3.978	89.8	50.88	48.82	3.78
	2	3.981	85.8	48.28		

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	3	3.975	84.3	47.31		

NA1 + H_2O_2 in water

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	3.963	82	45.81	45.70	0.30
	2	3.965	81.9	45.75		
	3	3.961	81.6	45.55		
8	1	3.97	72	39.32	39.08	2.31
	2	3.969	72.8	39.84		
	3	3.969	70.1	38.08		
16	1	3.961	86.2	48.54	48.35	3.57
	2	3.95	88.4	49.97		
	3	3.943	83.1	46.53		
24	1	3.948	87.1	49.12	47.39	4.28
	2	3.965	85.2	47.89		
	3	3.959	81	45.16		
32	1	3.984	78.5	43.54	41.18	5.29
	2	3.97	71.9	39.25		
	3	3.962	74.2	40.75		
40	1	3.952	76.1	41.98	42.87	1.83
	2	3.944	77.9	43.15		
	3	3.947	78.4	43.47		
48	1	3.961	86	48.41	47.26	2.81
	2	3.963	84.7	47.57		
	3	3.986	82	45.81		
56	1	3.982	75.2	41.39	40.92	1.49
	2	3.961	74.8	41.13		
	3	3.948	73.4	40.23		
64	1	3.944	88.4	49.97	48.48	3.15
	2	3.947	86.2	48.54		
	3	3.964	83.7	46.92		
72	1	3.965	85	47.76	45.29	4.75
	2	3.979	79.7	44.32		
	3	3.979	78.9	43.80		

*NA1 + **1a** + H_2O_2 in batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	3.962	81.50	45.49	45.75	0.65
	2	3.969	82.40	46.07		
	3	3.958	81.80	45.68		
8	1	3.947	70.20	38.15	37.87	2.73
	2	3.940	68.00	36.72		
	3	3.943	71.10	38.73		
16	1	3.963	79.50	44.19	42.89	2.64
	2	3.971	76.60	42.3		
	3	3.988	76.40	42.17		
24	1	3.978	76.00	41.91	41.22	4.80
	2	3.965	71.50	38.99		
	3	3.970	77.30	42.76		
32	1	3.961	67.80	36.59	36.65	1.17
	2	3.946	67.30	36.26		
	3	3.936	68.60	37.11		
40	1	3.943	70.80	38.54	37.72	1.90
	2	3.965	69.00	37.37		
	3	3.967	68.80	37.24		
48	1	3.968	66.80	35.94	36.85	2.38
	2	3.968	69.50	37.69		
	3	3.964	68.30	36.91		
56	1	3.954	66.50	35.74	37.58	7.90
	2	3.960	66.90	36.00		
	3	3.953	74.60	41.01		
64	1	3.940	69.20	37.50	34.51	16.15
	2	3.946	69.90	37.95		
	3	3.963	54.70	28.08		
72	1	3.967	53.20	27.10	26.30	5.05
	2	3.974	53.10	27.04		
	3	3.970	49.60	24.77		

*NA1 + **1b** + H_2O_2 in batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	3.967	82.0	45.81	46.12	1.27
	2	3.972	81.9	45.75		
	3	3.961	83.5	46.79		
8	1	3.952	67.6	36.46	37.61	2.87

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	2	3.943	69.6	37.76		
	3	3.946	70.9	38.6		
16	1	3.964	81.6	45.55	45.23	3.43
	2	3.965	83.2	46.59		
	3	3.984	78.5	43.54		
24	1	3.975	83.3	46.66	47.67	2.51
	2	3.965	86.9	48.99		
	3	3.971	84.4	47.37		
32	1	3.960	72.7	39.77	39.69	0.52
	2	3.950	72.8	39.84		
	3	3.939	72.2	39.45		
40	1	3.943	75.8	41.78	40.48	3.47
	2	3.968	74.1	40.68		
	3	3.964	71.5	38.99		
48	1	3.985	73.4	40.23	43.56	13.12
	2	3.969	88.7	50.16		
	3	3.961	73.5	40.29		
56	1	3.971	58.7	30.68	35.85	12.50
	2	3.960	70.9	38.6		
	3	3.949	70.4	38.28		
64	1	3.943	66.0	35.42	33.49	10.14
	2	3.942	57.0	29.57		
	3	3.962	66.1	35.48		
72	1	3.965	59.3	31.07	31.55	2.62
	2	3.989	61.5	32.5		
	3	3.970	59.3	31.07		

*NA1 + **1a** + H_2O_2 in semi-batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	3.964	82.2	45.94	45.79	0.70
	2	3.953	82.3	46.01		
	3	3.959	81.4	45.42		
8	1	3.945	65.2	34.9	35.38	1.74
	2	3.945	65.6	35.16		
	3	3.945	67	36.07		
16	1	3.984	76.5	42.24	39.77	5.61
	2	3.957	69.8	37.89		

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	3	3.974	71.8	39.19		
24	1	3.971	67.5	36.39	32.08	15.75
	2	3.965	52.3	26.52		
	3	3.952	62.8	33.34		
32	1	3.96	56.6	29.31	23.86	20.85
	2	3.945	41.6	19.57		
	3	3.944	46.4	22.69		
40	1	3.943	52.6	26.71	19.16	34.78
	2	3.961	33.2	14.11		
	3	3.954	37.1	16.65		
48	1	3.973	41.7	19.63	12.53	51.02
	2	3.962	22.6	7.23		
	3	3.961	28	10.73		
56	1	3.951	40.8	19.05	11.14	61.99
	2	3.965	17.2	6.35		
	3	3.953	23.8	8.01		
64	1	3.943	18.5	7.23	5.95	19.44
	2	3.941	14.8	5.64		
	3	3.967	14.1	4.98		
72	1	3.959	17.4	6.38	5.23	20.13
	2	3.947	20.5	4.98		
	3	3.952	18.3	4.32		

*NA1 + **1b** + H_2O_2 in semi-batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	3.963	82.7	46.27	45.88	0.79
	2	3.942	81.6	45.55		
	3	3.957	82	45.81		
8	1	3.949	68.2	36.85	36.98	0.46
	2	3.94	68.7	37.17		
	3	3.946	68.3	36.91		
16	1	3.962	79.5	44.19	44.14	2.36
	2	3.962	81	45.16		
	3	3.979	77.8	43.08		
24	1	3.973	81.8	45.68	40.18	12.09
	2	3.965	67.6	36.46		
	3	3.949	70.6	38.41		

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
32	1	3.959	58	30.22	30.24	3.75
	2	3.948	56.3	29.12		
	3	3.941	59.8	31.39		
40	1	3.94	59.9	31.46	30.40	3.05
	2	3.962	57.3	29.77		
	3	3.957	57.6	29.96		
48	1	3.972	50.7	25.48	25.48	0.75
	2	3.988	50.4	25.29		
	3	3.965	51	25.67		
56	1	3.948	45.9	22.36	22.34	8.01
	2	3.961	43.1	20.54		
	3	3.948	48.6	24.12		
64	1	3.945	35	15.28	14.57	6.31
	2	3.944	32.3	13.53		
	3	3.964	34.4	14.89		
72	1	3.957	29	11.38	11.21	1.44
	2	3.979	28.5	11.06		
	3	3.972	28.7	11.19		

NA1 at pH 8:

NA1 alone in water

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	3.966	81.8	45.68	45.70	0.09
	2	3.962	81.9	45.75		
	3	3.964	81.8	45.68		
8	1	3.958	75.8	41.78	39.16	5.79
	2	3.941	69.9	37.95		
	3	3.954	69.6	37.76		
16	1	3.967	92.4	52.57	51.90	1.32
	2	3.968	90.3	51.20		
	3	3.987	91.4	51.92		
24	1	3.967	80.5	44.84	44.32	1.34
	2	3.961	79.9	44.45		
	3	3.963	78.7	43.67		
32	1	3.956	85.5	48.09	48.37	0.54
	2	3.940	86.3	48.61		

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	3	3.952	86.0	48.41		
40	1	3.966	75.6	41.65	41.00	4.04
	2	3.972	71.7	39.12		
	3	3.987	76.5	42.24		
48	1	3.966	79.6	44.25	46.35	3.95
	2	3.962	84.2	47.24		
	3	3.964	84.7	47.57		
56	1	3.956	83.3	46.66	44.97	4.76
	2	3.941	77.0	42.56		
	3	3.951	81.8	45.68		
64	1	3.967	91.4	51.92	51.05	2.03
	2	3.970	90.5	51.33		
	3	3.988	88.3	49.90		

NA1 + H_2O_2 in water

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	3.965	82.1	45.88	45.81	0.14
	2	3.961	82	45.81		
	3	3.965	81.9	45.75		
8	1	3.955	68.4	36.98	38.41	3.49
	2	3.939	72.5	39.64		
	3	3.950	70.9	38.60		
16	1	3.964	92.6	52.70	50.10	5.06
	2	3.974	88.4	49.97		
	3	4.002	84.8	47.63		
24	1	3.964	75.9	41.85	43.11	7.81
	2	3.962	73.9	40.55		
	3	3.966	83.7	46.92		
32	1	3.957	77.2	42.69	42.43	4.78
	2	3.942	73.5	40.29		
	3	3.950	79.7	44.32		
40	1	3.963	72.9	39.90	42.78	5.85
	2	3.975	79.9	44.45		
	3	3.990	79.2	43.99		
48	1	3.966	73.6	40.36	43.32	10.46
	2	3.954	86.2	48.54		
	3	3.964	74.7	41.07		

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
56	1	3.960	74.7	41.07	40.31	1.63
	2	3.942	72.9	39.90		
	3	3.952	73	39.97		
64	1	3.965	87.7	49.51	43.45	23.78
	2	3.974	87.4	49.32		
	3	3.980	60	31.52		

*NA1 + **1a** + H_2O_2 in batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	3.965	85.1	47.83	48.19	1.68
	2	3.952	87.1	49.12		
	3	3.963	84.8	47.63		
8	1	3.960	53.7	27.43	27.75	1.47
	2	3.939	54	27.62		
	3	3.949	54.9	28.21		
16	1	3.964	76.6	42.30	43.30	2.41
	2	3.974	78	43.21		
	3	3.990	79.8	44.38		
24	1	3.969	74.1	40.68	41.31	1.43
	2	3.950	75.2	41.39		
	3	3.964	75.9	41.85		
32	1	3.960	68	36.72	36.72	0.18
	2	3.939	68.1	36.78		
	3	3.947	67.9	36.65		
40	1	3.966	69.1	37.43	37.26	0.53
	2	3.976	68.9	37.30		
	3	3.989	68.5	37.04		
48	1	3.968	65.5	35.09	35.37	2.41
	2	3.952	67.4	36.33		
	3	3.965	64.9	34.70		
56	1	3.954	66	35.42	35.92	1.35
	2	3.944	67.5	36.39		
	3	3.948	66.8	35.94		
64	1	3.965	55.2	28.40	33.10	13.20
	2	3.978	63.6	33.86		
	3	4.008	68.5	37.04		

*NA1 + **1b** + H_2O_2 in batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	3.969	83.4	46.72	46.72	0.97
	2	3.950	82.7	46.27		
	3	3.963	84.1	47.18		
8	1	3.961	55.1	28.34	29.03	2.34
	2	3.944	56.2	29.05		
	3	3.950	57.2	29.70		
16	1	3.964	81.4	45.42	45.99	1.10
	2	3.971	82.9	46.40		
	3	3.986	82.5	46.14		
24	1	3.970	81.6	45.55	45.44	0.82
	2	3.950	80.8	45.03		
	3	3.965	81.9	45.75		
32	1	3.961	72.4	39.58	36.01	9.22
	2	3.941	62.3	33.02		
	3	3.950	66.0	35.42		
40	1	3.964	70.0	38.02	38.99	2.20
	2	3.976	72.0	39.32		
	3	3.986	72.5	39.64		
48	1	3.965	50.5	25.35	27.73	8.33
	2	3.952	54.4	27.88		
	3	3.964	57.6	29.96		
56	1	3.959	70.5	38.34	38.13	0.59
	2	3.939	69.8	37.89		
	3	3.950	70.2	38.15		
64	1	3.966	64.4	34.38	34.40	0.67
	2	3.972	64.1	34.18		
	3	3.983	64.8	34.64		

*NA1 + **1a** + H_2O_2 in semi-batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	3.965	83.2	46.59	46.74	0.94
	2	3.953	84.2	47.24		
	3	3.962	82.9	46.40		
8	1	3.958	55.5	28.60	29.05	2.53
	2	3.938	55.6	28.66		
	3	3.946	57.5	29.90		

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
16	1	3.956	71.6	39.06	40.38	7.84
	2	3.980	79.2	43.99		
	3	3.973	70.1	38.08		
24	1	3.961	59.1	30.94	31.37	6.13
	2	3.952	63.0	33.47		
	3	3.961	57.2	29.70		
32	1	3.958	38.2	17.36	17.36	9.33
	2	3.940	40.7	18.98		
	3	3.943	35.7	15.74		
40	1	3.961	31.8	13.20	13.98	11.31
	2	3.973	35.8	15.80		
	3	3.977	31.4	12.94		
48	1	3.966	21.6	6.58	7.66	23.03
	2	3.952	26.4	9.70		
	3	3.964	21.8	6.71		
56	1	3.957	15.4	2.55	3.96	30.80
	2	3.939	18.6	4.63		
	3	3.943	14.7	4.69		
64	1	3.960	17.6	2.30	2.80	15.67
	2	3.980	20.3	3.12		
	3	3.983	19.6	2.98		

*NA1 + **1b** + H_2O_2 in semi-batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	3.964	87.5	47.83	48.19	1.68
	2	3.950	88.9	49.12		
	3	3.964	87.6	47.63		
8	1	3.958	57.4	29.83	29.53	0.92
	2	3.943	56.6	29.31		
	3	3.948	56.8	29.44		
16	1	3.962	82.8	46.33	45.07	3.06
	2	3.971	81.2	45.29		
	3	3.991	78.6	43.60		
24	1	3.963	71.0	38.67	38.67	0.67
	2	3.952	70.6	38.41		
	3	3.963	71.4	38.93		
32	1	3.954	61.6	32.56	31.46	3.51

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	2	3.940	58.2	30.35		
	3	3.949	59.9	31.46		
40	1	3.966	60.1	31.59	30.55	2.95
	2	3.972	57.7	30.03		
	3	3.998	57.7	30.03		
48	1	3.966	68.3	36.91	36.74	1.47
	2	3.955	67.1	36.13		
	3	3.961	68.7	37.17		
56	1	3.959	47.0	23.08	22.97	1.35
	2	3.942	47.2	23.21		
	3	3.949	46.3	22.62		
64	1	3.962	34.9	15.22	15.28	3.19
	2	3.975	35.8	15.80		
	3	3.984	34.3	14.83		

NA1 at pH 9:

NA1 alone in water

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	3.962	92.1	52.37	52.44	0.12
	2	3.948	92.2	52.44		
	3	3.961	92.3	52.50		
8	1	3.936	90.4	51.27	47.93	10.12
	2	3.945	88.7	50.16		
	3	3.948	76.7	42.37		
16	1	3.963	90.5	51.33	50.72	1.07
	2	3.983	88.9	50.29		
	3	4.03	89.3	50.55		
24	1	3.97	83.2	46.59	44.36	6.46
	2	3.953	81.3	45.36		
	3	3.961	74.8	41.13		
32	1	3.939	89.1	50.42	51.63	2.39
	2	3.946	90.9	51.59		
	3	3.949	92.9	52.89		
40	1	3.964	86.5	48.74	47.09	4.91
	2	3.981	85.5	48.09		
	3	3.994	79.9	44.45		

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
48	1	3.972	79.3	44.06	43.13	1.88
	2	3.953	77.1	42.63		
	3	3.96	77.2	42.69		
56	1	3.938	80.7	44.97	43.26	3.77
	2	3.946	77.8	43.08		
	3	3.949	75.7	41.72		
64	1	3.965	63.5	33.79	44.70	21.21
	2	3.984	89.9	50.94		
	3	4.001	87.5	49.38		

NA1 + H_2O_2 in water

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	3.973	95.3	54.45	54.52	1.49
	2	3.954	96.7	55.36		
	3	3.96	94.2	53.74		
8	1	3.937	86.5	48.74	48.93	0.47
	2	3.944	87.2	49.19		
	3	3.95	86.7	48.87		
16	1	3.965	88.7	50.16	50.46	2.50
	2	3.985	91.3	51.85		
	3	3.993	87.5	49.38		
24	1	3.965	81.4	45.42	45.14	0.96
	2	3.952	80.2	44.64		
	3	3.959	81.3	45.36		
32	1	3.938	91.5	51.98	49.80	3.99
	2	3.945	87.4	49.32		
	3	3.951	85.5	48.09		
40	1	3.965	73.7	40.42	41.87	3.17
	2	3.978	77.7	43.02		
	3	4.006	76.4	42.17		
48	1	3.964	81.7	45.62	43.54	4.14
	2	3.954	76.9	42.50		
	3	3.959	76.9	42.50		
56	1	3.937	73.8	40.49	40.68	0.82
	2	3.947	74.7	41.07		
	3	3.951	73.8	40.49		
64	1	3.962	87.8	49.58	48.48	2.59

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	2	3.979	86.5	48.74		
	3	3.995	84	47.11		

*NA1 + **1a** + H_2O_2 in batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	3.962	94.2	53.74	53.98	1.10
	2	3.953	95.6	54.65		
	3	3.965	93.9	53.54		
8	1	3.933	80.9	45.10	41.03	8.63
	2	3.945	71.0	38.67		
	3	3.953	72.0	39.32		
16	1	3.960	84.8	47.63	45.34	4.46
	2	3.984	78.9	43.80		
	3	3.999	80.1	44.58		
24	1	3.967	74.1	40.68	40.59	1.44
	2	3.953	73.0	39.97		
	3	3.963	74.8	41.13		
32	1	3.938	73.0	39.97	40.81	2.23
	2	3.947	74.1	40.68		
	3	3.950	75.8	41.78		
40	1	3.962	74.5	40.94	40.59	1.21
	2	3.982	73.1	40.03		
	3	3.980	74.3	40.81		
48	1	3.964	70.0	38.02	38.00	2.30
	2	3.950	68.6	37.11		
	3	3.959	71.3	38.86		
56	1	3.938	71.3	38.86	38.23	1.70
	2	3.949	69.3	37.56		
	3	3.948	70.4	38.28		
64	1	3.963	58.2	30.35	30.37	1.83
	2	3.988	57.4	29.83		
	3	3.992	59.1	30.94		

*NA1 + **1b** + H_2O_2 in batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	3.965	98.1	56.27	55.25	1.72
	2	3.957	96.3	55.10		
	3	3.959	95.2	54.39		
8	1	3.933	76.5	42.24	40.46	5.30
	2	3.949	74.7	41.07		
	3	3.949	70.1	38.08		
16	1	3.962	85.1	47.83	47.03	1.48
	2	3.977	83.3	46.66		
	3	3.986	83.2	46.59		
24	1	3.965	77.3	42.76	42.28	1.03
	2	3.954	76.4	42.17		
	3	3.957	76.0	41.91		
32	1	3.936	74.7	41.07	41.57	1.25
	2	3.946	76.3	42.11		
	3	3.951	75.4	41.52		
40	1	3.969	78.0	43.21	41.00	5.91
	2	3.982	75.2	41.39		
	3	3.982	70.6	38.41		
48	1	3.962	71.1	38.73	39.17	1.09
	2	3.954	72.4	39.58		
	3	3.961	71.8	39.19		
56	1	3.934	72.1	39.38	38.80	1.59
	2	3.934	71.3	38.86		
	3	3.953	70.2	38.15		
64	1	3.962	66.2	35.55	35.44	0.87
	2	3.982	65.5	35.09		
	3	3.991	66.4	35.68		

*NA1 + **1a** + H_2O_2 in semi-batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	3.967	94.2	53.74	53.31	1.02
	2	3.955	93.8	53.48		
	3	3.964	92.6	52.70		
8	1	3.933	64.4	34.38	36.74	5.65
	2	3.942	70.4	38.28		
	3	3.941	69.3	37.56		
16	1	3.954	74.8	41.13	41.28	4.18
	2	3.993	72.5	39.64		
	3	4.003	77.8	43.08		

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
24	1	3.968	54.2	27.75	29.46	5.13
	2	3.955	57.7	30.03		
	3	3.961	58.6	30.61		
32	1	3.938	38.2	17.36	21.95	18.47
	2	3.945	47.6	23.47		
	3	3.946	50	25.03		
40	1	3.957	30.8	12.55	15.78	17.82
	2	3.988	37.8	17.10		
	3	3.978	38.7	17.69		
48	1	3.97	21.1	6.25	8.94	26.40
	2	3.954	26.7	9.89		
	3	3.965	27.9	10.67		
56	1	3.936	15.1	4.63	7.08	30.12
	2	3.947	23.9	8.07		
	3	3.946	24.6	8.53		
64	1	3.958	6.1	2.35	4.30	40.34
	2	3.983	8.9	4.89		
	3	4.018	11.6	5.67		

*NA1 + **1b** + H_2O_2 in semi-batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	3.965	95.3	54.45	54.69	2.12
	2	3.954	97.6	55.95		
	3	3.959	94.1	53.67		
8	1	3.938	73.4	40.23	40.01	0.50
	2	3.945	73	39.97		
	3	3.946	72.8	39.84		
16	1	3.957	77.6	42.95	43.17	1.14
	2	3.988	77.4	42.82		
	3	3.987	78.8	43.73		
24	1	3.963	68	36.72	36.03	1.99
	2	3.949	65.8	35.29		
	3	3.958	67	36.07		
32	1	3.939	61.1	32.24	33.02	2.18
	2	3.946	62.5	33.15		
	3	3.96	63.3	33.66		
40	1	3.964	58.5	30.55	29.90	5.14

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	2	3.988	54.8	28.14		
	3	3.993	59.2	31.00		
48	1	3.963	49.5	24.70	25.03	1.46
	2	3.955	50.6	25.42		
	3	3.96	49.9	24.96		
56	1	3.935	44	21.13	22.38	4.99
	2	3.944	47.3	23.27		
	3	3.95	46.5	22.75		
64	1	3.961	35.9	15.87	16.00	0.81
	2	3.982	36.1	16.00		
	3	3.993	36.3	16.13		

NA3 at pH 7:

NA3 alone in water

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.138	198.4	50.92	50.05	2.47
	2	4.136	189.3	48.63		
	3	4.133	197.1	50.59		
8	1	4.116	161.4	41.64	41.70	0.14
	2	4.119	161.6	41.69		
	3	4.118	161.9	41.76		
16	1	4.121	171.6	44.19	42.69	3.05
	2	4.118	162.5	41.91		
	3	4.124	162.7	41.96		
24	1	4.110	153.2	39.58	41.04	3.25
	2	4.108	160.3	41.36		
	3	4.104	163.6	42.19		
32	1	4.096	151.1	39.05	39.17	0.58
	2	4.099	152.6	39.43		
	3	4.096	151.0	39.03		
40	1	4.101	151.8	39.23	39.60	0.89
	2	4.096	154.6	39.93		
	3	4.096	153.4	39.63		
48	1	4.138	128.5	33.38	33.97	1.87
	2	4.121	133.5	34.64		
	3	4.133	130.5	33.88		
56	1	4.123	135.3	35.09	34.71	1.02

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	2	4.128	132.5	34.39		
	3	4.131	133.6	34.66		
64	1	4.162	142.8	36.97	37.75	2.05
	2	4.153	149.0	38.52		
	3	4.154	145.9	37.75		
72	1	4.140	144.4	37.37	37.32	0.30
	2	4.161	143.7	37.19		
	3	4.160	144.5	37.40		

NA3 + H_2O_2 in water

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.133	193.7	49.74	49.73	0.27
	2	4.131	193.1	49.59		
	3	4.133	194.2	49.86		
8	1	4.118	159.4	41.13	41.16	0.27
	2	4.118	160	41.28		
	3	4.118	159.1	41.06		
16	1	4.119	162.5	41.91	42.01	0.41
	2	4.12	162.5	41.91		
	3	4.118	163.7	42.21		
24	1	4.104	152.2	39.33	40.47	2.47
	2	4.101	159.6	41.18		
	3	4.101	158.5	40.91		
32	1	4.095	150.8	38.98	38.94	0.48
	2	4.093	149.8	38.73		
	3	4.094	151.3	39.10		
40	1	4.094	157	40.53	39.07	3.26
	2	4.098	147.8	38.22		
	3	4.098	148.7	38.45		
48	1	4.124	133.2	34.56	34.43	0.33
	2	4.122	132.3	34.34		
	3	4.124	132.5	34.39		
56	1	4.125	132.7	34.44	34.80	1.73
	2	4.132	132.8	34.46		
	3	4.117	136.9	35.49		
64	1	4.159	142.1	36.79	36.74	1.37
	2	4.139	143.8	37.22		

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	3	4.169	139.8	36.22		
72	1	4.161	141.9	36.74	36.32	2.71
	2	4.181	135.7	35.19		
	3	4.15	143	37.02		

*NA3 + **1a** + H_2O_2 in batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.136	195.1	50.09	49.82	2.12
	2	4.117	197.6	50.72		
	3	3.525	189.4	48.66		
8	1	3.529	147.4	38.12	37.81	1.28
	2	3.529	147.1	38.05		
	3	3.53	143.9	37.25		
16	1	3.531	121.2	37.11	37.63	1.24
	2	3.532	123.4	37.79		
	3	3.533	124.1	38.00		
24	1	3.533	119.2	36.49	36.47	0.17
	2	4.131	119.3	36.52		
	3	4.115	118.9	36.40		
32	1	3.526	113.6	34.76	34.84	1.25
	2	3.528	115.4	35.31		
	3	3.53	112.6	34.45		
40	1	3.531	112.6	34.45	34.50	0.59
	2	3.531	112.2	34.33		
	3	3.532	113.5	34.73		
48	1	3.533	120.9	37.01	34.89	8.62
	2	3.533	118.3	36.21		
	3	4.138	102.9	31.45		
56	1	4.115	117.3	35.90	34.32	8.83
	2	3.526	118.4	36.24		
	3	3.529	100.9	30.83		
64	1	3.53	118.1	36.15	36.33	0.56
	2	3.531	118.6	36.30		
	3	3.532	119.4	36.55		
72	1	3.532	119.6	36.61	35.31	3.22
	2	3.532	113.9	34.85		
	3	3.533	112.7	34.48		

*NA3 + **1b** + H_2O_2 in batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.135	192.8	49.51	49.67	0.40
	2	4.134	193.2	49.61		
	3	4.131	194.3	49.89		
8	1	4.119	154.7	39.95	39.92	0.20
	2	4.120	154.2	39.83		
	3	4.119	154.8	39.98		
16	1	4.121	159.9	41.26	41.44	0.94
	2	4.120	159.6	41.18		
	3	4.121	162.4	41.89		
24	1	4.101	146.9	38.00	38.92	2.09
	2	4.106	151.7	39.20		
	3	4.108	153.1	39.55		
32	1	4.095	147.1	38.05	38.33	1.75
	2	4.095	146.3	37.85		
	3	4.073	151.3	39.10		
40	1	4.099	152.9	39.50	38.30	2.82
	2	4.100	146.9	38.00		
	3	4.101	144.5	37.40		
48	1	4.119	131.3	34.08	34.38	1.94
	2	4.120	135.5	35.14		
	3	4.120	130.6	33.91		
56	1	4.125	129.3	33.58	33.93	1.26
	2	4.123	132.6	34.41		
	3	4.122	130.2	33.81		
64	1	4.144	141.0	36.52	36.12	1.70
	2	4.144	136.6	35.41		
	3	4.153	140.6	36.42		
72	1	4.152	140.5	36.39	35.91	1.15
	2	4.144	137.5	35.64		
	3	4.166	137.8	35.71		

*NA3 + **1a** + H_2O_2 in semi-batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.138	197.3	50.64	49.69	2.51
	2	4.119	195.4	50.16		
	3	3.527	187.9	48.28		
8	1	3.529	145	37.52	37.23	1.04
	2	3.53	144.4	37.37		
	3	3.531	142.1	36.79		
16	1	3.531	113.8	34.82	34.56	2.03
	2	3.532	110.4	33.77		
	3	3.532	114.7	35.10		
24	1	3.533	102.7	31.39	30.64	2.16
	2	4.137	98.7	30.15		
	3	4.12	99.4	30.37		
32	1	3.527	81.1	24.72	23.92	6.97
	2	3.529	72.3	22.00		
	3	3.53	82.1	25.03		
40	1	3.531	76.9	23.42	22.43	5.53
	2	3.531	69.2	21.04		
	3	3.532	75	22.83		
48	1	3.532	61.7	18.72	18.74	6.59
	2	3.532	57.8	17.52		
	3	4.139	65.8	19.99		
56	1	4.121	59.3	17.98	17.03	8.57
	2	3.528	50.8	15.35		
	3	3.529	58.6	17.76		
64	1	3.53	52.3	15.82	15.18	10.23
	2	3.531	44.5	13.41		
	3	3.531	53.9	16.31		
72	1	3.532	46.7	14.09	13.39	13.03
	2	3.532	38	11.40		
	3	3.533	48.6	14.67		

*NA3 + **1b** + H_2O_2 in semi-batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.137	198.6	50.97	50.52	1.02
	2	4.138	197.3	50.64		
	3	4.136	194.6	49.96		
8	1	4.118	155.0	40.03	40.02	0.04
	2	4.112	155.0	40.03		

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	3	4.118	154.9	40.00		
16	1	4.125	155.7	40.21	40.16	0.63
	2	4.125	154.4	39.88		
	3	4.121	156.4	40.38		
24	1	4.106	140.4	36.37	37.20	1.99
	2	4.111	146.1	37.80		
	3	4.109	144.6	37.42		
32	1	4.096	132.6	34.41	34.30	0.69
	2	4.092	131.1	34.03		
	3	4.092	132.8	34.46		
40	1	4.102	137.9	35.74	34.25	3.90
	2	4.102	130.4	33.86		
	3	4.102	127.6	33.16		
48	1	4.124	109.1	28.52	28.45	0.23
	2	4.128	108.8	28.44		
	3	4.127	108.6	28.39		
56	1	4.132	104.0	27.24	26.86	1.41
	2	4.128	101.0	26.48		
	3	4.129	102.5	26.86		
64	1	4.167	105.0	27.49	27.55	1.16
	2	4.173	104.1	27.26		
	3	4.155	106.6	27.89		
72	1	4.173	88.1	23.25	26.05	9.34
	2	4.139	104.0	27.24		
	3	4.143	105.7	27.66		

NA3 at pH 8:

NA3 alone in water

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.132	194.8	50.01	49.83	0.32
	2	4.103	193.6	49.71		
	3	4.123	193.8	49.76		
8	1	4.114	133.6	34.66	34.51	3.43
	2	4.096	137.4	35.61		
	3	4.099	128.0	33.26		
16	1	4.116	166.8	42.99	43.01	0.07

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	2	4.137	166.8	42.99		
	3	4.168	167.0	43.04		
24	1	4.132	156.5	40.41	40.52	0.52
	2	4.104	156.4	40.38		
	3	4.121	157.9	40.76		
32	1	4.113	148.6	38.42	38.80	0.98
	2	4.095	151.6	39.18		
	3	4.103	150.1	38.80		
40	1	4.118	150.7	38.95	39.33	0.85
	2	4.141	152.7	39.45		
	3	4.185	153.2	39.58		
48	1	4.133	135.9	35.24	35.47	0.91
	2	4.106	138.3	35.84		
	3	4.123	136.3	35.34		
56	1	4.111	127.7	33.18	32.83	0.94
	2	4.096	125.4	32.60		
	3	4.099	125.8	32.70		
64	1	4.118	141.7	36.69	36.57	3.85
	2	4.141	135.4	35.11		
	3	4.156	146.6	37.92		
72	1	4.156	141.4	36.54	36.12	4.10
	2	4.102	135.9	35.23		
	3	4.117	141.5	36.58		

NA3 + H_2O_2 in water

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.133	193.6	49.71	49.94	0.43
	2	4.106	195.3	50.14		
	3	4.121	194.6	49.96		
8	1	4.111	133.8	34.71	34.94	0.62
	2	4.095	134.8	34.96		
	3	4.100	135.5	35.14		
16	1	4.117	164.4	42.39	41.96	1.37
	2	4.133	160.1	41.31		
	3	4.163	163.6	42.19		
24	1	4.132	158.0	40.78	40.51	0.68
	2	4.106	155.8	40.23		

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	3	4.120	156.9	40.51		
32	1	4.107	149.1	38.55	38.66	0.26
	2	4.098	149.9	38.75		
	3	4.101	149.6	38.68		
40	1	4.115	150.4	38.88	38.96	0.67
	2	4.137	149.9	38.75		
	3	4.164	151.9	39.25		
48	1	4.134	138.3	35.84	35.96	0.69
	2	4.108	139.9	36.24		
	3	4.120	138.1	35.79		
56	1	4.106	129.6	33.66	33.61	0.83
	2	4.096	128.2	33.31		
	3	4.099	130.4	33.86		
64	1	4.116	142.8	36.97	36.79	1.13
	2	4.137	143.3	37.09		
	3	4.170	140.2	36.32		
72	1	4.158	140.9	36.78	36.40	1.12
	2	4.106	140.5	36.45		
	3	4.126	139.5	35.97		

*NA3 + **1a** + H_2O_2 in batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.135	195.1	50.09	50.16	0.40
	2	3.533	196.3	50.39		
	3	3.536	194.8	50.01		
8	1	3.536	112.8	34.51	35.06	1.39
	2	3.535	115.8	35.44		
	3	3.533	115.1	35.22		
16	1	3.532	117.9	36.09	35.98	2.63
	2	3.532	114.3	34.98		
	3	3.532	120.4	36.86		
24	1	4.133	111.3	34.05	34.58	1.44
	2	3.534	113.2	34.64		
	3	3.536	114.5	35.04		
32	1	3.536	121.6	37.23	34.77	6.21
	2	3.534	108.6	33.21		
	3	3.533	110.7	33.86		

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
40	1	3.532	117.9	36.09	34.94	5.36
	2	3.533	107.2	32.78		
	3	3.533	117.5	35.96		
48	1	4.131	110.1	33.68	34.85	3.13
	2	3.534	117.1	35.84		
	3	3.536	114.5	35.04		
56	1	3.536	116.9	35.78	35.23	2.39
	2	3.533	112.0	34.26		
	3	3.533	116.5	35.65		
64	1	3.532	116.2	35.56	35.74	0.72
	2	3.532	117.7	36.03		
	3	3.533	116.4	35.62		
72	1	3.534	116.8	35.69	35.85	0.71
	2	3.532	118.3	36.14		
	3	3.531	116.9	35.71		

*NA3 + **1b** + H_2O_2 in batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.132	194.2	49.86	50.12	0.53
	2	4.106	196.3	50.39		
	3	4.120	195.2	50.11		
8	1	4.107	128.3	33.33	33.11	0.68
	2	4.096	127.4	33.11		
	3	4.099	126.5	32.88		
16	1	4.117	156.3	40.36	40.63	1.24
	2	4.144	159.7	41.21		
	3	4.164	156.1	40.31		
24	1	4.133	159.0	41.03	40.07	2.20
	2	4.112	154.4	39.88		
	3	4.117	152.1	39.30		
32	1	4.102	144.9	37.50	37.00	1.55
	2	4.100	143.4	37.12		
	3	4.100	140.4	36.37		
40	1	4.111	149.4	38.62	38.73	0.49
	2	4.153	150.7	38.95		
	3	4.163	149.4	38.62		
48	1	4.135	134.2	34.81	35.65	2.59

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	2	4.109	136.9	35.49		
	3	4.120	141.5	36.64		
56	1	4.102	119.7	31.17	30.56	4.86
	2	4.100	110.5	28.87		
	3	4.100	121.6	31.65		
64	1	4.109	129.7	33.68	33.87	0.91
	2	4.142	131.9	34.23		
	3	4.154	129.8	33.71		
72	1	4.110	129.4	33.12	33.41	1.21
	2	4.125	132.2	33.87		
	3	4.104	128.6	33.24		

*NA3 + **1a** + H_2O_2 in semi-batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.13	195.6	50.21	50.08	1.37
	2	3.534	197.5	50.69		
	3	3.536	192.1	49.34		
8	1	3.536	113.5	34.73	34.58	0.62
	2	3.533	113.3	34.67		
	3	3.533	112.2	34.33		
16	1	3.531	106.2	32.47	32.49	1.43
	2	3.532	107.8	32.97		
	3	3.533	104.8	32.04		
24	1	3.532	90.8	27.71	27.93	6.52
	2	4.134	97.7	29.85		
	3	3.534	86.0	26.23		
32	1	3.536	77.2	23.51	23.72	5.53
	2	3.536	82.4	25.12		
	3	3.533	74.0	22.52		
40	1	3.532	68.5	20.82	21.22	6.14
	2	3.531	74.5	22.68		
	3	3.532	66.4	20.17		
48	1	3.533	57.9	17.55	18.41	9.91
	2	3.533	67.5	20.51		
	3	4.132	56.7	17.18		
56	1	3.535	49.8	15.04	16.04	13.73
	2	3.536	61.2	18.57		

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	3	3.536	48.1	14.52		
64	1	3.533	44.3	13.35	14.29	11.86
	2	3.532	53.7	16.25		
	3	3.532	44.1	13.28		
72	1	3.532	37.5	11.24	12.50	14.33
	2	3.533	48.2	14.55		
	3	3.533	39	11.71		

*NA3 + **1b** + H_2O_2 in semi-batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.133	197.2	50.62	50.10	0.94
	2	4.109	193.6	49.71		
	3	4.115	194.6	49.96		
8	1	4.098	127.7	33.18	33.38	1.04
	2	4.096	130.1	33.78		
	3	4.104	127.7	33.18		
16	1	4.109	156.1	40.31	39.81	1.09
	2	4.117	153.4	39.63		
	3	4.178	152.9	39.50		
24	1	4.132	143	37.02	37.80	1.93
	2	4.108	146.5	37.90		
	3	4.116	148.8	38.47		
32	1	4.092	130.5	33.88	34.13	0.68
	2	4.097	131.6	34.16		
	3	4.101	132.3	34.34		
40	1	4.112	127.4	33.11	33.04	0.42
	2	4.148	126.5	32.88		
	3	4.170	127.5	33.13		
48	1	4.130	115.3	30.07	30.69	2.50
	2	4.117	116.8	30.45		
	3	4.116	121.2	31.55		
56	1	4.091	107.2	28.04	28.01	1.72
	2	4.097	105.1	27.51		
	3	4.104	108.9	28.47		
64	1	4.11	101.3	26.56	26.76	1.29

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	2	4.144	101.3	26.56		
	3	4.163	103.7	27.16		
72	1	4.152	100.2	25.48	25.53	2.28
	2	4.105	99.6	24.97		
	3	4.118	101.1	26.13		

NA3 at pH 9:

NA3 alone in water

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.135	196	50.31	50.33	0.20
	2	4.109	196.5	50.44		
	3	4.12	195.7	50.24		
8	1	4.092	146.6	37.92	38.46	1.12
	2	4.099	149.7	38.70		
	3	4.102	149.9	38.75		
16	1	4.119	161.2	41.58	41.12	0.84
	2	4.154	158.7	40.96		
	3	4.176	162.4	41.89		
24	1	4.134	159.9	41.26	39.09	0.84
	2	4.109	160.4	41.38		
	3	4.117	157.8	40.73		
32	1	4.095	149.8	38.73	40.34	0.47
	2	4.099	152.4	39.38		
	3	4.1	151.5	39.15		
40	1	4.117	155.7	40.21	35.80	0.46
	2	4.153	155.9	40.26		
	3	4.166	157.1	40.56		
48	1	4.131	138.1	35.79	34.92	1.78
	2	4.114	137.5	35.64		
	3	4.115	138.8	35.97		
56	1	4.094	133.1	34.54	37.15	1.39
	2	4.099	133.3	34.59		
	3	4.099	137.5	35.64		
64	1	4.114	141.3	36.59	37.15	1.39
	2	4.138	144	37.27		
	3	4.167	145.3	37.60		

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
72	1	3.536	148.1	38.30	38.17	0.41
	2	3.535	147.8	38.22		
	3	3.534	146.9	38.00		

NA3 + H_2O_2 in water

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.135	197.7	50.74	51.00	0.45
	2	4.109	199.1	51.09		
	3	4.115	199.4	51.17		
8	1	4.092	146.2	37.82	37.86	0.50
	2	4.1	145.7	37.70		
	3	4.096	147.2	38.07		
16	1	4.116	160.6	41.43	41.06	0.81
	2	4.134	158	40.78		
	3	4.184	158.8	40.98		
24	1	4.138	160.1	41.31	40.77	2.22
	2	4.109	153.8	39.73		
	3	4.116	160	41.28		
32	1	4.096	149.7	38.70	39.00	0.68
	2	4.099	151.6	39.18		
	3	4.098	151.4	39.13		
40	1	4.117	152.7	39.45	40.16	1.59
	2	4.15	156.3	40.36		
	3	4.171	157.6	40.68		
48	1	4.138	137.6	35.66	35.73	0.87
	2	4.111	139.2	36.07		
	3	4.114	136.8	35.46		
56	1	4.097	137.9	35.74	35.30	1.64
	2	4.099	133.5	34.64		
	3	4.098	137	35.51		
64	1	4.12	136.7	35.44	36.73	3.55
	2	4.138	141.7	36.69		
	3	4.149	147.1	38.05		
72	1	3.536	149.9	38.75	38.11	1.49
	2	3.535	146.5	37.90		
	3	3.535	145.6	37.67		

*NA3 + **1a** + H_2O_2 in batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.136	197.9	49.86	50.15	0.54
	2	4.134	197.6	50.21		
	3	4.132	198.7	50.39		
8	1	3.531	100.6	31.27	31.83	4.41
	2	3.533	110.6	33.43		
	3	3.533	101.7	30.80		
16	1	3.533	88.4	33.74	34.51	3.42
	2	3.533	85.1	33.92		
	3	3.531	85.7	35.87		
24	1	3.534	75.2	33.46	32.81	1.85
	2	3.533	68.8	32.72		
	3	3.537	69.6	32.26		
32	1	3.531	62.4	33.80	33.66	1.60
	2	3.532	56.0	33.06		
	3	3.533	58.0	34.11		
40	1	3.533	56.7	36.37	33.33	8.36
	2	3.533	50.2	30.90		
	3	3.531	51.8	32.72		
48	1	3.534	49.0	31.55	30.79	7.63
	2	3.533	43.6	32.66		
	3	3.537	43.4	28.15		
56	1	3.532	34.9	28.70	27.78	2.87
	2	3.533	29.3	27.40		
	3	3.533	21.9	27.25		
64	1	3.532	27.9	27.68	27.50	0.59
	2	3.533	23.8	27.37		
	3	3.532	24.6	27.44		
72	1	3.534	23.5	27.90	27.59	1.03
	2	3.533	19.9	27.53		
	3	3.536	20.3	27.34		

*NA3 + **1b** + H_2O_2 in batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.136	196.3	50.39	50.67	0.54
	2	4.11	198.5	50.94		
	3	4.114	197.4	50.67		

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
8	1	4.094	141.8	36.72	37.40	2.03
	2	4.098	143.9	37.25		
	3	4.099	147.8	38.22		
16	1	4.12	152.3	39.35	39.29	0.21
	2	4.148	151.7	39.20		
	3	4.169	152.2	39.33		
24	1	4.14	155.4	40.13	39.41	1.68
	2	4.109	150.2	38.83		
	3	4.117	152	39.28		
32	1	4.107	145	37.52	37.90	0.87
	2	4.101	147.1	38.05		
	3	4.096	147.4	38.12		
40	1	4.117	154.4	39.88	39.45	0.94
	2	4.138	151.8	39.23		
	3	4.176	151.9	39.25		
48	1	4.137	133.3	34.59	34.50	0.60
	2	4.11	133.5	34.64		
	3	4.113	132	34.26		
56	1	4.103	131.9	34.23	35.12	2.55
	2	4.102	135.4	35.11		
	3	4.099	139	36.02		
64	1	4.121	138.4	35.87	35.67	1.55
	2	4.136	135.1	35.04		
	3	4.163	139.3	36.09		
72	1	3.534	116.9	35.78	35.72	0.38
	2	3.534	116.2	35.56		
	3	3.534	117	35.81		

*NA3 + **1a** + H_2O_2 in semi-batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.138	194.2	50.79	50.83	0.28
	2	4.139	195.6	50.72		
	3	4.139	196.3	50.99		
8	1	3.530	102.3	30.74	31.88	5.31
	2	3.532	109.3	33.83		
	3	3.532	100.8	31.08		
16	1	3.533	110.3	26.97	26.35	2.06

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	2	3.536	110.9	25.95		
	3	3.533	117.2	26.14		
24	1	3.530	109.4	22.89	21.66	4.96
	2	3.535	107.0	20.92		
	3	3.534	105.5	21.16		
32	1	3.530	110.5	18.94	17.83	5.68
	2	3.532	108.1	16.96		
	3	3.533	111.5	17.58		
40	1	3.533	118.8	17.18	16.00	6.55
	2	3.531	101.1	15.17		
	3	3.532	107.0	15.66		
48	1	3.531	103.2	14.80	13.67	7.19
	2	3.535	106.8	13.13		
	3	3.536	92.2	13.07		
56	1	3.530	94.0	10.44	8.52	23.66
	2	3.533	89.8	8.71		
	3	3.533	89.3	6.42		
64	1	3.533	90.7	8.28	7.52	8.95
	2	3.531	89.7	7.01		
	3	3.533	89.9	7.26		
72	1	3.532	91.4	6.92	6.22	9.79
	2	3.535	90.2	5.81		
	3	3.537	89.6	5.93		

*NA3 + **1b** + H_2O_2 in semi-batch mode:*

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
0	1	4.135	197.5	50.69	50.68	0.57
	2	4.118	198.6	50.97		
	3	4.116	196.3	50.39		
8	1	4.098	133.3	34.59	34.18	1.08
	2	4.1	130.5	33.88		
	3	4.098	131.2	34.06		
16	1	4.132	148.6	38.42	37.95	1.21
	2	4.117	146.6	37.92		
	3	4.115	144.9	37.50		
24	1	4.099	143.7	37.19	37.70	1.52
	2	4.102	145.3	37.60		

Timepoint (h)	Replicate	RT	PA	Concentration (mg/L)	Average concentration (mg/L)	RSD concentration (%)
	3	4.098	148.2	38.32		
32	1	4.13	129.4	33.61	34.29	2.01
	2	4.119	134.9	34.99		
	3	4.12	132	34.26		
40	1	4.096	129.5	33.63	33.80	1.12
	2	4.112	129.1	33.53		
	3	4.096	131.9	34.23		
48	1	3.535	122.7	37.57	37.28	0.69
	2	3.532	121.1	37.08		
	3	3.534	121.5	37.20		
56	1	3.536	98.9	30.22	29.98	1.19
	2	3.537	98.7	30.15		
	3	3.533	96.8	29.57		
64	1	3.534	88.3	26.94	26.34	2.28
	2	3.536	84.4	25.74		
	3	3.536	86.4	26.35		
72	1	3.533	85.9	26.20	25.78	1.83
	2	3.535	84.8	25.86		
	3	3.537	82.9	25.27		