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INVESTIGATING THE BIODEGRADATION OF THE EMERGING POLLUTANT PARACETAMOL BY BACTERIA COMMUNITIES

Erasmus Mundus Masters in

Chemical Innovation and Regulations (ChIR)

Work Supervised

by

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UNIVERSITY OF ALGARVE

Faculty of Science and Technology

2016

University of Algarve

Faculty of Science and Technology

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A Thesis submitted to the Department of Environmental Science and Technology of the University of Algarve in Partial Fulfilment of the Requirements for the Award of the Erasmus Mundus Master in Chemical Innovation and Regulation, supervised by Pr. Maria Clara Costa and Co-supervised by Jorge Carlier (Laboratory of Environmental Technologies - Centre of Marine Sciences - CCMAR)

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DEDICATION

This piece of work is dedicated to my mother **NEBA ESTHER LUM** who has been so inspirational in teaching me how to strive for excellence

ACKNOWLEDGEMENT

My heartfelt gratitude goes to the Erasmus Mundus program of the European Commission who for the life time opportunity and international vision it offered to me. Similarly, my immense thanks go to the EMMC-ChIR management team, directors and most specially to the coordinator Isabel Cavaco for their enormous efforts, support and contributions to make this piece of work a success.

I wish to express my profound gratitude to my supervisor, Pr. Maria Clara Costa and Co-supervisor Jorge Carlier for taking the challenge of accepting and integrating me into the investigating team of the Laboratory of Environmental Technologies and Centre of Marine Sciences (CCMAR). I feel honoured and thankful for their confidence, motivation, hard work, encouragement, patience, enthusiasm, friendship, immense knowhow, creativity, close supervision and follow up.

Special thanks goes to Vera Gomes, Ana Luis and other lab mates for their expertise, constant moral and physical support which served a great deal as well as providing some useful contributions to this work.

I also wish to extend my gratitude to the following friends and classmates Wei Wang, Asnake Gudisa, Miguel Brion, Luis and Bethel Anuchi for their constant moral and psychological support which made me press on and paved my way against all possible challenges faced.

My heart felt gratitude goes to the NEBA PAUL Family of Ntarikon-Bamenda, Cameroon for the good moral ethic they instilled in me. To my fiancé, Ngangwa Loise, I wish to express special thanks for her love, advice, and encouragement she provided daily though thousands of miles away.

Most importantly, I wish to express special thanks to God almighty, the custodian of all knowledge and wisdom for his guidance.

ABSTRACT

Pharmaceutical active compounds are an important group of emerging pollutants that have raised an increasing interest in the scientific community due to their ubiquitous presence in the environment and their difficult degradation. Some of these drugs are extensively used as non-prescription drugs and after their intake, are excreted with urine and faeces either as active substance or metabolites. These substances come into wastewater treatment plants (WWTP) where some compounds are not efficiently removed, being able to reach surface, groundwater and subsequently, drinking water. Microorganisms (single isolates) have the potential to degrade a wide range of xenobiotics and recalcitrant contaminants. The tendency is more reinforced in their communities due to a great synergistic interaction between members of the consortium. In this study, the overall objectives aimed at investigating bacterial biodegrading communities from two different wastewater treatment plants (a passive lagoon system and an activated mud treatment system with aeration) for their abilities in effectively biodegrading and mineralizing paracetamol (APAP) and determining the optimum conditions required to achieve the outcome.

The study examined the aerobic biodegradation of paracetamol by microbial communities from WWTPs in Faro using residual water and minimum salt medium (MSM) as growth media. From an obscure 25 ºC incubated aerobic aerified (110mL/min) bioreactor with paracetamol as the only carbon and energy sources, the biodegradability of paracetamol was evaluated by direct sample analysis after a 24, 48, 72 and 120hrs growth period. An elution gradient HPLC analysis for paracetamol biodegradations and the identification of its associated metabolite respectively showed a 99.9% elimination within 72hrs and a complete degradation after 120hrs for aerified samples with residual water and 97% elimination within 120hrs for aerified samples with MSM. Tentative identified peaks corresponded to the following metabolites: 4-aminophenol, hydroquinone and p-benzoquinone. The Hach-spectrophotometry analysis of the chemical oxygen demand (COD mg/L) showed a progressive decrease in the values within most batch samples hence suggesting a possible usage of the paracetamol during the process. IC_{50} measurements by UV-vis spectrometer produced concentration values far to be toxic for the organisms. In a nutshell, the sludge contains aerobic microorganisms capable of totally degrading APAP and the resulting metabolites to obtain energy without any other source of carbon and energy. Degradation is faster with aeration but slower without.

Keywords:

emerging pollutants, biodegradation, bacterial communities, paracetamol, wastewater

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LISTS OF ABRREVIATIONS AND ACRONYMS

AEOS	alkoxylates
AERO	aerified aerobic culture
APAP	paracetamol
BOD	biological oxygen demand
CEC	chemicals of emerging concern
CFU	colony forming units
COD	chemical oxygen demand
CO2	carbon dioxide
EDTA	ethylenediaminetetraacetic acid
EP	emerging pollutants
EPPs	emerging pharmaceutical pollutants
GSH	glutathione
HPLC	high pressure liquid chromatography
HQ	hydroquinone
MC	microbial community
MSM	mineral salt medium
MWW	municipal wastewater
NAPQI	N-acetyl-p-benzoquinone imine
NPEO	nonylphenol ethoxylates
NSAID	non-steroid anti-inflammatory drugs
OPA	orthophosphoric acid
p-AM	para-aminophenol
p-BQ	para-benzoquinone
PCP	personal care products
RT	retention time
SPE	solid phase extraction
SS	suspended solids
TDS	total dissolved solids
TOC	total organic carbon
TSS	total suspended solids
WWT	wastewater treatment
WWTP	wastewater treatment plant

1. INTRODUCTION

1.1 Objectives and content

Paracetamol is ubiquitous in natural environment and has been detected worldwide in surface waters, wastewater, and drinking water at concentrations capable of causing adverse environmental impacts, thus it is a recalcitrant molecule that cannot totally be removed in wastewater treatment plants (WWTPs) by conventional treatment processes due to its high solubility and hydrophilicity. It has been found in concentrations as high as 6 mg/L in European WWTP effluents and at a maximum concentration of 10mg/L in US waters [1].

Its occurrence in treated wastewaters, drinking waters and/or general environment causes recognized negative effects within the receiving water bodies and on human health [2]. Microorganisms, particularly bacteria are reputed in exercising and playing an important role in the biodegradation of organic compounds [3].

Taking into account the unfriendly environmental impacts caused by photolytic degradations and the well know advantages of consortia over pure cultures [4], the present work tries to investigates bacterial biodegrading communities from two different types of WWTPs (a passive lagoon system and an activated sludge treatment system with aeration) for their capacity of effectively eliminating. The work lays claim to the stated objectives

- investigating bacteria biodegrading communities capable of effectively eliminating the emerging pollutant paracetamol,
- evaluating the most suitable conditions in which the degradation effectively occurs with a relatively limited cost and finally,
- The sub goal of the research aimed at determining the possible metabolites generated from the degradation.

1.2 Pollutants

Over the past century, evolution in science has led to major breakthroughs that have expanded and vastly improved human life in the area of exploration and development of pharmaceuticals. In Europe, America, ocean continents, and other continents, lots of regulations and legislations have been promulgated and are being scrupulously respected vis-à-vis chemical pollutants or wastes. Though certain nations or companies within nations do not follow some of the legislations rightly (e.g. Volkswagen (VW) carbon dioxide (CO_2) emission level malpractices of 2015), a large majority of these legislations and regulations are being respected. The principal aim of these legislations and regulations is to improve on the sustainability of the

planet Earth upon which human existence largely depends. On the contrary, an inadequate or insufficient implementation of these legislations and regulations would be catastrophic to the planet and its inhabitants. With progressive enforcements of these efforts, not all substances have regulations or legislation limiting their exposure or release into the environment. Chemical pollutants that do not have an established regulation and legislation are commonly classified under the class of pollutants referred to as emergent pollutants.

Pollution is a significant problem facing the environment nowadays. As the world's population continues to grow, the amount of potentially toxic substances that are released into the ecosystem also increases. Environmental pollutants can be derived from a number of sources. Knowing what the different types of pollution and their origins, can help us understand the potential impact these pollutants have on our health and that of the planet as a whole

a) Soil Pollutants

Soil pollution is the pollution of the Earth's land surfaces. According to Green Pack, the most common types of soil pollutants are heavy metals such as cadmium, chromium, copper, zinc mercury, pesticides or herbicides, organic chemicals, oils and tars, explosive or toxic gases, combustible or radioactive materials, biologically active compounds and asbestos [4]. These pollutants enter the soil through poor agricultural practices, industrial runoffs, mining, landfill leakage, littering or the improper or illegal dumping of household or industrial waste materials.

b) Air Pollutants

Air pollution is the pollution of the Earth's atmosphere. The U.S. Environmental Protection Agency identifies six types of common air pollutants. They include ozone, particulate matter, carbon monoxide, nitrogen oxides, sulphur dioxide and lead. These and other air pollutants typically enter the atmosphere through industrial processes related to the generation of heat and power, incineration of solid wastes and transportation. According to the University of the Western Cape, emissions from vehicles are estimated to be responsible for approximately 60% of all air pollution alone and 80% of air pollution in cities [5].

c) Water Pollutants

Water pollution is the pollution of the Earth's oceans and other water sources. According to the Minnesota Center for Environmental Recovery, common types of water pollutants include mercury, nitrates, phosphorous, faecal coliform and bacterial pollution. These and other types of pollutants enter the water supply through industrial waste runoff, sewage treatment plants, feedlots, urban and agricultural runoff, septic systems and the illegal dumping of solid waste.

d) Noise Pollutants

Noise pollution is a form of air pollution related specifically to the types of sound present in the atmosphere. The Environmental Protection Agency defines a noise pollutant as any sound that interferes with normal activities or disrupts, or diminishes one's quality of life. Noise pollutants can be present in the home, school, work or the community at large. Different types of noise pollutants may include sounds generated by aircraft, trains, boats, automobile traffic, construction, industrial manufacturing, vehicle alarms or even loud music.

1.3 Problematic of pollution

The five main axis on which pollution exhibits its effects are summarised below.

a) Environmental degradation

Primary casualty resulting from pollution increased the amount of CO_2 into the atmosphere leading to the production of smog, which can restrict sunlight passage unto the earth surface, hence preventing photosynthesis. SO_2 and NO_2 if mixed with rain water can produce acid rains. Oil spill may lead to death of several aquatic species and wildlife species.

b) Human health

A decrease in air quality leads to several respiratory diseases including asthma and lung cancer, chest pain, throat and inflammation cardiovascular diseases [6]. Water pollution on its part may lead to skin irritation and rashes (reference). On a similar note, noise pollution leads to stress, sleep disturbances and hearing loss [7].

c) Global warming

Greenhouse gases (GHG) such as CO_2 is the principal cause of global warming [8]. On a daily basis, new industries are gaining space, new vehicles are produced and used, more and more tress are being felt to build houses due to an increased population density, all these indirectly or directly lead to an increase in CO_2 release into the environment. The increased CO_2 results to the melting of ice-caps which increases sea level hence posing danger for inhabitants.

d) Ozone layer depletion

Human activities, chemicals e.g. chlorofluorocarbon (CFC) released into the atmosphere have contributed enormously to the depletion of ozone layer.

e) Infertile land

The continuous use of insecticides, pesticides and fungicides on soil render them infertile for plant growth. In case of rainfall over these lands, water may become contaminated with these chemicals, which will evidently affect 60% of the aquatic species living in water.

Knowledge on emissions, environmental fate, acute and chronic toxicity of pollutants is widely recognised as a basic need required to assess environmental risks. The environmental fate of most pollutants of chemical origin comprises the portioning between water and suspended solids, partitioning and uptake into biota adsorption to sediments, volatilization from water and breakdown (biotic and abiotic).

1.4 Pollutants removal mechanisms

Pollutants of pharmaceuticals origins can be removed from the aqueous phase in wastewater treatment plants through several processes. The most commonly used examples are:

1.4.1 Biodegradation

Biodegradation term is often applied in relation to ecology, waste management and mostly associated with environmental remediation or bioremediation [9].

"Degradation" means decay, and the "bio-" prefix means that the decay is carried out by a huge assortment of living microbial organisms notably: bacteria, fungi, insects, worms, and other organisms that eat or breakdown dead material or products thus converting them into new forms [9]. In simple terms, Biodegradation is the biological catalysed reduction in complexity of chemical compounds [10]. Biodegradation is nature's way of recycling wastes or breaking down organic matter into nutrients used by other organisms. In nature, there is no waste because everything gets recycled. The waste products from one organism become the food for others, providing nutrients and energy, while breaking down the waste organic matter. Some organic materials will break down much faster than others, but all will eventually decay.

When biodegradation is complete, the substances are converted to inorganic substances and the process is called “Mineralization”. However, in most cases the term biodegradation is more extensive and generally used to describe any biologically mediated change in a substrate.

Biodegradation is thus a very important process in the transformation of organic pollutants (pharmaceuticals inclusive) in Wastewater treatment plants (WWTPs). Alongside other co-metabolic breakdown processes of organic compounds, it facilitates a gain in energy to the microorganisms or bacteria carrying out the degradation process. In WWTP setups, biodegradation as earlier mentioned can be partial or complete. It is thus worth determining the outcomes that arise when each case scenario is attained. For complete breakdown, the final products are water (H_2O) and carbon dioxide (CO_2), whereas partial breakdown results in the transformation of pharmaceuticals into metabolites. Taking into account that these metabolites can be persistent, it is important to determine their fate within biological systems.

Equation 1.1 [11] can describe the rate of breakdown of any particular pharmaceutical with a pseudo first order reaction.

Equation 1.1: Relationship between degradation rate and Concentration

$$\frac{\delta Ci}{\delta t} = K_{biol,i} * SS * Ci$$

Where: Ci total concentration of pharmaceuticals

t time (days)

$K_{biol, i}$ specific biological degradation rate constant of pharmaceutical i (L/Gss/d)

SS Suspended solids concentration (g/l)

From Equation 1.1, the degradation rate is proportional to the concentration of the pharmaceutical and SS. Most bacterial exists in suspension in liquid media, the lower the SS value the fewer is there a possibility for these organisms to be in suspension. Based on the $\mathbf{K}_{biol,I}$ constant obtained from aerobic batch tests, pharmaceutical compounds can be classified as follows based on their degree of removal from conventional WWTP [11].

- K< 0.1 L/g SS/day No removal (less than 20%)
- 0.1< k < 10 L/g SS/day Partial removal 20 to 90%
- K< 10 L/g SS/day More than 90% removal.

By harnessing these natural forces of biodegradation, humans can reduce wastes and clean up environmental contaminants with hazardous effects. Using one of the biodegradation methods known as composting (common in most living hoods), organic wastes are easily converted to valuable resources. Applying the same process in wastewater, it is easy to demonstrate the ease to which wastewater accelerates natural forces of biodegradation. In this case, the purpose is to break down chemical substances of pharmaceutical origins in order to limit environmental pollution and contamination. Thus, in biodegradation it is possible to use microorganisms to breakdown persistent pharmaceutical wastes within wastewater treatment plants (WWTP). Most often, the inefficiency of the breakdown in the treatment plant is due to the relatively short period these substances turn to stay within the system.

1.4.2 Bioremediation

Bioremediation is the use of living organisms, primarily microorganisms, to degrade environmental contaminants into less toxic forms. It uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and/or the environment.

Bioremediation has the potential of complete degradation or transformation of hazardous organic pollutants into harmless products. The use of microorganisms in bioremediation is not limited to detoxification of organic compounds. Some microorganisms can transform cations of heavy metals into less toxic or less soluble forms. The principle is based on biologically degrading and detoxifying of soil, groundwater, wastewater and air from hazardous substances. Examples of these hazardous substances include: organic pollutants (oil products, pesticides, detergents, polymers, organic solvents, personal care products (PCP)), pharmaceutical products fertilizers, heavy metals (e.g. Hg, Cd, Pb), toxic elements and compounds (Ar and HCN) and toxic gases (H_2S). Most often, these products are effectively eliminated using microorganisms. Bioremediation process can be divided into three phases or levels notably:

1.4.2.1 Natural attenuation

This is a process whereby native microorganisms without any human augmentation degrade or reduce contaminants concentration in the environment through biological processes. When the environment is polluted with chemicals, nature can work in four ways to clean it up [12].

i. Tiny bugs or microbes that live in soil and groundwater use some chemicals for food. When they completely digest the chemicals, they change them into water and harmless gases.
ii. Chemicals can stick or sorb to soil, which holds them in place. This does not clean up the chemicals, keeps them from polluting groundwater and leaving the site.
iii. As pollutants move through soil and groundwater, they mix-up with clean water hence rendering them less hazardous.
iv. Chemicals like oil and solvents evaporate and change their physical forms within the soil. They can be destroyed by sunlight if they escape from the surface.

1.4.2.2 Bio-stimulation

This is the addition of nutrients, trace minerals, electron acceptors/donors or oxygen unto a system in order to enhance the biotransformation of a wide range of contaminants, hence improving its effectiveness and accelerating biodegradation.

1.4.2.3 Bio-augmentation

Bio-augmentation is a process whereby microorganisms are added unto a system in order to speed up the rate of degradation of a contaminant. The supplemental organisms should be more efficient than the native flora to degrade the target contaminant [13]. Studies have shown that abiotic factors such as temperature, pH, moisture and organic content alongside biotic factors

such as antagonistic interactions, exogenous and indigenous carbon source competition turn to influence immensely bio-augmentation [14]. Due to these enormous variabilities, bio-augmentation is considered the last option for remediation. It should be implemented only when natural or bio-attenuation and bio-stimulation have both failed [15]. It is worth noticing that bioremediation has proven to be cost effective and beneficial with respect to chemical and physical methods vis-a-vis managing wastes and environmental pollutants.

1.4.3 Sorption

Defined as a physical and chemical process by which one substance becomes attached to another. In WWTP, sorption to sludge can be an important removal mechanism especially when a pharmaceutical is persistent and has a high sorption potential. Lipophilicity and electrostatic characters are important aspects considered for compounds that are sorbed to the sludge. Two different kinds of sorption mechanisms, adsorption and adsorption can occur.

1.4.3.1 Absorption

Absorption is related to hydrophobic interactions of aliphatic and aromatic groups of a compound with the lipid fractions of the solids [16]. The hydrophobic character of a compound can be indicated by the K_{ow} value. K_{ow} is the partition coefficient between octanol and water for a specific compound. The higher the log K_{ow} value, the more hydrophobic the substance is. Three (3) groups can be distinguished for their sorption behaviour based on the Log K_{ow} values (Jones 2005).

- Log Kow < 2.5 Low sorption potential
- Log Kow > 2.5 but < 4.0 Medium sorption potential
- Log Kow > 4.0 High sorption potential

1.4.3.2 Adsorption

Adsorption is related to electrostatic interactions with the substance and the surface of microorganisms or adsorption materials. Knowing fully well that sludge is negatively charged, it will obviously attract positively charged molecules. This parameter affects most often pharmaceuticals of acidic nature. The pKa value indicates the acidity of a pharmaceutical. The lower this value, the more acidic a compound is, hence decreased adsorption affinity.

1.5 Wastewater

The manner in which organic compounds and pollutants enter the environment depends on their pattern of usage and mode of applications e.g. disposal of industrial, municipal and agricultural wastes, excretion of pharmaceutical an accidental spill. Once in the environment

they can be widely distributed. Because most emerging pollutants are from human use, their emissions are an issue for some wastewater processes. Therefore, the study of these pollutants occurrence and elimination within wastewater system is vital because most WWTP serve as reservoir of these pollutants.

1.5.1 Definition and characterisation

Wastewater is contaminated liquid effluents generated from institutions (residential, industrial and commercial), surface waters, ground waters and discharged into the community environment through various pathways [17]. By composition, it is made up of ninety-nine percent (99%) water by weight and one percent (1%) dissolved organic, inorganic substances and microorganisms. Wastewater from residencies, commercial places, institutions and farms are commonly referred to as domestic wastewaters or sewage. This water is divided into two groups; Blackwater basically made up of wastewaters from toilets (urine, faeces etc.) and Grey water refers to household wastewater originating from bathrooms and laundries [18] Grey water constitutes the largest flow of wastewater whereas industrial wastewater varies in flow and composition depending on the type of industry. A combination of sewage and industrial wastewater is referred to as Municipal wastewater (MWW) [17]. Wastewater is characterized in terms of its physical, chemical and biological compositions [19].

- Physical composition is made up of parameters such as total solid content [sub divided into total dissolved solids (TDS) and total suspended solids (TSS)] colour, odour temperature, particle size distribution, density, turbidity transmittance and conductivity.
- Chemical parameters are mostly associated with the organic content of wastewater and are made up of biochemical oxygen demand (BOD), chemical oxygen demand (COD), total oxygen demand (TOD) and total organic carbon (TOC).
- Inorganic parameters include salinity, acidity, pH, hardness, alkalinity as well as the concentration of ionized metals such as Fe and Mn
- The bacteriological parameters are faecal coliforms, pathogens and viruses [17].

1.5.2 Wastewater treatment plant processes

Wastewater treatment processes include chemical, biological and physical units to remove chemical and biological contaminants. Generally, wastewater treatment processes can be broken down into four basic steps with each specific step targeting the removal of contaminants at different levels. These steps include:

- Preliminary
- Primary

- Secondary and
- Tertiary (Advanced)

The primary and secondary unit operations and processes are commonly referred to as conventional wastewater treatment. Immediately after the secondary or conventional treatment comes the tertiary or advanced treatment, which is aimed at improving the quality emanating from the secondary wastewaters. The advanced process on its part produces an effluent that can be used as a substitute of freshwater sources for household and industrial needs [20, 17].

Collectively referred to preliminary treatment, the primary and secondary steps target the removal through screening and gravity of objects, oils, grease, fats, rags and grits [20]. By acting as a precursor to secondary treatment, the primary treatment partially removes suspended solids (SS) and organic matter found in wastewaters by means of physical operations such as screening and sedimentation. The secondary treatment units or process are designed to be capable of removing biodegradable dissolved and colloidal organics and SS that escaped the primary treatment unit. Three types of technologies are used to breakdown organic matter with agitation and aeration activated sludge, Trickling filters and Lagoon system [17].

The activated sludge process uses a variety of mechanism to re-utilize dissolved oxygen to promote the growth of a biological flock that substantially breaks down and removes organic material followed by a forceful settling-out of the solid flocks. Re-circulating bacteria containing "activate sludge" back to the aeration basin increase the rate of organic breakdown or decomposition [17]. After the conventional treatments, many physical-chemical processes have been investigated as tertiary treatment of secondary effluents. Most prominent ones are photocatalytic degradation of recalcitrant compounds using UV/ visible radiation and TiO_2 as photo-catalysis, ozonation [21] and adsorption, mostly with activated carbon [19]. Tertiary treatment is always performed by a sequence of coagulation, flocculation, membrane filtration and disinfection [22]. Despite all these physical-chemical process analysis and investigations within these stages of treatment, a considerable number of research works have reported and are still reporting the occurrences of some contaminants after treatment [23]. The most prominent one reported and which is becoming a real environmental issue now are emerging pollutants with pharmaceuticals having the highest occurrence.

1.6 Emerging Pollutants.

1.6.1 Occurrence and fate of EPs

Emerging pollutants are compounds that are not currently covered by existing water quality regulations, have not been studied before, and are thought to be potential threats to environmental ecosystems, human health and safety. They consist of a diverse group of compounds including personal-care products (PCP), surfactants, steroids and hormones, pharmaceuticals, industrial additives, flame retardants just to name a few. See Table 1.1

Table 1.1: Classes of Emerging Pollutants. A modification of Lapworth *et al*., 2012

Compounds	Examples
Drugs of abuse	Amphetamine, cocaine, tetrahydrocannabinol
Flame retardants	C_{10}–C_{13} chloroalkanes, hexabromocyclododecane,
Industrial additives and agents	Chelating agents (EDTA), aromatic sulfonates
Fragrances, insecticides, soaps, antimicrobials, sun-screen	Polycyclic and macrocyclic musk; triclosan
Pharmaceuticals	APAP, diclofenac, diazepam,
Analgesics & anti-inflammatory drugs, human and veterinary	Carbamazepine, bezafibrate, iopromide, oopamidol
Surfactants and surfactant metabolites	Alkyl-phenol ethoxylates, (nonylphenols & octylphenols), Alkylphenol carboxylates
New classes	Nanomaterials, swimming-pool disinfectants by-products

They are micro pollutants that are increasingly being sent into the environment. They are mostly referred to contaminants of emerging concern (CECs). Some common ones include alkyl-phenols, flame-retardants, hormones, personal care products, pharmaceutical steroids and pesticides. A majority of these CECs goes into the WWTP system via household usage paths like: bathing, laundry, cleaning, human wastes disposal and unused pharmaceuticals. On the other hand, most WWTP are designed to effectively undertake a secondary treatment process (activated sludge) in order to treat biological oxygen demand (BOD) and total suspended solids (TSS). These plants to some extent treat or disinfect the sludge wastes to inactivate or remove pathogens. Others are designed to be used as an advance treatment plant for other pollutants notably nutrients. WWTPs are often not designed to specifically remove CECs though at a certain level, they do eliminate some. Due to such outcomes, it is possible to find some of these CECs in our drinking water [11].

1.6.1.1 Occurrence of EPs

EPs reach the environment through diverse routes of transportation and distribution. Their different physical and chemical properties notably hydrophobicity, hydrophilicity, water solubility and vapour pressure determine their behaviour within the environment. The major sources of environmental relevant emerging contaminants are primarily WWTP effluents, seconded by terrestrial run-offs (roofs, pavements, roads etc.) and atmospheric deposition.

Veterinary drugs used for treatment and prevention of diseases in farming are deliberately introduced into the environment when manure is sprayed on agricultural field. These drugs and their metabolites are prone to contaminate soil and groundwater.

On a similar note, pharmaceuticals enter the aquatic systems after ingestion and subsequent excretion in the form of the non-metabolized parent compounds or as metabolites through WWTP [24]. If there is a possibility of these pharmaceuticals to pass through wastewaters, then there will evidently reach streams and rivers. They can reach groundwater after leaching and as well reach surface waters by run-offs from fields treated with digested sludge.

On another hand PCPs such as fragrances discharged through shower wastes passes through WWTP to reach the environment [25]. Commonly used examples of PCPs are nonylphenols and ethoxylates (AOEs). Nonylphenols are products from the degradation of 4-nonylphenols ethoxylates (NPEOs): Alkoxylates are ionic surfactants extensively used in cleaning products and in industrial processes. NPEOs are commonly present in detectable amounts within WWTP alongside AEOs due to their massive domestic and industrial uses; AOEs and nonylphenols are common in water, suspended particulate freshwater material, marine, estuarine environments and sediments [26]

1.6.1.2 Sources, translocation and fate of EPs

Nowadays, many households associated operations and human activities are responsible for the huge production of different wastewater streams. With growing technology and an increasing insufficient space required for expansion, the existing combined sanitary system are designed such that, streams originating from households are collected within the same piping system and send to the conventional WWTP (Figure 1.1). The content of these streams can be separated by taking into account their concentration and composition [27]. Ninety percent (90%) of EPs derive their sources from household usages and each section of these households contributes differently and significantly to their continuous discharge in to the environment.

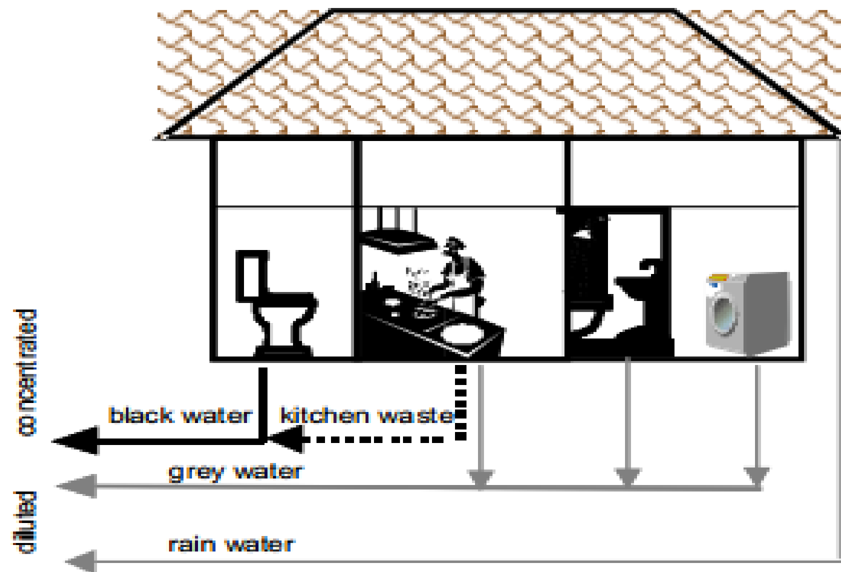


Figure 1.1: Wastewater streams produced in households

The waste referred to as black water originates from toilets and is the most concentrated streams because it consists of faeces, urine and flush water [28]. This waste contains a considerable amount of organic compounds, fractions of the nutrients from domestic wastewater, a vast majority of microorganisms and emerging pollutants.

Grey water on its part is a combination of sub-streams originating from shower, laundry and kitchen though relatively diluted compared to the black water. Be it grey or blackwater, the final destination of these substances are the aquatic environment. It has a high potential of reuse because it is the major fraction (70%) of domestic wastewater and relatively low in pollution.

1.6.2 Source separation sanitary concept

In the conventional sewage systems, not all the pharmaceuticals are removed from the WWTP. In order to limit the release of pharmaceuticals into the environment, additional treatment steps could be introduced for this purpose or the removal of pharmaceuticals could be integrated in the new source separated sanitation concept. The concept is considered as an optional possibility to reuse nutrients and clean water from micro-pollutants like pharmaceuticals. The principle entails collecting wastes from two separated domestic waste streams having different characteristics: for example, a concentrated black water stream of urine, faeces and a low concentrated grey water stream made up of shower, kitchen and laundry water [29]. The latter contains an important part of nutrients, pharmaceuticals, pathogens and a large part of Carbon Oxygen Demand (COD) in a relatively small volume whereas the COD concentration and the nutrient content in the grey water is relatively small. With no-mix toilet systems, faeces could

be separated from urine in Blackwater hence enabling a reduction in the release of the 75%Nitrogen and 50% phosphorus content of urine originating from total household's wastewater into the environment. Thus, this can serve as an alternative for a better water pollution control with respect to nutrient removal and reuse [11].
The advantage of this concept in relation to pharmaceuticals is that a good majority of the pharmaceuticals found in the blackwater are excreted out in high concentrations. In addition to this, seventy percent (70%) of the pharmaceuticals excreted will be present in the urine [11] which originally is a very small waste stream (1.5L/person/day).

1.7 Microorganism communities

Microorganisms do not exist in isolation in the environment but form complex communities among themselves as well as with their hosts. Different forms of interaction established by these organisms do not only shape the composition of these communities but also define how these communities are established and maintained. Microbial communities (MC) are at the heart of all ecosystems and could be defined as the assemblage of multi-microbial species in which organisms live together in a contiguous environment and interact with each other. The idea behind their study aim to analyse how biological assemblages are structured, what are their functional interactions and how the community structure changes in space and time. This has been partially fulfilled thanks to a growing number of discoveries based on their metabolic abilities to accumulate, metabolize and degrade compounds such as cellulose, alkanes and plastics [30]. This MC research has recently been taking hold in the field of synthetic biology and an increasing awareness of their importance to human existence. For example, the composition of the gut community has been shown to have an effect on various pathologies [31] as well as physiological traits such as obesity. A variety of these communities possesses very complex interactions among their members with a vast of them not very understood. These interactions can highly be dynamic hence forcing them to have alternative roles along with proportion within the same population. System biology has immensely facilitated a detailed study of these interaction complexities [32], as well as to identify the composition of a community through a high throughput 16s rRNA gene sequencing technique [33].
Despite these numerous complexities, MCs have been deployed for several industrial uses. For example, the production of biofuels from lignocellulose material, bio-mining and bioremediation. Similarly, they have been found to be beneficial for the production of natural products such as vitamin C precursors [34]. These communities are in ninety-nine percent composed of microorganisms with different forms, shapes and origins. Several microorganisms

such as fungi, yeasts, can be used in bioremediation processes. Despite the numerous microorganism, bacteria were the focal choice for this study

1.7.1 Bacteria

Bacteria are the most populous of the microorganisms used in wastewater treatment. These singled celled organisms directly breakdown the polluting matter in wastewater. Bacteria can be sub-divided into two major groups notably Heterotrophs and Autotrophs. Whatever the group, they possess an almost identical mechanism of metabolism. (Figure 1.2)

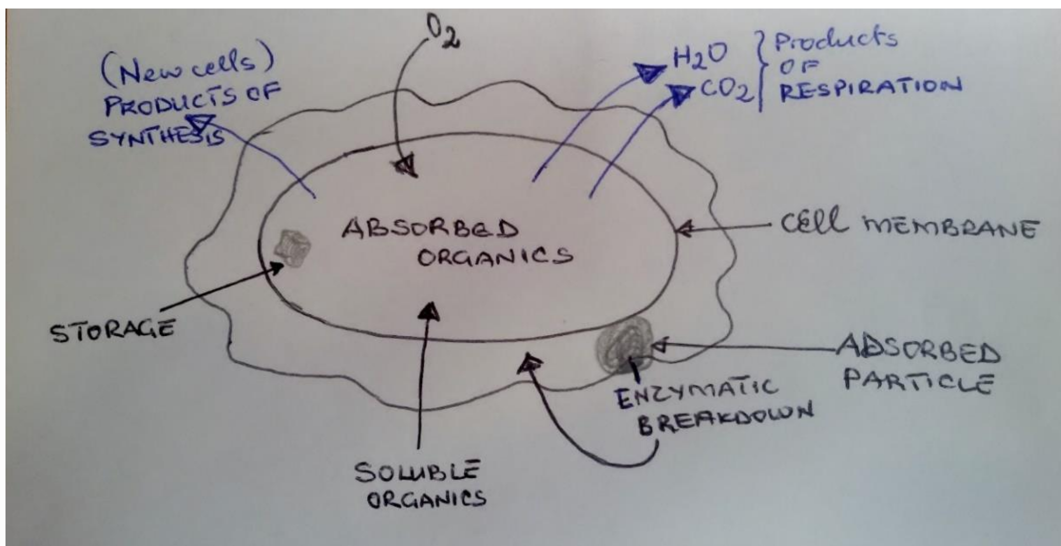
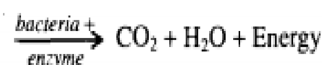


Figure 1.2: Metabolism and Transportation mechanism in Bacteria cells CRS Group 1978 - modified

Heterotrophs are capable of breaking down organic matter such as carbohydrates, proteins and fats. These breakdowns can be characterised by biological oxygen demand (BOD) and chemical oxygen demand (COD) in wastewater. The ease with which these compounds biodegrade facilitates high growth rates for the microorganism. Two main equations can be used to summarise this breakdowns processes.

Equation 1.2

(i) Organic matter + O_2



$$\xrightarrow[enzyme]{bacteria\ +} CO_2 + H_2O + \text{Energy}$$

(ii) Organic matter + P + NH_3 + O_2 + Energy

$$\xrightarrow[enzyme]{bacteria\ +} \text{New cell material}$$

Internal and external enzymes are used to breakdown the substrate (food) into consumable forms that are easily used up for maintenance and propagation of life. These forms or microorganisms can live under variable conditions. Bacteria living in the presence of oxygen are termed aerobes and anaerobes for those living in the absence of oxygen. Facultative microorganisms or bacteria are those that can live under both conditions.

1.7.1.1 Methodology and classification

Most bacteria are very small in shape and sizes of the order of a few micrometres in length. The most common are the rod-like and spherical shapes corresponding respectively to bacillus and coccus forms. The rod shapes vary from very short rod that are similar to cocci to very long filament. Bacteria also form spiral and corkscrews, oval commas and elaborately branched structures. The cocci often exist as streptococci or tetrads. Another criterion for distinguishing bacteria is based on the cell wall structure. This cell wall gives different coloration when stained with reagents called Gram staining. Thin cell walls stain red corresponding to gram-negative bacteria and thicker cell wall that stain violet are gram-positive bacteria.

An alternative classification identifies and classifies another division under a separate kingdom referred to as Achaea. They include many interesting bacteria with unusual metabolic capabilities such as those that produce methane. Achaea differs in many ways from the bacteria known as Eubacteria commonly used in the classroom.

1.7.1.2 Bacteria growth and growth curve

With an existent variable morphology, bacteria share one major characteristics known as binary fission: a process whereby a single cell or colony of cells is capable of doubling their original number and genetic content. The process leads to the generation of daughter cells called clones or colonies when a mass of cell is formed. This is known as colony forming units (CFU). The mathematics of bacteria growth stems from a single division into two daughter cells with loss of original parent in a series expressed as follows: 1, 2, 4, 8, 16, 32, 64... or 1, 2^1, 2^2, 2^3, 2^4...

This common growth pattern is referred as exponential serial growth, which leads to fast population increment. For example, bacteria with a generation time of thirty minutes can result to an almost sixteen-fold population increment in 24hours. The growth becomes limited as the population density increases. For a bacterium newly inoculated into a fresh growth medium, growth overtime can be graphed as cell number against time. This curve typically has four distinct phases, Lag, Exponential (Log), Stationary and Death phases [35]. See Figure 1.3

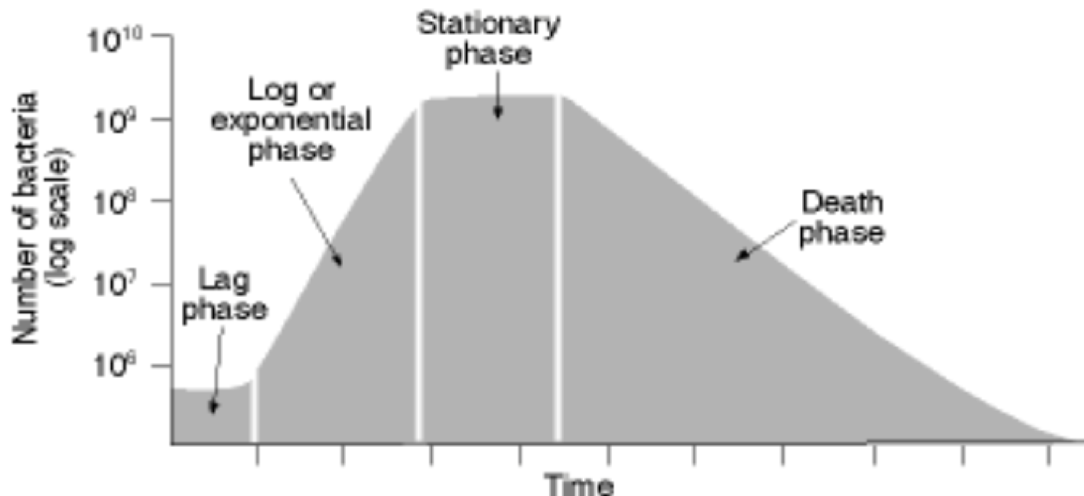


Figure 1.3: Bacteria growth curve and constituent phases of growth.

At the level of the first phase or lag phase, cells are characterised by no increase in cell number but are actively metabolising and preparing for cell division. These phases maybe short if and only if the growth medium supplies are rich in cell requirements and long if the milieu lacks these nutrients. Alternatively, if cells are just diluted from one medium to a relatively freshly tube of the same medium, this phase may be absent because cells won't need to change their metabolisms. The time it takes the culture to double is called the 'generation time'. The generation time can be easily obtained from the exponential phase of a growth curve. This is done by plotting the log of the cell number versus time. This plot will yield a straight line when the cells are in exponential growth. The generation time can be read directly from the graph using two points on the straight line that represent a two-fold increase in the cell number. Once cells start active metabolism, DNA replication begins and eventually, cell division give open up the second phase of growth: the log or exponential phase. At this phase, the substrate or medium is highly consumed by cells until it becomes limited because they are growing and doubling at a constant rate.

The stationary phase is characterised by a drop in the metabolic rate of the bacteria due to a drop in cell division. At this point, the substrate left is used to sustain live and not for growth. Environmental factors, nutrients depletion and accumulation of wastes are among the changes that slow the growth and decrease in metabolic rate. If cells at this stage are inoculated within a fresh medium, they regain exponential growth. As the substrate within becomes more and more limited, bacteria die off and others feed on them (cryptic growth or endogenous respiration). Here, bacteria cells do not divide or quickly lose their duplicative abilities even if they are placed in a fresher medium.

Death phase is by itself an exponential phase where the cells die quickly. This stage could be slowed in order to maintain maximum cell viability by slowly lowering the temperature. These four phases are the rate-determining step for bacteria growth.

1.7.1.3 The factors affecting growth

The activities of microorganisms and bacteria in particular are greatly affected by chemical and physical conditions of their environments. Different organisms react to their environment in different ways. Microorganisms show varying sensitivity to different types of factors. A deadly factor to a specific specie may be beneficial to the development of another specie. Factors capable of affecting growth actually affects the generation time of the organisms. These factors include temperature, pH, oxygen, salt concentration and nutrients.

- **Temperature**

Different bacteria species have different temperature requirements for growth Psychrophiles grow best at cooler temperatures ranging from -5 to 20ºC. They can be found growing in refrigerators, snow, Arctic and Antarctic, and deep oceans are slow growing bacteria commonly found at temperature below 20ºC. Examples such as *Staphylococcus aureus* and listeria species can grow in refrigerated food and cause food-borne illnesses. Mesophiles have as best temperature growth ranges from 20 to 40 ºC with optimum at 25ºC. They are the most disease-causing forms of bacteria. Thermophiles on their part grow in hot spring and thermal vents prefer warmer temperature ranging between 40 to 80ºC. Hyperthermophiles on their part can be found in geysers and volcanoes where they grow at temperature above 80ºC

- **pH**

Each organism has a pH range within which growth is possible and most have well defined optima ph. A majority of natural environments have a pH value range of 5 to 9: a range that harbours numerous microorganism has their optima growth. Based on their optimum pH, bacteria can be placed in one of the following group neutrophils, acidophiles and alkaliphiles. Neutrophils are bacteria that thieve at pH of 6-8 with optima situated at 7. These are common disease causing bacteria in humans because they are capable of living in the human systems that are characterised by an optima pH of 7.4

Acidophiles on their part are made up of Achaea and certain types of bacteria. They thrive in acidic environment such as sulphuric acid pools where pH ranges from 1 to 5. They have the ability to pump H^+ out of their system in order to prevent the destruction of essential molecules such as DNA.

Alkaliphiles are classes of extremophiles microbes capable of surviving in alkaline (pH 8-11) environments. They are obligate alkaliphiles because they require high pH to survive. Facultative alkaliphiles are those that require high pH but also grow in normal conditions and halo-alkaliphiles corresponding to those that require high salt content to survive [36]

- **Salts**

Many bacteria thrive in high salt environments. These salt-loving bacteria are called halophiles. Many halophiles belong to the bacteria kingdom called Achaea. They have active mechanisms to pump out salt, keeping the inside of the cell at a normal salt concentration. They exist anywhere with a concentration of salt 5 times greater than the concentration of salt in oceans such as the Great Salt Lake, lake Utah and Owens Lake. Halophiles can be classified as slightly moderate, or extreme with respect to their halo-tolerance. Slightly halophiles prefer 0.3 to 0.8M (seawater has as concentration 0.6M), moderate halophiles 0.8 to 3.4M and extreme halophiles 3.4 to 5.1M salt content [37]. Halophiles require sodium chloride for growth, in contrast to halotolerant organism that are capable of growing in the presence or absence of saline conditions.

- **Oxygen**

Microbes and bacteria display a great diversity in their ability to use and tolerate oxygen. This is simply because oxygen (O_2) can be essential and toxic to life. Aerobes prefer O_2 and anaerobes do not and facultative swabs between the two conditions.

Aerobic and anaerobic bacteria are identified by growing them in test tubes of THGL broth [38].

Figure 1.4 : The effects of Oxygen on the growth of various types of Bacteria

The following observations arise and correspond to specific condition.

1. Obligate Aerobes need oxygen because they cannot ferment or respire anaerobically hence gather at the top of the test tube because oxygen concentration is high.

2. Facultative Anaerobes grow with or without oxygen because they can metabolise energy with or without oxygen. They are found to aggregate at the top of the tube because aerobic respiration generates more energy than fermentation [39].
3. Obligate Anaerobes are poisoned by oxygen and therefore gather at the bottom of the tube where oxygen concentration is lowest [40]
4. Aero-tolerant organisms do not require oxygen because they can metabolise their energy anaerobically. Unlike obligate aerobes, they are not poisoned by oxygen and can be found evenly spread within the tube.
5. Microaerophiles assemble at the upper part of the test tube but not at the top. These microbes require oxygen, but at concentrations lower than those found in the atmosphere.

Thus, too little or excessive oxygen in an environment influences the biological activities of any microorganisms with bacteria not exclusive.

1.8 Pollutants of pharmaceutical origins

Pharmaceuticals products and their metabolites are subclasses of organic contaminants that have been detected in wastewater and surface waters throughout the world [41, 42]. Continuously, these classes of pollutants are being introduced into the aquatic environment via human, industrial, agricultural and municipal activities. [42]. With the recent advancement in analytical techniques of trace pharmaceutical residues, many studies have demonstrated the widespread occurrence of pharmaceuticals in water environment at minute concentrations. Despite the fact that the concentrations most often detected vary between Nano-grams (ng/l) to Micrograms per litre (µg/l) range, it is worth noting that, these molecules if sensitively active, would eventually affect aquatic organisms [43]. Numerous adverse effects varying from acute and chronic damage, accumulation in tissues, reproductive damage, inhibition of cell proliferation, and behavioural changes, have been documented at low concentration levels of these pharmaceutical products send into the environment [42]. Studies aimed at elucidating a clear cut understanding of pharmaceutical transformation in surface water or water milieu as a whole have focused more on photo-degradation than microbial-mediated degradation as an alternative pathway for their elimination. The outcome of these researches have produced reports capable of elucidating the ease to which photo-degradation can be used to degrade pharmaceuticals like sulfamethoxazole, diclofenac [44] over others like carbamazepine, levofloxacin, cimetidine and Clofibric which are resistant to the process [45]. Summarily speaking, photo degradation of pharmaceuticals varies structurally with dissimilar compounds.

1.8.1 Pharmaceutical in the Environment

The increasing consumption of pharmaceuticals and development of analytical tools with very low detection limits to determine these trace compounds in various environmental matrices have led to a gradual increase of these compounds in the environment via WWTP effluents or application of manure in farm fields. Pharmaceutical end up in the aquatic system simply because, WWTPs are not designed to efficiently remove these wastes. With the increasing use of pharmaceuticals worldwide and the shortcomings of photo degradation, it is very important to analyse, develop and optimize environmental friendly approaches for photo degradation.

1.8.2 Use of Human pharmaceuticals

Within the interval of time between 2002 and 2006, the "Farmacotherapeutish kompas" (CVZ) agency reported a considerable increase in the consumption of pharmaceuticals and attributed the increase to the increase in growth and the aging of the population. [46]. Table 1.2 (below) classifies the drugs into various classes depending on their functional users. Anti-infective and cardiovascular seen as the most used within the chosen period. These usages have greatly witnessed an increase as the years go by.

Table 1.2: Drug classes and users (x1000) in Netherlands (CVZ 2006)

	2002	2003	2004	2005	2006
Alimentary tract and metabolism	2910	3004	2769	2969	3441
Blood and blood forming organs	1655	1663	1667	1673	1944
Cardiovascular system	2676	2759	2910	2982	3630
Dermatological	3421	3465	3193	3166	3484
Genito urinary system and sex hormones	2774	2703	1419	1412	1594
Systematic hormonal preparations	828	854	890	927	927
Anti-infective for systematic use	3840	3826	3775	3945	4229
Antineoplastic & immunomodulation agents	145	157	169	180	221
Musculo-skeletal system	3403	3423	3322	3136	3369
Nervous system	3584	3598	3345	3345	3345
Anti-parasitic agents, insecticides repellents	144	148	161	162	170
Respiratory system	3149	3064	3033	3099	3481
Sensory organs	1785	1802	1759	1755	2137
Others	34	37	40	43	43

Table 1.3: Annual consumption of different classes of prescribed drugs for different countries.

Data are expressed in Tons/year and represents the most (top 20) sold drugs per nation. Data in bracket indicates nation ranking of the drug, [a]Huschek et al.2004, [b] Sattleberger (1999), [c]Stuer-Lauridsen et al.2000, [d]Khan and Ongerth (2000) [e]Jone et al. (2000), [f] Calamari et al. (2003) and [g]IMS Health Incorporated or its affilates. All rights reserved. MIDAS-02/03/05.

Compounds		Germany 1999[a]	Germany 1999[a]	Germany 1999[a]	Austria 1997[b]	Denmark 1997[c]	Australia 1998[d]	England 2000[e]	Italy 2001[f]	Switzerland 2004[g]
Analgesics antipyretics and anti-inflammatory										
	Acetylsalicylic acid	902.27(1)	862.60(1)	836.26(1)	78.45(1)	0.21(7)	20.4(9)			43.8(3)
	salicylic acid	89.70(12)	76.98(17)	71.76(17)	9.57(11)					5.30(6)
	PARACETAMOL	**654.42(2)**	**641.82(2)**	**621.65(2)**	**35.08(2)**	**0.24(6)**	**295.9(1)**	**390.9(1)**	-	**95.20(1)**
	Naproxen				4.63(16)		22.87(7)	35.07(12)		1.07(12)
	Ibuprofen	259.85(5)	300.09(5)	344.89(5)	6.7 (13)	0.03 (19)	14.2 (13)	162.2 (3)	1.9 (15)	25.00 (4)
	Diclofenac	81.79 (16)	82.20 (14)	85.80 (14)	6.14 (15)			26.12 (16)		4.50 (7)
Beta-Blockers										
	Atenolol							28.98 (13)	22.07 (4)	3.20 (9)
	Metoprolol	67.66 (18)	79.15 (16)	92.97 (11)	2.44 (20)					3.20 (10)
Anti-lipidemic										
	Gemfibrozil						20 (10)			0.399 (18)
	Bezafibrate				4.47 (17)				7.60 (8)	0.757 (15)
Neuroactive										
	Carbamazepine	86.92 (13)	87.71 (13)	87.60 (12)	6.33 (14)		9.97 (18	0.35 (8)		4.40 (8)
	Diazepam					0.21 (8)				0.051 (21)
Anti-acidic										
	Ranitidine	85.41 (15)	89.29 (12)	85.81 (13)			33.7 (5)	36.32 (10)	26.67 (3)	1.60 (13)
	Cimetidine							35.65 (11)		0.063 (20)
Diuretics										
	Furosemide					3.74 (1)		6.40 (19)		1.00 (14)

1.8.3 Pharmaceutical polluants, fate, occurrence and effects

As earlier indicated, the main route of these pharmaceutical to reach the environment is via sewage and wastewater treatment plants effluents. Figure 1.5 (below) shows a schematic summary of the routes that pharmaceutical can take to reach environment.

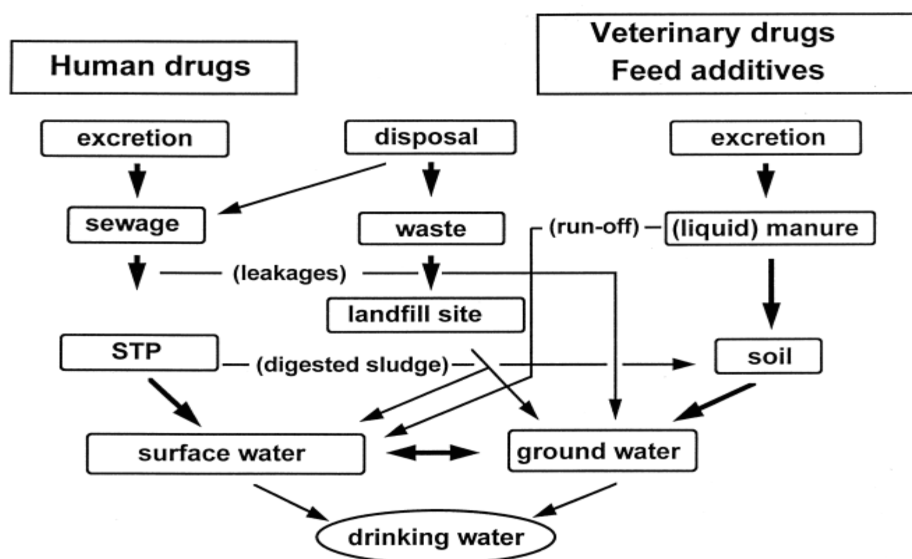


Figure 1.5 : Common pathways for pharmaceuticals to reach the environment (Reemtsma 2006). This research focuses on the highlighted pathway

From Table 1.4 (below), Fent et al., 2006 gives an overview of concentration of pharmaceuticals found in influents and effluents that have been reported by many authors and different researches. The classification took into account several factors, which can be different in many countries. It illustrates the detection of pharmaceuticals in the µg/L range in the influent and effluent of WWTPs and their differences.

Table 1.4: Measured influent and effluent concentration of pharmaceuticals common in wastewater treatment. (Fent 2006 & Petrovic 2005)

Compounds	Influent conc. (µg/l)	Effluent conc.(µg/l)
Acetylsalicylic acid	3.2	0.6
Salicylic acid	57 - 330	0.05 - 3.6
Ibuprofen	2 - 38.7	0 - 4
Diclofenac	3.0	2.5
Carbamazepine	0.7 - 1.5	0.7 - 1.5
Metoprolol	-	0.08 - 0.73
Clofibric	0.15 - 1	0 - 0.88
Bezafibrate	0.42 - 5	0 - 0.84
Fenofibric	0.44	0.22 - 0.4

The environmental effects of pharmaceuticals are not easy to detect. However, effects in minor periods have been determined for aquatic organisms though occurring at several mg/l concentrations. For example, Ibuprofen with an LC_{50} (96hr) has been determined for a 173mg/l concentration for the bluegill sunfish [47]. Due to a continuous addition unto the already existing low environmental concentrations of these pharmaceuticals, chronic effects are much more likely to be observed within aquatic life though difficult to predict because of the long time required for their effects to become clearly visible. Exceptions to this are endocrine disruptors that are capable of disturbing organisms' functions at very low concentrations. Despite the fact that a few of these pharmaceutical effects on aquatic life have been investigated and reported, a large majority are still undetermined. Some of the possible effects suggested so far are:

- Mixed or synergic effects occurs when the effect caused by a single can't be evaluated because it has been merged with several others e.g. the cardiovascular drug verapamil is capable of increasing intercellular concentration of other pharmaceuticals in organisms **[48]**
- Behavioural effects are identified in a change of behaviour due to a change in chemical signalling pattern caused by these pollutants. They can interfere in information sharing or transfer between or within organisms **[49]**.
- Effects cause by metabolites are thought to be more frequent. Pharmaceuticals may degrade to metabolites which have bioactive or persistent properties e.g. clofibric acid is a metabolite of clofibrate and is quite persistent and classified as hazardous to aquatic life **[50]**.

1.8.4 Paracetamol (APAP)

The systematic IUPAC nomenclature of APAP is N-(4-hydroxyphenyl) ethanamide.

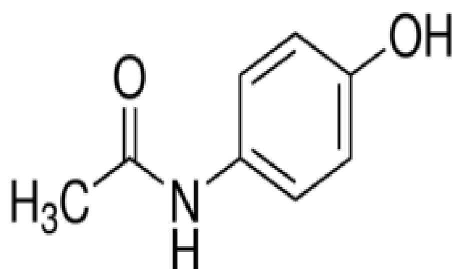


Figure 1.6: Chemical structure of Paracetamol

It possesses the following properties: molecular formula $C_8H_9NO_2$, Molar mass 1.263g/cm^3, boiling point 420 ℃, melting point of 169 ℃ and a water solubility ranging from 7.21 to 12.78mg/ml for a temperature range of 0 to 20 ℃ [51].

N-(4-hydroxyphenyl) ethanamide also known as Paracetamol in the United Kingdom, Tylenol as a Brand name in the United States was introduced in medicine in 1893 [52]. It is a widely used over-the-counter analgesic and antipyretic drug. It is an active metabolite of acetanilide and phenacetin. APAP exhibit a limited side effect and an efficient anti-inflammatory advantage over other analgesics. Out of the gastro toxicity effects exhibited by it and many other non-steroid anti-inflammatory drugs (NSAID), a long-term administration of APAP have less side effects than other NSAIDs though capable of inducing liver damage at doses greater than the recommended daily therapeutic dose of 4g [53].

Paracetamol and structural analogues are ubiquitous in the natural environment and easily accumulate in aquatic environments and have been detected in surface waters, wastewater, and drinking water throughout the world. APAP has as well been reported to be capable of accumulating within environmental waters and exhibiting some toxic effects (associated with other pharmaceuticals) within aquatic lives. Progressively, they are becoming one of the emerging pollutants of great concern to aquatic environmental life.

1.8.4.1 Paracetamol occurrence in the environment

Table 1.5: Paracetamol occurrence in the environment

Surface water		Wastewater influents		Wastewater effluents		Drinking water
Conc. (µg/l)	Reference	Conc. (µg/l)	Reference	Conc. (µg/l)	Reference	
< 0.1	Stackelberg 2004	1.2 - 10	Kolpin 2002	0 – 0.6	Ternes 1998	Low ppb levels (Han et al., 2006)
0 - 0.25	Gros 2006	6	Ternes 1998	0 – 5.9	Gros 2006	
0.11	Kolpin 2002	29- 246	Gomez 2007	1.9	Brun 2006	

Just like the other pharmaceutical products and drugs, APAP consumption worldwide has increased enormously. An increment of fivefold was noticed between 1978 and 1988 in Nordic countries. Similarly, from 1994 to 1995, the rate in developed countries exceeded 20g/person/year [54]. APAP is one of the most prescribed analgesics in most clinical settings. In 2006, it was dispensed at a level of over 140tons that is approximately 45g/person/year [55]. In the United Kingdom, alone, approximately 3.2 x 109 tablets are consumed every year

corresponding to an average of 55 tablets /person/year [56]. With this level of consumption, they evidently easily accumulate in the aquatic environment due to their high solubility and hydrophilicity characters. Thus, they have been detected in surface waters, wastewaters abd drinking water worldwide. It has been found in concentration of up to 6µg/l in European sewage effluents [23], 10µg/l in US natural waters [57] and more than 65µg/l in Tyne River in the UK [58]. In hospital effluents and WWTP, concentration as high as 150µg/l have been exceeded. However, effective removal within this treatment centres results in the detection of low ng/l amount in the effluents. The table below gives a summary of those variable concentrations detected and reported by many authors

The widespread use of APAP raises concern of whether or not this compound persists during treatment of wastewater and drinking water. In the treatment process, of drinking using chlorination, Bedner and Maccrehan (2006) reported that 11 different chlorination products were observed including the toxic N-acetyl-p-benzoquinone imine (NAPQI) and 1,4 Benzoquinone. Though their occurrence is in small quantities, their synergic effects with other pharmaceuticals present makes them deserve further considerations [59]

1.8.4.2 Structure, and characteristic of Paracetamol

Of all the pharmaceuticals present in wastewater and actively playing the roles of emerging pollutants, APAP was chosen for this particular study based on following facts commonly reported in literature:

- High rate of consumption worldwide
- Reported eco-toxicity that is both acute and chronic
- Reported re-occurrence in the environment
- Physical – chemical properties (hydrophilicity /hydrophobicity)
- Available validated analytic methods
- Susceptibility to biodegrade. The Octanol-water partitioning coefficient (Kow) of APAP is 2.88 (Log Kow = 0.34) with a pKa = 9.71 at T= 25ºC. This is indicative to a less hydrophobic tendency hence its removal by absorption is limited. APAP will thus be less absorbed in sludge but will be present more in the water phase.

Commonly used to treat pain and fever with a mode of administration being either oral or intravenous. Its 85-90% excreted through urine [60] and has as metabolites APAP-glu, APAP-sulphate, APAP-GSH and NAPQI. APAP is the most used over the counter analgesics and antipyretic drug. It is available in different dosage forms: tablets, capsules, drops, elixirs, and suspensions. Its saturated aqueous solution has a pH of 6.0, which decreases in acid and

alkaline conditions due to the hydrolysis of the “imide” bond into p-aminophenol and acetic acid Equation 1.3 [61].

Equation 1.3: Hydrolysis pathway for Paracetamol

The primary organ of metabolism is the liver. Three metabolic pathways are are shown in Figure 1.7 (below). Glucuronidation accounts for approximately 40% of the metabolism, sulfation 20-40% and GSH conjugation less than 15% [62]. The hepatic cytochrome P450 enzymes found in the liver form the metabolite NAPQI. The three pathways yield products that are inactive, non-toxic which are eventually excreted by the kidney. The only exception is noticed in the third pathway where the intermediate NAPQI produced is toxic and responsible for the paracetamol toxicity.

Figure 1.7: Main pathways of Paracetamol metabolism in Animals

Chemical oxidation processes easily treat contaminated wastewater. Though these chemical methods may be available for treatment, the harsh reaction condition, secondary pollutants generation, and high operational cost associated with these methods have often made them not a desirable choice. Biodegradation of paracetamol by microbial communities could be considered as an environmentally friendly and low-cost option compared to the use of pure culture and photocatalytic degradation that are commonly practiced.

Many bacteria members notably *Pseudomonas* have been reported for their abilities to degrade Aromatic compounds of environmental concern [63]. However, microbial consortium from activated sludge collected from Nascent-Faro wwtp has not been reported to the best of our knowledge. Without discrediting the efficiency of pure bacterial cultures vis-à-vis biodegradation of environmental recalcitrant compounds and APAP specifically, the choice of using consortium stem from the fact that microbial mixed cultures are generally considered to be potentially more efficient in the biodegradation of recalcitrant compounds than pure cultures [64].

With the increasing change in life styles and medical challenges worldwide, there is no doubt of an increase in concentration of the recent forms of pollutants referred to as emerging pollutants. These pollutants have very diverse origins. They range from personal care products (PCP) to pharmaceuticals. Due to their incomplete elimination in wastewaters and a growing presence in surface water, advanced research methods are necessary to be tested and used to overcome the problem. Bearing in mind the elaborated advantages of microbial consortia over pure cultures [65], it is worth evaluating feasible small scale research procedures and practices that can be applicable within large scale set-ups to solve this novel environmental hazard

Table 1.6: Summary of overall experimental batch tests performed during entire study

INOCULUM	THIOGLYCOLATE MEDIUM			MINERAL SALT MEDIUM (MSM)			RESIDUAL WATER		
	Anaerobic	Aerobic without aerification	Aerobic with aerification	Anaerobic	Aerobic without aerification	Aerobic with aerification	Anaerobic	Aerobic without aerification	Aerobic with aerification
WWTP lagoon inactivated sludge (Faro East)	√√√√	√√√√		√√√√	√√√√				
WWTP activated sludge (Faro North)						**√√√√**		**√√√√**	**√√√√**

2. MATERIAL AND METHOD

2.1 Sources of bacteria inoculum

In order to obtain anaerobic and aerobic bacterial communities necessary to assess APAP biodegradation for the study, two samples were collected from two different sources. The first was obtained from a deep anaerobic passive wastewater treatment lagoon system in North-east of Faro. Secondly, activated sludge was obtained from an aerated WWTP tank located in the North-west area of Faro, Portugal. Additionally, wastewater, which obviously contains small quantities of bacteria, were also collected from this WWTP of Faro-North and served as aqueous medium for growth in some of the experimental assays.

2.2 Enrichment of bacteria Inocula

Table 2.1 summarises the several experiments performed to assess paracetamol biodegradation. In some cases, the sludge was used directly as inoculum source, without any previous enrichment (unenriched) and in others a previous enrichment from the sludge was performed using the media indicated.

Table 2.1: Summary of enriched and unenriched bacteria inoculum

CONDITIONS TESTED		INOCULUM	
Medium	Conditions for respiration	wwtp lagoon sludge (Faro East)	wwtp activated sludge (Faro North)
THIOGLYCOLATE MEDIUM	Anaerobic	ENRICHED	-
	Aerobic without aerification	ENRICHED	-
	Aerobic with Aerification	ENRICHED	-
MINERAL SALT MEDIUM (MSM)	Anaerobic	ENRICHED	-
	Aerobic without aerification	ENRICHED	UNENRICHED
	Aerobic with Aerification	-	UNENRICHED
RESIDUAL WATER	Anaerobic	-	
	Aerobic without Aerification	-	UNENRICHED
	Aerobic with Aerification	-	UNENRICHED

2.2.1 Enrichments of bacteria inoculum from sludge using thioglycolate

Using safety laboratory practices, 10% (w/v) (20g) of sludge was weighed into two sterile falcon tubes on a scale balance. Similarly, a 10% (w/v, 20ml) of residual water (wastewater) was measured using a measuring cylinder into two similar falcon tubes. Into two 250ml culture flasks, 20g and 20ml of sludge and residual water were respectively mixed in each flask. The operation was carried out within a lamina-flow hood. Thioglycolate (THGL) growth medium was added in both flasks to the 250ml mark. In one flasks, paraffin-oil was added and the flask sealed with aluminium-caps in order to create anaerobic condition of growth and in the latter, cotton was used to serve as lid thus creating aerobic conditions. The aerobic flasks were placed in a lab shaker over night at a speed of 10 rpm. The anaerobic flask was kept in ambient condition for a 24 hr period. After 2 hrs growth, 5ml of the culture was collected into a falcon tube, mixed with some glycerol and froze. The remainder was used for corresponding batch tests i.e. anaerobic enriched inocula was used for anaerobic test cultures and vice versa for aerobic.

2.2.2 Enrichments of bacteria inoculum using mineral salt medium

Knowing fully well that the microbial organisms cannot grow in a milieu composed exclusively of mineral salt medium (MSM) without any alternative carbon source, frozen inoculum prepared and preserved during the THGL enrichment process (Section 2.2.1) was collected and thawed for 10minutes under room temperature. The culture was grown once more overnight in THGL under anaerobic and aerobic growth conditions. After 24 hrs, the samples were obtained and centrifuged for 10 minutes, the supernatant were discarded while the pellets are re-suspended using MSM medium (not THGL). A doubled 10 mins centrifugations of the re-suspended samples using MSM led to the total elimination of THGL. After the final washing (last centrifugation), the pellets were re-suspended in a 30ml MSM medium. A 2ml of this was used as inoculum for both anaerobic and aerobic cultures. The washing was done independently for both case scenarios i.e. aerobically and anaerobically.

2.3 Biodegradation experiments

Biodegradation experiments were performed in triplicates. Batch tests were composed of APAP, growth media with/without 10% (v/v) activated or inactivated sludge (sludge from Lagoon WWTP- Table 1.6). This was done for 3 different microbial respiration conditions:

1. Sealed batches for anaerobic conditions
2. Open batches for low or moderate oxygenated aerobic conditions

3. Aerified open batches with a 110ml/min of air was used to highly oxygenate the batches submitted under aerobic conditions.

Negative controls (without bacteria) were performed to evaluate APAP degradation by non-biological means. Sampling for HPLC analysis and associated metabolites was done after 1, 2, 3 and 7 days.

2.3.1 Stock of paracetamol solution

Standard stock solution of APAP (1000μg/ml) was prepared by dissolving 0.05g of APAP (obtained from Sigma Aldrich with a purity ≥ 99.99%) in each of the prepared growth media previously described. The volume was made to the 50 ml mark of the volumetric flask by using the media in which the compound was primarily dissolved. By diluting proper volumes of the stock, working volumes were obtained.

Table 2.2: Volume of stock solution required to prepare the pre-determined concentration of APAP

Paracetamol conc. (mg/l)	0	10	50	100	150	250
Volume of stock (μL)	0	10	500	1000	1500	2500

2.3.2 Thioglycolate (THGL) medium

This growth medium had as composition the following masses per litre of water: 15g of pancreatic digest of casein, 5 g of yeast extract, 5.5 g of dextrose (Glucose), 2.5 g of NaCl, 0.5 g of L-cystine,0.5 g of sodium thioglycolate, 0.001 g of resazurin, 0.75 g of agar. 59.5 g of THGL acquired from Sigma Aldrich was weighed and dissolved in 2 litres of distilled water. After adjusting the pH of the solution to 7.03 using diluted NaOH, the medium was autoclaved at 121ºC for 15 minutes and kept to cool. The medium was stored in the fridge at 4ºC prior usage. The choice of this nutrient medium was due to its ability to facilitate the growth of both anaerobic and aerobic organisms [39, 38].

2.3.2.1 Anaerobic batch experiments

The anaerobic experiment performed consisted of the following:

1. A culture flask with a mix of pharmaceutical, sludge, paraffin-oil and growth medium sealed at the top with an aluminium iron foil in order to create the anaerobic condition
2. Another batch culture with a mix of APAP, paraffin-oil and autoclaved growth medium. This served as the control with the main target being to evaluate alternative degradation, interaction processes between the pharmaceutical APAP and the environment in within

which they have been submitted. Table 2.3 gives a resume of volumes added within each flask

Table 2.3: Growth media volume and inoculum mixed per culture batch

Test	Medium + APAP added	Sludge
Biodegradation test	19ml	1ml
Control	20	0

All experiments were performed in triplicates. Mother sample flasks of 250ml were used to dissolve 0.05g of APAP (which corresponded to a concentration of 50mg/g). 19ml of this mother sample mixture comprising of APAP and medium ONLY was distributed into 12 smaller flasks each. The distribution was independently done for the four predetermined concentrations (0, 10, 50 and 100, 5000 and 10000 mg/l). Table 4.1 (Appendix) for details. Unto each recipient flask, 1ml of inactivated sludge enriched using THGL was added except for the negative controls. Paraffin-oil was added into each flask to create anaerobic conditions. Sampling was done using sterilised syringes, 0.22µm pore seize filters and chromatographic flasks. For day 0, 5ml of the mother sample was collected, filtered and preserved. It served as the initial concentration of APAP at the start of the experiment. Every 24hours, 5ml of each sample was collected, filtered and stored in the fridge for HPLC analysis of APAP biodegradation. This was done within an interval of 24h, 48h, 72h and 168h. All reaction bottles were covered with aluminium foil and samples stored in the dark to prevent any photolytic degradation of APAP.

2.3.2.2 Aerobic batch experiment without aerification (AERO 3)

Experimental procedures involving AERO 3 were identical to those of anaerobic culture described above. Here, all experiments were performed in triplicates. Mother sample flasks of 250ml volume were used to dissolve 0.05g of APAP, which corresponded to a concentration of 50mg/g. 19ml of this mother sample mixture comprising of APAP and medium ONLY was distributed into 12 smaller flasks each. The distribution was independently done for the four predetermined concentrations (0, 10, 50 and 80 mg/l). See **Figure 4.1** (Appendix) for details. Unto each recipient flask, 1ml of inactivated sludge enriched previously using THGL (Section 2.2.1) was added except for the negative controls. Contrary to the anaerobic culture, paraffin-oil is **not** added in the culture reactors. Here the airflow rate was kept as low as 2ml/min. Samples were placed in a darkroom to prevent any possible photolytic degradation.
Same methodology of sampling as described in Section **2.3.2.2 (above)** was used here.

2.3.3 Mineral Salt Medium (MSM)

The mineral salt medium (MSM) used for the experimental contained the following per 2 litres of deionized water, 1.0g of K_2HPO_4,1.0g of KH_2PO_4, 20.0mg of NaCl, 0.4 g of $MgCl_2.6H_2O$, 0.04 g of $CaCl_2$, 1.53 mg of ZnSO4, 0.78 g of $CoCl_2.6H_2O$, 0.73 mg of MnSO4, 20 mg of EDTA, 0.64 mg of $(NH_4)_6Mo_7O_{24}.4H_2O$. Using an electronic balance, these substances were carefully weighed and dissolved using deionized water in a 2 litres volumetric flask. The pH of the resultant mixture was adjusted to 7.20 using a 0.5M NaOH solution. The medium was autoclaved at 121℃ for 15 minutes and allowed to cool. It was further stored in the fridge at 4℃ for subsequent usage.

2.3.3.1 Anaerobic batch cultures

Experimental procedures involving MSM were identical to those of anaerobic culture described earlier (Section 2.3.2.1). Here, all experiments were performed in triplicates. The major difference is noticed in the inoculum used. The inoculum used was obtained from the anaerobic MSM enrichment process (Section **2.2.2**). Each recipient flask received a 1ml of the inoculum except for the negative controls. Paraffin-oil was added in the culture reactors to create anaerobic condition for growth. Here the airflow rate was as low as 2ml/min. Samples were placed in a darkroom to prevent any possible photolytic degradation of APAP. Just as in THGL, biodegradation of APAP was tested by sampling each member flask of the triplicate every 24 h and a week after.

2.3.3.2 Aerobic batch experiments

The experimental procedure followed here was identical to those previously described in section 2.3.2.2. Here, THGL is simply replaced by MSM as medium for growth and the inoculum for inoculation was obtained from the aerobic MSM enrichment process. Recipient flasks received 1ml of inoculum except the negative control experiments. Sampling in both cases i.e. aerobic and anaerobic was done using sterilised syringes, 0.22µm pore seize filters and chromatographic flasks. For day zero, 5ml of the mother sample was collected, filtered and preserved. It served as the initial concentration of APAP at the start of the experiment. Every 24hours, 5ml of each sample was collected, filtered and stored in the fridge for HPLC analysis of APAP biodegradation. This was done within an interval of 24h, 48h, 72h and a 168h. All reaction bottles were covered with aluminium foil and samples stored in the dark to prevent any photolytic degradation of APAP.

2.3.3.3 Aerobic aerified batch experiments (AERO 1)

A 1800ml mother sample solution of MSM was mixed with 0.9g of APAP (conc. 50mg/l) in a 2litre flask. 600ml of the sample was collected and poured into three flasks of 250ml (200ml/flask). A 10% (sludge) is to 90 %(growth media) ratio was used in determining the exact volume of additional sludge required. Thus, 134ml was extracted from 1200ml mother sample solution and replaced with 134ml of sludge: restitution the total volume of 1200ml. This 1200ml composing of paracetamol, residual water and activated sludge was further distributed into two triplicates that is six 250ml culture flasks (200ml/flask). In addition to the measured APAP biodegradation, COD (mg/L) for each flask was measured using a spectrophotometer for an identical interval of time.

2.4 Residual water (wastewater: AERO 2)

Residual water as a growth medium was composed basically of small quantities of bacteria because it was obtained from the WWTP in Faro NORTH on the 11 may 2016.

2.4.1 Aerobic batch experiments with aerification

Using residual water as growth medium (AERO 2), an identical approach with slight variations and changes in measurements and procedures was followed. Residual water and activated sludge were mixed in the proportion of 9:1 (90 parts of volume of residual water: 10 parts of volume of sludge) in a 2l reaction flask. This mixture served as the mother sample solution prior to distribution. The total volume of the mother sample was 1800ml. 600ml was collected and distributed into three flasks (negative control samples). Unto the 1200ml left, a mass of 0.06g of paracetamol was weighed, dissolved and homogenised. The resultant was distributed into the six 250ml growth culture medium. Aeration for both case was provided by means of air pumps at a velocity of 110ml/min aiming at maintaining dissolved oxygen concentration within required concentration.

2.4.2 Aerobic experiments without aerification

Using residual water as growth medium (AERO 2), an identical approach with slight variations and changes in the procedures was followed for aerobic culture batches without aerification. Sample preparation followed the same procedure explained above (section2.4.1). Differences were observed at aerification level. Here, aerobic batch samples (3 flasks) were left exposed in the dark without any external source of air. In a nutshell, a net total of 18 flasks were used for the overall set up that is AERO1 and AERO2. Of this 18 flasks, 12 were submitted to aerification and 6 not aerified. All experimental predetermined conditions were performed in

triplicates and growth was exclusively carried out in the dark. Table 2.4 summaries the content of each experiment and gives an overview of the respective volumes added to each flasks.

Table 2.4: Volume of media and components added in aerobic batch tests

Test	Batch Cultures	Sludge vol. (ml)	Medium + APAP (ml)	Total volume (ml)
AERO 1	Biodegradation test	22	178	200
	Control	0	200	
AERO 2	Biodegradation test	22	178	200
	Control	22	178	
AERO 3	Biodegradation test	2	18	20
	Control	0	0	

Sampling was done using sterilised syringes, 0.22µm pore seize filters and chromatography flasks. On day 0 (immediately after experimental set-up), 5ml of the mother sample was collected, filtered and preserved for future analysis. COD values were measured as well in Day 0 (AERO1 and AERO2). This served as the initial parameters for APAP at the start of the experiment. Every 24hours, samples were collected, filtered and stored in the fridge for HPLC analysis and metabolites detection. This was done within an interval of 24h, 48h, 72h and 168h. All reaction bottles were covered with aluminium foil and samples stored in the dark to prevent any photolytic degradation of APAP.

2.4.3 Instruments

Advanced Scientific Instrument KNAUER™ (Smartline UV detector 2600 Smartline Manager 5000) HPLC equipment was used to analyse the biodegradation of APAP, BRUNKE™ SCION 456-GC TQ serve for the identification of biodegradation products, HACH-LANGE™ DR-2800 spectrophotometer was used to measure concentration and the optical densities of samples Sample pH was measured using CRISON™ GLP21. Chromatographic analysis of paracetamol degradation was performed using an HPLC Reverse Phase Xbridge-C18 column (functional group composition: 1,2-bis (siloxy) ethane ($O_3SiCH_2CH_2SiO_3$) – Hybrid technology) purchased from WATERS™. The separation was performed at room temperature on an Xbridge-C18 Column (5µm, 4.6 x 250mm). TERUMO™ Syringe and PES Syringe filters of 0.2µm were used respectively to collect and filter samples prior to HPLC analysis. OASIS MCX (Mixed-mode Cation exchanger -225mg) columns were used in the Solid Phase Extraction (SPE) step to concentrate samples for GC-MS analysis. All chemical substances for

the study were of analytical high-grade purity. Purity standards > 99% of paracetamol (CAS nº 103-90-2), 4-aminophenol (CAS nº 123-30-8), p-benzoquinone (CAS nº 106-51-4). Hydroquinone (CAS nº 123-31-9) and 4-nitrophenol (CAS nº 100-02-7) were obtained from Sigma Aldrich. Thioglycolate fluid medium, Methanol, Acetonitrile (HPLC grade) and MSM component were all obtained from VWR Chemical PROLABO™ alongside a high purity orthophosphoric acid (OPA), EDTA, K_2HPO_4, NaCl, $(NH_4)_6Mo_7O_{24}.4H_2O$. Milli-Q water was filtered using 0.22µm. The methods or approaches implemented or applied followed biosafety aseptic techniques so as to avoid the contamination of samples under investigation.

2.4.4 IC50 determination procedure

IC50 is the quantitative measure of how much a particular drug or substance is needed to inhibit a given biological process by half its initial value. In course of determining the IC50 value for paracetamol, predetermined concentration of paracetamol ranging from 0 to 12g/l were chosen (0, 4, 6, 8, 10 and 12g/l). Masses corresponding to the respective predetermined concentrations were weighed and dissolved in a sterilised 150ml flask. The volume was made to the 100ml marked using THGL. The mix served as mother sample per concentration value. From each mother solution, a 10ml redistribution was carried out for both anaerobic cultures and marked using THGL. The mix served as mother sample per concentration value. From each mother solution, a 10ml redistribution was carried out for both anaerobic cultures and aerobic cultures. Unto each flask, a 1ml inoculum prepared using THGL was added. This gave a final volume of 11ml/flask/conc. Both positive and negative controls were performed

Table 2.5: Experimental test for IC50 determination

Concentration (g/l)	Flasks	
	Anaerobic	Aerobic
4	√√√	√√√
6	√√√	√√√
8	√√√	√√√
10	√√√	√√√
12	√√√	√√√

IC_{50} determination was done by measuring at 600 nm the Optical density values of each duplicate using Hach-Lange spectrophotometer DR-2800.

2.5 Analytical methods

2.5.1 APAP analysis

Sampling in the anaerobic test culture flasks were taken using a plastic syringe of mark TERUMO® equipped with a needle. By so doing, the closed system stayed close thence preventing any oxygen flow into and out of the flask. APAP degradation and mineralization was analysed using an HPLC Reverse Phase Xbridge-C18 column (250mm x 46mm, 5µm particle size) connected to a Guard column Xbridge-C18 column (4.6 x 20mm, 5µm particle size) purchased from Waters®. The mobile phase for APAP separation was composed primarily of CH_3CN / H_2O (25/75, v/v) and further changed to K_2HPO_4-$KH_2PO_4.3H_2O$ / CH_3OH. Using CH_3CN / H_2O, the separation was performed in an isocratic mode with a flow rate of 1ml/min and column maintained at room temperature. The injection volume was 5µl alongside a total run time of 4 minutes. Metabolites were not detected under such conditions. With K_2HPO_4-$KH_2PO_4.3H_2O$ / CH_3OH, the mode of separation was by elution gradient. The metabolites Hydroquinone (HQ), p-Benzoquinone (p-BQ) and 4-aminophenl (4-AP) were tentatively detected and matched to standards. Table 2.6 summarises the different chromatographic conditions used

Table 2.6: Mobile phase variation during sample separation by chromatography

Run time (mins)	%CH_3OH	%Buffer	Detection (ƛnm)
0	20	80	234 247 254 301 ↓
8	50	50	
10	50	50	
11	20	80	
15	20	80	

The buffer (pH 4.88)-methanol mobile phase was conditioned such that from 0-8 minutes, the percentage of the organic modifier varies from 20% - 50% and stayed constant for 2minutes then decreases to 20% for 1minute then for an additional 4minutes. The flow rate of 0.8ml/min stayed constant for the entire separation run that lasted for 15 minutes. Four different wavelengths were used for the analysis notably, 234, 247, 254 and 301 nm.

2.5.2 Solid phase Extraction

In order to concentrate the samples for GC-MS analysis, 5% NH_4OH in pure CH_3OH and 2% HCOOH were prepared. The former was prepared by mixing slowly 40ml of CH_3OH with 10ml (2%) of NH_4OH and the latter by mixing 49ml of Milli-Q water with 1ml of acid.

2.5.3 HPLC mobile phase

The mobile phase used were acetonitrile/water (CH_3CN/H_2O) for a first case and phosphate buffer/CH_3OH for a second. CH_3CN/H_2O solution was prepared by diluting CH_3CN (HPLC grade) with Milli-Q water in a 1:4 ratios that is 25 ml of acetonitrile to 75 ml of water. Phosphate buffer on its part was prepared by dissolving 13.5g of KH_2PO_4 and 0.124g of K_2HPO_4 in a 1500ml of Milli-Q water. The pH was adjusted to 4.88 with OPA (85%). Both samples were filtered and stored.

2.5.4 Chemical oxygen demand (COD) analysis

Chemical oxygen demand (COD) was determined exclusively for AERO 1 and AERO 2. Measurements were done using the HACH method [66]. Tubes with pre-determined amounts of potassium dichromate, sulphuric acid and catalyst were used. 2ml of each flask content were mixed and homogenised within tubes. The tubes were placed in the AccuBlock™ Digital Dry Bath that served as the digester. The temperature and time for digestion were fixed at 148ºC and 120 minutes respectively. At the end of the 120 minutes, samples were allowed to cool for 5 minutes and COD values measured. The Hach-Lange spectrophotometer DR-2800 was used to measure the COD values at a wavelength of 605nm before (Day 0) and after (24 hours) preparation.

2.6 Paracetamol metabolites analysis

4-aminophenol, p-benzoquinone and hydroquinone were the biodegradation products of APAP that were analysed and tentatively identified using GC-MS and an elution gradient mode HPLC. the choice of these metabolites were done based on the fact that they are the most prominent constituents of the degradation pathway of APAP [67, 68]. Prior to this analysis, samples were concentrated using OASIS® SPE columns. Samples are retained in the column and then eluted. Firstly, SPE columns are conditioned with 2ml CH_3OH then equilibrated with 2ml Milli-Q water. 2ml of already filtered sample was loaded onto the column using a syringe. Column was then washed using 2% Formic (HCOOH) acid and a first elution performed by passing twice a 500µl methanol (2 x 500µl CH_3OH) through the column to elute APAP. A second elution was performed by passing a similar 2 x 500µl 5%NH_4OH/CH_3OH solution in order to elute metabolites, salt and phosphates.

The identification of metabolites was performed using an Agilent 6890 Network GC System, 5973 Inert Mass Selective Detector (GC-MS (BRUNKE™ SCION 456-GC TQ) equipped with a DB5-MS capillary column (25m x 0.25mm internal diameter, 0.25µm film thickness, Agilent). Helium was used as a carrier gas, while the following conditions were fixed for metabolites determination. The Oven temperature was programmed at 50°C for 5 minutes, ramped to 280 ℃ at 5 ℃/min, and held for 5 minutes. Injection temperature was 180 ℃. The ionization source temperature was set at 220 ℃. The mass spectra were obtained at 70eV. Metabolites were tentatively identified by GC-MS by matching retention times and ion spectra with authentic standards and NIST library data.

3. RESULTS AND DISCUSSION

3.1 RESULTS

Batch tests were performed in order to determine the biodegradation of the emerging pharmaceutical compound; paracetamol. All experimental batch tests were operated in the dark and in the absence of Iron ions (Fe^{3+}) in the media because when exposed to visible light or Fe^{3+}, APAP is easily degraded to other components. In order to be certain that no degradation takes place, it was necessary to store the samples away from light. In total, 4 different sets of batch tests were performed notably, batch tests for the determination of IC_{50} value of APAP, chemical oxygen demand (COD) for the wastewater used, concentration of APAP and metabolites arising due to biodegradation. Apart from experiments performed to determine COD (which aimed at determining the degree of organic contaminants within experimental samples), all other tests aimed analysing the biodegradation of APAP and all other factors thought to be associated with it. Analysis gave results expressed in peak areas (PA) which were further transposed into concentration through a calibration curve (APAP quantification).

3.1.1 Background concentration

In order to determine whether the sludge and residual water used for the experiment contributed to the total concentration of paracetamol measured, a mixture of both residual water and activated sludge was made without any APAP and air and further analysed in the HPLC using an elution gradient method. The method uses phosphate buffer/methanol as mobile phase.

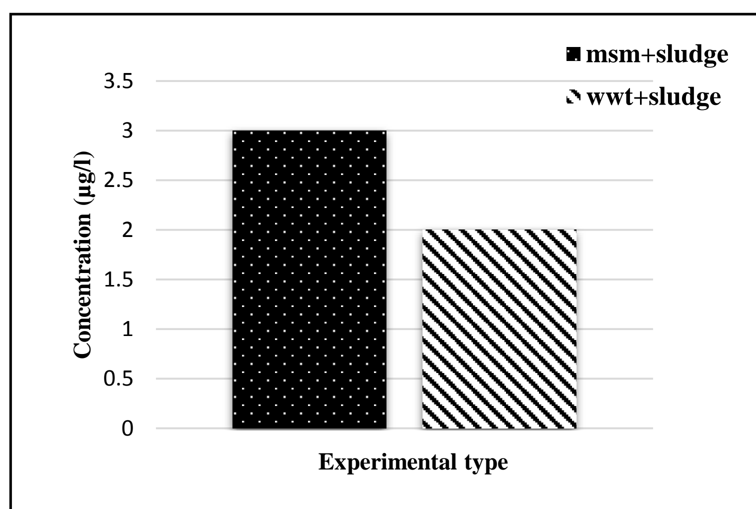


Figure 3.1: Background concentration of APAP from mixed wastewater, activated sludge (obtained from Faro-North) and growth media.

Figure 3.1 shows the likely presence of very small quantity of paracetamol in the sludge or within the wastewater treatment plant system from which it was obtained. This correlates the

theoretical analysis found in literature review (Ternes 1998). These minimal quantities detected in the sludge therefore testify APAP's persistence and partial elimination from WWTPs systems. From the analysis, it could be concluded that passage duration for APAP and any other pharmaceutical within WWTP system acts as an important and crucial factor to be considered during the biodegradation and subsequent elimination process.
Table 3.1 and Table 3.2 summarises the overall results obtained from the biodegradation of APAP. These results were evaluated under diverse experimental conditions for predetermined periods.

3.1.2 Paracetamol quantification

The calibration curve was obtained by plotting peak areas versus concentration. Table of values (eight data points) for MSM and THGL media are respectively displayed in Figure 4.2**A** and **Figure 4.2B** (Appendix). Figure 4.6 show the standard curve with a good linearity and a correlation coefficient (R^2) of 0.997. This curve was used to quantify APAP by converting the obtained peak areas into their corresponding concentrations.

Table 3.1: Summary of the experimental set-ups and removal percentages of APAP in each respective assay (≤ 24 Hours)

CONDITIONS TESTED				INOCULUM		
Medium	**Conditions for Respiration**	**[APAP] (mg/L)**	**Max. Period (days)**	**Absence**	**wwtp lagoon sludge (Faro-East)**	**wwtp activated sludge (Faro North)**
THIOGLYCOLATE MEDIUM	Anaerobic	10 50 80 5000 10000	1 1 1 1 1	17±3% 20±5% 2.2±5% 0±0.1% 13.2±4%	24±8% 21.8±3% 11.6±8% 4.4±2% 2.2±0.1%	NT
	Aerobic without aerification	10 50 80 5000 10000	1 1 1 1 1	40±7% 22.4±11% 28±6% 32±8% 22.1±1%	55±12% 25.7±2% 33±7% 38.4±9% 24±3%	NT
	Aerobic with Aerification	NT	NT	NT	NT	NT
MINERAL SALT MEDIUM (MSM)	Anaerobic	10 50 80	1 1 1	0.7±8% 14±5% 0.3±6%	6.1±9% 23.7±5% 8±2%	NT
	Aerobic without aerification	10 50 80	1 1 1	1.9±0.9% 11.7±3% 0±1%	10.4±9% 11.9±4% 9.4±5%	NT 0% NT
	Aerobic with Aerification	10 50 80	NT 1 NT	NT 0±0.1% NT	NT NT NT	NT 2.9±2% NT
RESIDUAL WATER	Anaerobic	NT	NT	NT	NT	NT
	Aerobic without Aerification	50	1	NT	NT	10.8±8%
	Aerobic with Aerification	50	1	NT	NT	69.9±2%
NT means not tested						

Table 3.2: Summary of the experimental set-ups and removal percentages of APAP in each respective assay (≥ 3days).

CONDITIONS TESTED				INOCULUM		
Medium	**Conditions for Respiration**	**[APAP] (mg/L)**	**Max. Period (days)**	**Absence**	**wwtp lagoon sludge (Faro-East)**	**wwtp activated sludge (Faro North)**
THIOGLYCOLATE MEDIUM	Anaerobic	10 50 80 5000 10000	7 7 7 3 3	43.2 ± 12% 37 ± 10% 25 ± 9% 0 ± 0.8% 11 ± 1%	55 ± 16% 56.9 ± 0.7% 33 ± 9% 37.3 ± 13% 25 ± 11%	NT
	Aerobic without aerification	10 50 80 5000 10000	7 7 7 3 3	31 ± 7% 25 ± 14% 8 ± 0.3% 36 ± 9% 20 ± 11%	44 ± 5% 43.7 ± 5% 16.1 ± 5% 36 ± 4% 22 ± 1%	NT
	Aerobic with Aerification	NT	NT	NT	NT	NT
MINERAL SALT MEDIUM (MSM)	Anaerobic	10 50 80	7 7 7	0 ± 18% 13.5 ± 5% 17.3 ± 6%	6.1 ± 13% 23.7±5% 20.8 ± 4%	NT
	Aerobic without aerification	10 50 80	7 7 7	2 ± 0.9% 3.2 ± 2% 0.3 ± 10%	10.4 ± 0.1% 11.9 ± 3% 9.4 ± 5%	NT 97 ± 2% NT
	Aerobic with Aerification	10 50 80	NT 3 NT	NT 0 ± 1% NT	NT NT NT	NT 97 ± 2% NT
RESIDUAL WATER	Anaerobic	NT	NT	NT	NT	NT
	Aerobic without Aerification	50	3	NT	NT	88.3 ± 6%
	Aerobic with Aerification	50	3	NT	NT	99.9 ± 2%
NT means not tested						

Anaerobic and unaerated Aerobic biodegradation of APAP batch culture

The anaerobic experiments were performed at the same temperature, pressure and growth conditions using 2 different growth media and 2 different concentrations (THGL for low and high concentration of APAP and MSM for low concentration only). The difference between the two tests was the concentration of APAP used alongside the presence and absence of anaerobic culture conditions. See Table 3.1 and Table 3.2 (above).

3.1.3 Determination of half maximal Inhibitory Concentration (IC50) of APAP

IC_{50} indicates the quantitative measure of how much a particular drug or substance is needed to inhibit a given biological process by half. For paracetamol, IC_{50} values were determined by performing anaerobic and aerobic (unaerated) experiments that consisted of THGL as medium of growth, 20% sludge and variable concentrations of APAP. Negative (without bacteria) and positive controls were performed for each concentration tested in order to evaluate the extent to which the microbial communities (sludge) are capable of degrading the pharmaceutical. A 2ml sample were collected and the optical density was measured using UV-vis spectrophotometer. The optical values measure is summarised below (Table 3.3**)**.

Table 3.3: Determination of IC_{50} values of APAP by UV-vis spectrophotometry (λ=600nm) for both aerobic and anaerobic culture conditions using THGL as medium for growth

Conc. (g/l)	O.D. 24h (24Hrs growth)		O.D. zero (day 0)	O.D. 24H - O.D. zero		Average ± st. dev
	replicate 1	replicate 2		replicate 1	replicate 2	
0	0.830	0.885	0.146	0.684	0.739	0.71 ± 0.04
4	0.653	0.620	0.085	0.568	0.535	0.55 ± 0.02
6	0.399	0.481	0.115	0.284	0.366	0.33 ± 0.06
8	0.305	0.334	0.116	0.189	0.218	0.20 ± 0.02
10	0.205	0.180	0.115	0.090	0.065	0.08 ± 0.02
12	0.072	0.110	0.089	-0.017	0.021	0.002 ± 0.03

Optical density (OD) values were measured at 600nm using a spectrophotometer at the beginning of the experiment and 24 hours after. Experiments were performed in duplicates from very low to very high concentrations. The reading obtained in day zero for each corresponding experiment was subtracted from that obtained 72Hrs after and the average and standard deviation error values determined. A graphical plot of optical density against concentration gave a curve with decreasing slope. An extrapolation of half the optical density value (y-axis) gave a specific concentration value (x-axis). This corresponded to the IC_{50} value

for the drug. For the anaerobic cultures, the value obtained was 6.21g/l and 5.98gl/l for aerobic batch cultures. See **Figure 4.5** (Appendix). The results showed a gradual decrease in concentration of the drug with respect to time.

3.1.4 Batch cultures with thioglycolate as growth medium

Focus was based on the experiments that were capable of showing some degree of degradation. The entire experiments were run for a duration of 14days. In the 1st 3days, the concentration of APAP were frequently analysed to determine their respective elimination rates. After 7days, additional breakdown was monitored for samples with lower concentration only. The results of the anaerobic batch experiments with THGL (as medium for growth) and high concentration of APAP are shown in Figure 3.2 (**a-d**) below. The graphs show the rate of biodegradation of high concentration of APAP within different batch cultures. Time factor was taken into consideration during sampling and served as a parameter to monitor the use of APAP as the only source of carbon, nitrogen and energy for the microorganisms in question. Similarly, the concentration of APAP in the negative controls (without sludge) are plotted in the graph.

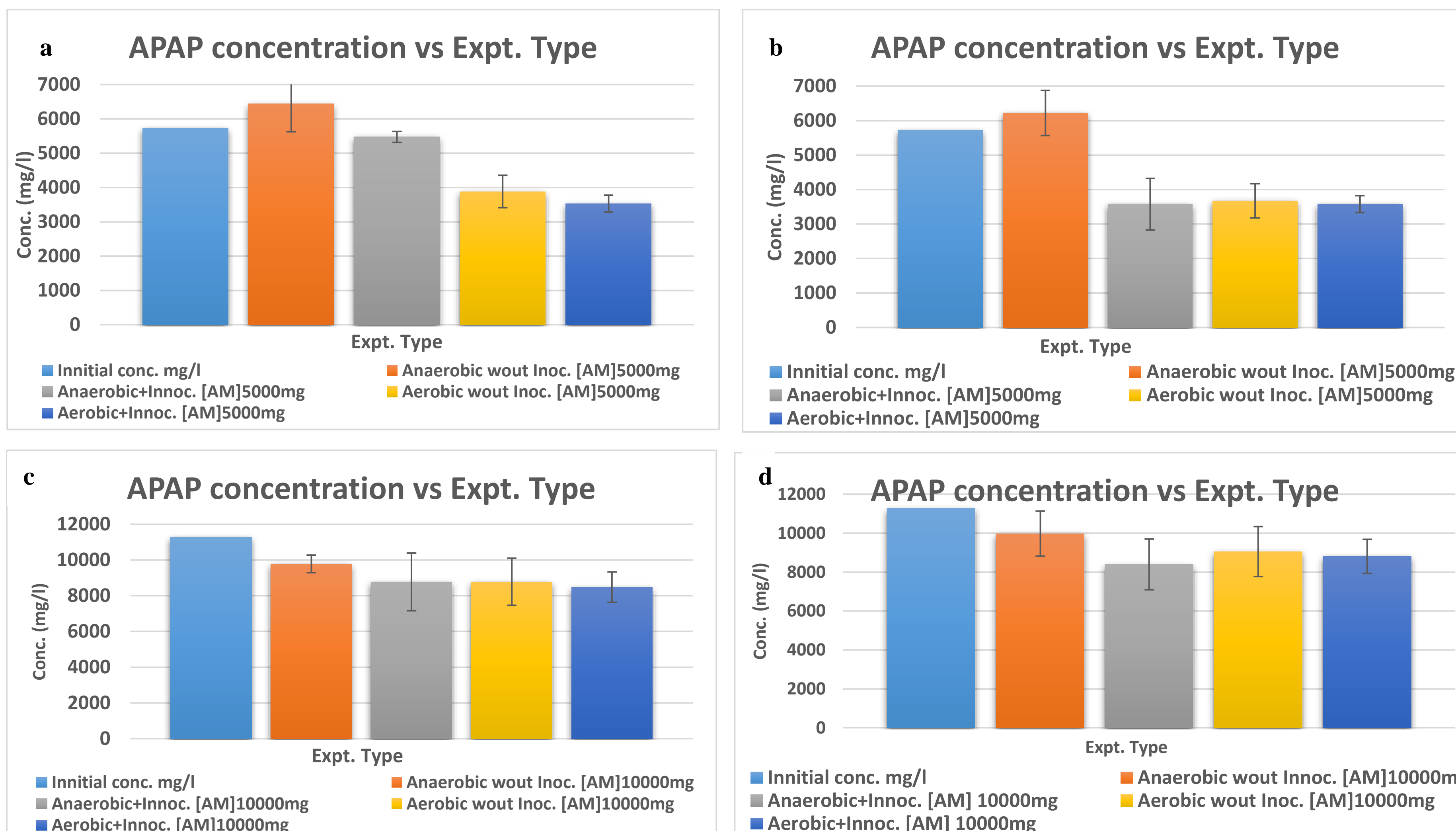


Figure 3.2: Biodegradation profile of paracetamol using thioglycolate medium

a, **b**, **c** and **d** respectively represents 24 and 72hrs biodegradation analysis of [APAP] = 5000mg/l and 10000mg/l with and without inoculum. Data points are given as mean ± standard deviations (n=3)

From Figure 3.2 (**a** and **b**), starting with a concentration of 5000mg/l we noticed that there was no substantial breakdown of APAP after 24hrs of anaerobic and aerobic (no aeration) incubation. However, eliminating the experimental errors observed in the anaerobic culture without inoculum and some cultures with inoculum, a slight decrease in concentration (approx. < 5%) of APAP in batches with inoculum was noticed against a relatively no visible change in the negative control experiments. Similarly, a slight decrease was observed for the 10000mg/l samples with the anaerobic culture (showed a slight higher level than the aerobic culture). This could be attributed to decomposition because the concentration in the controls stayed relatively constant over the entire sampling period of 72 hours. The initial concentration (i.e. d= 0) of APAP expected to be 5000mg/l (Figure 3.2**a** & **b**) or 10000mg/l (Figure 3.2**c** & **d**) was never obtained with exactitude in all of the batches be due to some experimental errors. Its therefore possible to link the observed minimal elimination of APAP in the biodegradation test to biological breakdown processes. A similar observation was noticed with 72hrs samples. Here, the supposed breakdown witnessed an insignificant increment. The results brought to mind two major points vis-à-vis APAP biodegradation; APAP used was either toxic to the organism at one point or the organisms were unable to biodegrade the compound within such conditions or better still, the bacteria feed on alternative sources of energy present in the media.

On a similar plan, experiments with low concentration of [APAP] were performed using THGL and mineral salt medium (MSM) as growth media. The experiments were performed under identical growth conditions as the previous but sampling was continued for an additional 10days (a frequent daily sampling for 3 days and 7days after the last sampling) This was to determine whether APAP could be eliminated when bacteria have no other alternative source of nutrient or long incubation period led to nutrient depletion thus compelling organisms to use APAP.

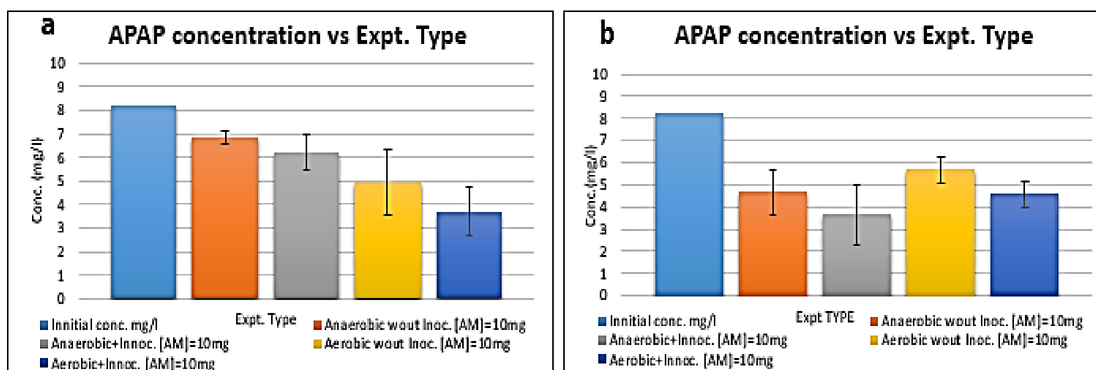


Figure 3.3: Biodegradation analysis profile of paracetamol (10mg/l) with and without inoculum using Thioglycolate as growth medium.
'**a**' & '**b**' respectively represents 24 and 72hrs results obtained and expressed in data points given in mean ± standard deviations (n=3)

Still using THGL as culture medium with low APAP concentration, we still noticed an insignificant decrease in 24hrs batch tests and a possible increased 72hrs after. The outcome of the results is not very much different from the suggested reasons witnessed and tentatively analysed in (Figure 3.3).

3.1.5 Batch cultures with Mineral Salt Medium as culture medium

Analysis using MSM medium were performed so as to evaluate the impact caused by the culture medium during growth of microbial communities or breakdown of APAP. MSM prepared for the culture milieu was completely made up of salts (no carbon source added). This is contrary to THGL that contain dextrose (glucose) which possibly served as an alternative source of carbon or energy for the organisms. This supposition was eliminated when MSM was used as culture media. Taking into account the previous results obtained with large concentrations of APAP using THGL as medium of growth and literature reported facts vis-à-vis the concentration at which they often appear in the environment, analysis using MSM were done at low concentrations (only) of APAP: notably 10mg/l, 50mg/l and 80mg/l and 350mg/l (see **Figure 4.4** (Appendix). Culture batches were developed with and without (negative control) inoculum. Samples were all stored in dark to avoid light degradation of the pharmaceutical. Figure 3.4**c** & **d** (below) respectively represents 24hrs and 1680hrs (one week) results for 10mg/l concentration.

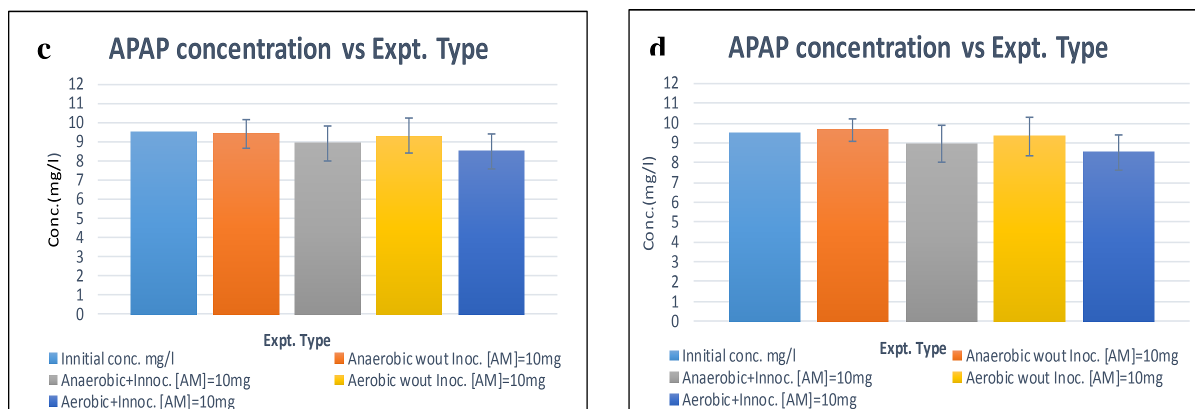


Figure 3.4: APAP (10mg/l) mineralization profile with/without bacteria using minimal salt medium (MSM) as the culture medium for growth.

c & **d** respectively represents 1day and 7days results obtained and expressed in data points given in mean ± standard deviation (n=3)

Using MSM as growth medium, no major biodegradation of the pharmaceutical was observed. The concentrations obtained in the negative controls showed no significant difference to those with inoculum. After a day and a week of incubation, the anaerobic and aerobic (unaerated) batch cultures with inoculum showed some possible decrease in concentration compared to the initial sample and that of the negative controls. This insignificant decrease witnessed no considerable change for the 1week samples Figure**d**. A similar observation was noticed for all concentrations tested (50mg/l, 80mg/l and 100mg/l). (Figure **4.4**). In the negative controls, the concentration stayed constant. The expected initial concentration (10mg/l) in day zero (d=0) was not achieved due to some experimental errors.

The insignificant decrease noticed previously (MSM and THGL batch culture media) and likely associated to a decomposition caused by the microbial community was believed to be retarded by other sources of carbon and energy furnished by the growth media. However, future studies concluded that MSM and THGL did not affect the breakdown of APAP as well as the growth of the community. The conditions of growth and the nature of inoculum samples were noticed to probably play some major in hampering or not the biodegradation process of the pharmaceutical.

3.1.6 Chromatographic conditions for paracetamol separation from MSM and Thioglycolate batch cultures.

For both MSM and THGL experimental set-ups, High Pressure Liquid Chromatography (HPLC) was used for separating APAP and its constituents within the samples. A relatively fast isocratic separation mode consisting of acetonitrile: water (25:75 v/v) with a flow rate of 1.0 ml/min and a UV-vis detection at a wavelength of 244nm was first used for separation. A

modification of the separation technique led to, an elution gradient mixed mode separation technique which consisted of a phosphate buffer-methanol as mobile phase (flow rate: 0.8ml/min) and four separation channels (ƛ = 235nm, 254nm, 294nm and 301nm) for absorption of APAP and its metabolites. Both techniques were performed using an Xbridge-C18 column (4.6 x 20mm,5µm) maintained at room temperature. Both experiments had a total run time of 15minutes and an injection volume of 20µl. The retention time (RT) for APAP in the first case scenario was 3.53minutes at 244nm (Figure 3.5) whereas that for the second method was 7.61 minutes at 254nm (Figure 3.6).

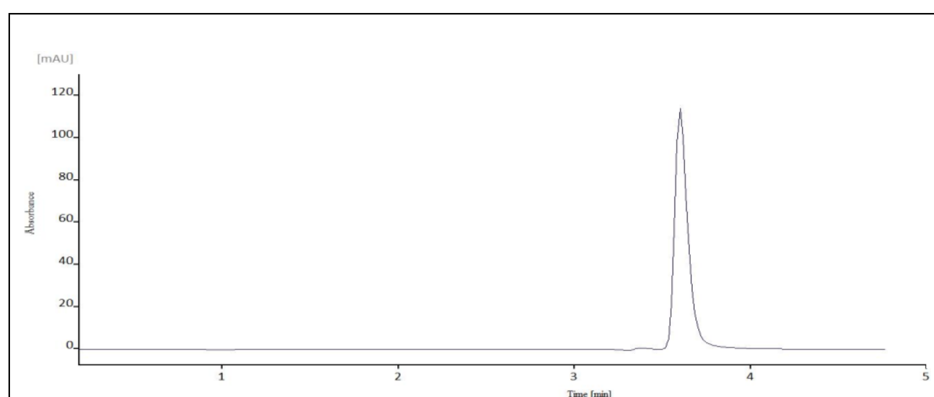


Figure 3.5: Chromatogram of APAP separation using CH_3CN/H_2O (25:75 v/v) as mobile phase for HPLC separation. APAP RT was 3.51minutes

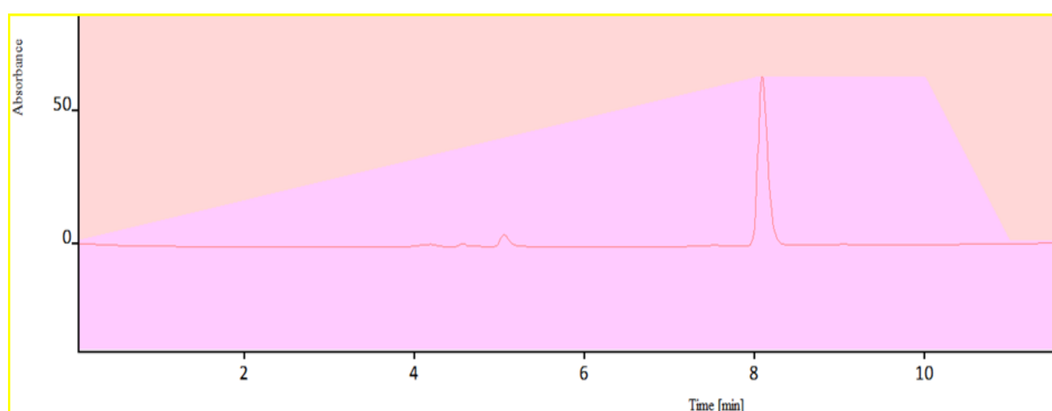


Figure 3.6: Representative chromatograms of APAP (50mg/l) showing peak appearances. APAP RT was 7.61minutes. other peaks probably correspond to metabolites

3.1.7 Chemical Oxygen demand (COD) measurement and significance

The Chemical Oxygen Demand was measured from activated sludge samples only (AERO1 and AERO2). This was done at 600nm using Hach-method (Table 3.4)

Table 3.4: Chemical Oxygen Demand (COD) variations with concentration of organics (drug) by aerobic bacteria

Sample ref. #	Samples Content	$COD_{initial}$ (mg/l)	COD_{final} (mg/l)	% Removal
S_1	Residual water +Sludge +Para+ air	1095	180	84
S_2	Residual water +Sludge - Para + air	940	276	70
S_3	Residual water +Sludge + Para - air	940	216	77
S_4	MSM medium +Sludge + Para + air	503	102	80
S_5	MSM medium -Sludge + Para + air	184	154	16
S_6	MSM medium +Sludge + Para - air	503	123	76

The percentage removal for the respective batch conditions after a 72 hours' incubation was determined from the formulae below:

Equation 3.1

$$\% \text{ Removal } \frac{S_0 - S_1}{S_0} \times 100$$

Where:
S_0 = initial COD concentration
S_1 = final COD concentration

After 3days of investigation and sampling, we noticed a considerable decrease (relative to initial) in the concentration of organic matter. This was more pronounced in the samples that contained sludge. Samples with no sludge witnessed a decrease but relatively less than those with sludge. Similarly, samples with air showed an advanced biodegradation level compared to those lacking. S_1 and S_4 showed very high concentration of COD in day1 because of the additional concentration of APAP added. on another hand, S_5 showed the least due to the absence of sludge in the batch. In general, the initial COD concentration for the residual water medium are higher than those of MSM medium. Residual water serving as milieu for growth showed higher concentrations of organic substances simply because it originates from WWTP systems. S_4, S_5 and S_6 showed reduced values because the medium of growth was made up entirely of mineral salts. Figure 3.8**Error! Reference source not found.** graphically summarises the variation of the COD (mg/l) with experiments performed.

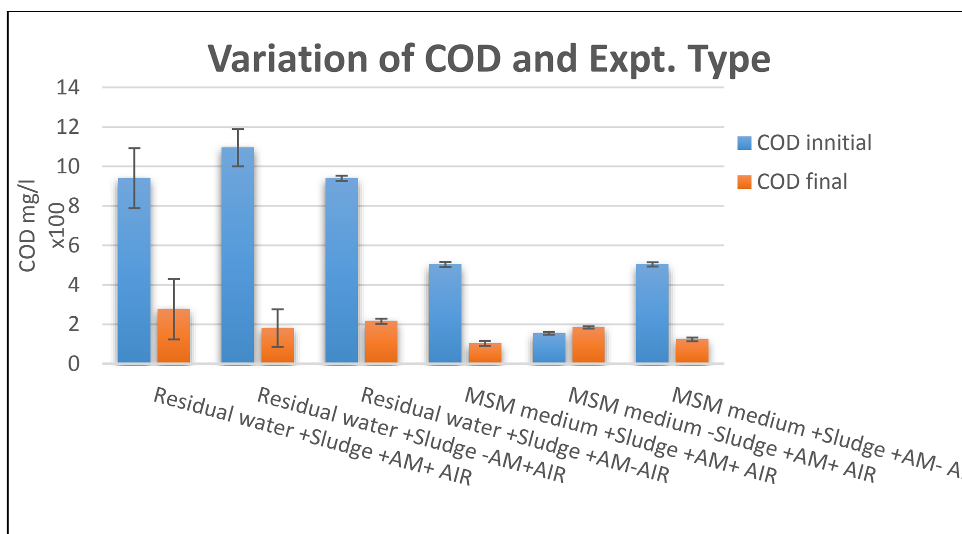


Figure 3.7: Measure (mg O2/L) in residual water & MSM assays both composed of sludge ±APAP. Initial & Final values were obtained just after culturing and 24 hrs after incubation

3.1.8 Biodegradation of paracetamol in aerated batch tests

The aerobic aerated biodegradation experiments were performed under standard environmental conditions of temperature and pressure. The tests are abbreviated as AERO1 and AERO2. (see Table 3.2(above) for summary of the respective tested concentrations and conditions. An elution gradient HPLC analysis of the respective samples resulted to the following observation

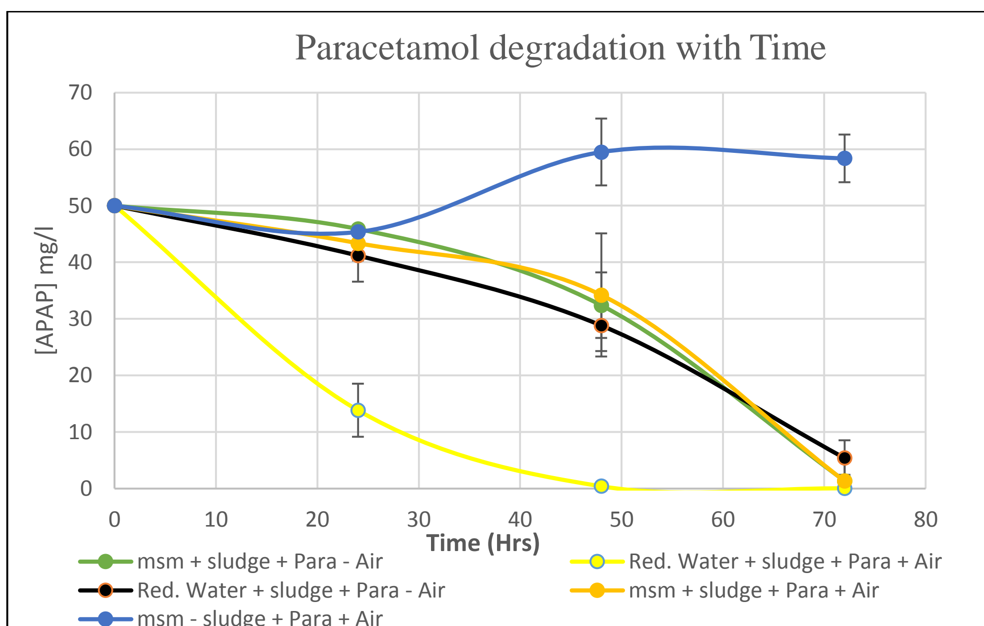


Figure 3.8: Biodegradation profile of APAP within aerated and non-aerated batch tests. Complete degradation was observed after 72hours.

AERO1 sample flasks containing Residual water, Air, Sludge and APAP showed a sharp decrease in APAP concentration reaching the zero value barely after 48hours of incubation. MSM with identical conditions (AERO2) takes a longer time (72hours) to reach the mark thus suggesting a possible role of the media in the biodegradation process. On another hand, samples (AERO1) lacking air or sludge turn to witness degradation though at a slower rate. After 72hours of incubation, the concentration does not reach the zero mark. An identical scenario is observed for AERO2 samples which do not contain air. Degradation was noted to be incomplete after 72hours. Meanwhile, the batch tests samples deficient in sludge showed a relative small or no degradation even with the presence aeration. After 72hours of incubation, analysis showed a considerable amount of APAP still present. An extended growth for a one-week period gave no additional change in the concentration. The variability of the daily observed concentration and removal percentages are summarised in the table below.

Table 3.5: Overall percentage removal of APAP from daily biodegradation analysis

Experimental batches	Concentration (mg/l)					
	Day 0	Day 1	Day 2	Day3	Average ± Ste dev	%Removal
Residual water +Sludge +APAP: NO AIR	46.24	41.18	28.82	5.4	25.13 ± 18	45.65
Residual water +Sludge +APAP + AIR	46.24	13.89	0.43	0.06	4.8 ± 2	89.63
Residual H2O +Sludge +APAP: NO APAP	0.01	0.05	0.01	0.03	0.03 ± 0.02	0
MSM + Sludge +APAP + AIR	44.72	43.38	34.22	1.35	26.32 ± 22	41.15
MSM + NO Sludge +APAP +AIR	44.72	45.89	32.4	1.38	26.56 ± 22	40.62
MSM + Sludge +APAP + NO AIR	44.72	45.88	59.49	58.38	54.58 ± 7	0

The obtained values simply ascertain the previous observation. In a nutshell, we noticed an increased biodegradation rate for sludge and containing samples and a slower though existing biodegradation rate for sludge deficient samples.

3.1.9 Possible metabolite identification

The identities of the probable metabolites of APAP found were tentatively matched by GC-MS analysis of concentrated samples. By comparison with commercially available authentic standards and by matching against the NIST spectra library, the supposed metabolites present were tentatively identified. After a 3days incubation in aerobic aerified experimental conditions and HPLC analysis, chromatogram produced showed in addition to the peak of APAP, 3 other peaks. The peak appearances were proportional to the disappearance of APAP. Figure 3.9

(below) shows a representative chromatogram for the biodegradation of APAP into its metabolites. The peak areas obtained were evaluated with time for each reaction conditions and rate of occurrence determined **Figure 4.3** (Appendix). After running standards of most occurring metabolites of APAP in the GC-MS, samples were then concentrated by SPE and analysed in the GC-MS. The NIST matched of the samples that followed didn't correspond to any previously ran standards. The metabolites were alternatively determined by HPLC. HPLC chromatograms revealed two pronounced and a less prominent metabolite peaks (RT 4, 5 and 7.4mins)

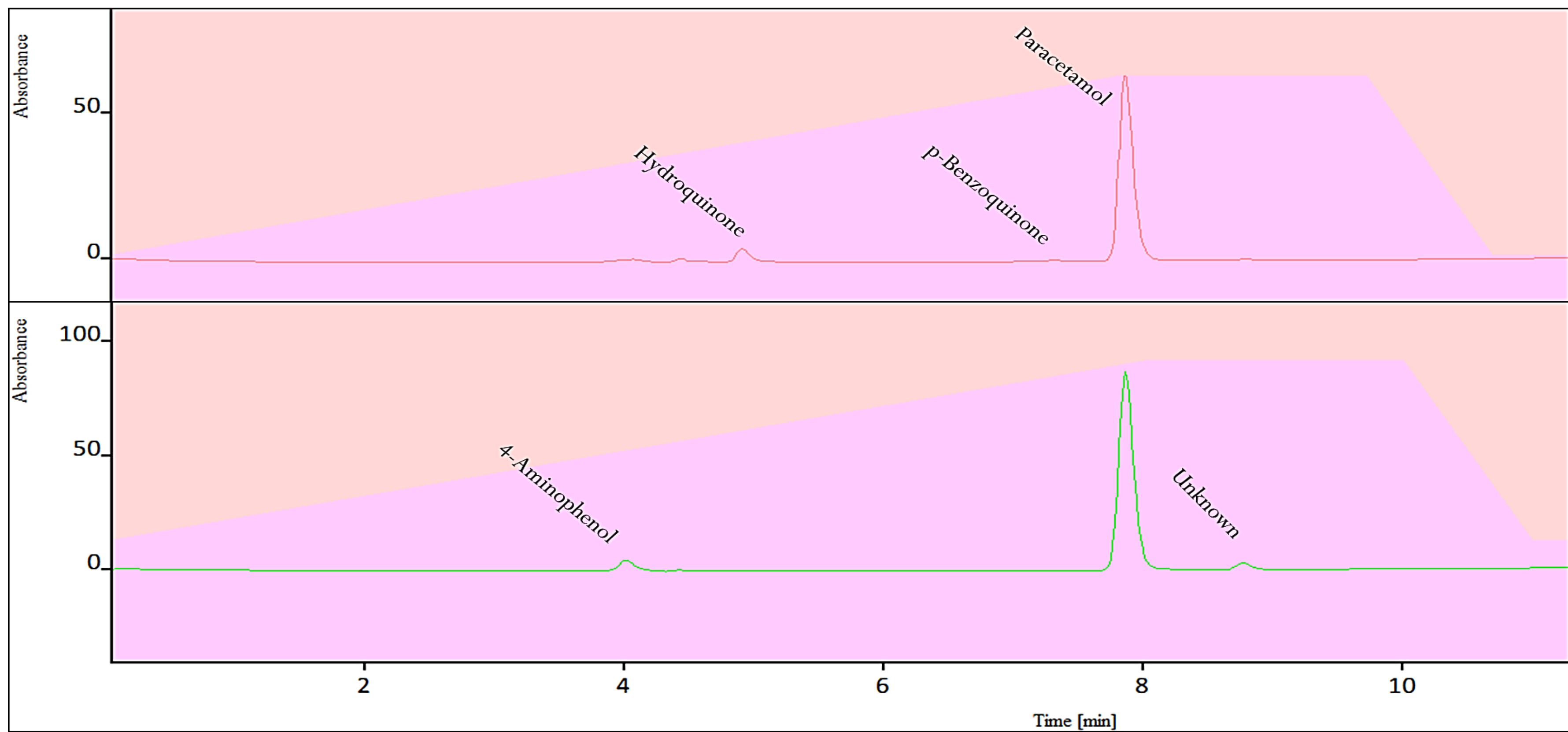


Figure 3.9: Chromatograms representing Paracetamol biodegradation and possible metabolites present. Paracetamol retention time was 7.5mins whereas, 4.0, 5.0 and 7.4 corresponded to metabolites peak.

3.2 DISCUSSION

The biodegradation of APAP and many other pharmaceuticals requires in most cases sludge enrichments and submissions of microbial communities to diverse growth conditions. In this study, the microbial community used was conditioned to utilize APAP as the only source of carbon, nitrogen and energy at one half and evaluating the degradation capabilities within batch cultures that contain alternative sources of nutrients on another part. The overall results summarizing the biodegradation of APAP in all batch tests experiments performed are presented 1n **Table 3.1** and **Table 3.2** (above).

Using microbial consortia obtained respectively from activated sludge (Nascent Faro wwtp) and inactivated sludge (Lagoon wwtp) and grown with APAP supposed to serve as the sole source carbon (C), nitrogen (N) and energy, the results show that at very high and low concentration values of APAP, degradation is virtually very slow or inexistent during the test period. Using THGL as a culture medium for their growth, we were capable of noticing some degree of degradation in anaerobic culture without inoculum. When compared to batch samples containing inoculum, a possible significant difference in degradation abilities was noticed. Samples in anaerobic batch cultures with MSM as medium for growth showed almost unnoticeable percentage breakdown of APAP. In a nutshell, the removals of APAP achieved in the different assays inoculated with microorganisms from the anaerobic lagoon of the Faro east WWTP were always below 60% and were similar to, or just slightly higher than, the removals obtained in the negative controls (without microbial inoculum) of the respective experiments. This suggests that those removals were mainly not caused by microbial activity. Moreover, in those experiments in which the removal of APAP was mainly due to other causes than microbial activity, we see a trend suggesting higher removals within the most complex media (THGL medium), reaching values of up to 56.9%, higher than those obtained with the simplest media (MSM), for which all removals were below 25%. This difference in biodegradation of APAP observed were to some extend conclusively suggested to probably be due to the components that make up the media. THGL is generally termed a complex medium because it is made up of different organic and chemical substances. These substances are capable of chemically reacting with APAP and breaking it down. This therefore, was used as an evidence to back-up the hypothesis of chemical degradation.

On the contrary, when the activated sludge from the Faro Northwest WWTP was used as the microbial inoculum, the final removal of APAP was always higher than 85%, while the

negative control experiments (MSM without microbial inoculum – dark blue line in Figure 3.8 (above)) showed no removal. This suggests that microbial activity had a major role in the removal of APAP in this case. Looking more in detail to these experiments along with time (Figure 3.8), we observed that in the MSM media inoculated with activated sludge (with aeration (orange-yellow line) and without aeration (green line)) or residual water inoculated with activated sludge (without air supply (black line)), the degradation of APAP was relatively slower (< 15% removal in 24 h, about 25% in 48 h and > 85% in 72 h)compared to batch experiments containing residual water, activated sludge and air (bright yellow line) (about 75% removal in 24 h and 100% after 48 h). Two putative causes can be associated with this:

1. a more complex composition of residual water compared to MSM, thus contributing to something else other than biological removal of APAP and/or
2. an additional presence of microorganisms in the residual water involved in the degradation of APAP.

The linear functions representing the relation between the concentration of this drug and the growth rate of bacteria for both conditions tested are very similar and have relatively good relation coefficients (R^2):

For Aerobic growth : $y = - 0.0631x+0.7326$ ($R^2=0.9783$)

For Anaerobic growth: $y= 0.0575x+0.7352$ ($R^2=0.9788$)

Therefore, the IC50 values for both conditions are also similar: 5.98mg/L and 6.26mg/L. These values represent the theoretical concentrations of APAP capable of causing a decrease of 50% in the bacterial growth within the respectively aerobic and anaerobic cultures. This thus justifies that the concentrations tested for the experiments were not toxic to our organism.

The COD values measured in this work and presented in Figure 3.8 provided some clues on the effective biodegradation process of APAP. The initial COD values in Residual water with close to 50 mg/L of APAP were about 1000mgO_2/L and in the MSM with similar concentration of APAP were about 450mgO_2/L if inoculated with activated sludge and about 200mgO_2/L in the absence of activated sludge. This led to the following observations that

- The tested residual water had a COD of approximately 450mgO_2/L,
- A paracetamol concentration of approximately 50mg/L generated an approximated COD value of 200mg/l and lastly,
- The activated sludge inoculum contributed approximately 250mgO2/L of COD.

Twenty-four hours after the start of the experiment the COD values in the assays inoculated with activated sludge dropped to about 20% the initial values in both tested media (residual

water or MSM), independently of the presence or absence of 50mg/L of APAP. This suggests that the degradation of organic matter achieved with activated sludge is not affected by the presence of APAP up to this concentration (~50 mg/L) and that was indeed expected as this concentration is about only 0.8% of the IC_{50} value (~6g/L) estimated for this drug in this work (the concentration causing a drop of 50% in the bacterial growth). Contrarily, after the same 24hrs hours, the COD value in MSM with an approximate 50mg/L of APAP and without activated sludge remained equal to the initial value, confirming the results showing no removal of APAP in this assay.

Knowing fully well that the regulatory limits on COD in wastewater from pharmaceutical factories is typically 150mg/l O_2, whereas the limit for discharge of wastewater in the receiving water bodies is 125mg/l O_2; regulated by decree law number N° 152/97 of 19June (Directive no 91/271 CEE, of 21 May) and taking into account the background concentration of paracetamol, COD values measured showed a decreased for all experiments though not substantially for most cases. Despite this decrease, the concentration remained above the regulatory limit after 72 hours of culture.

Characterisation of the compounds that showed a peak area at 4.0, 5.0 and 7.4 revealed that the hydrolysed product of paracetamol was p-aminophenol (p-AM), hydroquinone (HQ), p-benzophenol (p-BQ) which progressively increased with decreased in the concentration of APAP. This result was supported by the fact that when a standard solution of p-AM, HQ and p-BQ were injected in HPLC under the same conditions as the samples, identical peaks with a 99% RT similarity were obtained. See **Figure 4.7** (Appendix). **Figure 4.3** (Appendix) shows the variation in metabolite peak area with time as reaction proceeded. Peak 1 which corresponded to p-AM witnessed an increase whereas peak 2 witnessed an increased then a subsequent decreased in some samples. This may be due to an imminent conversion into an alternative product. Peaks p-BQ were very small because p-BQ is quickly converted into HQ through polymerisation process within a short reaction period. This is easily observed within experimental batches. The batch samples coloration changed from pale yellow to dark brown. Metabolites standard ran in GC-MS showed clear peaks of relative abundance. Sample analysed using GC-MS did not match with any of the expected metabolites issued from biodegradation. The was attributed to be due to the sample concentration steps. Samples were concentrated using a strong acid and base. These substances could possibly react with the samples. Also, this was probably due to parameters set up for separation in the system. The HPLC elution gradient separation mode easily identified the metabolites p-BQ, HQ,4-AM by comparing with standards.

4. CONCLUSIONAND FUTURE PERSPECTIVES

4.1 Conclusion

The activated sludge contain aerobic microorganisms capable of totally degrading APAP and the resulting metabolites to obtain energy without any other source of carbon and nitrogen.
Degradation is faster under aeration conditions but relatively slower without aeration. This degradation temporarily produces the following metabolites usually associated with APAP degradation: 4-aminophenol, p-BQ and HQ.
50 mg/L of APAP was capable of generating a COD value of about 200mgO_2/L and concentrations of this drug up to this value does not affect the degradation of organic matter in residual water with activated sludge based treatments. The estimated IC50 values showed that APAP could only cause a decrease of 50% in bacterial growths if and only if the approximate concentration of 6mg/L was reached for both aerobic and anaerobic conditions. Thus, problems in WWTPs caused by APAP affecting the biodegradation of organic matter are not expected because this concentration is hardly ever reached. The ability to breakdown APAP by some anaerobes or aerobes found in the consortium simply demonstrates that microbial consortium if submitted to appropriate conditions of growth and survival are capable of eliminating these re-occurring emerging pollutants within water environment and the environment as a whole. Biodegradation was in another point well pronounced when the appropriate conditions were put in place. Therefore, the application of microbial communities obtained from activated sludge from the WWTP system in Nascent-Faro-North efficiently biodegraded APAP hence attesting.

4.2 Future perspectives

In order to validate the above mentioned findings, very high predetermined concentrations of APAP will be mixed with residual water, activated sludge and conditioned for growth in the presence and absence of light within small batch culture medium. Several parameters such as TOC, temperature, pH, TSS will be monitored so as to evaluated the efficiency of the sludge under different culture conditions and evaluate the impact each parameter play in the biodegradation path.

Furthermore, we will be carrying out genetic extraction, identification and classification of various microorganisms found in the consortium using molecular biology kits and techniques in order to isolate, characterise and classify the specie (s) capable of significantly influencing the biodegradation process and elaborating the specific traits that make them.

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Appendix

Table 4.1: Summary of experimentation with conditions

	EXPERIMENTAL CONDITIONS		INNOCULUM	
	Respiratory conditions	**[APAP] Tested (mg/l)**	**wwtp lagoon (Faro-East)**	**Activated sludge (Faro North)**
Thioglycolate medium	Anaerobic	10 50 80 5000 10000	√√√	
	Aerobic without aerification		√√√	
	Aerobic with aerification			
Mineral salt medium (MSM)	Anaerobic	10 50 80	√√√	
	Aerobic without aerification		√√√	
	Aerobic with aerification			√√√
Residual water	Anaerobic	50		
	Aerobic without aerification			
	Aerobic with aerification			√√√

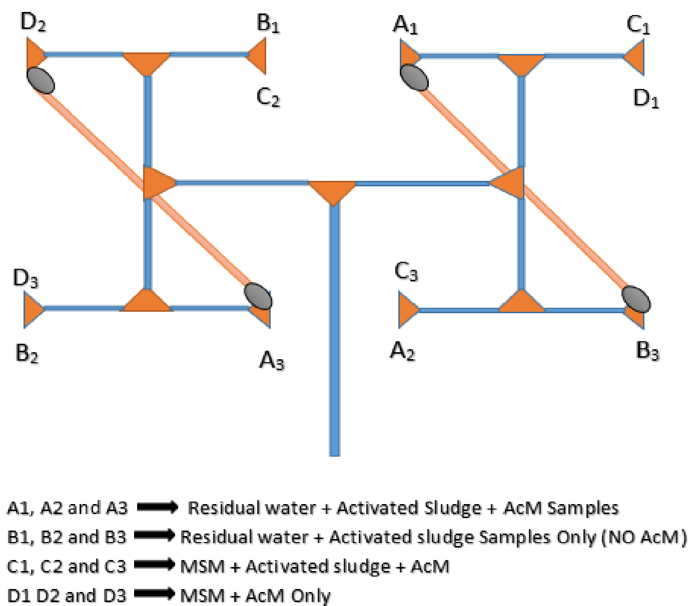
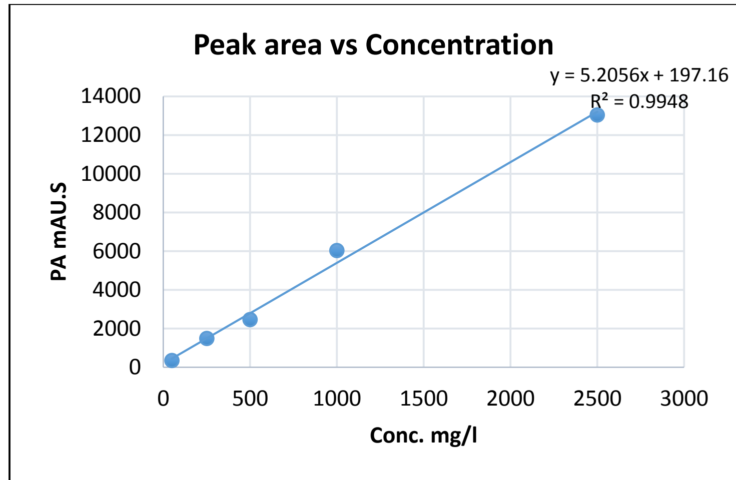


Figure 4.1: Experimental design for AERO 1, AERO2 and AERO 3. Air flow rate was fixed at 110ml/min and culture was operated in the dark. In a 250ml flasks volume, 200ml of culture samples were tested

Conc. (mg/ml)	Peak Area (mAU.S)
50	352.20
250	1495.47
500	2462.02
1000	6029.87
2500	13030.34

A



Conc. (mg/ml)	Peak Area (mAU.S)
5	90.98
10	183.68
50	711.97
100	1495.53
250	4250.86
500	7128.83
750	10831.61
1000	13788.62

B

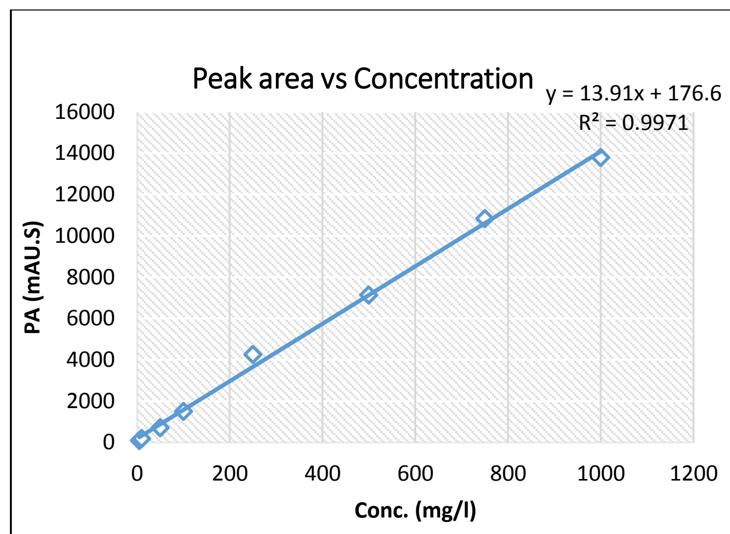
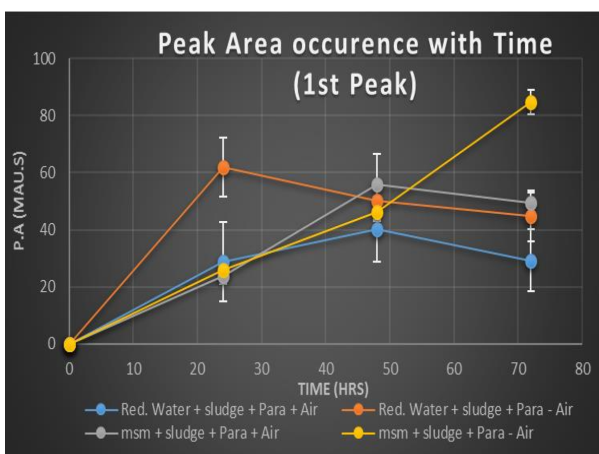


Figure 4.2: Standard curves for APAP quantification
A&B Standard curve made with THGL and MSM as GM respectively



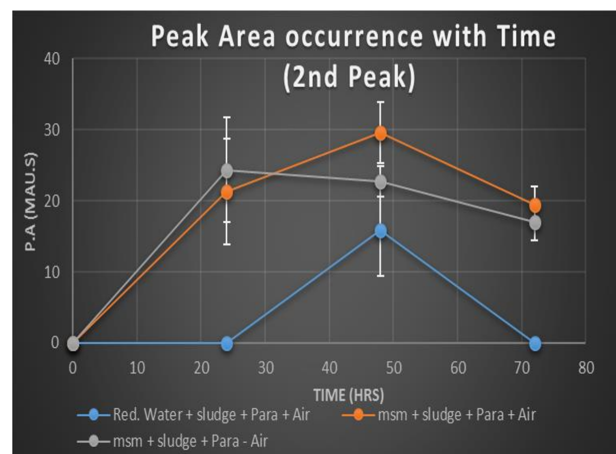
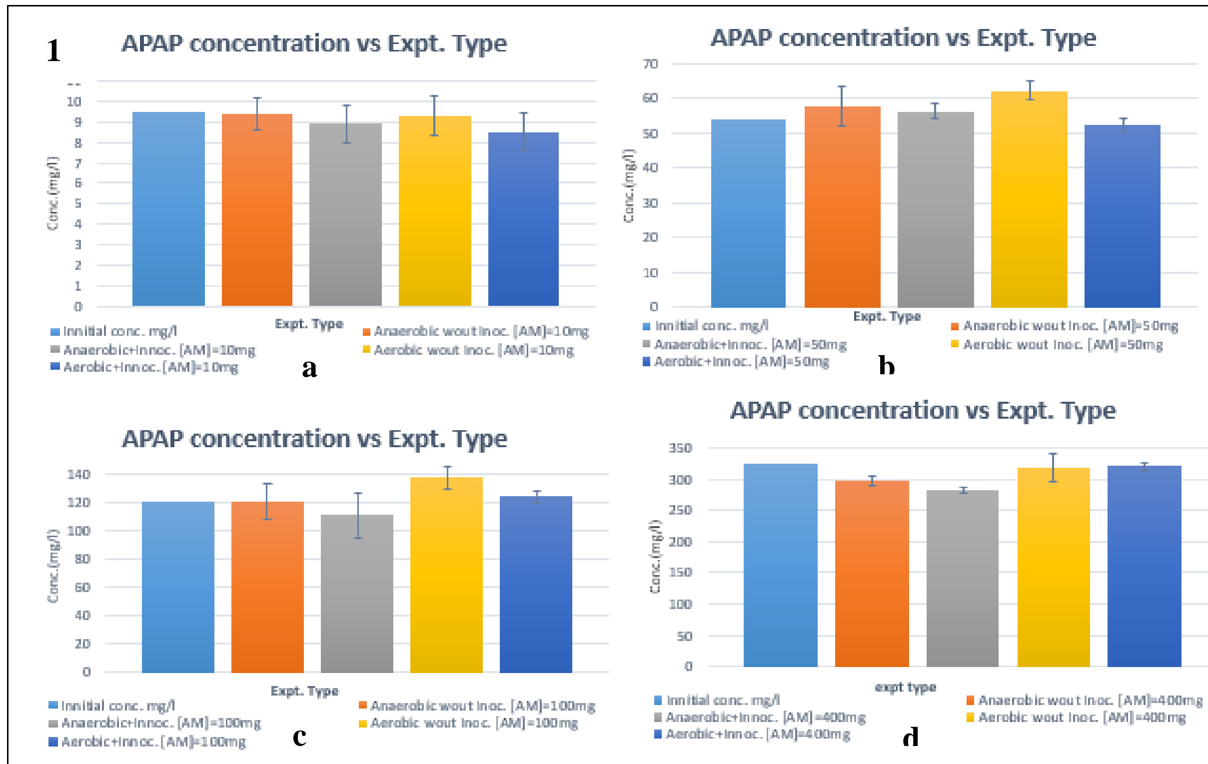


Figure 4.3:Time dependent variations of peak areas per corresponding batch samples for possible APAP metabolites detected



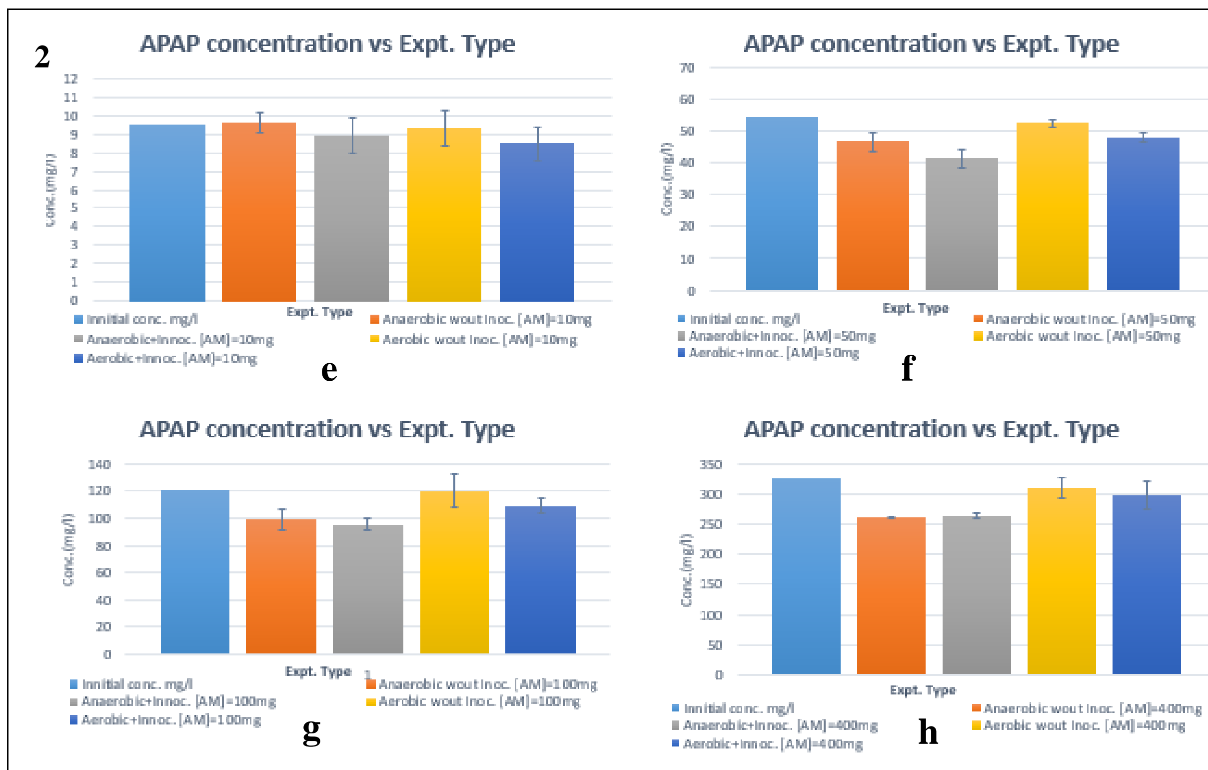
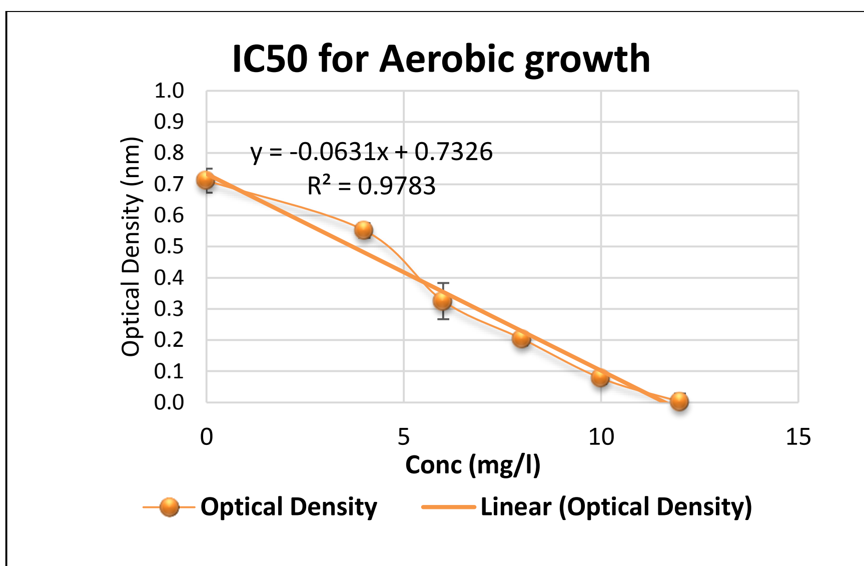


Figure 4.4: One-day (1) one-week (2) analysis of APAP removal in MSM tests culture. Graphs 'a-h' represents the degree of biodegradation of the respective concentrations 10mg/l, 50mg/l, 80mg/l and 350mg/l in both cases



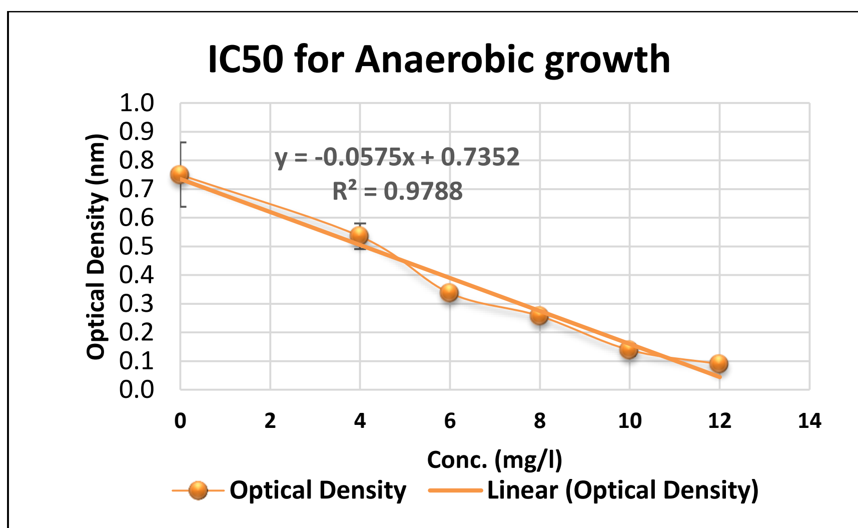


Figure 4.5: Graphical representation of IC_{50} values of APAP obtained by UV-vis spectrophotometry (ƛ=600nm). Anaerobic & Aerobic batches showed IC_{50} values of 6.23mg/l & 5.98mg/l respect..

Table 4.2: Biodegradation concentrations of APAP variation with time within aerified batch studies

REACTION BATCHES	CONCENTRATION ± SD (mg/l)			
	Day 0	**Day 1**	**Day 2**	**Day 3**
Red water + Sludge + APAP - AIR	46.24	41.18±4	28.82±4	5.40±3.14
Red water + Sludge + APAP + AIR		13.90±4	0.43±0.20	0.06±0.01
Red water + Sludge - APAP + AIR		0.05±0.01	0±0.00	0±0.00
MSM + Sludge + APAP + AIR	44.72	43.38±1	34.22±5	1.98±1
MSM – Sludge + APAP + AIR		45.88±1	49.49±5	48.38±4
MSM + Sludge + APAP - AIR		45.89±1	32.4±5	1.38±0.03

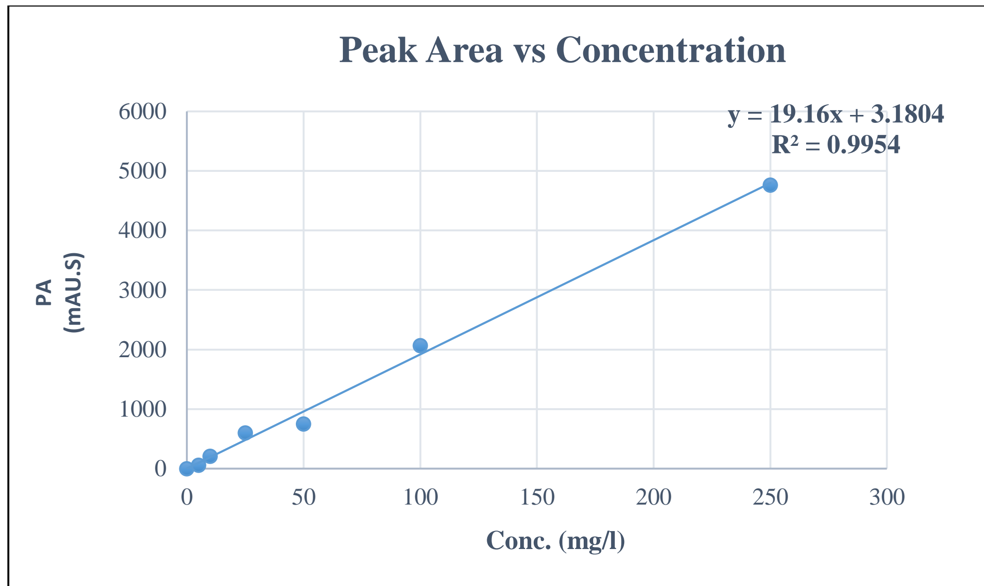
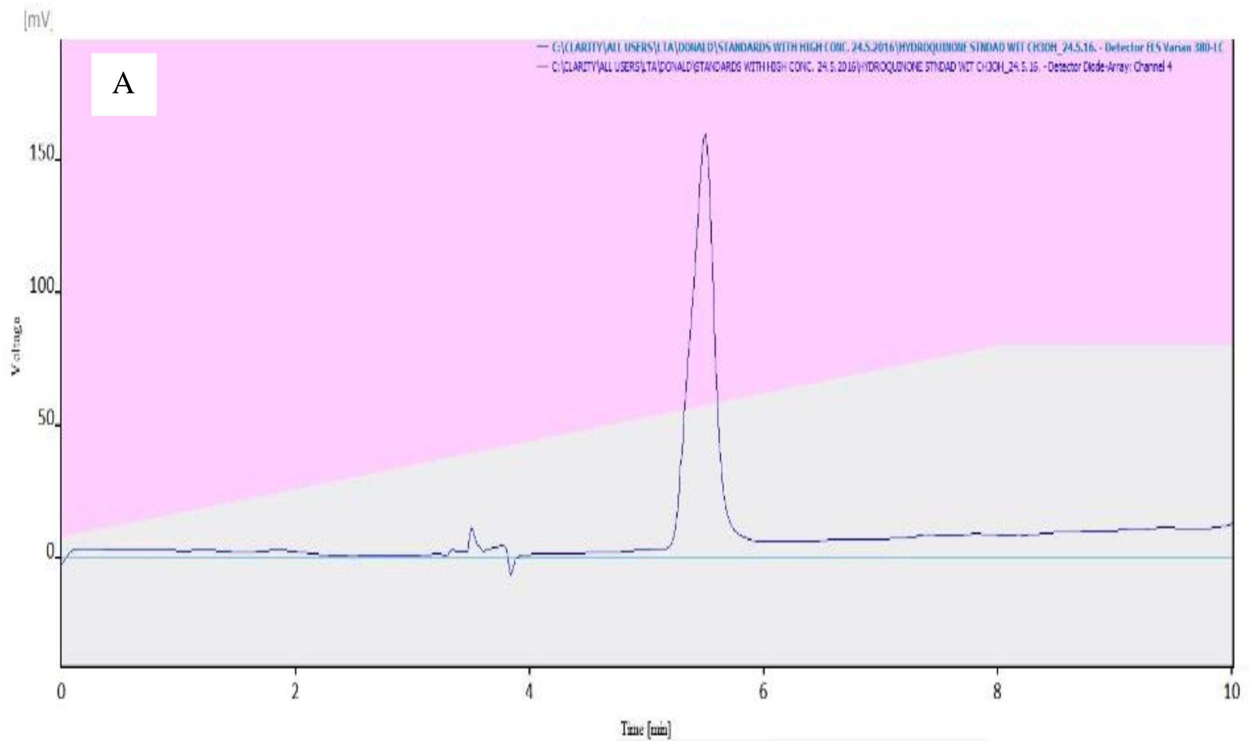
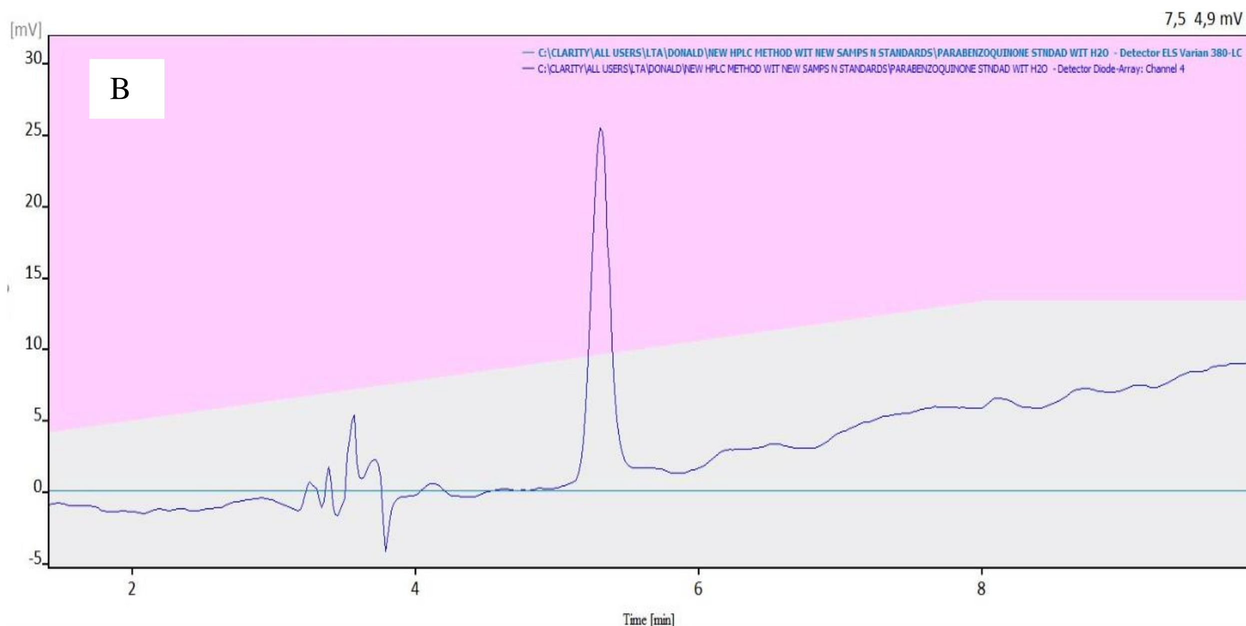


Figure 4.6: Calibration curve for APAP quantification in aerobic batches





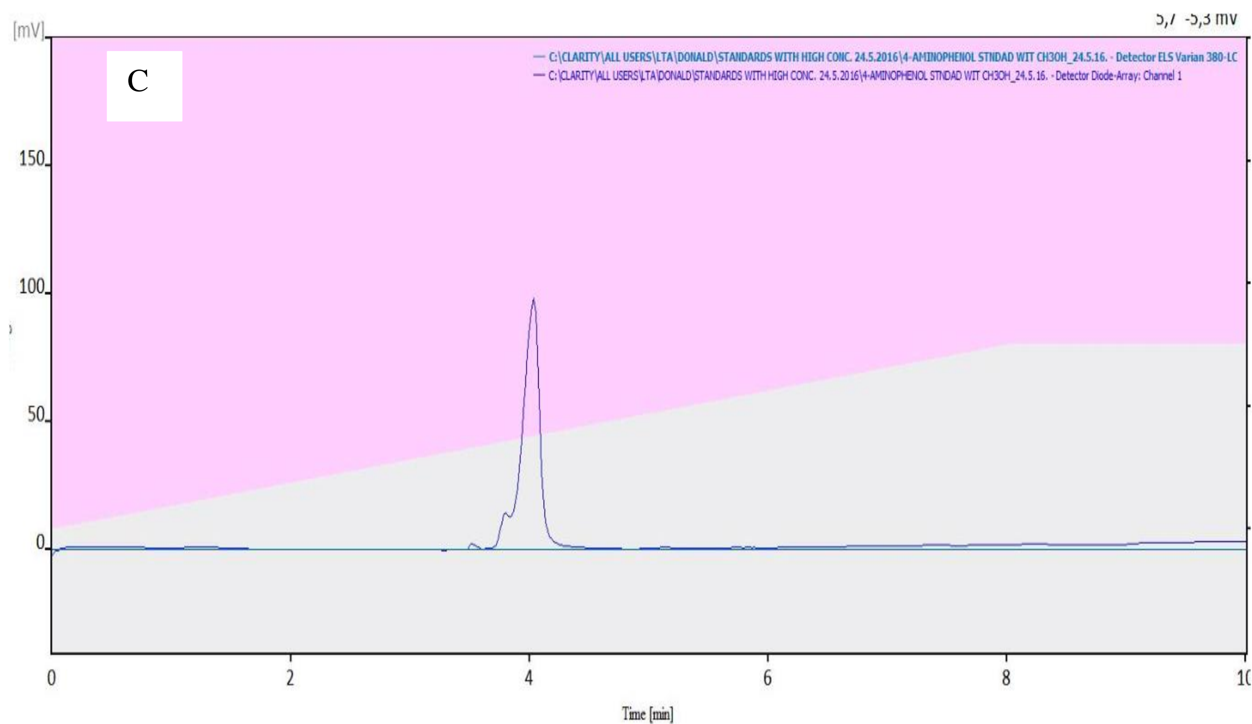
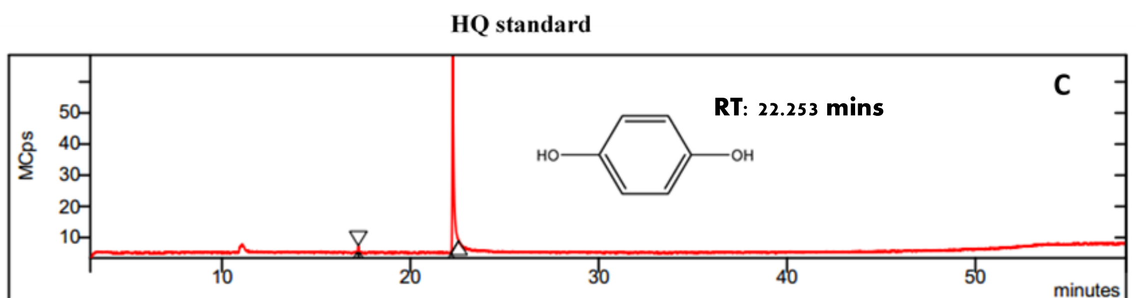


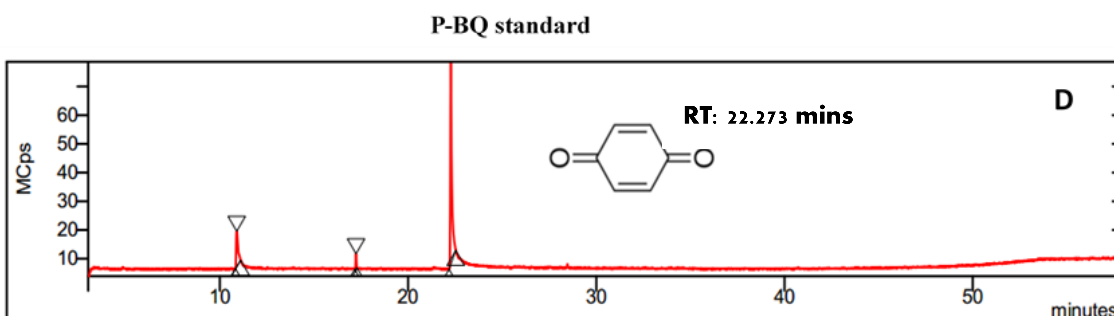
Figure 4.7: Representative chromatograms for standards of HQ, p-BQ & 4-AM (100mg/l) showing peak appearances. RT obtained were 5.0, 7.4, & 4.0 minutes for HQ (A), p-BQ (B) and 4-AM (C), respectively. Other peaks correspond to growth medium

HQ standard



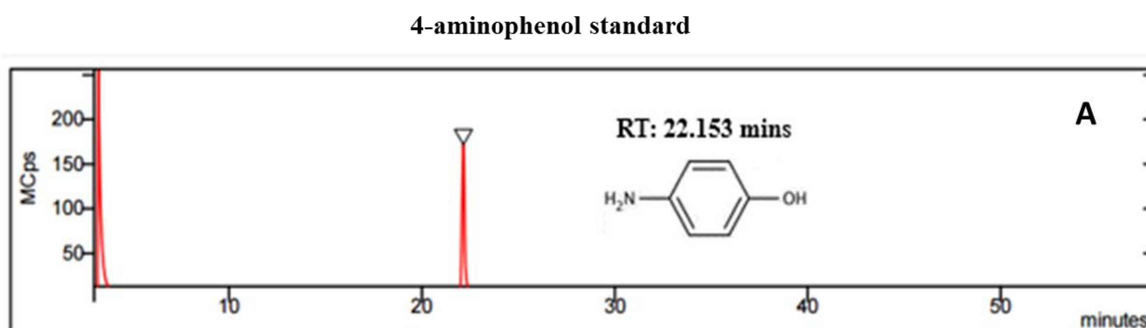
#	RT	Peak Name	Res Type	Area	Amount	R.Match
1	17.237	Hydroxylamine, O-decyl-	TIC	7.996e+6	2.349	803
2	22.252	Hydroquinone	TIC	3.325e+8	97.651	919

P-BQ standard



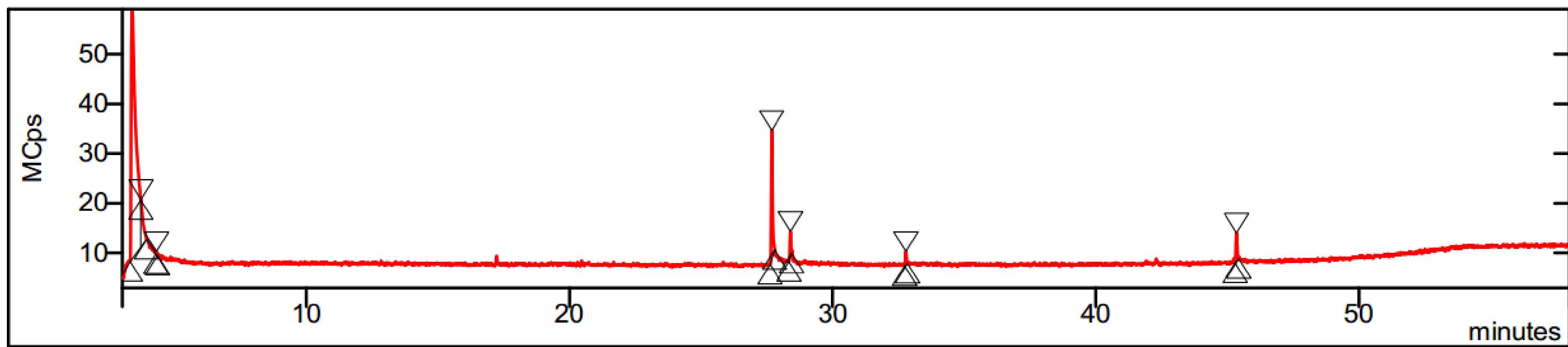
#	RT	Peak Name	Res Type	Area	Amount	R.Match
1	10.896	p-Benzoquinone	TIC	8.673e+7	18.368	762
2	17.236	Dodecane	TIC	1.515e+7	3.208	873
3	22.273	Hydroquinone	TIC	3.703e+8	78.424	917

4-aminophenol standard



#	RT	Peak Name	Res Type	Area	Amount	R.Matcl
1	3.224	Acetamide, 2,2,2-trifluoro-N	TIC	2.299e+9	59.741	817
2	17.241	Tridecane, 3-methyl-	TIC	4.492e+6	0.117	807
3	22.153	Phenol, 4-amino-	TIC	1.541e+9	40.039	951
4	53.870	Ethyl 2-acetamido-3,3,3-trif	TIC	3.997e+6	0.104	563

Figure 4.8: Standards of principal metabolites that originate from paracetamol biodegradation. C, D and A corresponds to Hydroquinone, para-benzoquinone and 4-aminophenol respectively. Data points are given in as mean ± standard deviation (n=3)



#	RT	Peak Name	Res Type	Area	Amount	R.Match
1	3.392	Formamide	TIC	6.130e+8	77.516	851
2	3.721	Threo-3-bromo-2-pentanol	TIC	5.422e+7	6.856	835
3	4.306	2-Formylhistamine	TIC	1.077e+6	0.136	818
4	27.684	1-Dodecanol	TIC	8.289e+7	10.483	906
5	28.401	Butylated Hydroxytoluene	TIC	1.260e+7	1.594	899
6	32.783	2-Propenoic acid, tridecyl e	TIC	7.721e+6	0.976	832
7	45.350	9-Octadecenamide, (Z)-	TIC	1.929e+7	2.439	898

Figure 4.9: Constituents of sample after GC-MS analysis.

Experimental set-up

Coloration of batch culture with MSM 3days after

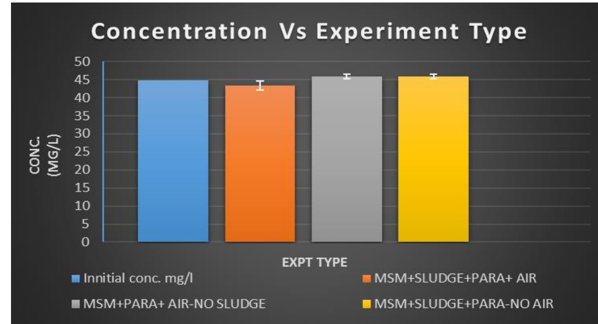
Batch culture coloration with Residual H_2O 3days after

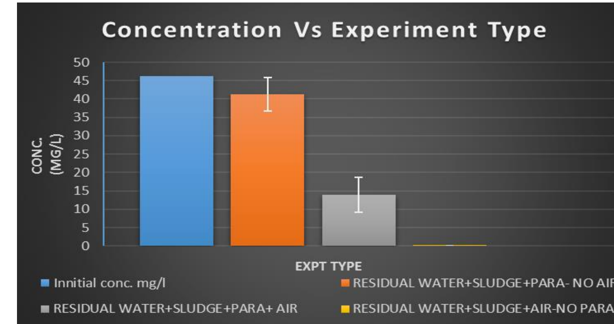
Figure 4.8: Experimentation set-up displaying colour variations of samples with different media

Graphical expression of APAP conc. in aerified MSM culture batches
A

Graphical expression of APAP conc. in aerified red.H2O culture batches
B

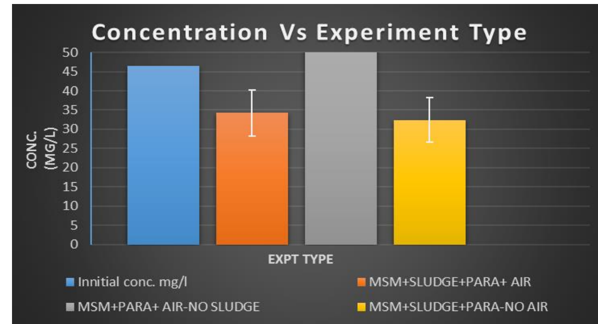
APAP conc. obtained 24Hrs

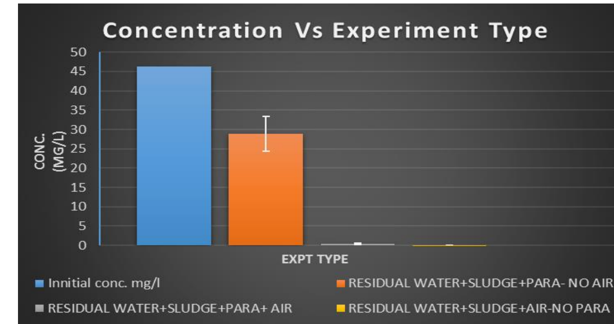




Conc. of APAP 24hrs after culture

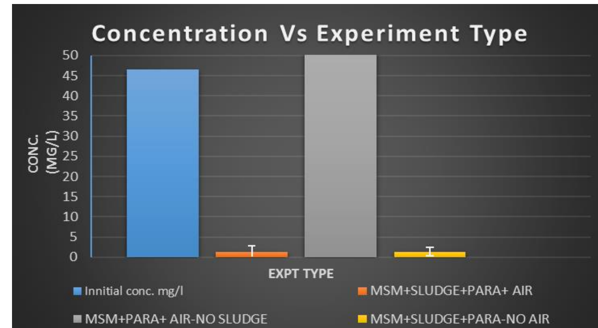
APAP conc. after

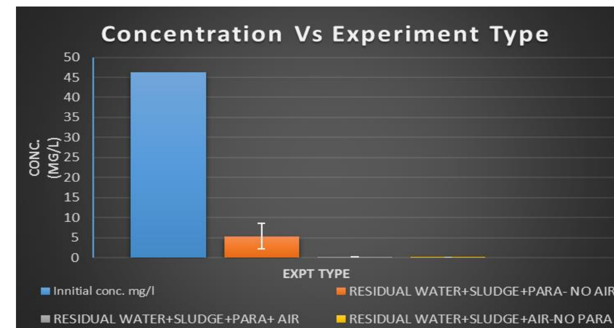




48Hrs of cultured

APAP concentration left after 72hrs of





72Hrs of culture

Figure 4.10: Biodegradation of Paracetamol in aerified batch cultures. 'B' represents biodegradation using residual water as medium for growth whereas 'A' shows degradation in MSM medium.