FATMA M.A. EL-SAYED ISMAIL

BIODEGRADATION OF PARACETAMOL AND ITS INTERMEDIATE METABOLITE HYDROQUINONE BY BACTERIAL STRAINS ISOLATED FROM TWO MINES OF THE IBERIAN PYRITE BELT

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BIODEGRADATION OF APAP AND ITS INTERMEDIATE METABOLITE HQ BY BACTERIAL STRAINS ISOLATED FROM TWO MINES OF THE IBERIAN PYRITE BELT

Declaration of Authorship

I declare that I am the author of this work, which is original. The work cites other authors and works, which are adequately referred in the text and are listed in the bibliography.

FATMA M.A. ELSAYED ISMAIL

ACKNOWLEDGMENT

This work has been accomplished through the sustain and bless I got from The Creator- exalted be him- from whom I expect the acceptance, support, and reward.

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Finally, I would love to let my parents, my grandmother, my aunt, and my brothers, especially Mohammad, know that I had gone through this project to make them proud of me, and crown their invaluable efforts to support me in whatever I decide. May the creator prepare the best for them in this worldly life and the hereafter as well.

FATMA

ABSTRACT

The main objective of the current study was to isolate bacterial strains able to biodegrade the emerging pollutants paracetamol (APAP) and hydroquinone (HQ), amongst the most worldwide prescribed drugs, also frequently detected in wastewater treatment plants influents and effluents and the environment.

The most promising microbial consortia of Poderosa and Lousal mines for APAP removal were selected based on the previous reports of PROBIOMA project (European Regional Development Fund ERDF - Interreg V-A Spain-Portugal program (POCTEP)). The ability of the selected microbial consortia to remove APAP from the Mineral Salt Medium (MSM) at an initial concentration of about 500 mgL^{-1} (MSM-APAP (500 mgL^{-1})), under dark shaking conditions of 160 rpm at 25 °C, was confirmed using UV-vis molecular spectroscopy.

Subsequently, the isolation step from selected samples proceeded from three successive enrichment cultures using MSM-drug (500 mgL^{-1}) under the aforementioned conditions by spreading first on LB-drug and then on MSM-drug (500 mgL^{-1}) agar plates and resulted in seven isolates able to utilize APAP as sole carbon source, and identified according to 16S rRNA gene sequence analysis as members of genera *Aeromonas*, *Bacillus* (two isolates), *Niallia*, *Paraburkholderia*, *Rhizobium*, and *Variovorax*, as well as one HQ utilizing isolate (*Mycolicibacterium* sp.).

The HPLC analysis of APAP removal, in MSM-APAP (500 mgL^{-1}) under the same culture conditions, by the two putative APAP biodegrading *Bacillus* sp. isolates revealed that *Bacillus* sp. (PDE3.1) showed maximal APAP %removal of 63 ± 3 after 18 days, while *Bacillus.* sp. (PLC2.1) showed %removal of only 8 ± 1 at the end of the experiment after 21 days. The key metabolites of APAP degradation (4-aminophenol and HQ) were detected through GC-MS analysis in the experiment with *Bacillus* sp. (PDE3.1) at very low concentrations.

Then, the seven potential APAP biodegrading bacterial isolates were tested for APAP removal in MSM at a lower concentration of 50 mgL^{-1}. *Rhizobium* sp. (PDE3.3) and *Paraburkholderia* sp. (PLA3.3) seemed the most promising where APAP %removal was 49±4 and 47.9±0.9, respectively. Later, the co-culture of the three most promising isolates (*Rhizobium* sp.

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(PDE3.3), *Paraburkholderia* sp. (PLA3.3), and *Bacillus* sp. (PDE3.1) didn't improve the %removal compared with the pure cultures, while the co-culture with the seven potential APAP biodegrading isolates did not show removal capacity. *Mycolicibacterium* sp. (HPB1.1) showed at least 88% removal of HQ from MSM-HQ (50 mgL^{-1}) after four days; hence, was checked for APAP removal in MSM-APAP (50 mgL^{-1}) and showed APAP %removal of 41.6±0.1. Overall, some bacterial strains isolated from Poderosa and Lousal mines showed removal capacity; hence, more efforts should be directed at investigating if biodegradation is the main removal mechanism involved, and at exploring the biodegradation potentials of The Iberian Pyrite Belt mines associated bacteria.

Key words: APAP, HQ, bioremoving bacteria, bioprospecting

RESUMO

As crescentes preocupações ambientais sobre a incapacidade das estações de tratamento de águas residuais (ETARs) para remover completamente os produtos farmacêuticos e outros poluentes das águas residuais, são apoiadas por estudos que reportam as ETAR como a principal fonte de poluentes emergentes no ambiente aquático. Desde a década de 80 que a aplicação da tecnologia de bioaumentação em sistemas de ETAR convencionais usando estirpes bacterianas com as capacidades de biodegradação desejadas tem recebido atenção com o objetivo da remoção completa dos medicamentos contaminantes das águas residuais antes da liberação no meio ambiente. Por outro lado, ambientes extremos sempre foram considerados como uma fonte valiosa de estirpes microbianos com extraordinários potenciais metabólicos considerados como mecanismos de adaptação às condições circundantes de habitats tão extremos, permitindo que esses micróbios extremófilos dominem e se sobreponham a outras comunidades microbianas. O principal objetivo do presente estudo foi isolar e identificar estirpes bacterianas com potencial metabólico para biodegradar o poluente emergente paracetamol (APAP) e seu metabólito hidroquinona (HQ) em ETARs. A escolha do APAP como modelo de estudo deve-se à sua classificação entre os medicamentos mais prescritos no mundo, à sua deteção frequente em afluentes e efluentes de ETARs e no meio ambiente, bem como sua inclusão na Lista Modelo da Organização Mundial da Saúde de Medicamentos Essenciais. Para monitorização da remoção de APAP e HQ foram utilizados diferentes métodos de análise em diferentes fases do trabalho: absorvância de UV-vis em leitor de placas, HPLC com detetor de UV-vis e GC-MS. A análise espectral de UV-vis em leitor de placas foi usada para monitorizar APAP e HQ nas culturas de enriquecimento dos consórcios microbianos utilizados para os isolamentos, o HPLC foi utilizado nos estudos de remoção com os isolados selecionados e o GC-MS para detetar produtos da degradação do APAP. Para a construção de retas de calibração a usar nas análises das amostras fizeram-se os seguintes testes e/ou afinações: no leitor de placas foram selecionados comprimentos de onda com picos de absorbância em soluções padrão dos compostos APAP e HQ; no HPLC determinaram-se os tempos de retenção destes compostos e também do metabolito 4-aminofenol com um método

previamente utilizado para estes compostos em trabalhos anteriores já publicados pelo grupo onde o trabalho foi efetuado, no GC-MS testou-se um método previamente utilizado pelo grupo para análises de outro fármaco (17α-etinilestradiol) e determinaram-se os tempos de retenção dos compostos em estudo neste trabalho. Os consórcios microbianos mais promissores das minas Poderosa e do Lousal para biodegradação de APAP foram selecionados com base nos relatórios anteriores do projeto PROMIOMA. A capacidade dos consórcios microbianos selecionados de biodegradar APAP em meio mineral MSM suplementado com uma concentração de 500 mgL^{-1} (MSM-APAP (500 mgL^{-1})), no escuro sob condições de agitação de 160 rpm a 25 °C, foi confirmada por análise de absorbância de UV-vis. Subsequentemente, a etapa de isolamento fez-se a partir de três culturas de enriquecimento sucessivas em MSM-APAP (500 mgL^{-1}) nas condições descritas, por espalhando primeiro em placas de LB-agar e depois em placas de MSM-agar-APAP (500 mgL^{-1}), e resultou em sete isolados potencialmente degradadores de APAP identificados de acordo com análises das sequências do gene ARNr 16S como membros dos géneros *Aeromonas*, *Bacillus* (dois isolados), *Niallia*, *Paraburkholderia*, *Rhizobium* e *Variovorax*. Foi também selecionado um isolado degradador de HQ identificado como *Mycolicibacterium* sp.. Estes géneros, exceto o *Bacillus*, são relatados pela primeira vez para a biodegradação de APAP. As análises por HPLC da biodegradação do APAP, em MSM-APAP (500 mgL^{-1}) nas mesmas condições de cultivo, pelos dois *Bacillus* sp. revelaram que o isolado *Bacillus* sp. (PDE3.1) mostrou uma percentagem máxima de remoção de APAP de 63±3 após 18 dias, enquanto o isolado *Bacillus*. sp. (PLC2.1) mostrou apenas 8±1% de remoção no final da experiência. Nenhum dos principais metabólitos da degradação do APAP (HQ e 4-aminofenol) foi detetado por análise de HPLC, no entanto eles foram detetados na análise por GC-MS, ainda que em concentrações baixas, na experiência com o *Bacillus* sp. PDE3.1, sugerindo que a biodegradação do APAP pode ter ocorrido através da descarboxilação inicial do APAP em 4-aminofenol no qual o grupo amina é depois substituído pelo grupo hidroxilo produzindo-se HQ. Os sete isolados bacterianos potencialmente biodegradadores de APAP foram também testados quanto à biodegradação de APAP em MSM numa concentração mais baixa: 50 mgL^{-1}. Nestes testes, os isolados *Rhizobium* sp. (PDE3.3) e *Paraburkholderia* sp.(PLA3.3) pareceram os mais promissores, com com % de remoção de APAP de 49±4 and 47,9±0,9, respectivamente. Posteriormente, uma co-cultura dos três isolados mais promissores (*Rhizobium* sp. (PDE3.3), *Paraburkholderia* sp.

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(PLA3.3) e *Bacillus* sp. PDE3.1) não melhorou a remoção de APAP em comparação com as culturas de isolados puros, enquanto uma co-cultura com todos os sete isolados potencialmente biodegradadores de APAP não apresentou capacidade degradadora. Por outro lado, o isolado *Mycolicbacterium* sp. (HPB1.1) revelou pelos menos 88% de remoção de HQ após quatro dias numa experiência em MSM-HQ (50 mgL^{-1}). Portanto, este promissor isolado foi também testado para biodegradação em MSM-APAP (50 mgL^{-1}), tendo sido comprovada a remoção de 41,6±0,1% do APAP inicial com uma concentração residual de 29,34±0,05 mgL^{-1}. Por fim, o isolado *Mycolicibacterium* sp. (HPB1.1) foi selecionado para trabalhos futuros e foi cultivado em meio LB (pH 7) a 25°C sob condições de agitação (160 rpm) para estudo da cinética de crescimento da cultura ao longo de 68 h, com base na densidade ótica a 600 nm (OD_{600}) de amostras em intervalo de 2 h. As células cresceram exponencialmente sem fase lag visível e o crescimento exponencial, associado ao crescimento mais rápido com coeficiente de determinação R^2 de 0,99, estendeu-se até 36 h onde foi alcançado uma OD_{600} máxima de 1,27. A taxa de crescimento específico μ durante o crescimento exponencial foi calculada como 0,047 h^{-1}. Depois, uma fase de desaceleração ou aceleração negativa de crescimento parece seguir-se ao crescimento exponencial e estender-se das 36 h até às 42 h, onde a fase estacionária parece começar e durar até às 68 h, havendo depois uma diminuição da OD_{600}.

Palavras-chave: APAP, HQ, bactérias bioremoçãos, bioprospecção

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A	**UV absorbance**
A 260/230	Ratio of absorbance at 260 nm to absorbance at 230 nm
A 260/280	Ratio of absorbance at 260 nm to absorbance at 280 nm
Å	Angstrom
bp	Base pair(s)
°C	Degree Celsius
DNA	Deoxyribonucelic acid
EC_{50}	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
eV	Electronvolt
GC-MS	Gas chromatography-Mass spectrometry
g	Gram
HPLC	High performance liquid chromatography
h	Hour
kV	Kilovolt
LOD	Limit of detection
LOQ	Limit of quantification
M	Molar
m	Meter
mAU.s	Milli absorbance unit. Second
mg	Milligram
mgL^{-1}	Milligram per liter
min	Minute
mL	Milliliter
mM	Millimolar
mm	Millimeter
MSTFA	N-Methyl-N-(trimethylsilyl)trifluoroacetamide

LIST OF ABBREVIATIONS

m/z	Mass to charge ratio
µg	Microgram
µg mL^{-1}	Microgram per milliliter
µgL^{-1}	Microgram per liter
µL	Microliter
µM	Micromolar
µm	Micrometer
N	Normal
NCBI	The National Center for Biotechnology Information
ngL^{-1}	Nanogram per liter
ng mL^{-1}	Nanogram per milliliter
ng/(mgh^{-1})	Nanogram per (milligram per hour)
NIST	National Institute of Standards and Technology
nm	Nanometer
PCR	Polymerase chain reaction
R^2	Coefficient of determination
rpm	Revolutions per minute
s	Second
16S rRNA	16 Svedberg ribosomal ribonucleic acid
TAE	Tris-Acetate-EDTA
UHPLC	Ultra-High performance liquid chromatography
UV-vis	Ultraviolet-visible
V	Volt
v/v	Volume per volume
w/v	Weight per volume

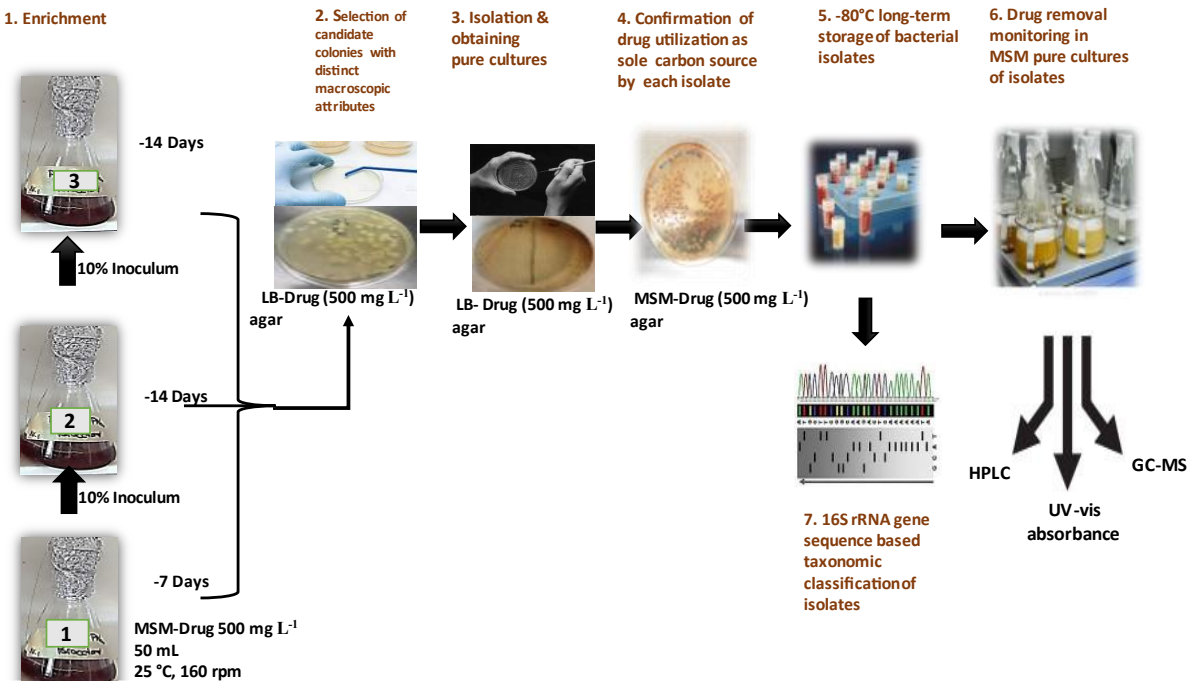
STUDY OBJECTIVES

This study was performed in the framework of BIOEXTREMEDEGRAD project (reference PTDC/CTA-AMB/7782/2020, funded by The Portuguese Foundation for Science and Technology, 2021-2024), which has the general aim of contributing to maintain water quality in the environment, through biomining for new bacterial strains (and their genetic material) that can be used as tools for the development of new strategies to realize total degradation of APAP and HQ as emerging pollutants. The work plan and specific objectives included:

1. Verifying the analysis of APAP and HQ and their degradative metabolites by UV-vis molecular spectroscopy, HPLC with UV-vis absorbance, and GC-MS analyses.
2. Evaluation of APAP and HQ removal ability of the selected Poderosa and Lousal mines microbial consortia in selective liquid medium.
3. Isolation of bacterial strains by plating the obtained enriched liquid cultures on non-selective nutritive medium supplemented with the drug, and selection of strains with different macroscopic characteristics for streaking on the same medium.
4. Subsequent streaking the selected isolates on selective medium to confirm their drug utilization ability as sole carbon source.
5. Taxonomic classification of APAP and/or HQ potentially biodegrading isolates using 16S rRNA gene sequence analysis.
6. Studying the removal of APAP and HQ by the bacterial isolates using a selected chromatographic method.
7. Discussion of the biodegradation results and contextualization of known degradative capacities of the identified taxa.

The work plan and objectives are graphically summarized as shown in the figure below.

STUDY OBJECTIVE



The work plan for studying the potential biodegradation of APAP and HQ by bacterial isolates isolated from the enrichment cultures of Poderosa and Lousal mines microbial consortia.

1. INTRODUCTION AND REVIEW OF LITERATURE

1.1. Problem definition

In spite of the intensive use of the non-steroidal anti-inflammatory drugs (NSAIDs), especially the analgesic paracetamol (APAP), and their metabolites continuous release into the environment, especially aquatic environments, and although some light was already thrown on their biodegradation pathways and respective genetic ground, these chemicals are still detected in the effluents of treatment plants. Therefore, there is still an urging need for the isolation of new biodegrading microbial strains with the focus on the identification of biodegradation genes, operons, and the factors possibly regulating their expression; thus, increasing the knowledge available for the development of new tools aiming to improve the bioremediation of these chemicals (Żur et al., 2018). In fact, the known NSAIDs biodegrading microorganism are relatively limited, especially those capable of utilizing polycyclic drugs (Domaradzka et al., 2015; Żur et al., 2018).

1.2.The environmental concerns about the emerging pollutants and micropollutants

Over the last two decades, environmental concerns have surpassed the classical pollutants such as polychlorinated biphenyls, dioxins, and organochlorine and organophosphorus pesticides, and have extended to emerging pollutants introduced into the environment mainly through domestic activities (Daughton & Ternes, 1999; Ternes et al., 2004).

Emerging pollutants can be synthetic or naturally existing chemicals or microbes not commonly detected but can be introduced into the environment; hence, cause proven or disputed negative effects on the ecosystem and/or human health (Haddaoui & Mateo-Sagasta, 2021). Emerging pollutants include pharmaceuticals, pesticides, industrial chemicals, surfactants, and personal care products consistently monitored in groundwater, surface water, municipal wastewater, drinking water, as well the food chain (Ziylan-Yavas et al., 2022; Mohapatra et al., 2021). Pharmaceutical emerging pollutants include endocrine-disrupting drugs, analgesics, antibiotics, hormones, anti-inflammatory, antidiabetic, and antiepileptic drugs, and other pharmaceuticals (Mohapatra et al., 2021). The true threat behind those

pollutants is that their environmental and human toxicological effects have not yet been fully revealed, also in many times those compounds cannot be tested for in municipal water. Moreover, the toxicity and chemical attributes of the end products resulting from the treatment of such pollutants in drinking water treatment systems is not determined yet (Rosenfeld & Feng, 2011).

With this respect, APAP and its degradation product hydroquinone (HQ) are considered not only emerging pollutants but also as micropollutants detected in the environment at concentrations ranging from μgL^{-1} to below ngL^{-1} (Kim & Zoh, 2016). Determination of the concentration of such contaminants in drinking water is problematic largely due to the limits of quantification and detection. Thus, to check pharmaceutical levels in drinking water, sophisticated analytical techniques should be applied (Wiest et al., 2018). Also, it is worth mentioning that up to now and for most of the organic micropollutants including APAP, there are no release guidelines or monitoring recommendations. Furthermore, the interactions among pharmaceutical contaminants in the environment are entirely overlooked (Luo et al., 2014). Kosma et al. (2010) reported 11 pharmaceuticals including APAP and ibuprofen in the municipal and hospital wastewater treatment plants of Ioannina City, located in Western Greece. Later, Ashfaq et al. (2017a) reported APAP as the contaminant showing the highest concentration among the 11 formulated drugs considered in the environmental matrices (sludge, solid waste, and soil samples) in the vicinity of pharmaceutical formulation units of Shiekhupura, Lahore, Pakistan.

1.3.Occurrence and fate of pharmaceutical pollutants in water systems

In 2014, a global environmental review of pharmaceuticals, authorized by Germany's environment ministry (IWW, 2014), emphasized that of the 713 pharmaceuticals tested for, 631 were mainly found in lakes and rivers, groundwater, soil, manure and even drinking water. They were detected in 71countries across all continents (Figure 1.1).

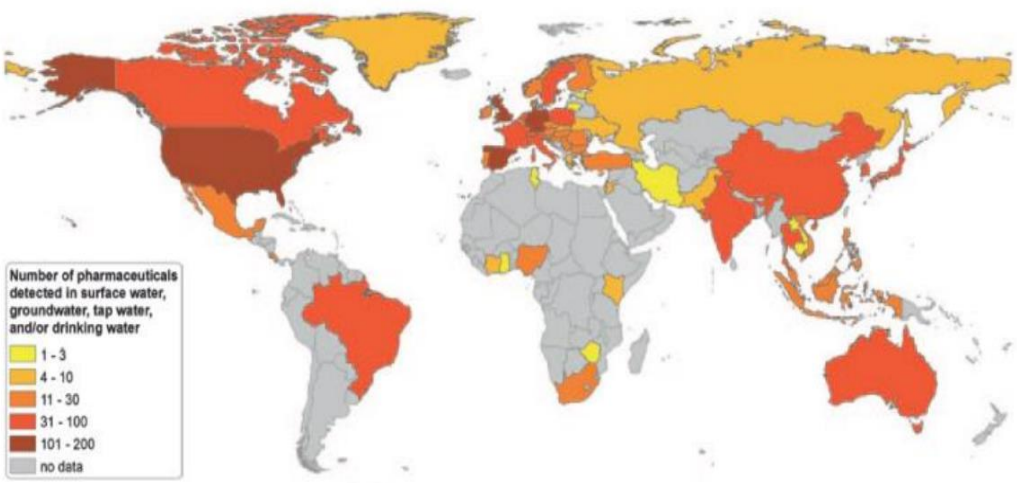


Figure 1.1. Global occurrence of pharmaceuticals in water (IWW, 2014).

Figure 1.2. describes the main processes controlling the migration and fate of pharmaceuticals in water systems including sorption, photodegradation, hydrolysis, and biodegradation. Normally, photodegradation and biodegradation dominate, while hydrolysis affects only some pharmaceuticals, especially antibiotics. Several factors impact the level of each process including pharmaceutical types and structure, sunlight, water depth, organic matter content, water physico-chemical properties, sediment characteristics, and microbial abundance and type; accordingly, pharmaceutical compounds undergo mineralization, partial degradation, or totally resist degradation (Bavumiragira et al., 2022).

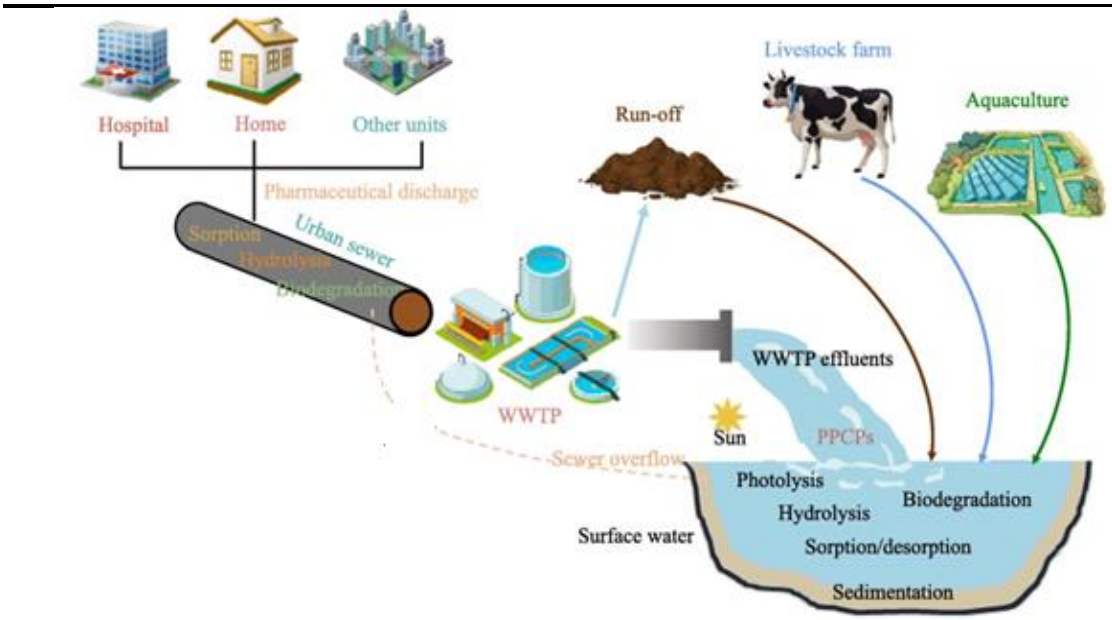


Figure 1.2. Fate and transport of pharmaceuticals in water systems (Bavumiragira et al., 2022).

1.4. APAP and HQ contamination levels, ecotoxicity, and ecological risk assessment

APAP (Figure 1.3) is frequently shortened as *N*-acetyl-*p*-aminophenol (APAP), also recognized as acetaminophen (ACN), and has the IUPAC name *N*-(4-hydroxyphenyl) ethanamide. Other names include acetaminophen, 4-acetaminophenol, 4′-hydroxyacetanilide, and *N*-acetyl-*p*-aminophenol. Currently, it is one of the most broadly used non-prescribed anti-inflammatory drug (Żur et al., 2018; Shabani et al., 2021). Besides the medicinal applications, APAP and its main degradation product, 4-aminophenol, are also partly used in the manufacture of azo dyes and photographic materials (Zhang et al., 2013).

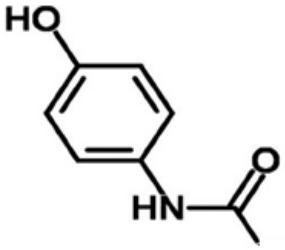


Figure 1.3. The chemical structure of APAP (Żur et al., 2018).

1.4.1. APAP toxicity

Although APAP is generally safe, it is one of the most widespread drugs responsible for intoxication and liver impairment. In the liver, APAP is mostly and rapidly converted by the conjugating enzymes uridine 5′-diphosphoglucuronosyltransferase and sulfotransferases to non-toxic compounds, before excretion by kidneys and biliary. The residual APAP is oxidized by phase I cytochrome P450 isoenzymes to *N*-acetyl-*p*-benzoquinone-imine(NAPQI) detoxified by glutathione (GSH) to form cysteine- and mercapturate- APAP conjugates. In case of APAP overdose, GSH reserves are consumed up; hence, NAPQI accumulates causing covalent modification of thiol groups of proteins, nucleic acids damage, oxidation of membrane lipids, cell necrosis, and death (Pu et al., 2016; Van Wijk et al., 2017).

Based on the former EU Directive 93/67/EEC (Commission of the European Communities, 1996), APAP was categorized as harmful to the aquatic organisms based on EC_{50} concentration between 11 and 100 mgL^{-1}, and as very toxic to the aquatic organisms based on EC_{50} concentration < 1 mgL^{-1} after long-term exposure. The current legal act governing the accountability from public authorities to industry regarding evaluating and managing the risks modeled by chemicals and providing appropriate safety information for their users is 1907/2006 The European Regulation on Registration, Evaluation, Authorization and Restriction of Chemicals – REACH (Regulation (EC) No 1907/2006).

1.4.2. APAP levels in aquatic environments and toxicity

APAP has been disclosed in various environments including surface water at concentrations as high as above 65 μgL^{-1} in the Tyne River, England (Roberts & Thomas, 2006), higher than 78 μgL^{-1} in the Danube River (Grujić et al., 2009), and at almost 5 ngL^{-1} in different surface water (Esterhuizen-Londt et al., 2016), 12-64 μgL^{-1} and 1.57-56.9 μgL^{-1} in wastewater from Korea, Spain, and Western Balkan Region (Bosnia and Herzegovina, Croatia, and Serbia), up to 483 $\mu g\ kg^{-1}$ in sludge and up to 81 $\mu g\ kg^{-1}$ in soil (Luo et al., 2014; Ashfaq et al., 2017a, b). Besides, APAP can easily accumulate in aquatic environments and shows no sorption in aquifer sand investigations (De Gusseme et al., 2011).

APAP and other NSAIDs have been detected in sewage effluents at nanograms per liter levels, and in natural waters at micrograms per liter levels; however, varied negative effects for such

low levels have been reported including reproductive or DNA damage, accumulation in tissues, oxidative stress, lipid peroxidation, and behavioral changes observed in algae, microcrustaceans, mollusks, or teleost fish (Islas-Flores et al., 2013; Gómez-Oliván et al., 2014; Minguez et al., 2016; Islas-Flores et al., 2017).

Oliveira et al. (2015) and Ramos et al. (2014) underlined that the reports available concerning the chronic effects of pharmaceuticals, calculated for environmentally pertinent levels, showed that most of pharmaceuticals at these concentrations are not responsible for lethality. Therefore, assessment of the toxicity based on sub-lethal concentration will be far better than using organisms representing various trophic levels. At present, the most frequently considered biomarkers, used as early markers for even very low concentration of pollutants, are enzymatic markers of different metabolic pathways including oxidative stress or neuronal function. Guiloski et al. (2017) showed the disruption of hypothalamic-pituitary-gonadal axis and severe changes of hematological parameters such as mild blood congestion, leukocyte infiltration, and decrease of hemoglobin and hematocrit in male fish of *Rhamdia quelen* after exposure to environmental levels of APAP in a semi-static bioassay over 21 days. Freitas et al. (2015) studied the impacts of ecologically relevant concentrations of 25 μgL^{-1} of APAP on Polychaete *Diopatra neapolitana* regenerative tissue capacity. The results exposed that APAP considerably reduce the regenerative capacity in a dose-dependent behavior. Du et al. (2016) highlighted substantial time-dependent and concentration-dependent negative effects of APAP with 58.3% mortality of *Daphnia magna*. Likewise, APAP triggered 50% mortality of *D. magna* after 21 days at the concentration of 5.32 ± 0.32 mgL^{-1}.

1.4.3. Toxicity of APAP intermediate metabolites 4-aminophenol and HQ

4-Aminophenol or para-aminophenol (Figure 1.4) is a possible intermediate product of APAP biodegradation and even considered as dead-end metabolite defined according to Mackie et al. (2013) as the metabolite that can be produced but not consumed, or consumed but not produced by an organism, and in both cases has no known transporter. 4-Aminophenol is reported for its considerable nephrotoxicity, mutagenicity, and teratogenicity, as well as DNA cleavage induction in mouse and human lymphoma cells (Guzik et al., 2013a).

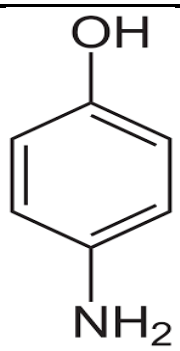


Figure 1.4. The chemical structure of 4-aminophenol (Żur et al., 2018).

HQ (Figure 1.5) is widely spread in the environment due to its extensive utilization in varied activities. For example, it can be used as a developing agent in photography, dye intermediate. Also, it can act as a stabilizer in paints, varnishes oils, and motor fuels. Moreover, HQ has been used as an antioxidant in the rubber and food industry. Since 1950s and for five decades, HQ was used in cosmetic skin lightening products in European Union countries and in 1960s it became commercially presented as a medical product. HQ is also involved in nails and hair dyes (O'Donoghue, 2006). Parallel, HQ is used as a component of high molecular aromatic compounds such as resin, an intermediate, or appear as a degradation product generated by transformation of aromatic compounds including APAP. HQ results normally from the advanced oxidation of several aromatic compounds especially phenol whose early stages of its degradation results in HQ and *ρ*-benzoquinone which are proven to be more toxic and less degradable than phenol (Santos et al., 2004).

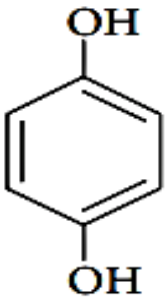


Figure 1.5. Chemical structure of HQ (Enguita & Leitão, 2013).

Although HQ gives a negative response in the standard bacterial gene mutations studies, such as Ames test using *Salmonella typhimurium* strain TA 98, TA 100, TA 1535, and TA 1537 (Shiga et al., 2010); however, in yeast cells it increased homologous recombination (Sommers & Schiestl, 2006). Concerning its effect on soil microbial growth, HQ is the most toxic dihydroxybenzene (Chen et al., 2009).

HQ, as a highly redox-active chemical, is known for its extreme toxicity to aquatic organisms including *Pimephales promelas*, *Brachydanio rerio*, *Daphnia magna*, *Desmodesmus armatus*, *Synechocystis* sp., *Nostoc* sp., and *Microcystis aeruginosa* (OECD SIDS, 2012; Bahrs et al., 2013). A 48 h EC_{50} value of 0.15 mgL^{-1} was reported for the marine *Daphnia magna*, while 24 h LC_{50} values ranging from 0.22 to 0.28 mgL^{-1} were reported for *Brachionus plicatilis* (Guerra, 2001). *Photobacterium phosphoreum* focused studies inferred that HQ toxicity is one hundred and one thousand times higher than catechol and resorcinol toxicity, respectively (Kaiser & Palabrica, 1991).

As postulated by some studies, the toxicity of HQ may have been undervalued considering the positive data from a limited number of experimental animals and clinical trials. HQ is reported for induction of mononuclear cell leukemia, renal tubular cell tumors, and liver cancer in rodents (Kari et al., 1992). Tsutsui and colleagues tried to prove that HQ has genotoxic effect on mammalian cells in culture, which showed higher frequencies of DNA gaps, breaks, and sister chromatid exchanges in addition to chromosome aberrations (Tsutsui et al., 1997). Moreover, in mice based in vivo trials, HQ was proven for inducing allergic immune response, as well as impaired innate immune response against bacteria (Ribeiro et al., 2011; Lee et al., 2002). Even in cultured human cells, DNA strand breaks were induced by HQ in the presence of copper (II) ions, and assumingly attributed to peroxide production (Hiraku & Kawanishi, 1996).

1.5. Assessment of the different pharmaceutical pollutants and APAP elimination strategies

1.5.1. APAP elimination strategies and studies

Since the capacity of wastewater treatment plants (WWTPs) is gradually decreasing, due to the buildup of increasing levels of pollutants, including APAP and their metabolites, which accordingly accumulate in the environment. Hence, novel removal technologies in physical,

chemical, biological, and nanotechnology-based have been developed (Wang & Wang, 2016). However, the conventional method of chlorination as well as the advanced methods including membrane bioreactors, nanofiltration, reverse osmosis, and carbon nanocomposites with magnetic properties are not sufficiently tailored for pharmaceutical removal (Kim et al., 2018; Tiwari et al., 2017; Żur et al., 2018). Both current and advanced methods mostly convert APAP to its metabolites which are even more toxic (Chopra & Kumar, 2020).

Various approaches have been applied for removal of the pharmaceutical pollutant APAP including activated sludge, activated sludge coupled with disinfection, sand filtration, or trickling filter, membrane bioreactor, membrane bioreactor coupled with ultrafiltration modified Bardenpho process, powdered activated carbon, graphene, ozone oxidation, and Fenton oxidation (Snyder et al., 2007; Kasprzyk-Hordern et al., 2009; Kosma et al., 2010; Kovalova et al., 2012; Nielsen et al., 2013; Al Qarni et al., 2016; Rajbongshi et al. 2016; Yang & Tang, 2016; Wang & Wang, 2016).

Bioremediation can be considered as a collective strategy involving utilization of biological systems, mainly microbes and plants, fungi, and enzymes to clean-up contaminated spots through oxidization, immobilization, or transformation of the contaminants. Its main objective is decreasing pollution levels to undetectable, nontoxic, or acceptable (i.e., within limits set by regulatory agencies) levels (Vishwakarma, 2020). Microbial biodegradation of pollutants as an eco-friendly bioremediation is mostly considered as an effective, sustainable, cost-effective approach for the removal of APAP and other micropollutants compared with physical and chemical approaches like UV oxidation, fixation, and solvent extraction which may be associated with toxic by-products, complexity, safety and regulatory issues, inefficiency, and high cost (Mohapatra & Phale, 2021).

To boost the effectiveness of degradation, three strategies have been suggested (Figure 1.6a): natural attenuation or bioattenuation (using the ability of the intrinsic microbial community to degrade the contaminant), bioaugmentation (the addition of living cells capable of degradation or plasmid-mediated bioaugmentation) and biostimulation (the addition of limiting nutrients to support microbial growth) (Kang et al., 2009). Biostimulation also involves manipulating the environmental conditions for bioremediation through the addition of electron acceptors as oxygen, nitrogen, carbon, and phosphorous, as well as controlling pH and temperature (Adams et al., 2015). The goal to achieve through biodegradation is the complete conversion of

pollutants into CO_2 and H_2O, and it has been proven possible for some cases can be achieved (Chen et al., 2010; Hasan et al., 2011).

Even after considering the advantages of bioremediation as the most promising environmental remediation approach, it is characterized by some possible disadvantages, including the possibility of destruction of bioremediation additives by indigenous microbiota, low susceptibility of some contaminants to biodegradation, the potential hazards of genetically engineered microorganisms at the end of bioremediation, production of intermediate metabolites of high or undetermined toxicity or persistence, challenges of applying field operations, and presence of co-pollutants may hinder the biodegradation course. Moreover, is the biodegrading microbe's requirement of specific conditions such as optimal temperature, pH, additional nutrients, and convenient contaminants levels to stimulate the biodegradation enzymes. Also, such microorganisms usually must be adapted to increasing concentrations of contaminations; thus, expanding the bioremediation time (Kumar et al., 2011; Singh & Kumar, 2016). Hence, there is a continuous need for more investigation and development in this field.

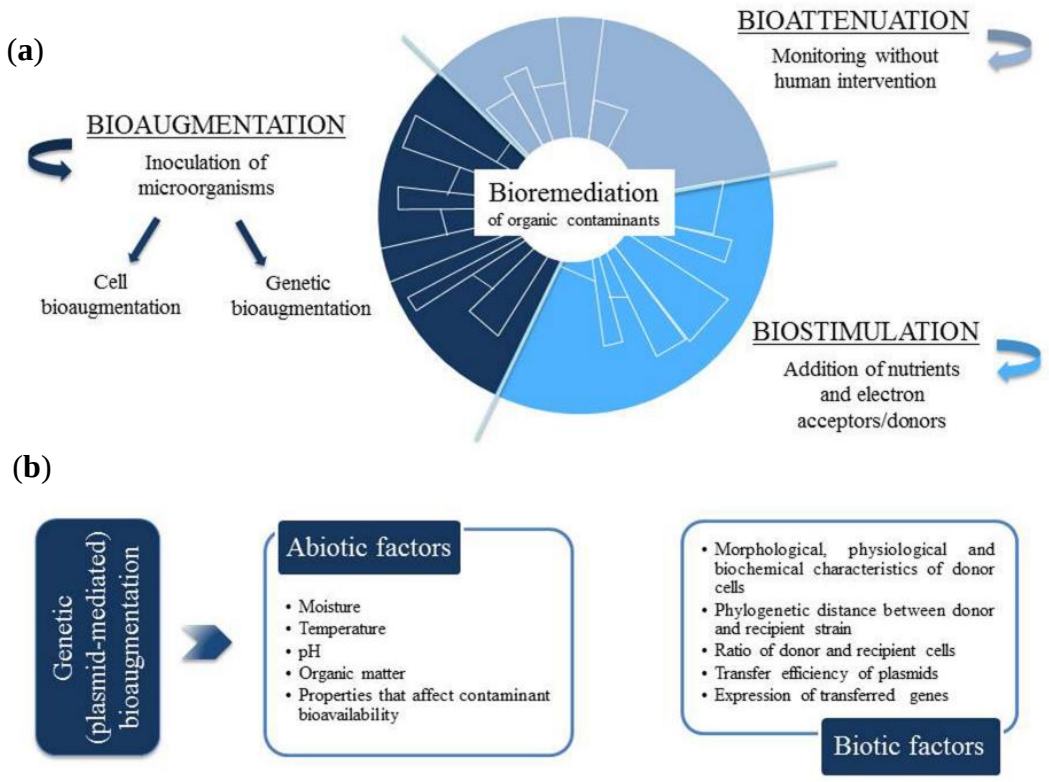


Figure 1.6. (a) Approaches for bioremediation of organic contaminants, (b) factors regulating the effectiveness of plasmid-mediated bioaugmentation (Garbisu et al., 2017).

1.5.2. Plasmid-mediated bioaugmentation as a future alternative

For the studies on bacterial horizontal gene transfer (HGT), many times they were centered on antibiotic and metal resistance, while insufficient reports addressed the horizontal transfer of genes responsible for biodegradation of organic pollutants in natural environments (Christensen et al., 1998; Top et al., 1998; Dejonghe et al., 2000; Aspray et al., 2005; Overhage et al., 2005; Musovic et al., 2010).

In the technology of plasmid-mediated bioaugmentation, donor bacteria having self-transmissible catabolic plasmids are introduced into the contaminated matrix to stimulate, through HGT, contaminant degradation by the indigenous bacterial community (Top et al., 2002; Ikuma & Gunsch, (2010, 2012)). Plasmid-mediated bioaugmentation seems superior to cell bioaugmentation as bacteria, assumed to biodegrade the pollutants after acquiring the plasmids of the degradation genes, are expected to be already adapted to survival in the

contaminated site; hence, one of the principal weaknesses for the effective application of cell bioaugmentation which is the low survival of the inoculated microbial cells, can be overcome. The success of the plasmid-mediated bioaugmentation requires a proper selection of the plasmid donating bacteria and deep knowledge of the indigenous bacterial populations to enhance the possibility of acquisition of the plasmid by the native bacteria and the expression of the target genes, in addition to other biotic and abiotic factors (Figure 1.6b). Accordingly, plasmid-mediated bioaugmentation in its best helps increasing the number and diversity of native bacteria able to metabolize the contaminants. Plentiful pathways involved in the degradation of organic contaminants have been discovered in mobile genetic elements (Top et al., 2002; Jussila et al., 2007).

Several reports have discussed plasmid-mediated bioaugmentation including laboratory and field studies. In 2015, Gao et al. conducted an effective plasmid-mediated bioaugmentation in a dichlorodiphenyltrichloroethane (DDT) contaminated soil located in Cixi, Zhejiang (China), using *E. coli* TG I (pDOD-gfp) as donor strain. Parallel, the catabolic plasmid pDOD from *Sphingobacterium* sp. D-6 was conjugally transferred to soil bacteria including representatives of *Cellulomonas*, and accelerated DDT degradation. Besides, varied studies have reported the use of the GFP (green fluorescence protein) monitorization system to monitor plasmid transfer from donor cells to native soil bacteria in soil slurries (Ikuma et al., 2012) as well as field contaminated soil (Gao et al., 2015).

1.6. Strategies for APAP biodegradation studies

There are studies focused on the taxonomic characterization of microbial populations existing in consortia, enriched selectively from environmental samples in the presence of the APAP, capable of degrading the drug and/or its intermediate metabolites. Other studies are focused on the isolation of strains capable of degrading APAP, as well as studies evaluating the combination in co-cultures of a certain number of pre-selected isolates (i.e., artificial consortia). The knowledge of the dynamics of microbial populations in the consortia, while the biodegradation of chemicals occurs allows the development of strategies to attempt optimizing the operating conditions of bioreactors to try to favor certain taxonomic groups to increase the effectiveness of water treatment. On the other hand, the isolation of strains allows studying the molecular mechanisms of biodegradation of chemicals and their genetic bases, opening the

way for the development of biotechnological strategies also to increase the efficiency of bioreactors.

1.6.1. APAP biodegradation studies using enriched microbial consortia

Numerous recent authors emphasized that the biodegradation of contaminants by microbial consortia, where the degradation process occurs through the combined action of various species, is more successful compared with using pure bacterial cultures due to metabolic diversity (Villaverde et al., 2017). In this regard, APAP degradation by bacterial consortia, enriched selectively from environmental samples, has been considered in several reports. For example, Palma et al. (2018) reported APAP biodegradation by sulfate reducing consortium of *Flavobacterium*, *Dokdonella* and *Methylophilus* enriched under anaerobic conditions for seven days in Postgate B medium from the sludge sample collected in the anaerobic lagoon system of Faro East wastewater treatment plant. In addition, Yang et al. (2020) studied both aerobic and anaerobic biodegradation of APAP in mangrove sediments and reported that the addition of $NaNO_3$, Na_2SO_4 and $NaHCO_3$ increased APAP degradation, with $NaNO_3$ giving the best results. Sixteen microbial genera were recognized as the main contributors of microbial communities associated in anaerobic APAP degradation in Mangrove sediments with addition of $NaNO_3$ and Na_2SO_4. Three (*Arthrobacter*, *Enterobacter,* and *Bacillus*) of the 16 microbial genera were identified in the isolated APAP degrading bacterial strains. Also, De Gusseme et al. (2011) demonstrated APAP complete removal after 16 days by a microbial consortium in a membrane bioreactor was at a relatively high concentration of 100 μgL^{-1} as well as an environmentally relevant concentration of 8.3 μgL^{-1}.

1.6.2. APAP biodegradation studies using isolates

A varied panel of bacterial strains have been disclosed during the last two decades for their ability to biodegrade APAP through using it as a sole carbon source, including members of *Pseudomonas*, *Stenotrophomonas*, *Burkholderia*, *Delftia*, *Rhodococcus* (Żur et al., 2018). Similarly, *Staphylococcus* and *Bacillus* strains were reported (Chopra & Kumar, 2020). For fungi, the study of Edrees et al. (2017) described fungal isolates of *Aspergillus niger* and *Fusarium oxysporium* as APAP degrading strains. Also, *Brevibacterium*

frigoritolerans, Corynebacterium nuruki, *Enterococcus faecium,* and *B. cereus* have been revealed by Palma et al. (2021) for APAP degradation.

- **Using pure cultures**

Pseudomonas moorei KB4 strain was proven for APAP biodegradation at concentrations of 50 mgL^{-1} (Żur et al., 2018). Hu et al. **(**2013**)** reported APAP degradation by *Pseudomonas aeruginosa* strain with the capacity to use APAP as sole carbon and energy source and degrade it at concentrations up to 2.2 gL^{-1} in mineral salt medium (MSM). Similarly, in De Gusseme et al. (2011) report, *Delftia tsuruhatensis* isolated from the membrane bioreactor showed APAP biodegradation (From 10.325 ± 0.027 to 0.263 ± 0.034 mgL^{-1}) in MSM after 48 h. Later, *Br. Frigoritolerans, C. nuruki, E. faecium,* and *B. cereus* have been revealed by Palma et al. (2021) for APAP degradation with removal of 97±4%, 97±6%, and 86.9±0.8% respectively from liquid MSM supplemented with 200 mgL^{-1} of APAP at 28 °C in the dark.

- **Using co-cultures**

Recent reports on APAP biodegradation included not only the utilization of pure cultures but also the co-culture (mixed culture) strategy as for, Zhang et al. (2013) reported APAP degradation by pure culture as well as a co-culture of *Stenotrophomonas* and two *Pseudomonas* strains isolated from membrane bioreactor, where the co-culture showed a more efficient APAP removal of 4 gL^{-1} from MSM whilst pure cultures removed APAP completely at a concentration of 0.4, 2.5, and 2 gL^{-1}, respectively. The degradation and mineralization potential of the consortium was confirmed by oxygen consumption rate as well as GC-MS analysis of intermediate metabolites. Chopra & Kumar (2020) reported *Staphylococcus sciuri* strain DPP1, *B. subtilis* strain DPP3, *B. paralicheniformis* strain DKP1, *E. faecium* strain DKP2 and DDP2 as APAP degrading strains able to use it as the sole carbon source. Interestingly, the co-culture of the five strains has removed 1200 mgL^{-1} of APAP from MSM within 70 h, while individual strains took 10 days. The intermediate products such as 4-aminophenol, benzamide, I-2-methylpentanoic acid, methylene-3-vinyl cyclohexane, and 1,5-hexadiene were detected by GC–MS.

Interestingly the recent report of, Shabani et al. (2021) discussed the bioremediation of APAP and 4-aminophenol, in a dual-chamber fungal microbial fuel cell (FMFC), using the pure

culture of fungus *Trichoderma harzianum* and mixed culture of bacteria and fungi (*T. harzianum* and *Pseudomonas fluorescens*) separately. The biodegradation products were detected electrochemically. These experiments revealed that the biodegradation rate in 0.1 M phosphate buffer saline (pH 7.2) supplemented with 300 mgL^{-1} APAP was enhanced in bacterial-fungal biofilm about five times (7 h) higher than in the pure culture of fungus.

1.6.3. APAP intermediate metabolites biodegrading strains

Compared with the number of APAP biodegrading strains, a fewer number of microbial strains have been proven for their ability to biodegrade 4-aminophenol. Khan et al. (2006) reported 4-aminophenol biodegradation was induced by *Pseudomonas* sp. ST-4 strain by growing it in the presence of 4-aminophenol in the co-metabolic culture with glucose at concentrations up to 400 mgL^{-1}; hence, the induced cultures were able to remove 50 mgL^{-1} of 4-aminophenol. Later in 2009, Khan et al. demonstrated maximal removal of 4-aminophenol by *Pseudomonas* sp. strain ST4, at pH 8, temperature 30°C and glucose concentration of 15 mM after 72 h.

Also, compared with APAP, fewer studies were directed to bacterial HQ biodegradation especially-anerobic-bacteria. *Moraxella*, *Pseudomonas*, *Sphingomonas*, *Burkholderia*, *Variovorax*, *Azospirillum*, *Brachymonas*, and *Cupriavidus* have been described for their ability to utilize HQ which may be a product of not only APAP but also other phenolic compounds such as 4-chlorophenol, 4-fluoro-, 4-bromo-, 4-iodo-, and 4-nitrophenol degradation (Enguita & Leitão, 2013).

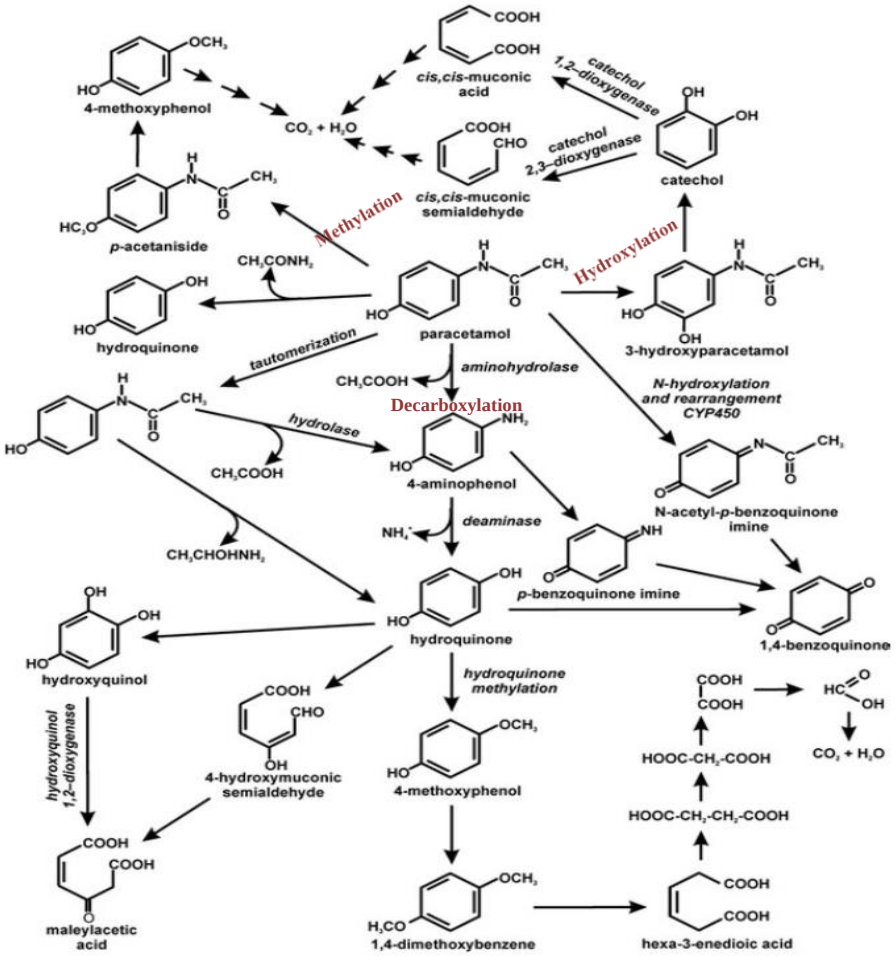
In the study of Zhao et al. (2017), hydroquinone was removed most effectively (80% – 90% in a single cycle) in both phenol enriched and non-phenol enriched acetate-fed granules bioreactors at HQ concentration up to 100 mgL^{-1} after a single aerobic/anaerobic cycle of 3 h. HQ biodegradation had been reported in early studies by several fungi including *Cryprtococcus* sp., *Trichosporon* sp., *Candida* sp., and *Exophiala jeanselmei* (Middelhoven, 1993). The dilemma of the production of the toxic metabolic intermediates like 4-aminophenol and HQ can be surpassed through utilize the mixed biofilm of fungi and bacteria (Shervington & Sakhnini, 2000); hence, allow mineralization of APAP as was suggested by Shabani et al. (2021).

1.7. The biodegradation pathways of APAP

Up to the present time, the biodegradation pathways of APAP biodegradation are not sufficiently described. 4-Aminophenol and HQ are known as the two key metabolites produced during APAP biodegradation as confirmed by the studies of Hu et al. (2013) and Zhang et al. (2013), and as will be explained.

Two graphical summaries of the most reported pathways for APAP degradation, summarized by Żur et al. (2018) and Guzik & Wojcieszyńska (2019) are shown in Figure 1.7. They agree mainly about the hydroxylation, methylation, and decarboxylation pathways; however, Guzik & Wojcieszyńska (2019) also highlighted the formation of HQ and the tautomerization pathways of APAP biodegradation, while Żur et al. (2018) clarified the catechol pathways as well.

As early as 2006, Ivshina et al. identified 4-aminophenol, HQ, and pyrocatechol, and referred to them as the main products of APAP biodegradation in *Rhodococcus* sp. Such Oxidative deamination of APAP was affirmed through the reports of De Gusseme et al. (2011), Wu et al. (2012), and Zhang et al. (2013) as well as Hu et al. **(**2013**)** who reported APAP degradation by *P. aeruginosa* strain exhibiting the ability to degrade APAP at concentrations up to 2.2 gL^{-1} and utilize it as the sole carbon and energy source. In these cases, eight intermediates including 4-aminophenol, HQ, formic acid, lactic acid, oxalic acid, succinic acid, nitrate, and nitrite have been detected. Accordingly, the authors suggested two degradation pathways in this strain: (1) the initial hydroxylation of APAP resulting in HQ, acetamide, and ring opening and (2) the initial decarboxylation to 4-aminophenol, in which amino group is then replaced by the hydroxyl group; thus, producing HQ. The similar catabolic degradation pathways were suggested by Zhang et al. (2013).


4-methoxyphenol
cis,cis-muconic acid
catechol 1,2-dioxygenase
catechol
catechol 2,3-dioxygenase
cis,cis-muconic semialdehyde
p-acetaniside
Methylation
Hydroxylation
hydroquinone
paracetamol
3-hydroxyparacetamol
tautomerization
aminohydrolase
Decarboxylation
N-hydroxylation and rearrangement CYP450
hydrolase
4-aminophenol
N-acetyl-p-benzoquinone imine
deaminase
p-benzoquinone imine
1,4-benzoquinone
hydroxyquinol
hydroquinone methylation
hydroxyquinol 1,2-dioxygenase
4-hydroxymuconic semialdehyde
4-methoxyphenol
maleylacetic acid
1,4-dimethoxybenzene
hexa-3-enedioic acid

(a)

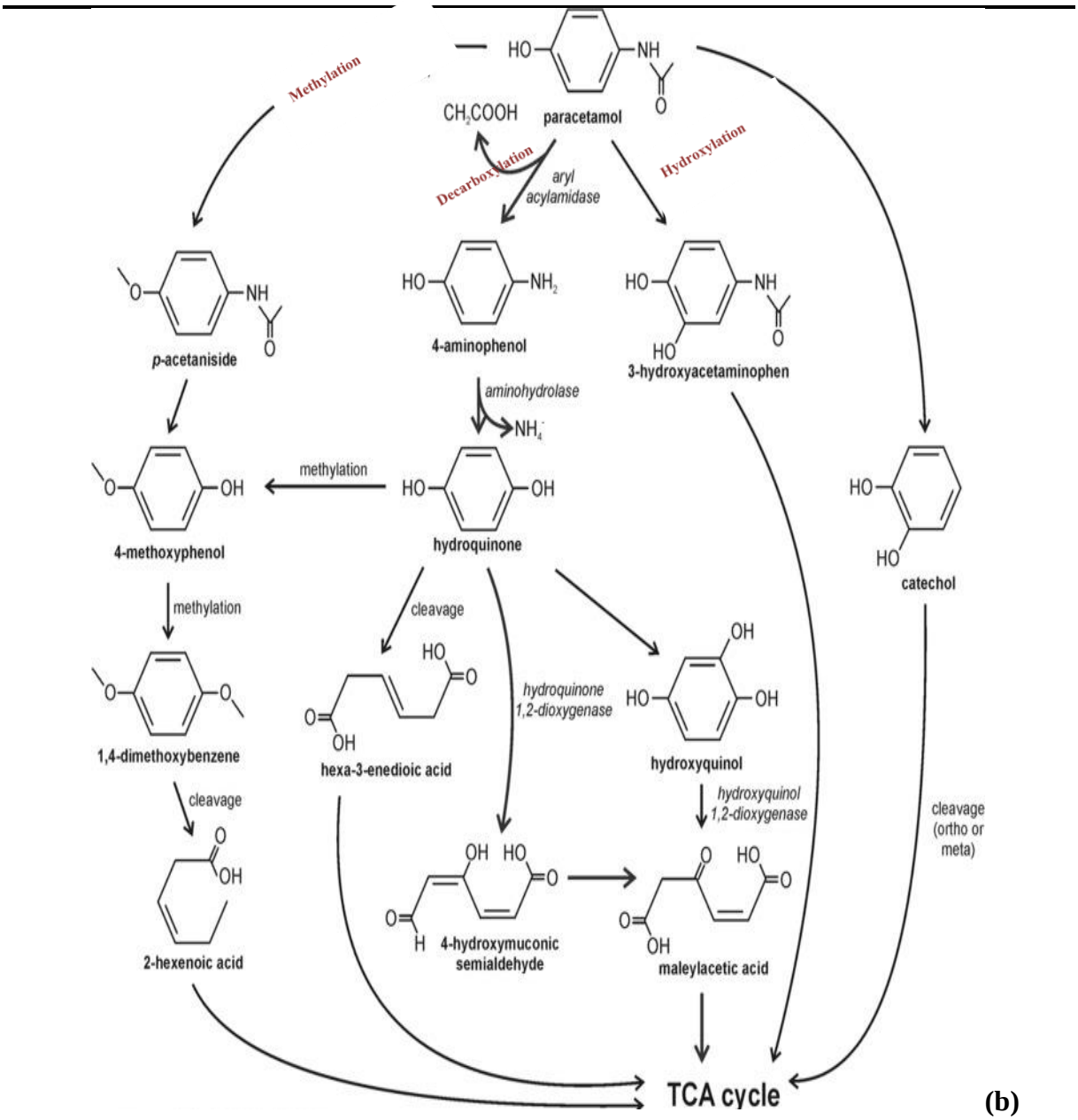
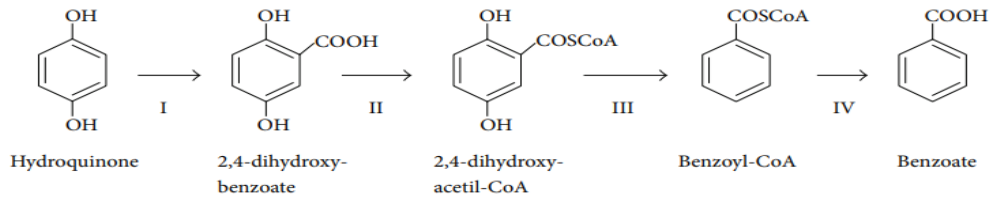


Figure 1.7. Summarization of the most common biodegradation pathways of APAP described in literature, (a) adapted from (Guzik & Wojcieszyńska, 2019); (b) TAC: tricarboxylic acid, adapted from Żur et al. (2018).

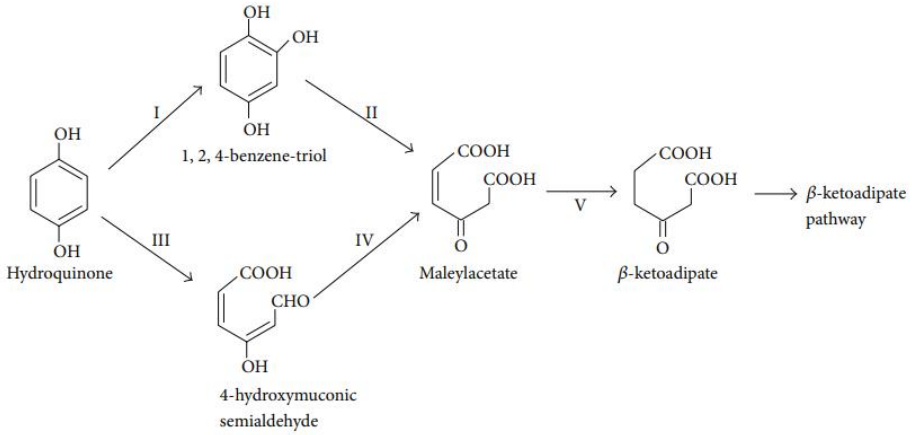
Like APAP, 4-aminophenol the first hydrolytic product is hardly biodegradable and can possibly inhibit degradation metabolic pathway; thus, retard or completely stop the bioremediation. Additionally, for APAP, co-contaminants occurring in the polluted environments such as 4-hydroxybenzoate or 4-chlorophenol can possibly compete with 4-aminophenol resulting from degradation of APAP, because the same enzymes are involved in degradation of *para*-substituents (Guzik et al., 2013b).

For HQ anaerobic biodegradation (Figure 1.8a), first occurs the carboxylation into gentisate (2,5- dihydroxybenzoate) through the action of an uncharacterized carboxylase induced by hydrocarbon as a sole carbon and energy source (Gorny & B. Schink, 1994a, b). Subsequently and as normally occurring in anaerobic biodegradation of phenolic compounds, the classical step of CoA group addition to gentisate by CoA-ligase using Acyl-CoA as a donor follows (Glockler et al., 1989). Later, the prominent step of reductive decarboxylation of gentisyl-CoA, by an oxygen sensitive enzyme which removes the two hydroxyl groups in one step, follows producing finally benzoate which in turn is biodegraded through the anaerobic benzoate pathway to be finally degraded through the beta-oxidation (Gorny & Schink, 1994b; Valderrama et al., 2012). More intense research should be directed to expose such enzyme activity. The anaerobic biodegradation of HQ in nature was described through a few reports which highlighted sulfate-reducing bacteria from the genus *Desulfococcus* (Gorny & Schink, 1994a, b) and dehalogenating bacteria isolated from soil consortia together with filamentous fungi (Milliken et al., 2004a, b) as anaerobic biodegraders of HQ. The difficulty of anaerobic HQ biodegradation is due to the of two hydroxyl groups in para position in the benzene ring which makes it unlikely that any microorganism can conduct oxidative ring fission anaerobically.

Aerobic degradation of HQ (Figure 1.8b) is possible either through cleavage of the benzene ring by HQ 1,2-dioxygenase to 4-hydroxymuconic semi-aldehyde as was suggested for *P. moorei* KB4 strain (Żur et al., 2018), or transformation into 1,2,4-benzenetriol (hydroxyquinol) cleaved by hydroxyquinol 1,2-dioxygenase to maleylacetic acid as was suggested by Takenaka et al. (2003) for the 4-aminophenol degrading *Burkholderia* sp. AK-5 strain, with the degradation pathway involving1,2,4-trihydroxybenzene, 1,4-benzenediol, and maleylacetic acid.



(a)



(b)

Figure 1.8. Pathways for the biodegradation of HQ. (a) Anaerobic pathway: I: HQ carboxylase; II: HQ Acetyl-CoA transferase; III: benzoyl-CoA oxidoreductase; IV: benzoyl-CoA hydrolase, (b) two possible aerobic pathways. I: HQ hydroxylase; II: 1,2,4-trihydroxybenzene 1,2-dioxygenase; III: HQ dioxygenase; IV: 4-hydroxymuconic semialdehyde dehydrogenase; V: betaketoadipate oxidoreductase (Enguita & Leitão, 2013).

1.8. Genetic basis of bacterial degradation of APAP and its main intermediate metabolites 4-aminophenol and HQ

Despite the studies described above on the mechanisms of microbial degradation of APAP the genetic bases of APAP biodegradation are not sufficiently revealed. Because it was for long known that aryl acylamidase can hydrolyze the amide bond between aryl and acyl groups, Ko et al. (2010) studied the action of an aryl acylamidase identified in soil bacterium isolated using media with APAP as a sole carbon source. They overexpressed its gene in *Escherichia coli* and examined the enzymatic characteristics and kinetic parameters of the enzyme on various substrates (including APAP), suggesting potential biotechnological applications. Moreover,

recently Rios-Miguel et al. (2021) isolated from one APAP degrading bioreactor two *Pseudomonas* strains which in presence of APAP highly expressed two distinct amidase-like proteins. However, these amidase genes were not detected in the bioreactor metagenome, and a metataxonomic study on the bioreactor suggested that other uncharacterized amidases could be responsible for the first biodegradation step of APAP.

Over the last decades, studies focused on revealing the genetic foundation of bacterial degradation of aromatic compounds were mainly directed to aromatic hydrocarbons. Several important plasmids like TOL (Williams & Murray, 1974; Wong & Dunn, 1974; Williams & Worsey, 1976), NAH (Dunn & Gunsalus, 1973), or SAL (Chakrabarty, 1972) have been discovered with this respect. Moreover, a large conjugative 138-kb SAL-TOL recombinant plasmid, pKF439 was found on a *P. putida* strain (Furukawa et al., 1985). Additionally, in the study of Park & Kim (2000), which discussed nitrobenzene degradation by *P. putida* HS12, it was shown that the genes *nbzC*, *nbzD*, and *nbzE*, involved in the ring cleavage pathway of 2-aminophenol, which is a structural isomer of 4-aminophenol, as an intermediate metabolite, are localized on plasmid pNB1 in *E. coli*.

Returning to the specific case of APAP and its metabolites, according to Hu et al. (2014) and Chen et al. 2016a, b**,** microbial genes responsible for the degradation of HQ were revealed through 4-nitrophenol degradation by *P. putida* DLL-E4. Nine genes were proven to be involved in the degradation of 4-nitrophenol, specifically *pnpA*, *pnpR*, and *pnpC1C2DECX1X2*, while the four genes *pnpC1C2DE* were shown to be responsible for conversion of HQ to tricarboxylic acid cycle metabolites. Moreover, *pnpC* encode hydroxyquinol 1,2-dioxygenase which converts hydroxyquinol to maleylacetic acid, subsequently reduced to beta-ketoadipate by maleylacetic acid reductase encoded by *pnpE*. Regulation of 4-nitrophenol degradation genes by DLL-E4 is complicated. Glucose significantly enhances 4-nitrophenol degradation; however, inhibits HQ degradation. *pnpC1C2DECX1X2* operon is positively regulated by *pnpR* gene, which positively regulates its own expression and partially the expression of *pnpA* encoding 4-nitrophenol 4-monooxygenase.

Also, to better understand the genetic foundation of HQ degradation, comparative genomics was adopted by searching in NCBI database using BLAST on default settings with *E*-value cut-off of 1×10^{-5}. It was elucidated that the genes order is highly conserved, and even two

distinct gene clusters were detected based on *pnpC* which was present only in some but not all *Burkholderia* strains, while other strains of *Burkholderia*, *Pseudomonas*, and *Cupriavidus* have *pnpE* allowing hydroxyquinol degradation.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Microbial consortia

The bioprospection of bacterial isolates in this work was based on the five bacterial consortia LA, LC, PB, PDE, and PF previously obtained within the scope of a previous work (PROBIOMA project: European Regional Development Fund ER–F - Interreg V-A Spain-Portugal program (POCTEP)). The consortia were stored at -80ºC in Luria Bertani (LB) medium with 20% (v/v) glycerol at the Laboratory of Environmental Technologies of the Centre of Marine Sciences of the Algarve. LA and LC microbial consortia were isolated from Lousal mine, while PB, PF, and PDE microbial consortia were isolated from Poderosa mine.

2.1.2. Microbiological media

- **Enrichment medium composition**

The mineral salt medium (MSM), described by Zhang et al. (2013) and characterized by being free from any carbon source, was adopted for selective enrichment of APAP and HQ degrading bacterial isolates, with the modification of the addition of ammonium sulfate as nitrogen source. The modified MSM composition per liter of deionized water is as follows: 0.5 g of KH_2PO_4, 0.5 g of K_2HPO_4, 0.01 g of NaCl, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.02 g of $CaCl_2$, 0.339 mg of $MnSO_4$, 0.428 mg of $ZnSO_4$, 0.347 mg of $(NH_4)6Mo_7O_{24} \cdot 4H_2O$, 670 mg of $(NH_4)_2SO_4$, 0.4 mg of $CoCl_2 \cdot 6H_2O$, and 10 mg of EDTA.

- **Medium for bacterial growth**

LB broth medium (Oxoid, UK) was used for growing the seed cultures. LB agar was used for checking the growth and purity of cultures.

- **Cryopreservation medium**

LB broth supplemented with 20% glycerol (85%, Analar, CE) (v/v) was used for long term storage of the study isolates and consortia at -80 ºC.

2.1.3. Pharmaceutical reagents and intermediate metabolites

The study pharmaceuticals and metabolites were purchased from Sigma-Aldrich USA, Inc, and included APAP (BioXtra ≥ 99.0%, Sigma-Aldrich, USA); HQ (ReagentPlus®≥ 99%, Sigma-

Aldrich, China); 4-aminophenol ((≥ 98%, Sigma-Aldrich, Italy); and succinic acid certified reference material (Sigma-Aldrich, Sweden).

2.1.4. Stock solutions, reagents, buffers, mixtures, and kits

- **For pH adjustment of microbiological media before autoclaving**

- 100 mL of HCl (5 M) solution was prepared by adding 43.6 mL of HCl to 56.4 mL of deionized water.
- 100 mL of NaOH solutions (5 M) were prepared by dissolving 20 g of NaOH in 100 mL of deionized water.

- **For construction of drug standard curve**

For UV absorbance and HPLC analyses, six concentrations (1, 10, 20, 30, 40, and 50 mgL^{-1}) were prepared separately, as 25 mL aliquots in volumetric flasks, for APAP, 4-aminophenol, and HQ, using MSM as a solvent. For GC-MS analysis, a standard solution of a mixture of HQ, 4-aminophenol, and succinic acid (1, 2, 4, 6, 8, and 10 mgL^{-1}) using MSM as a solvent, were prepared. Standard solutions were stored at 2-8°C.

- **For HPLC analysis**

For this purpose, the HPLC grade chemicals listed as shown below were used:

- KH_2PO_4 (AnalaR Normapur, EC)
- $K_2HPO_4 \cdot 3H_2O$ (VWR Chemicals, Germany)
- Methanol (Sigma Aldrich, U.S.A)
- Phosphoric acid (Sigma Aldrich, U.S.A)

- **For GC-MS analysis**

- All reagents were purchased from Sigma-Aldrich, Germany. The silylation reaction mixture was prepared by mixing 45 µL of MSTFA with 5 µL of a freshly prepared solution consisting of 10 mg ammonium iodide, 250 µL MSTFA, and 15 µL β-mercaptoethanol.
- Absolute ethanol for sample dilution (Thermofisher, U.K)

- ***For genomic DNA extraction***

NZY microbial gDNA Isolation Kit (NZYTech, Lisbon, Portugal) was used for this purpose.

- **For 16S rRNA gene amplification**

- Forward primer (8F (also known as fD1): 5′- AGA GTT TGATCC TGG CTC AG -3′) (Weisburg et al., 1991)

- Reverse primer (1492R: 5′- GGT TAC CTT GTTACG ACT T-3′) (Lane, 1991)
- Ultrapure water obtained using Milli-Q® IQ Water Purification System (Merck, Germany)
- Supreme NZYTaq 2× Green Master Mix (Nzytech, Portugal)

➢ **For gel electrophoresis**

- 1% Agarose gel: 1 g agarose (Cambrex, Denmark) dissolved in 100 mL 1x TAE buffer followed by heating and stirring (Heidolph, Germany) till complete dissolution and gelling.
- 50x TAE buffer (AMRESCO, Solon, USA): 2 M Tris, 1 M Acetate, and 0.05 M Na_2EDTA (VWR Chemicals, EC), pH 8.3.
- SYBER Safe Premium (Invitrogen, USA)
- 100 bp NZYDNA Ladder VII (NZYTech, Portugal) (170 ng μL^{-1})

2.2. Methodology

2.2.1. Analytical methods for detection/monitorization of APAP, 4-aminophenol and HQ

The detection of APAP, 4-aminophenol, and HQ in MSM was tested using three different analytical methods (UV-vis molecular spectroscopy, HPLC, and GC-MS methods) aiming to select the detection strategy to follow for realizing the following specific objectives: screening of the enrichment cultures of bacterial consortia, studying the removal capacity of selected isolates, and detection of 4-aminophenol, and HQ the intermediate metabolic products of APAP biodegradation.

For the three implemented methods, the verification of some method validation parameters according to the instruments used in the current study included R^2, sensitivity, as well as LOD, and LOQ, measurement range, linearity range was conducted as guided by Konieczka & Namiesnik (2009). Sensitivity was expressed in terms of the slope of the calibration curve of each analyte, while measurement range was calculated as the interval between the LOQ and the highest standard concentration used in the calibration curve. For LOD and LOQ calculations, three replicates of the lowest three concentrations of the calibration curve were subjected to regression analysis (using MS-Excel 2010) for UV-vis spectrometry and HPLC methods, while for the GC-MS method two replicates were used. LOD was calculated as the average of $LOD_{residual}$ and $LOD_{intercept}$ calculated as shown in the following formulae:

$$LOD_{residual} = 3.3 \times SD_{residual} / b$$

$$LOD_{intercept} = 3.3 \times SD_{intercept} / b$$

where $SD_{residual}$ and $SD_{intercept}$ are the standard deviation of the regression model residuals and the curve intercept respectively, while b is the slope of the calibration curve. LOQ was

calculated as 3×LOD. The quality criterion for considering the LOD as correct was that the lowest concentration in the calibration curve was expected to be higher than the calculated LOD and lower than the calculated LOD multiplied by ten.

- **UV-vis spectrum scan**

UV-vis spectrum scan was conducted to check the possibility of monitoring the concentration of APAP, 4-aminophenol, and HQ in the microbial consortia enrichment cultures using UV-vis absorbance. For this purpose, six concentrations (1, 10, 20, 30, 40, and 50 mgL^{-1}) were prepared separately for APAP and HQ using MSM as a solvent. For, 4-aminophenol, the concentration range was from 1 to 40 mgL^{-1}. UV-vis absorption spectrum determination at 230-350 nm was applied using SYNERGY neo2 microplate reader (BioTek, USA) and Gen5 3.05 software. Measurements were in duplicates. The wavelength showing the maximum UV-vis absorbance (λ_{max}) was determined based on this step. A calibration curve was established at the specified λ_{max} using MS-Excel 2010 in the concentration range 10-50 mgL^{-1} for APAP and HQ, and 10-40 mgL^{-1} for 4-aminophenol for which the 50 mgL^{-1} standard solution was excluded from the calibration curve due to the observed brownish coloration and precipitation.

- **HPLC analysis**

HPLC analysis was conducted to check the possibility of monitoring the concentration of APAP, and HQ in cultures of the selected isolates, as well as detecting 4-aminophenol and HQ the intermediate metabolic products of APAP biodegradation. For this purpose, 1 mL of each standard solution was centrifuged at 4000 rpm for 20 min. Standard solutions of APAP and HQ were prepared as described in Section 2.1.4 for constructing standard curves for the subsequent step of quantitative analysis. Supernatants were membrane filtered before injection using polyether sulfone syringe filters (0.22 µm pore size).

HPLC conditions were applied as described by Palma et al., (2018) for the identification of APAP metabolic products but at isocratic mode and with a column of another brand. A reversed phase Surf Extreme C18 column (250 mm x 4.6 mm, 5 µm, functional group: a 100 Å pore-sized silica), purchased from ImChem, was used for separation. A Xbridge-C18 guard column (4.6 x 20 mm, 5µm) purchased from Waters was used at the entry of the separation column. Smartline HPLC set (KNAUER, Germany), with UV-detector 2600 was used and peaks were analyzed using Chromatography-Clarity SW software. The mobile phase consisted of phosphate buffer/methanol (80/20, v/v). The phosphate buffer solution (pH 4.6) was

prepared by dissolving 4.5 g KH_2PO_4 and 0.0314 g $K_2HPO_4 \cdot 3H_2O$ in 500 mL of ultrapure water, using phosphoric acid (85%) to adjust pH if necessary. Isocratic mode was applied with 1 mL min^{-1} flow rate. The column was maintained at room temperature. The total run time was 10 for APAP and HQ and 30 min for 4-aminophenol and the injection volume was 20 µL. The detection was at 245, 297, and 290 nm for APAP, 4-aminophenol, and HQ respectively.

- **GC-MS analysis**

The analytes in standard solutions of 4-aminophenol, HQ, and succinic acid (prepared as described in Section 2.1.4) were first derivatized by silylation using MSTFA reagent according to the modified method of Dalmázio et al. (2008). For this purpose, 50 µL of the silylation reaction mixture described in Section 2.1.4 was added to each sample previously dried with nitrogen gas using a sample concentrator SBHCONC71 (Barolworld, U.K), followed by vortexing for 1 s, and kept at 60 °C for 30 min.

The GC-MS analysis was conducted using SCION 456/GC gas chromatograph (Bruker, Germany) coupled to SCION TQ MS mass spectrometer (Bruker, Germany) equipped with a ZB-5 ms capillary column (30 m×0.25 mm×0. 25 µm, Phenomenex). The column oven temperatures were adjusted as follows: 70 °C for 5 min, ramped to 280 °C at 5 °Cmin^{-1}, and held for 5 min. The temperature was set at 250 °C for the injector, 260 °C for the transfer line, and at 220 °C for the ionization source. The electron ionization mode was chosen for operating the mass analyzer detector at 70 eV with a scan range of m/z 50-650. Finally, the metabolites were identified by matching the retention times and ion spectra with the authentic standards and NIST library data. Quantitative analysis was conducted based on peak areas of each target metabolite.

2.2.2. Screening/Enrichment of the microbial consortia for APAP and HQ removal

- **Origin of the microbial consortia**

The work aiming to isolate and select a collection of potentially degrading bacterial strains included the selective enrichment of the microbial consortia in liquid MSM supplemented with APAP or HQ as sole carbon source, isolation of potentially biodegrading bacterial strains using MSM-drug and LB-drug agar media, taxonomical classification of isolates, and studying the removal capacity by the bacterial isolates in liquid MSM. Based on the previous results obtained through PROBIOMA project according to which the most promising Poderosa and Lousal mines microbial consortia, with the highest APAP removal ability, were selected for

this work aiming to obtain potential APAP degrading bacterial isolates. The same consortia were used for isolating potential HQ degrading bacterial isolates. Figure 2.1 describes the map location of Poderosa mine in Spain (Figure 2.1a) and Lousal mine in Portugal (Figure 2.1b).

Figure 2.1. Map location of Poderosa (a) and Lousal (b) mines in Spain and Portugal, respectively, and field photo of Poderosa (c) and Lousal (d) mines (Fernandez-Caliani et al., 2021).

- **Thawing of frozen microbial consortia**

The cryotubes with previously selected microbial consortia stored at -80ºC were thawed on ice and 220 µL aliquot of each thawn microbial suspension was inoculated into 20 mL of LB medium in 100 mL Erlenmeyer flask, incubated at 25 °C under shaking conditions of 160 rpm for 24-48 h. The optical density of each culture at 600 nm (OD_{600}) was measured at the end of incubation period using DR2800 spectrophotometer (HACH LANGE GmbH, U.S.A). Incubation proceeded until heavy growth was obtained (OD_{600}~1).

- **Cryopreservation medium**

LB medium supported with 20% glycerol (v/v) was used as freezing medium for long term preservation of the study microbial consortia and isolates. For this purpose, 735 µL of each bacterial consortium culture (18 h) grown in 5 mL aliquot of LB medium in 10 mL vial, was mixed with 235 µL of sterile glycerol (85% v/v) in a sterile cryovial. Cryovials were stored at -80 °C.

- **Seed culture preparation**

Five milliliters of the grown culture obtained from each thawn microbial consortium was inoculated into two separate 45 mL aliquots of LB medium at 25 °C under shaking conditions of 160 rpm for 24 h. The grown cultures were used as seed cultures for studying the biodegradation of APAP and HQ in MSM. PB, PF, and PDE consortia represented Poderosa mine, while LC and LA consortia represented Lousal mine.

- **Enrichment medium preparation**

MSM medium was used for this purpose. After weighing the reagents listed in section 3.1.2 and adding deionized water, the pH was adjusted to 7.2-7.4 using 6 M NaOH, and 6 M HCl solutions. To aid dissolution of the reagents, the medium was mixed with a magnetic stirrer. Autoclaving proceeded at 121 °C for 20 min, after which media aliquots were allowed to cool down before APAP and HQ were added separately as the sole carbon source, at a concentration of 500 mgL^{-1}.

- **Enrichment cultures**

The grown seed cultures of Poderosa mine consortia (PB, PF, and PDE) and Lousal mine consortia (LA and LC) were centrifuged at 4000 rpm for 20 min. After discarding the

supernatant, pellets were washed by resuspension in 10 mL of sterile MSM followed by centrifugation. The pellets were resuspended in 3 mL of MSM. Each bacterial suspension was used to inoculate 100 mL Erlenmeyer flask containing 45 mL of MSM-drug (500 mgL^{-1}) (i.e., MSM supplemented with APAP or HQ at a concentration of 500 mgL^{-1}, so that OD_{600} of each corresponding enrichment flasks was gradually adjusted to ~1. Incubation proceeded at 25 °C under shaking conditions of 160 rpm in the dark (to avoid photodegradation of the drug). Uninoculated MSM were used as zero-time control. Incubation of the first enrichment cultures proceeded for one week after which two successive subcultures representing the second and third enrichment cultures (each inoculated with the previous enrichment culture) were scheduled under the same culture conditions. The second enrichment culture, was incubated for two weeks after which the third enrichment culture, was made and incubated for two weeks. The selective enrichment of strains able to utilize APAP and HQ as sole carbon source was conducted as described by (Wu et al., 2021; Lara-Moreno et al., 2021). At the end of the incubation period planned for each enrichment culture, 2 mL sample aliquots divided equally in two sterile Eppendorf tubes were taken for monitorization of the levels of each drug and another for pursuing the procedures of isolation of the potential biodegrading bacterial isolates, as will be shown in Section 2.2.3. The drug removal ability was assumed based on the ability of the investigated microbial consortia to utilize the tested drug as sole carbon source.

- **APAP detection/monitorization**

Based on the verification tests, it was decided that UV-vis spectrum scans of the samples were sufficient to monitor if the concentration of APAP in the enrichment cultures were decreasing at different rates than in the negative controls. HQ and 4-aminophenol showed very close λ_{max} of 290 nm and 297 nm respectively; hence, resulting in interference in UV-vis absorbance of each drug. Accordingly, at the end of the incubation time, the UV-vis absorbance of APAP in the supernatant of each enrichment culture was measured spectrophotometrically as described in Section 2.2.1 at 245 nm. For this purpose, 1 mL of each culture was centrifuged at 4000 rpm for 20 min, after which 10-fold dilution in sterile MSM was applied. Zero-time samples were also tested. % Drug removal was calculated in the removal experiments based on drug concentration in the zero-time sample.

2.2.3. Isolation of potential APAP and HQ biodegrading bacterial isolates from the enrichment cultures

For the first enrichment culture (7-day old), a loopful was spread, using sterile disposable plastic spreader, on LB plates, to confirm the bacterial growth, and on MSM-drug plates supplemented with each drug separately at a concentration of 500 mgL^{-1} to confirm the growth of isolates able to utilize APAP or HQ as sole carbon source; hence, considered potentially drug biodegrading.

For the isolation of separate bacterial isolates from the second and the third enrichment cultures (14 day old), a loopful of the second and the third enrichment cultures separately, was spread on LB plates, supplemented with the drug at a concentration of 500 mgL^{-1} (to maintain the potential biodegrading ability of the isolates) using sterile disposable plastic spreaders. After confirming the microbial growth on LB-drug plates, five 10-fold serial dilutions of each culture were spread on LB-drug plates to obtain morphologically distinct colonies (which is not achievable using MSM plates). During one week of incubation at 30 °C, the plates were checked daily, and morphologically distinct colonies were picked separately and streaked on MSM-drug (500 mgL^{-1}) plates to confirm the isolates ability to utilize the drug as sole carbon source. Pure colonies of each isolate were suspended into the cryopreservation medium in a cryovial for long term storage at -80 °C.

2.2.4. Taxonomic classification of isolates by 16S rRNA gene sequencing

- **Genomic DNA extraction from pure cultures**

Genomic DNA was extracted from a pure culture of each of the 15 potential isolates that were able to utilize APAP as sole carbon source as well as the HQ utilizing isolate, using NZY microbial gDNA isolation kit. Cultures were checked for degrading capacity by streaking on MSM-Drug (500 mgL^{-1}) agar plates, then one colony of each isolate was picked, inoculated into 5 mL of LB broth and incubated overnight at 30 °C. One milliliters of each culture was centrifuged at 4000 rpm for 20 min after which supernatants were discarded and DNA was extracted from cell pellets as described in the kit. Column extracted genomic DNA was eluted in 50 μL of the elution buffer. The purity of the extracted DNA was checked by measuring the absorbance ratios A260/A280 and A260/A230 using NanoDrop One^{c} spectrophotometer (Thermoscientific, USA) which was also used to determinethe concentration of the extracted DNA solution in ng μL^{-1} before the subsequent step of PCR. The concentration of the extracted DNA ranged from ~5 to 50 ng μL^{-1}.

- **PCR amplification of 16S rRNA gene**

The extracted genomic DNA was subjected to PCR using the thermocycler 2720 Thermal Cycler (Applied Biosystems, Foster City, USA). Universal primers for prokaryotes were used (8F: 5′- AGA GTT TGATCC TGG CTC AG -3′, 1492R: 5′- GGT TAC CTT GTTACG ACT T-3′). Primers were designed to amplify almost 1500 of the full length (~1600 bp) of the 16S rDNA. The PCR mixture consisted of 2 μL of the extracted DNA, 0.5 μL of forward primer (at 10 μM), 0.5 μL of reverse primer (at 10 μM), 7 μL of sterilized ultrapure water, 10 μL of Supreme NZYTaq 2× Green Master Mix. A negative control including all reaction mixture constituents except the DNA sample was also prepared. After a predenaturation step at 95 °C for 5 min, the PCR was carried out for 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 90 s; and with 7 min of final extension at 72 °C. The PCR amplification was confirmed by gel electrophoresis using ENDURO^{TM} electrophoresis system (Labnet, USA), which was carried out in 1% (w/v) agarose gel in 1x TAE buffer. The DNA was stained by adding 50 $\mu L L^{-1}$ of SYBER Safe Premium to the molten gel. The electrophoresis was conducted at 100 V (5 V per cm of gel length). The DNA ladder lane was loaded with 5 μL of the DNA ladder.

- **16S rRNA gene sequencing and taxonomic classification**

The amplified products were sequenced by the Sanger method in both directions, with the same primers that were used for PCR amplification. A capillary electrophoresis sequencing system (Genetic Analyzer, Model 3130xl, Applied Biosystems, Foster City, USA) was used for Sanger sequencing at the center for technologies and services of the Algarve Center for Marine Sciences. The taxonomic classification was based on the results obtained by nucleotide alignment with the "16S ribosomal RNA sequences (bacteria and archaea)" database, using the BLAST tool at the NCBI Web BLAST site (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2.5. Studying the potential biodegradation of APAP and HQ by the bacterial isolates

- **Inoculum preparation**

Frozen cultures of isolates were thawn as described earlier in 5 mL of LB broth with the modification that the LB broth was supplemented with 500 mgL^{-1} of APAP or HQ. Incubation proceeded for 48 or 72 h at 25 °C under shaking conditions of 160 rpm. Five milliliters of each grown culture was inoculated into two separate 100 mL aliquots of LB-drug (500 mgL^{-1}) medium dispended in 200 mL bottles and incubated under the same conditions. The grown

cultures were used as seed cultures for studying the potential biodegradation of APAP or HQ in MSM.

- **Removal cultures**

Each grown seed culture was used to inoculate 60 mL aliquots of MSM supplemented with APAP or HQ at a concentration of 50 or 500 mgL^{-1} dispensed in 200 mL bottles. Initial OD_{600} was adjusted to ~1. Incubation proceeded at 25 °C under dark shaking conditions of 160 rpm. Negative controls of uninoculated media were also tested. Trials were in duplicates.

- **APAP and HQ detection/monitorization**

Based on the results obtained from the method verification step (section 2.2.1), HPLC was chosen to study APAP and HQ removal capacity of the selected isolates, since it was found to be the best option, compared with UV-vis molecular spectroscopy or GC-MS analysis, in terms of combining procedures simplicity and quantification accuracy. Nevertheless, in one experiment samples were also collected for GC-MS for further attempts to detect intermediate metabolic products. %Removal was calculated in the removal experiments based on drug concentration in the zero-time sample. The results were presented as the average of the duplicate samples, and the error bars represent the average deviation (range) of the results. One milliliter of each culture was centrifuged at 4000 rpm for 20 min, membrane filtered using polyether sulfone syringe filters (0.22 μm pore size). Samples were stored at 2-8 °C until HPLC analysis time. HPLC analysis was conducted as described in Section 2.2.1.

- **Removal of 500 mgL^{-1} of APAP by PDE3.1 and PLC2.1 isolates**

The main goal of this experiment was to study APAP removal at a relatively high concentration of 500 mgL^{-1} in MSM. For this purpose, seed cultures of Poderosa mine isolate PDE3.1 and Lousal mine isolate PLC2.1 were grown as described earlier in this section and used to inoculate 60 mL aliquots of MSM supplemented with APAP at a concentration of 500 mgL^{-1} dispensed in 200 mL bottles. For PDE3.1, APAP concentration was detected after 0, 2, 4, 7, 14, and 18 days, while for PLC2.1, APAP concentration was detected after 0, 2, 4, 7, 14, and 21 days. At each time interval, 1 mL of each culture was centrifuged at 4000 rpm for 20 min after which 100 μL of the supernatant were mixed with 900 μL of sterile MSM in a sterile Eppendorf tube for the subsequent HPLC analysis, while 50 μL of each sample supernatant was stored in glass vial for the GC-MS. Samples for GC-MS were kept at -20 °C until analysis,

while HPLC were kept at 2-8 °C. For GC-MS analysis, only samples of *Bacillus* sp. (PDE3.1) were tested.

- **Removal of 50 mgL^{-1} of APAP by the potential APAP biodegrading isolates**

After checking APAP removal by the two Poderosa mine *Bacillus* sp. isolates PDE3.1 and PLC2.1 at 500 mgL^{-1} in MSM, the seven potential APAP biodegrading isolates, including PPDE3.1 and PLC2.1 and other five (selected avoiding taxa redundancy) were tested separately for APAP removal at a lower concentration of 50 $mgL^{-1.}$ The same culture conditions were applied as mentioned earlier in this section. One milliliter sample aliquots were taken as described earlier and stored at 2-8°C until the time for HPLC analysis. Also, the HQ removing Poderosa mine isolate HPB1.1 was investigated for APAP removal. The five isolates PLA3.1, PLA3.2, PLA3.3, PDE3.1, and PDE3.3 were tested in the same experiment in which samples were taken at time intervals of after 0, 5, 7, 14, and 23 days, while Lousal isolates PLC2.1, PLC2.3 as well as the HQ removing isolate HPB1.1 were tested separately for APAP removal, and samples were taken at time intervals of 0, 1, 4, 7, 14, and 21 days. For *Niallia* sp., the last sample aliquots were taken after 14 days.

- **APAP removal using the co-culture approach**

For one group of two bottles, a mixed inoculum of seed cultures of the seven potential APAP biodegrading isolates was used. For the second set of two bottles, the three most promising APAP removing isolates PDE3.1, PLA3.3., and PDE3.3 were used as inoculum. For this purpose, each seed culture was centrifuged at 4000 rpm for 20 min after which pellet was resuspended in 5 mL of MSM. The OD_{600} of the 10-fold dilution of each suspension was ranging from 1.5-1.6. Four hundred microliters of each suspension was added to each of the two 60 mL aliquots of MSM-APAP (50 mgL^{-1}) broth. Samples taken at time intervals of 0, 1, 4, 7, 14, and 21 days.

- **HQ removal by isolate HPB1.1**

Poderosa mine isolate HPB1.1 was investigated for HQ removal in MSM at a concentration of 50 mgL^{-1}. Samples were taken at time intervals of 0, 1, and 4 days.

2.2.6. Growth kinetics of the selected study strain

Kinetic parameters for batch culture of Poderosa isolate PHB1.1, degrading both APAP and HQ, were determined according to the method described by Hall et al. (2014). Inoculum (10%)

from previously prepared seed cultures of this strain grown in liquid LB medium (OD_{600} ~ 1) were used to inoculate 100 mL bottle containing 60 mL of liquid LB medium (pH7). Initial OD_{600} was adjusted at 0.10. Incubation proceeded under shaking conditions (160 rpm) at 30°C. At time intervals of 2 h, 1 mL sample aliquots were taken to measure the OD_{600} using spectrophotometer (HACH LANGE GmbH, U.S.A.). Based on OD_{600} values, incubation was extended until 68 h

3. RESULTS AND DISCUSSION

3.1 Analytical methods for detection/monitorization of APAP and HQ

As was explained in Section 2.2.1, this section was focused on testing three analytical methods (UV-vis molecular spectroscopy, HPLC, and GC-MS analysis methods) for detecting and/or quantifying APAP, HQ, and their metabolites with the aim of selecting a method for each phase of the work. The verification of some method validation parameters included R^2, sensitivity, linearity range, measurement range, as well as instrument LOD, and instrument LOQ and was conducted as described by Konieczka & Namiesnik (2009).

3.1.1. UV-vis spectrum scan

UV-vis spectrum scan was conducted to check the possibility of monitoring the removal of APAP, 4-aminophenol, and HQ in the microbial consortia enrichment cultures using UV-vis absorbance, and it included defining the UV-vis absorbance spectra (230-350 nm) and the wavelengths showing maximal UV-vis absorbance for APAP, 4-aminophenol, and HQ.

APAP, 4-aminophenol, and HQ showed maximum UV-vis absorbance at 245 nm, 297 nm, and 290 nm, respectively (Figure 3.1, 3.3, and 3.5). The close values of λ_{max} of 4-aminophenol and HQ resulted in interference which turns difficult the analysis of both drugs in the same solution by this method. Moreover, heavy precipitation was observed for 4-aminophenol in MSM. Hence, UV-vis molecular analysis was only selected for monitoring APAP removal in enrichment cultures. Calibration curves of APAP, 4-aminophenol, and HQ are shown in Figure 3.2, Figure 3.4, and Figure 3.6, respectively. The verified method validation parameters, according to the applied analytical equipment, are summarized in Table 3.1.

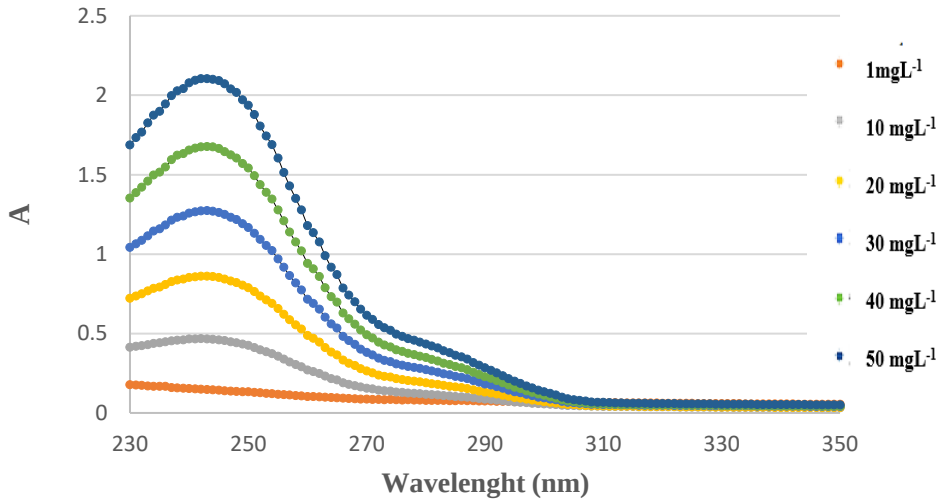


Figure 3.1. UV-vis absorbance spectra of APAP standard solutions in MSM at the concentration range of 1-50 mgL^{-1}.

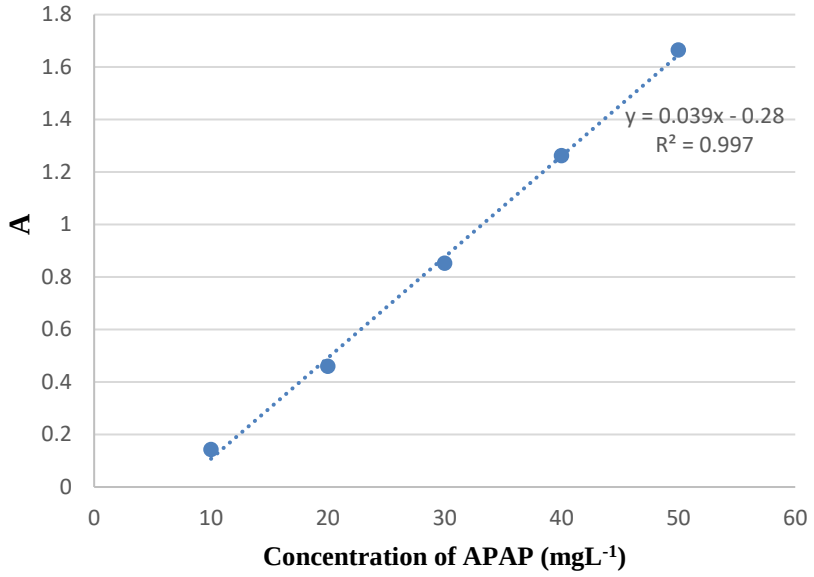


Figure 3.2. Calibration curve of APAP in MSM at the concentration range of 10-50 mgL^{-1} obtained at 245 nm.

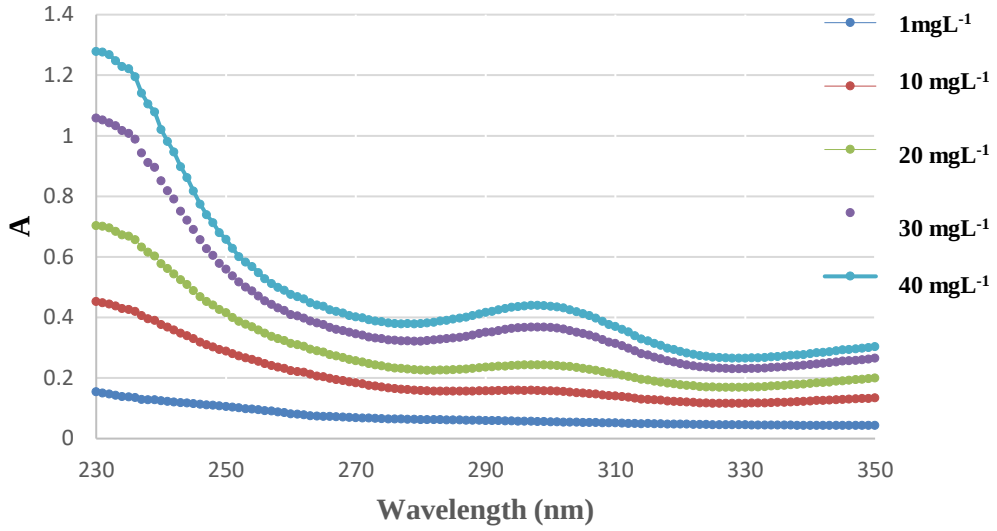


Figure 3.3. UV-vis absorbance spectra of 4-aminophenol in MSM at a concentration range of 1-40 mgL^{-1}.

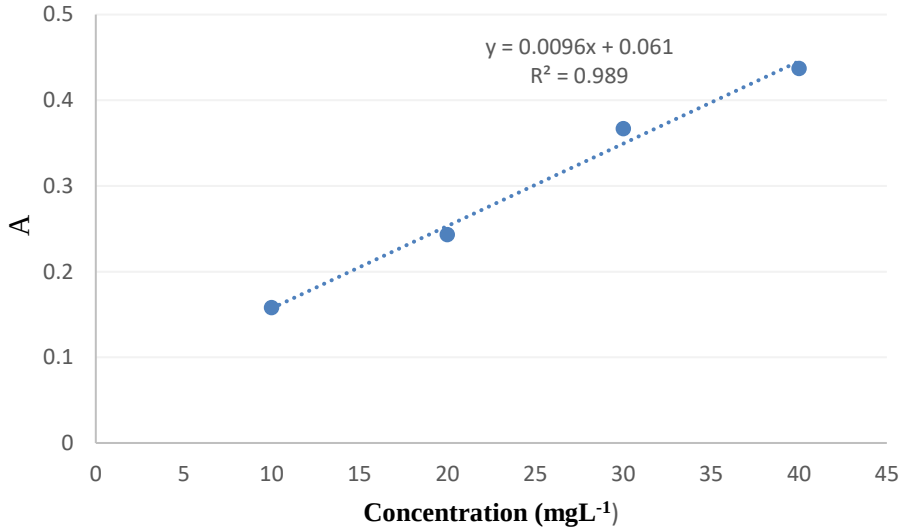


Figure 3.4. Calibration curve of 4-aminphenol in MSM at the concentration range of 10-40 mgL^{-1} obtained at 297 nm.

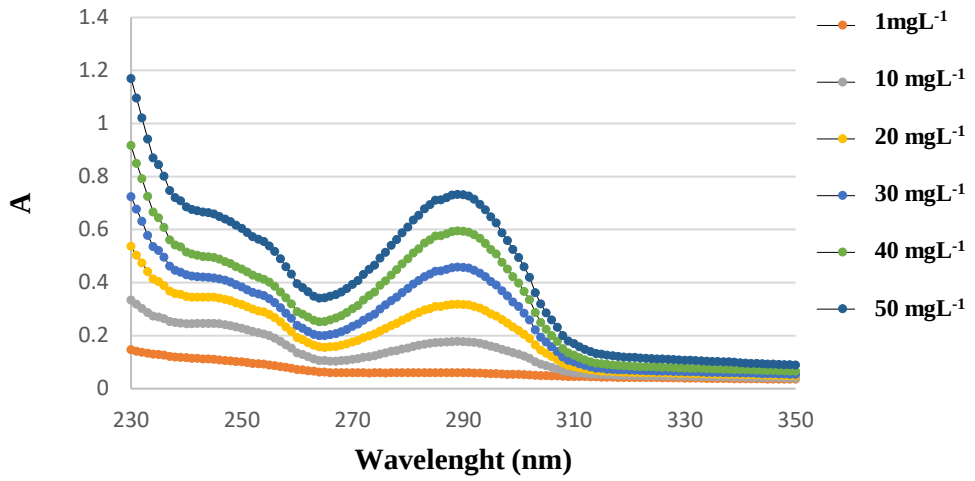


Figure 3.5. UV-vis absorbance spectra of HQ standard solutions in MSM at the concentration range of 1-50 mgL^{-1}.

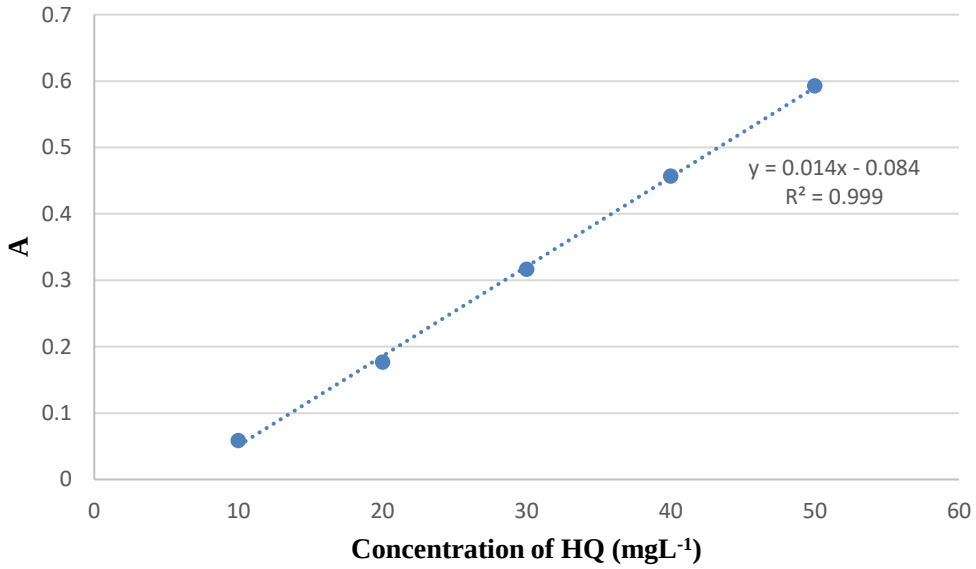


Figure 3.6. Calibration curve of HQ in MSM at the concentration range of 10-50 mgL^{-1} obtained at 290 nm.

Table 3.1. Verification parameters of the UV-vis absorbance analysis of APAP, 4-aminophenol, and HQ in MSM.

	APAP	*4-Aminophenol*	*HQ*
R^2	0.997	0.989	0.999
Linearity range (mgL^{-1})	10-50	10-40	10-50
Measurement range (mgL^{-1})	4.5-50	17.2-40	5.6-50
Sensitivity (signal/mgL^{-1})	0.04	0.01	0.01
LOD (mgL^{-1})	1.5	5.7	1.9
LOQ (mgL^{-1})	4.5	17.2	5.6

3.1.2. HPLC analysis

While UV-vis molecular spectroscopy was selected for monitoring APAP removal in enrichment cultures of the microbial consortia, HPLC analysis was intended in this work for monitoring APAP and HQ removal capacity of the selected bacterial isolates, as well as for detecting APAP biodegradation intermediate metabolic products, 4-aminophenol and HQ. This method was used due to its simplicity compared with GC-MS which requires a derivatization step. HPLC analysis option was confirmed based on method verification results; however, it couldn't detect successfully APAP biodegradation into the intermediate metabolic products, 4-aminophenol and HQ, as will be shown in Section 3.5. As shown in the chromatograms presented in Figure 3.7, APAP, 4-aminophenol, and HQ peaks were detected after 9.30, 3.93, and 6.23 min using phosphate buffer:methanol (80:20 v/v) mobile phase (pH 4.6) in the isocratic mode. Table 3.2 summarizes HPLC parameters of APAP, 4-aminophenol, and HQ analysis as reported by Chromatography-Clarity SW software. Calibration curves of APAP and HQ are shown in Figures 3.8 and 3.9, while the calibration curve of 4-aminophenol was not possible to build due to the observed inconsistent peak areas, probably due to the possible chemical instability of this compound in aqueous solutions, polymerization, and/or precipitation under the stated experimental conditions (Mitchel et al., 2001). The tested method verification parameters are summarized in Table 3.3.

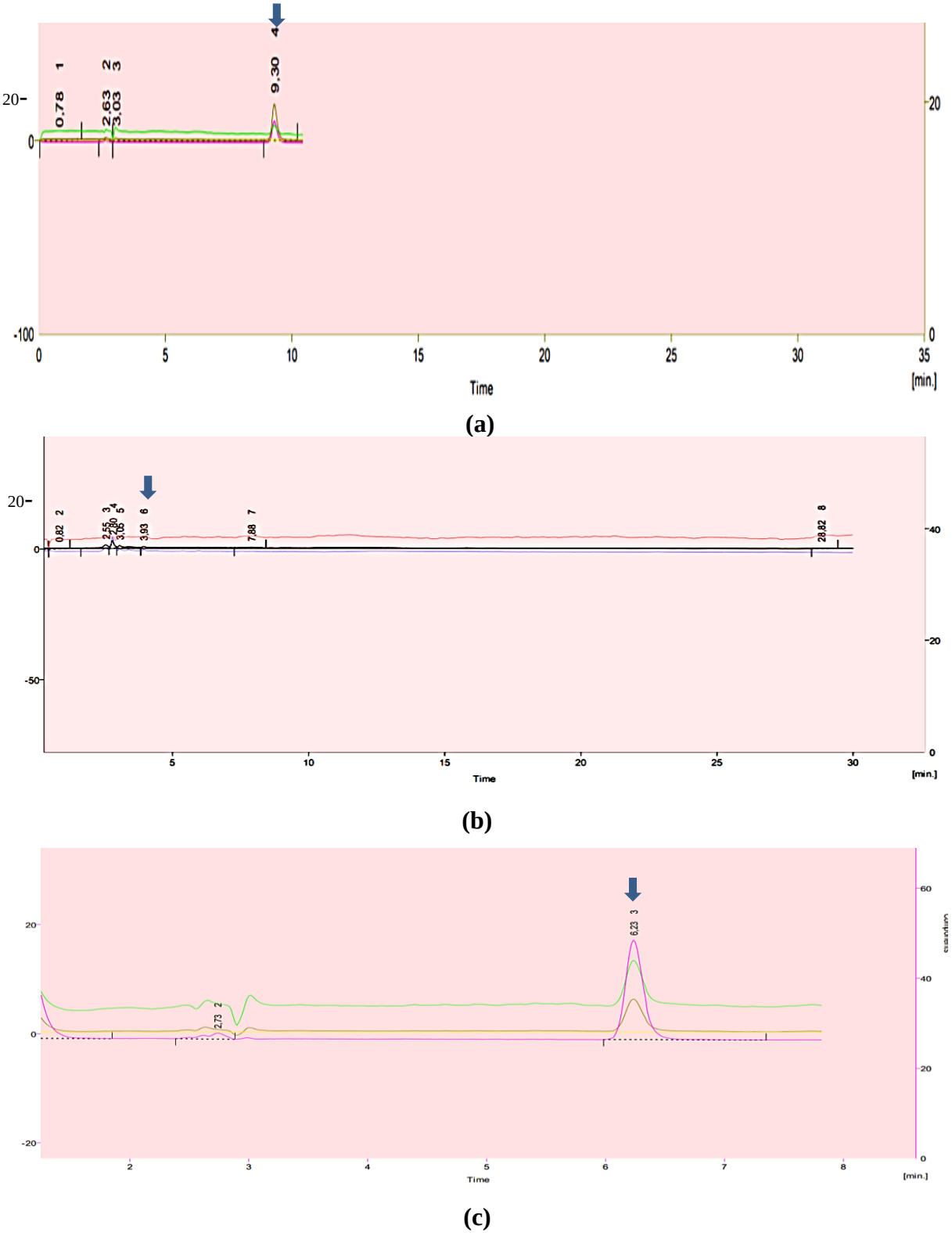


Figure 3.7. HPLC chromatogram of (a) APAP, (b) 4-aminophenol, and (c) HQ in 50 mgL^{-1} standard solutions. Arrows refer to the target peaks.

Table 3.2. HPLC analytical parameters of APAP, 4-aminophenol, and HQ standard solution (50 mgL^{-1}) detected using molecular UV-vis spectroscopy analysis.

	Wavelength (nm)	**Retention Time (min)**	**Peak area (mAU.s)**	**Peak height (mAU.s)**	**Area%**	**Height%**
APAP	245	9.30	275.16	18.77	53.5	85.9
4-Aminophenol	297	3.93	17.68	0.77	15.1	8.8
HQ	290	6.23	194.95	18.23	58.1	57.3

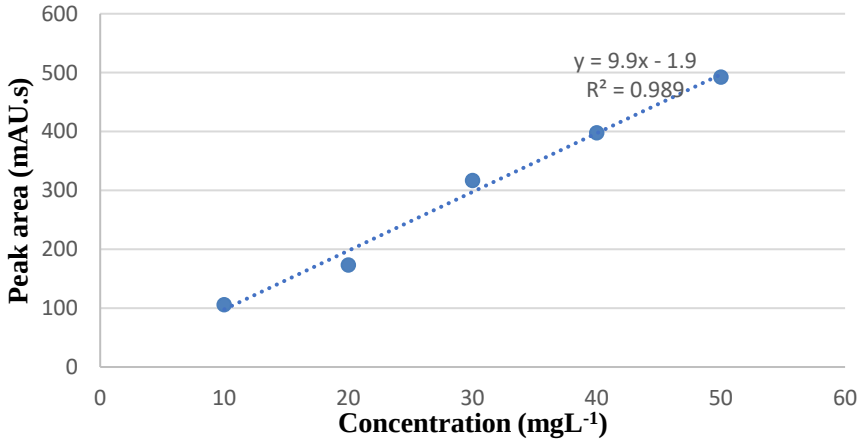


Figure 3.8. HPLC calibration curve of APAP in MSM at a concentration range of 10-50 mgL^{-1}.

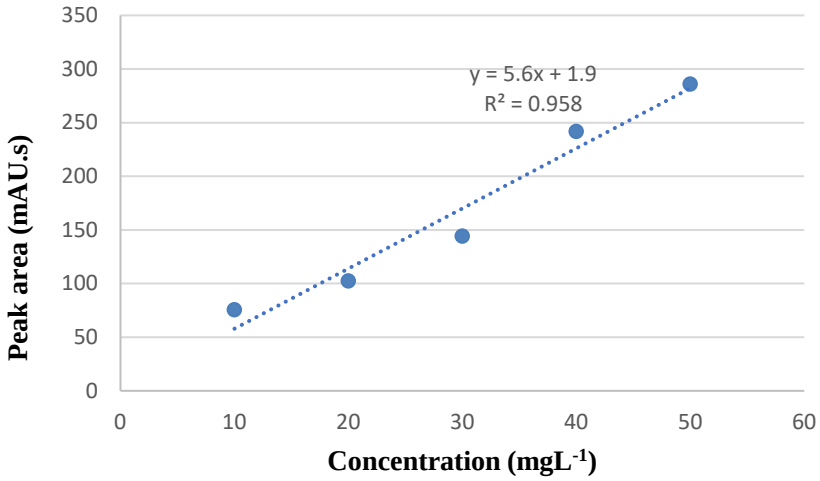


Figure 3.9. HPLC calibration curve of HQ in MSM at a concentration range of 10-50 mgL^{-1}.

Table 3.3. Verification parameters of the HPLC analysis of APAP and HQ in MSM.

	APAP	*HQ*
R^2	0.989	0.586
Linearity range (mgL^{-1})	10-50	10-50
Measurement range (mgL^{-1})	10-50	10-50
Sensitivity (signal/mgL^{-1})	9.9	5.6
LOD (mgL^{-1})	1.6	3.6
LOQ (mgL^{-1})	4.8	10.8

3.1.3. GC-MS

Due to the inability to monitor the intermediate metabolic products of APAP biodegradation, 4-aminophenol and HQ, using the HPLC analysis, GC-MS was used for this purpose. In this experiment, in addition to 4-aminophenol and HQ, the production of succinic acid as possible intermediate product of APAP biodegradation was also investigated (Hu et al. 2013, Zhang et al. 2013). As shown in Figure 3.10, 4-aminophenol, HQ, and succinic acid peaks were detected

at retention times of 24.59, 20.01, and 17.79 min. Calibration curves obtained with standards in the concentration range of 1-10 mgL^{-1} are shown in Figures 3.11-3.13. Accordingly, GC-MS analysis was later selected for detection and quantification of the aforementioned intermediate metabolites of APAP biodegradation by one of the Poderosa mine isolate *Bacillus* sp. (PDE3.1). The tested method verification parameters are summarized in Table 3.4.

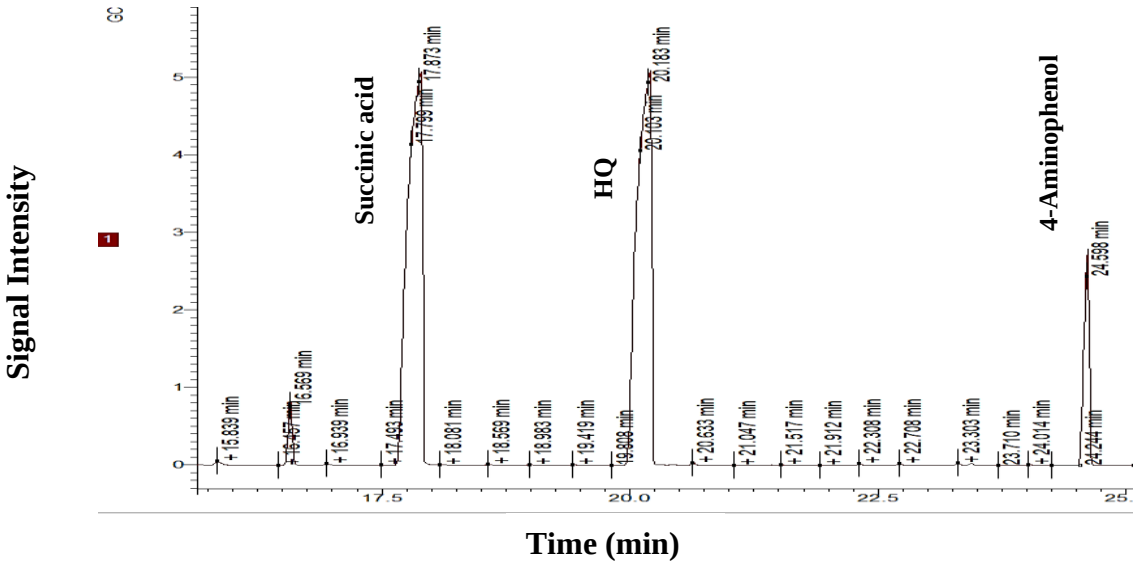


Figure 3.7. GC-MS Chromatogram of a standard solution consisting of a mixture of 4-aminophenol, succinic acid, and HQ standard solution (10 mgL^{-1}).

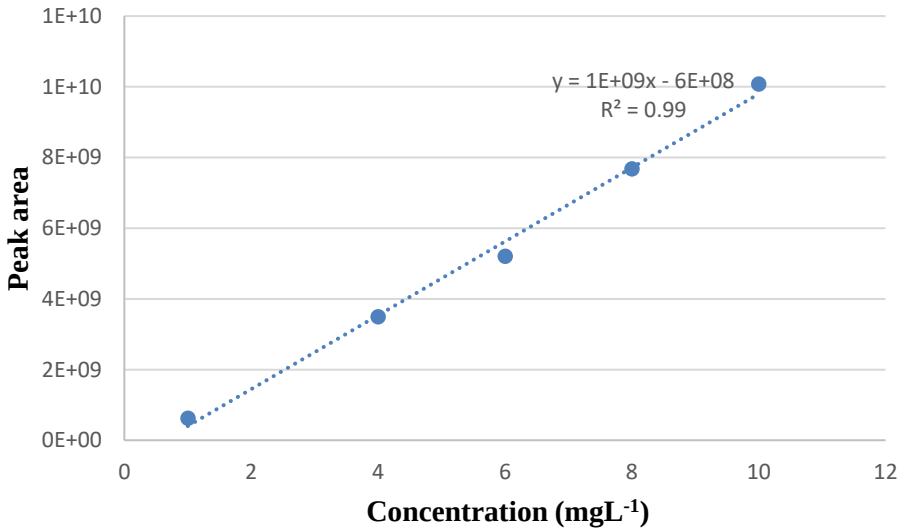


Figure 3.11. GC-MS calibration curve for 4-aminophenol in MSM at a concentration range of 1-10 mgL^{-1}.

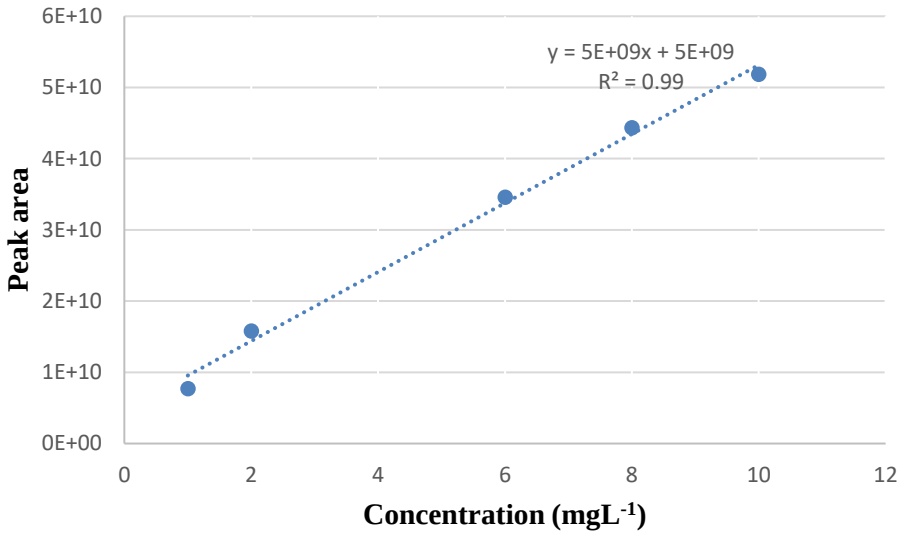


Figure 3.12. GC-MS calibration curve for HQ in MSM at a concentration range of 1-10 mgL^{-1}.

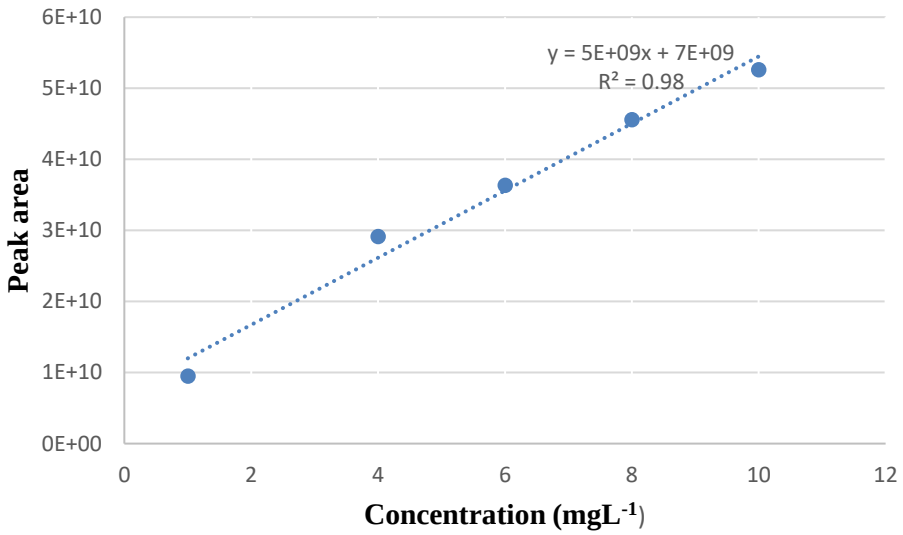


Figure 3.13. GC-MS calibration curve for succinic acid in MSM at a concentration range of 1-10 mgL^{-1}.

Table 0.4. Verification parameters of the GC-MS analysis of 4-aminophenol, HQ, and succinic acid in MSM.

	4-Aminophenol	*HQ*	*Succinic acid*
R^2	0.99	0.99	0.98
Linearity range (mgL^{-1})	1-10	1-10	1-10
Measurement range (mgL^{-1})	1-10	1-10	1-10
Sensitivity (signal/mgL^{-1})	1×10^9	5×0^9	5×10^9
LOD (mgL^{-1})	0.8	0.7	1.6
LOQ (mgL^{-1})	2.3	2.2	4.8

In conclusion, based on the aforementioned results of verifying the tested analytical methods, UV-vis molecular spectroscopy analysis was selected for monitoring APAP removal in the enrichment cultures of the microbial consortia. However, UV-vis molecular spectroscopy was not selective enough to monitor the removal of HQ or 4-aminophenol in the enrichment cultures. Subsequently, HPLC analysis was selected for monitoring APAP and HQ removal by the selected isolates due to method selectivity, simplicity and accuracy. However, HPLC analysis had relatively high limits of detection, which could prevent the detection of possible intermediate metabolites of APAP biodegradation, while were successfully detected using GC-MS analysis (as will be shown in Section 3.5.1) due to the higher sensitivity compared with HPLC.

3.2. Screening/Enrichment of the microbial consortia for APAP and HQ removal

During the course of the incubation period of each culture (one week for the first enrichment, and two weeks for the second and third enrichment) at 25 °C under dark shaking conditions of 160 rpm, deep brown coloration of MSM-APAP (500 mgL^{-1}) was observed for all enrichment cultures of all microbial consortia (indicate the consortia tested), except the consortium LC from Lousal mine. No coloration was observed for the negative control (Figure 3.14).

Such medium coloration is associated with APAP biodegradation according to De Guesseme et al. (2011) who reported a brown coloration of the synthetic mineral medium during the microbial degradation of APAP by two described isolates, due to the formation and/or accumulation of degradation intermediates, e.g., polymerization products of catechol.

According to UV-vis molecular spectroscopy analysis, the 7-day old first enrichment culture of consortium LA from Lousal mine showed only 6% APAP removal. On the other hand, the

14-day old second and third enrichment cultures of consortium LA showed the maximal APAP removal capacity as inferred by APAP %removal of 99.7 and 84.1 respectively. The results obtained in this work are not sufficient to explain the decrease in the removal capacity observed in the third enrichment cultures. (Figure 3.15). The higher APAP %removal reported for the second and third enrichment cultures compared with the first enrichment culture can be explained based on enhanced growth of APAP utilizing strains.

The enrichment cultures of Poderosa mine consortia PDE (7-day old first enrichment culture and 14-day old second enrichment culture), and PB (14-day old second and third enrichment cultures) showed maximal close values of APAP %removal of almost 22. The enrichment cultures of Poderosa mine consortium PF (14-day old second and third enrichment cultures) showed maximal APAP %removal of 15. For the enrichment cultures of Lousal mine consortium LC, even less APAP %removal was detected by UV-vis molecular spectroscopy analysis where maximal APAP %removal of almost 15 was reported for the first enrichment culture. Appendix 2 describes the residual concentration of APAP (mgL^{-1}) in each MSM enrichment culture at the end of the incubation period.

The next step of isolation was planned for all consortia based on the deep brown coloration observed in almost all cultures. It was suspected that there could be production of some compound(s) that could have absorption at wavelengths overlapping those of APAP.

In the case of HQ enriched cultures, also deep brown coloration was observed for Poderosa mine (Figure 3.16) as well as Lousal mine enrichment cultures, but also in the negative control medium, indicating that HQ was changing due to abiotic factors. In fact, it is known that oxygen can act as oxidant in the oxidative polymerization of HQ, leading to the formation of brown polymers (Zhang et al. 2012; Dang et al. 2014). Moreover, it was impossible to monitor HQ concentration in the enrichment cultures of the microbial consortia by UV-vis molecular spectroscopy due to the interference caused by this abiotic transformation (data not shown).

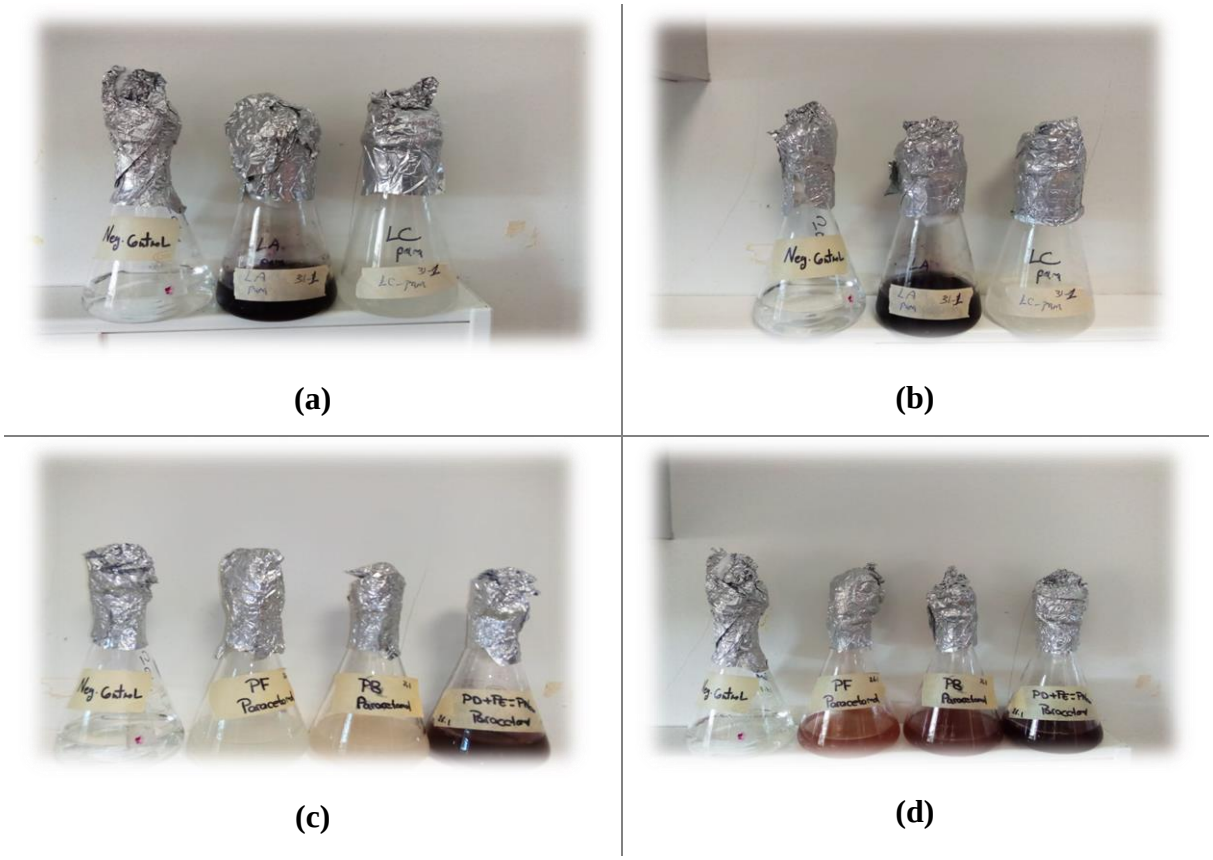


Figure 3.14. The first enrichment cultures of Lousal (LA and LC) and Poderosa (PF, PB and PDE) mines microbial consortia using MSM-APAP (500 mgL^{-1}) after one week (a and c) and after one month, (b and d). The negative control flasks are on the left.

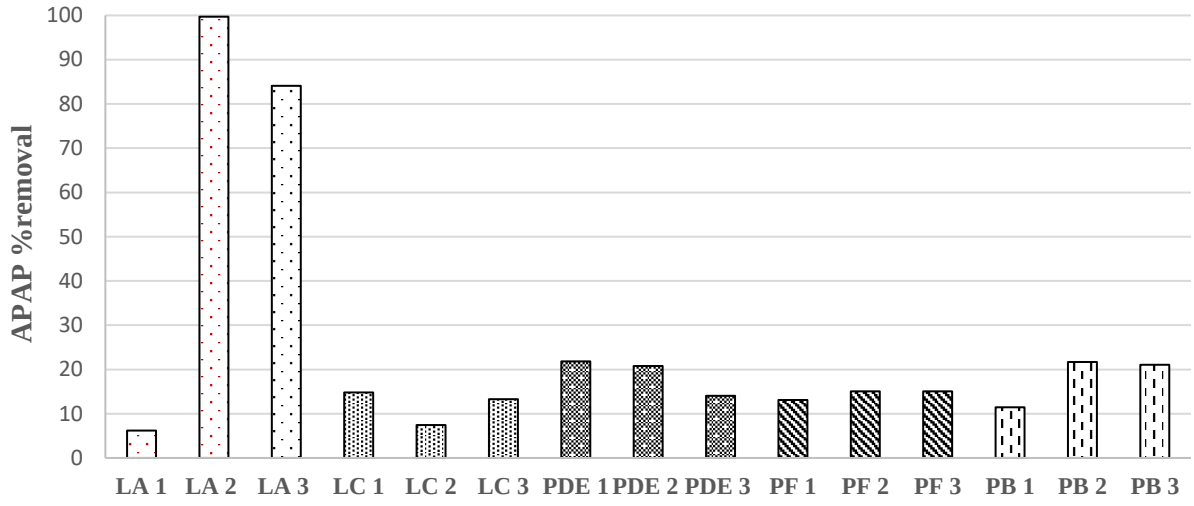


Figure 3.15. APAP %removal in MSM-APAP (500 mgL^{-1}) enrichment cultures of PDE, PB, and PF microbial consortia of Poderosa mine and LA and LC microbial consortia of Lousal mine using UV-vis absorbance (245 nm)**.** 1, 2, & 3: 7-day 1st enrichment culture, 14-day old 2nd enrichment culture, & 14-day old 3rd enrichment cultures, respectively.

Figure 3.16. The first enrichment cultures (7-day old) of Poderosa mine microbial consortia using MSM-HQ (500 mgL^{-1}) after one week, with the deep brown coloration of observed for MSM-HQ cultures.

3.3. Isolation of potential APAP and HQ degrading bacterial isolates from the enrichment cultures

As was clarified in Section 2.2.3, the growth of each microbial consortium in the first enrichment culture was confirmed by streaking on LB agar plates, while isolation from the second and third enrichment cultures proceeded by spreading five 10-fold serial dilutions of each culture on LB-drug (500 mgL^{-1}) plates to obtain morphologically distinct colonies whose biodegradation ability was demonstrated by streaking on MSM-drug (500 mgL^{-1}) plates where the drug was used as the sole carbon source. This strategy resulted in 15 bacterial isolates able to grow in the presence of APAP as sole carbon source. Concerning HQ, it was impossible to isolate any bacteria able to utilize HQ as sole carbon source from the second and third enrichment cultures; however, only one isolate able to utilize HQ as sole carbon source was isolated from the first enrichment of Poderosa mine microbial consortium PDE.

3.4. Taxonomic classification of selected isolates by 16S rRNA gene sequencing

3.4.1. 16S rRNA gene PCR amplification

For each of the 15 isolates that showed the ability to utilize APAP as sole carbon source and the isolate able to utilize HQ, following the PCR amplification of the 16S RNA gene using universal primers as described in Section 2.2.4, gel electrophoresis was applied to confirm the 16S rRNA gene amplification (Figure 3.17 & Figure 3.18). The blank reaction for the amplification of the 16S rRNA gene revealed a band corresponding to the size of the expected gene product, indicating bacterial contamination (during the PCR mixture preparation). Despite of that, since the blank's band was very weak comparing the samples' bands, sequencing the PCR products proceeded. The 16S rRNA gene amplification of HQ degrading isolate was carried out using a new aliquot of ultrapure water filtered through a syringe filter (0.22 μm pore size).

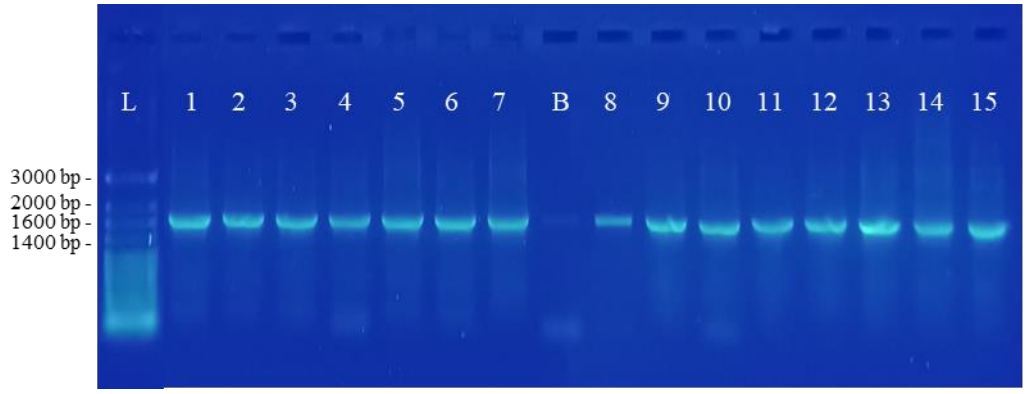


Figure 3.17. The PCR products of the 16S rRNA genes on 1% (w/v) agarose gel with 50 μLL^{-1} SYBER Safe Premium imaged after electrophoresis at 100 V in 1x TAE buffer at room temperature. L: DNA Ladder; B: blank; 1 to 15: bands of the amplified 16S rRNA genes of the APAP removing isolates PDE2.2, PDE3.1, PDE3.2, PLA2.1, PLA2.2, PLC2.2, PLC2.3, PLC2.1, PLA3.2, PDE3.3, PDE2.4, PLA3.3, PLA3.1, PLA2.4, and PLA2.3, respectively.

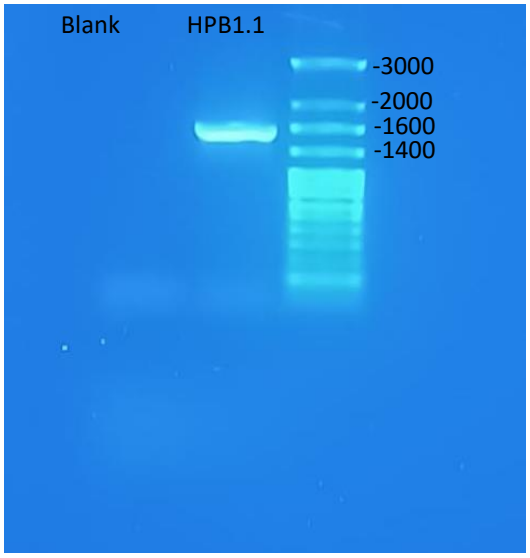


Figure 3.18. The PCR products of the 16S rRNA gene of the HQ utilizing isolate HPB1.1 on 1% (w/v) agarose gel with 50 μLL^{-1} SYBER Safe Premium imaged after electrophoresis at 50 V in 1x TAE buffer at room temperature.

3.4.2. 16S rRNA gene sequencing and taxonomic classification

The obtained 16S rRNA gene sequences were checked and trimmed to remove poor quality ends by visually inspection of chromatograms and then both forward and reverse sequences of same product were combined by sequence alignment using BioEdit 7.2 software. The sequences obtained are listed in Appendix 1.

The BLAST alignment with other sequences of related bacteria in the NCBI database performed to determine phylogenetic relationships to other bacteria showed seven distinct isolates phylogenetically related to members of genera *Bacillus* (two isolates),

Paraburkholderia, *Rhizobium*, *Aeromonas*, *Variovorax*, and *Niallia*, in addition to one HQ utilizing strain phylogenetically related to members of genus *Mycolicibacterium*.

For the studied isolates identified as members of genera *Aeromonas*, *Bacillus*, *Rhizobium*, and *Variovorax*, values of the 16S rRNA %sequence identity with the first closest neighbors, suggested by the NCBI BLAST alignment, are above 98.99, hence, above the cut-off value of 98.7 suggested for the definition of bacterial species as described by (Yarza et al., 2014). However, the sequences have also very high similarities with other closest neighbors of the same genus but assigned to different species. Therefore, the BLAST alignment was not significant enough to identify the studied isolates to the species level.

For the two *Bacillus* sp. isolates, *Rhizobium* sp., and *Variovorax* sp. isolates, the ranges of the %sequence identity with the closest suggested neighbors (same genus and varied species) were 99.86-100, 99.5-99.8, 99.62-99.85, and 98.99-99.64, respectively. Moreover, in some cases the isolates showed the same %sequence identity with two or more of the suggested species of the closest neighbors as for *Aeromonas* sp. isolate, which showed the same value of 99.88% identity with all the suggested closest neighbors (Appendix 1). Also, for isolates identified as members of *Mycolicibacterium*, *Niallia*, and *Paraburkholderia* it was impossible to identify the isolates to the species level for the same reason. However, the ranges of %sequence identity with the closest neighbors were 99-100, 97.55-99.19, and 98.5-100, respectively. The reason for keeping the two isolates belonging to genus *Bacillus* was because their first BLAST hits were different and because these isolates came from samples collected on different mines.

Table 3.5 shows the genotypic identification at the genus level of the study isolates based on BLAST alignment of 16s rRNA gene sequence of each isolate.

Table 3.5. Genus level identification of the potential APAP and HQ biodegrading bacterial isolates using 16S rRNA gene sequence analysis.

Isolate	*Genus*	*Source mine of the selected isolates*
PLA2.3, PLA2.4, **PLA3.1**	*Aeromonas*	Lousal
PLA2.1, PLA2.2, PLC2.2 **PPDE3.1**, PPDE3.2, PPDE2.2	*Bacillus*	Poderosa
PLC2.1	*Bacillus*	Lousal
HPB1.1	*Mycolicibacterium*	Poderosa
PLC2.3	*Niallia*	Lousal
PLA3.3, PPDE3.4	*Paraburkholderia*	Lousal
PPDE3.3	*Rhizobium*	Poderosa
PLA3.2	*Variovorax*	Lousal

NB: bold isolate code is to highlight the selected representative isolate among the replicate isolates

Interestingly and to our knowledge, members of genera *Varivorax*, *Paraburkholderia*, *Rhizobium*, and *Niallia* haven't been stated previously in APAP biodegradation or removal studies. Therefore, more studies can be directed to further explore the diversity of potential biodegrading bacteria from mines as representatives of extreme environments. Likewise, the HQ utilizing actinomycete isolate *Mycolicibacterium* sp., isolated from Poderosa mine, haven't been stated previously in the literature for APAP or HQ degradation, although actinomycetes generally are known for their unique metabolic potential concerning the production of bioactive compounds, as well as bioremediation (Devanshi et al., 2021). *Mycolicibacterium* has been reported recently by Zhang et al. (2021) as a producer of steroid-based drug intermediates. To our knowledge, *Mycolicibacterium* have not been reported in pharmaceutical biodegradation studies. However, it has been reported for polychlorinated biphenyls biodegradation (Steliga et al., 2020). Also, Naloka et al. (2021) highlighted *Mycolicibacterium* sp. as a part of an artificial consortium for degradation of fuel oil with 50% removal of high fuel oil concentration of 3000 mgL^{-1} in synthetic medium and contaminated fresh water. Table 3.6 summarizes some of the pharmaceutical biodegradation reports on each genus. Bako et al. (2021) highlighted polychlorinated biphenyls degradation in bioreactors by *Paraburkholderia* comprising mainly

environmental nonpathogenic strains. However, it was previously classified as *Burkholderia* comprising mainly pathogenic strains and hence, needs to be further investigated for environmental studies. *Rhizobium* sp. has been stated for phenol compound degradation (Wei et al., 2008) and in the study of Yang et al. (2020), it was suggested that *Rhizobium* was one of the16 microbial genera involved in anaerobic degradation of APAP in mangrove sediments. This can be explained based on the nitrogen fixing role played by *Rhizobium* in nature under anaerobic conditions which enhances the growth of other biodegrading bacterial populations as suggested by Teng et al. (2015). In the study of Patel et al. (2022), *Niallia circulans* strain has been reported for the azo dye Reactive Red 152 biodegradation.

Table 3.6. Literature reports describing the history of members of the bacterial genera reported in the current study concerning biodegradation of APAP, its key metabolites, or other pharmaceuticals.

Genus	***Drug***	***Experimental conditions & results***	***Authors***
Aeromonas	Gliclazide	*Aeromonas* sp. degraded gliclazide in MSM supplemented with 0.5 gL^{-1} of gliclazide, with a specific activity of 22.3 $ng/(mgh^{-1})$, in a batch culture experiment.	Ouartsi et al. (2018)
Bacillus	Ibuprofen	*B. thuringiensis* B1(2015b) degraded 20 mgL^{-1} after 6 days in MSM supplemented with 1 $mgL-^{1}$ of glucose, at 30 °C with shaking at 130 rpm.	Marchlewicz et al. (2017a, b)
	APAP	*B. subtilis* removed 1200 mgL^{-1} from MSM after 10 days at 30 °C under shaking conditions.	Chopra & Kumar (2020)
	APAP	*B. cereus* removed 200 mgL^{-1} from MSM at 28 °C after 144 h.	Palma et al. (2021)
Variovorax	Ibuprofen	*Variovorax* sp. Ibu-1 degraded 200 mgL^{-1} after 75 h in MSM.	Murdoch & Hay (2015)
	HQ	The genome of genus *Variovorax* has been reported to include genes for a family of HQ 1,2-dioxygenases but no biodegradation study is available.	(Enguita & Leitão 2013)
Mycolicibacterium	NR		
Niallia	NR		
Paraburkholderia	NR		
Rhizobium	NR		

NR: not reported earlier in the literature.

3.5. Studying the potential biodegradation of APAP and HQ by the bacterial isolates

Based on the earlier reported ability of the selected study bacterial isolates to utilize APAP as sole carbon source in MSM plates, their biodegradation ability was assumed; hence, it was intended in this section to monitor the drug removal as well as the levels of the intermediate

biodegradation metabolites in liquid MSM cultures as a function of time using HPLC analysis. Trials were duplicates. APAP %removal was calculated based on APAP concentration in the zero-time samples. Similarly, HQ removal was investigated in liquid MSM cultures of one isolate. The tables displaying the numerical data describing the detected levels of the investigated drugs and the intermediate metabolite in MSM cultures, with the average deviation for two replicates, are shown in Appendix 3, while the corresponding graphs are displayed in context.

3.5.1. Removal of 500 mgL^{-1} of APAP by PDE3.1 and PLC2.1

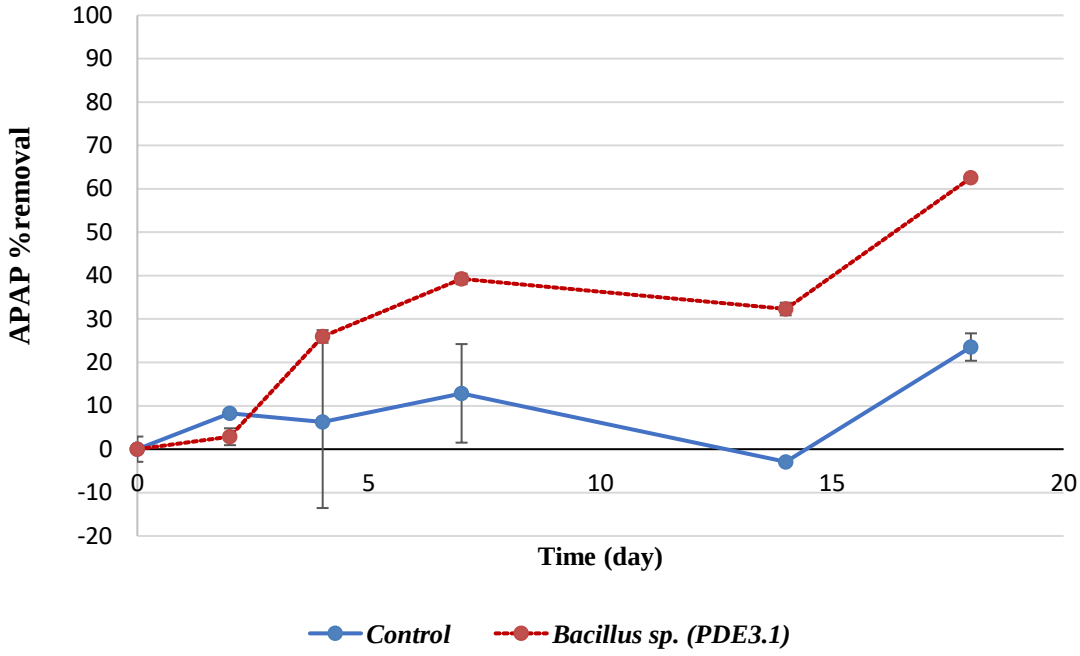
As mentioned earlier in Section 3.2.4, Poderosa mine isolate PDE3.1 and Lousal mine isolate PLC2.1, identified as two putative distinct *Bacillus* species, among the seven potential APAP biodegrading isolates, were studied for removal of APAP in MSM at a high concentration of 500 mgL^{-1}.

According to HPLC analysis, Poderosa mine *Bacillus* sp. isolate (PDE3.1), showed maximal APAP removal% of 63±1 was recorded after 18 days (% removal was calculated based on APAP concentration in the zero-time samples), while APAP %removal in the negative control (uninoculated medium) was 24±3 (Figure 3.19a & and Figure 3.20). Another interesting observation in the assay with *Bacillus* sp. (PDE3.1) is that removal seems to occur only in the first week, during which the APAP concentration decreases in the inoculated medium but not in the negative control, while in the following weeks the changes seem to be caused by abiotic factors since the drug concentration changes in parallel in the test and in the control. For Lousal mine *Bacillus.* sp. isolate (PLC2.1), APAP %removal was only 8±1 after 21 days, while no removal was observed in the negative control, instead an increase in APAP residual concentration was observed in the negative control, which may be explained based on a possible interference caused by APAP abiotic degradation products (Figure 3.19b & Figure 3.20).

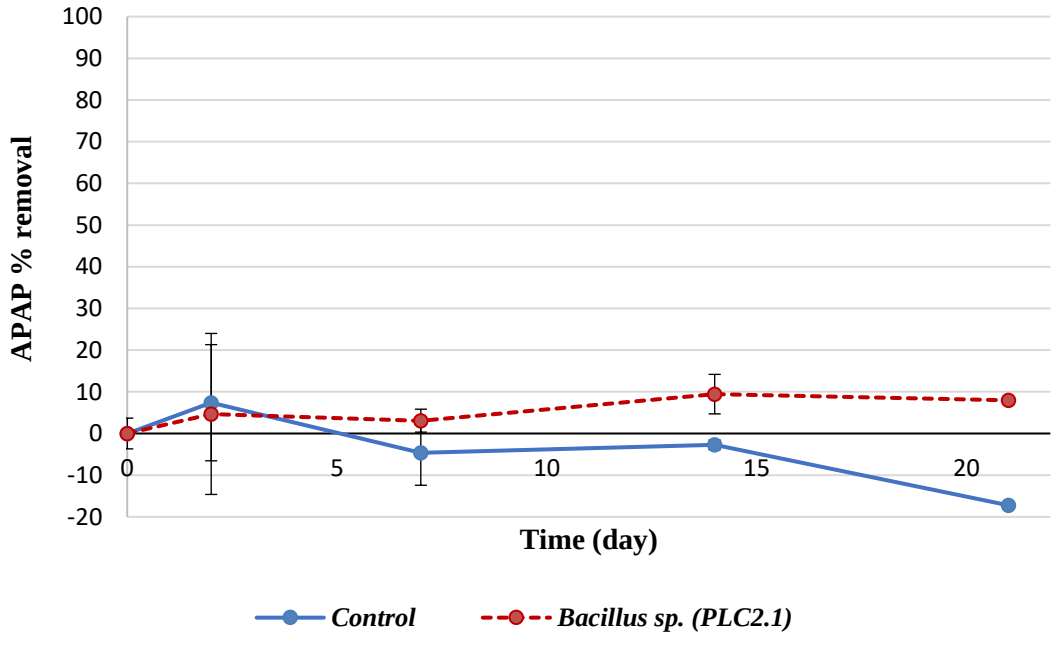
4-Aminophenol and HQ, the intermediate metabolites of APAP biodegradation, (Hu et al. 2013, Zhang et al. 2013) were not detected through HPLC analysis for both isolates during the study. Considering that 4-Aminophenol is liable to rapid oxidation in the presence of air and to photolysis due to its absorption in the environmental UV-vis spectrum with UV max value of 294 nm (Mitchel et al., 2001); hence, the concentration of 4-aminophenol as a primary product of APAP degradation is expected to decrease rapidly which makes the detection of its levels in the culture medium more difficult. Similarly, according to Serrano et al. (2019), the

inability to detect HQ can be explained based on the chemical instability of HQ, which results in its conversion into the carcinogenic p-benzoquinone and the production of polymeric compounds that lead to dark coloration. Moreover, HQ stability is affected by pH changes and is more stable at acidic pH values (Zhang et al. 2012; Dang et al. 2014).

The decrease in APAP concentration detected in the negative control samples during the biodegradation study can be explained based on the instability of APAP aqueous solutions due to oxygen and/or light induced oxidation. In fact, APAP degrades through several degradation pathways, as was stated in the report of Koshy & Lach (1961), resulting in coloration due to the formation of several compounds including the toxic benzoquino imines which may polymerize. Moreover, these compounds may negatively affect the biodegradation process by inhibiting bacterial growth, which in turn could explain why APAP removal from culture medium stops early or later during the course of the biodegradation experiment. According to the same study, another possible reaction explaining APAP instability in aqueous solutions is the deacetylation of the amino group generating 4-aminophenol which in turn gets quickly degraded producing p-benzoquinone imine. This comes in agreement with the reports of Dietlin & Fredj (2000) and Chiam et al. (2015), as well as The International Agency for Research on Cancer (IARC) monograph of APAP (IARC, 2000) which depicted APAP instability under humid conditions resulting in its hydrolysis and clarified that APAP degradation rate in aqueous solutions is boosted by temperature increase, exposure to light and oxygen, changes in pH outside the range of 5-6, and even by a slight contamination with 4-aminophenol. Those reports, in addition to APAP hydrolysis, highlighted the possibility of APAP decomposition into the toxic HQ and a quinone imines.



(a)



(b)

Figure 3.19. APAP %removal as a function of time in MSM-APAP (500 mgL^{-1}) cultures of (a) *Bacillus.* sp. (PDE3.1), and (b) *Bacillus.* sp. (PLC2.1), and in the negative control using HPLC analysis. The error bars represent the average deviation for two replicates. When error bars are not visible, it means they are smaller than the data point markers.

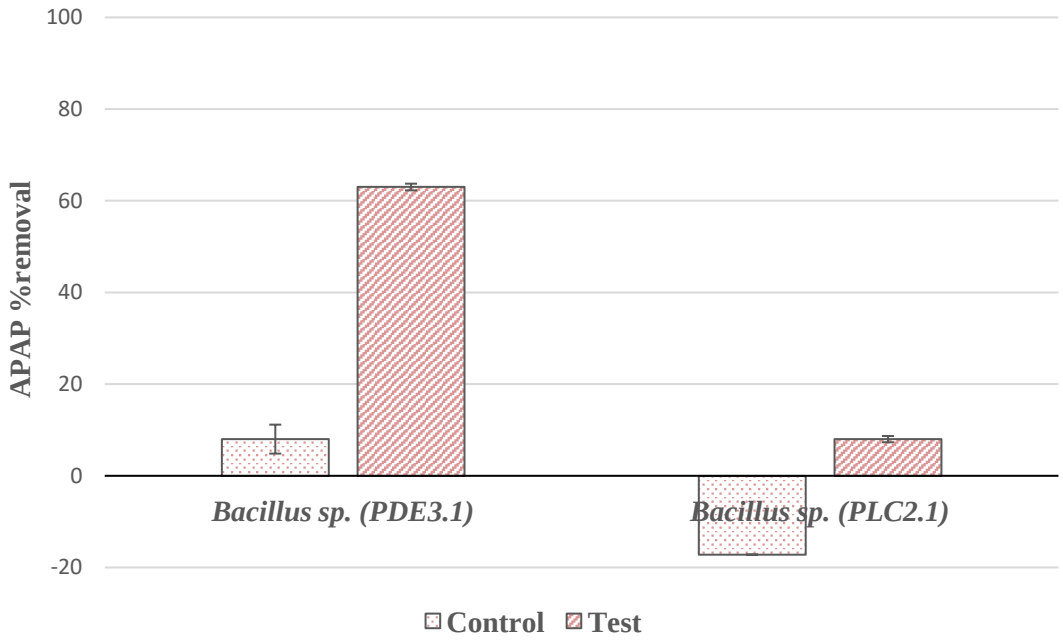


Figure 3.20. APAP %removal in MSM-APAP (500 mgL^{-1}) cultures of *Bacillus* sp. (PDE3.1) and *Bacillus* sp. (PLC2.1), and in the negative control after 18 and 21 days, respectively using HPLC. The error bars represent the average deviation for two replicates. When error bars are not visible, it means they are smaller than the data point markers.

3.5.2. GC-MS monitorization of APAP biodegradation products in Bacillus sp. (PDE3.1) cultures

Based on the proven ability of *Bacillus* sp. (PDE3.1) to remove APAP from MSM compared with *Bacillus* sp. (PLC2.1), the samples from *Bacillus* sp. (PDE3.1) experiment were selected for quantitative GC-MS analysis for the monitorization of the levels of the possible APAP intermediate degradation metabolites, since they were not detected through the HPLC analysis. Samples derivatization through silylation was conducted as described in Section 3.2.5 aiming the analysis of possible APAP biodegradation metabolites by GC-MS. 4-Aminophenol and HQ were selected as the two key intermediate metabolites for monitorization of APAP biodegradation as was stated in of Hu et al. (2013) and Zhang et al. (2013) reports, which highlighted also succinic acid as an intermediate metabolite indicating phenol ring fission. Hence, the presence of 4-aminophenol, HQ, and succinic acid were investigated based on the analysis of the derivatized standard solutions of a mixture of the investigated metabolites, at lower concentration range (1, 2, 4, 6, 8, and 10 mgL^{-1}).

According to the chromatograms of the standard solutions, the retention times of 4-aminophenol, HQ, and succinic acid were 24.6, 20.1, and 17.8 min, as mentioned earlier in

Section 3.2.3. Based on the aforementioned retention times, the target metabolites were checked in the derivatized test and negative control samples in the resultant chromatograms and the associated mass spectra using NIST library. Peak areas were reported for quantification in mgL^{-1}.

Interestingly, 4-aminophenol and HQ, the usually most important intermediate metabolites of APAP biodegradation, were detected only through GC-MS analysis at low concentrations (Figure 3.21), and were not detected in the negative control samples. Such observation implied APAP biodegradation by the *Bacillus* sp. (PDE3.1) through the 4-aminophenol pathway with the initial decarboxylation of APAP into 4-aminophenol in which amino group is then replaced by the hydroxyl group, thus, producing HQ (Żur et al., 2018).

Succinic acid was not detected in the culture supernatant at any time interval. Yet, 4-aminophenol appeared after 4 days at a concentration of almost 6 mgL^{-1} followed by gradual decrease until disappearance after 14 days with a subsequent slight increase (0.6 mgL^{-1}) detected at day 18. HQ appeared at extremely low concentrations below the verified LOD, and then increased to 0.28 mgL^{-1} after 18 days (Figure 3.21). Although 4-aminophenol and HQ detected levels were mostly below the LOD and LOQ, their detection was confirmed by the NIST library which identified the structure of the detected peaks as 4-aminophenol and HQ.

Since 4-aminophenol and HQ, the two key metabolite of APAP biodegradation, were not detected by the tested HPLC method, while were successfully detected at very low concentration through GC-MS analysis, it can be concluded that the second analytical method has higher sensitivity which allows lower detection and quantification limits compared with the former. In addition, there is the possibility of determination of the structural identity of analytes in the sample based on the m/z ratio (Awad et al, 2015). Indeed, GC-MS is characterized by a higher chromatographic resolution compared to HPLC or LC-MS based methods, a good retention of low molecular weight analytes that elute early in reverse-phase LC-MS methods, as well as having huge spectral data bases (Aretz & Meierhofer, 2016).

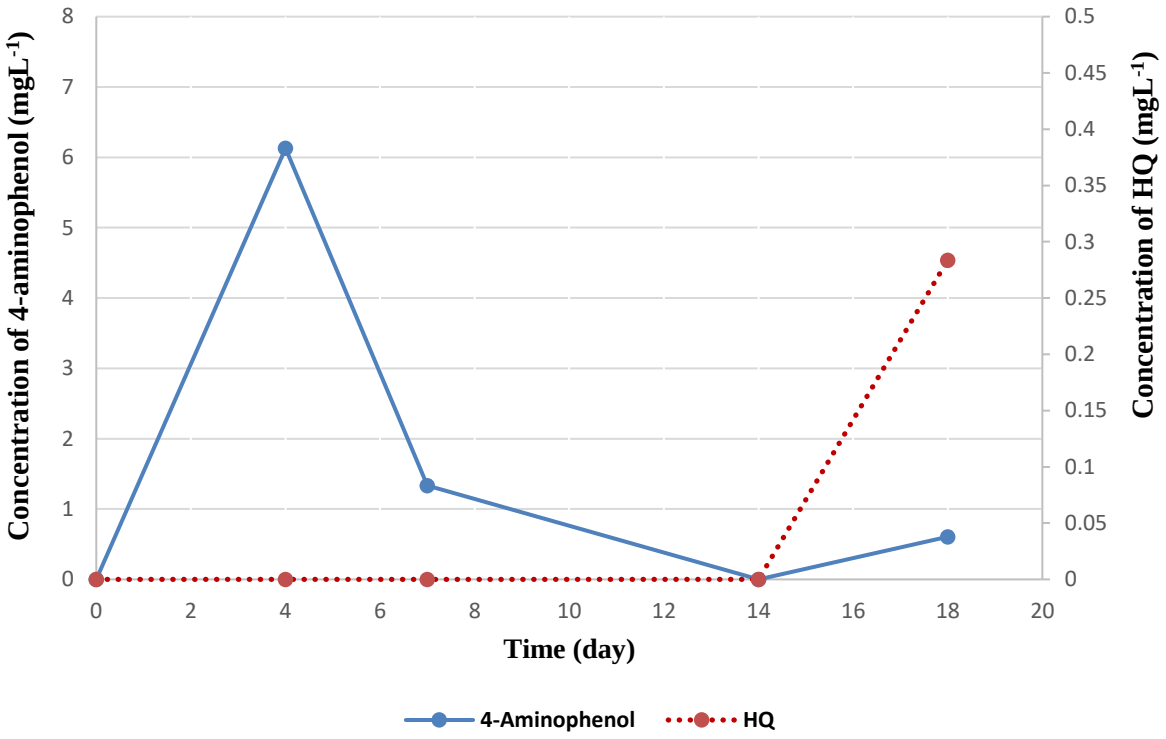


Figure 3.21. Concentration of 4-aminophenol and HQ, the metabolic intermediates of APAP biodegradation, in MSM-APAP (500 mgL^{-1}) culture of *Bacillus* sp. (PDE3.1), as a function of time as monitored by GC-MS analysis.

3.5.3. Removal of 50 mgL^{-1} of APAP by potential APAP degrading isolates

In this trial it was aimed to study APAP removal by each of the seven potential APAP degrading selected isolates as well as the potential HQ degrading isolate, separately using MSM supplemented with a concentration of 50 mgL^{-1} of APAP. Four independent experiments were conducted in which five isolates with common control were tested at a time (Figure 3.22), then each of the remaining isolates at a time (Figures 3.24-3.26). Figure 3.27 shows the APAP concentrations at the end of all experiments.

Generally, the utilization of APAP as sole carbon source by the isolates was associated with deep brown coloration of the MSM-APAP broth and plates as shown for *Bacillus* sp. (PDE3.1) in Figure 3.23, and as reported previously for the enrichment cultures of most of the investigated APAP removing microbial consortia.

In the first experiment, apparently there was no significant APAP removal until day 10 neither in the negative control nor in the tests with the isolates. Then, between day 10 and day 14 some removal occurred in the control, indicating abiotic degradation, but greater removal was observed in all tests, thus suggesting the biodegrading capacity of all isolates. Thereafter, APAP concentration decreased only in *Paraburkholderia* sp. (PLA3.3) cultures, indicating that in this case removal may have continued until the end of the experiment (after 23 days). Among the first five investigated isolates, *Rhizobium* sp. (PDE3.3) and *Paraburkholderia* sp. (PLA3.3) seemed the most promising for APAP removal where APAP %removal was 49±4 and 47.9±0.9, respectively, relative to the zero-time samples where APAP %removal was 21±1 (Figure 3.22).

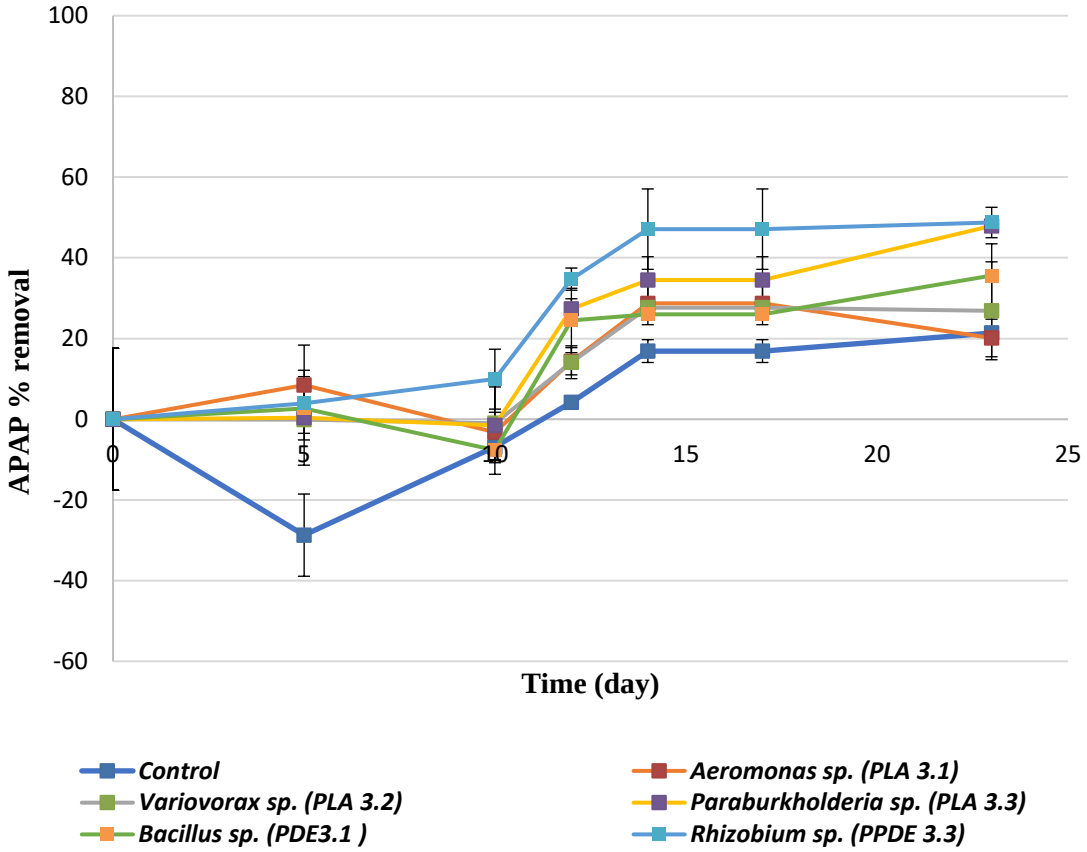
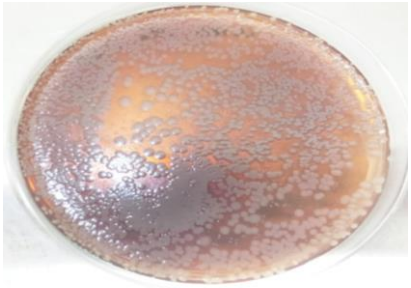


Figure 3.22. APAP %removal as a function of time in MSM-APAP (50 mgL^{-1}) cultures of five Poderosa and Lousal mines bacterial isolates separately, and in the negative control using HPLC analysis. The error bars represent the average deviation for two replicates. When error bars are not visible, it means they are smaller than the data point markers.

(a)



(b)

Figure 3.23. (a) Brownish coloration of MSM-APAP (500 mg L^{-1}) culture of Poderosa mine *Bacillus* sp. isolate (PDE3.1), compared with the negative control medium after 7-days of incubation at 25 °C at160 rpm, (b) Brownish coloration of MSM-APAP (500 mgL^{-1}) plate by the same isolate.

No APAP removal was observed for *Bacillus* sp. (PLC2.1) and *Niallia* sp. (PLC2.3) (Figure 3.24 & Figure 3.25, respectively). Interestingly, the HQ removing Poderosa mine *Mycolicibacterium* sp. isolate (HPB1.1) (as will be shown in Section 3.5.5) also showed APAP removal. As shown in Figure 3.26, after 21 days, APAP %removal was 41.6±0.1 in the MSM cultures, while in the negative control it was 13±1. As shown in Figure 3.27, *Mycolicibacterium* sp. (HPB1.1) was superior to *Bacillus* sp. (PLC2.1) and *Niallia* sp. (PLC2.3) in terms of APAP removal. Moreover, being able to remove HQ makes *Mycolicibacterium* sp. (HPB1.1) together with *Rhizobium* sp. (PDE3.3) and *Paraburkholderia* sp. (PLA 3.3) interesting for the future works of whole genome sequencing and search for genes responsible for degradative pathways.

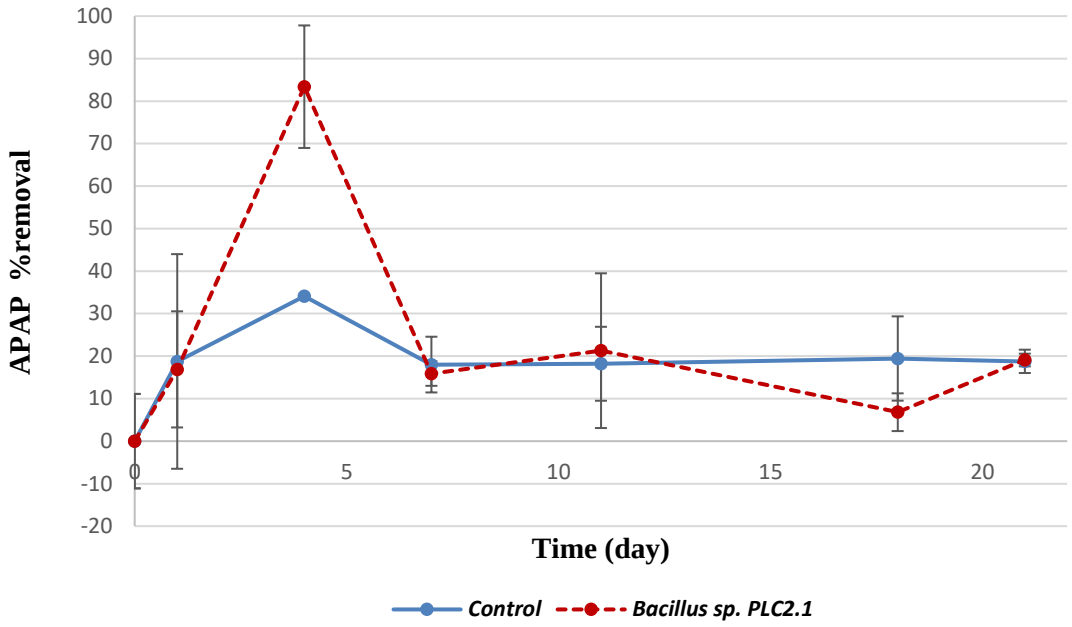


Figure 3.24. APAP %removal as a function of time in MSM-APAP (50 mgL^{-1}) cultures of *Bacillus* sp. (PLC2.1), and in the negative control using HPLC analysis. The error bars represent the average deviation for two replicates. When error bars are not visible, it means they are smaller than the data point markers.

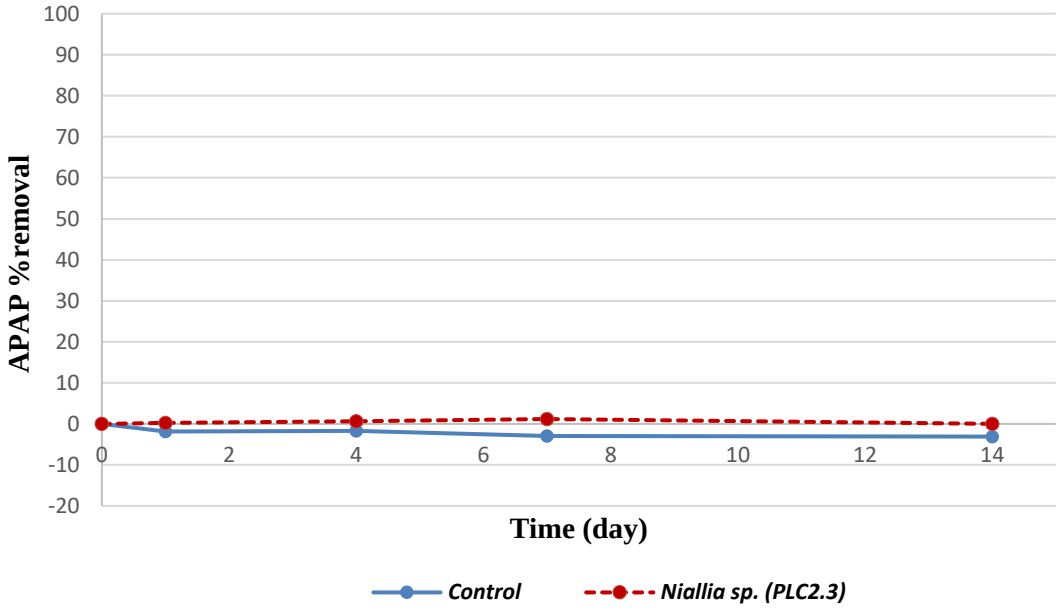


Figure 3.25. APAP %removal as a function of time in MSM-APAP (50 mgL^{-1}) cultures of *Niallia* sp. (PLC2.3), and in the negative control using HPLC analysis. The error bars representing the average deviation for two replicates are not visible as they are smaller than the data point markers.

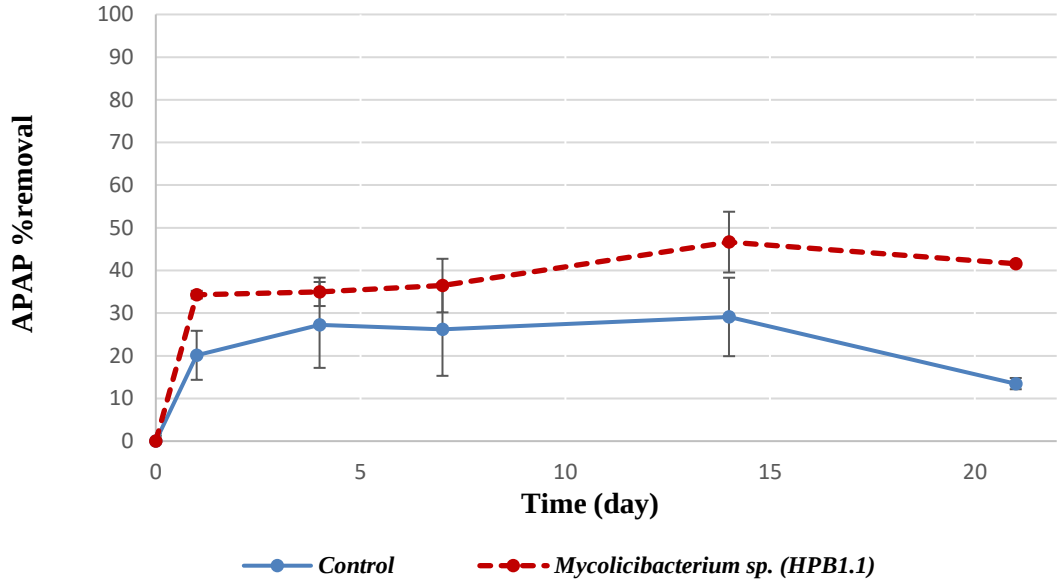


Figure 3.26. APAP %removal as a function of time in MSM-APAP (50 mgL^{-1}) culture of *Mycolicibacterium* sp. **(**HPB1.1), and in the negative control using HPLC analysis. The error bars represent the average deviation for two replicates. When error bars are not visible, it means they are smaller than the data point markers.

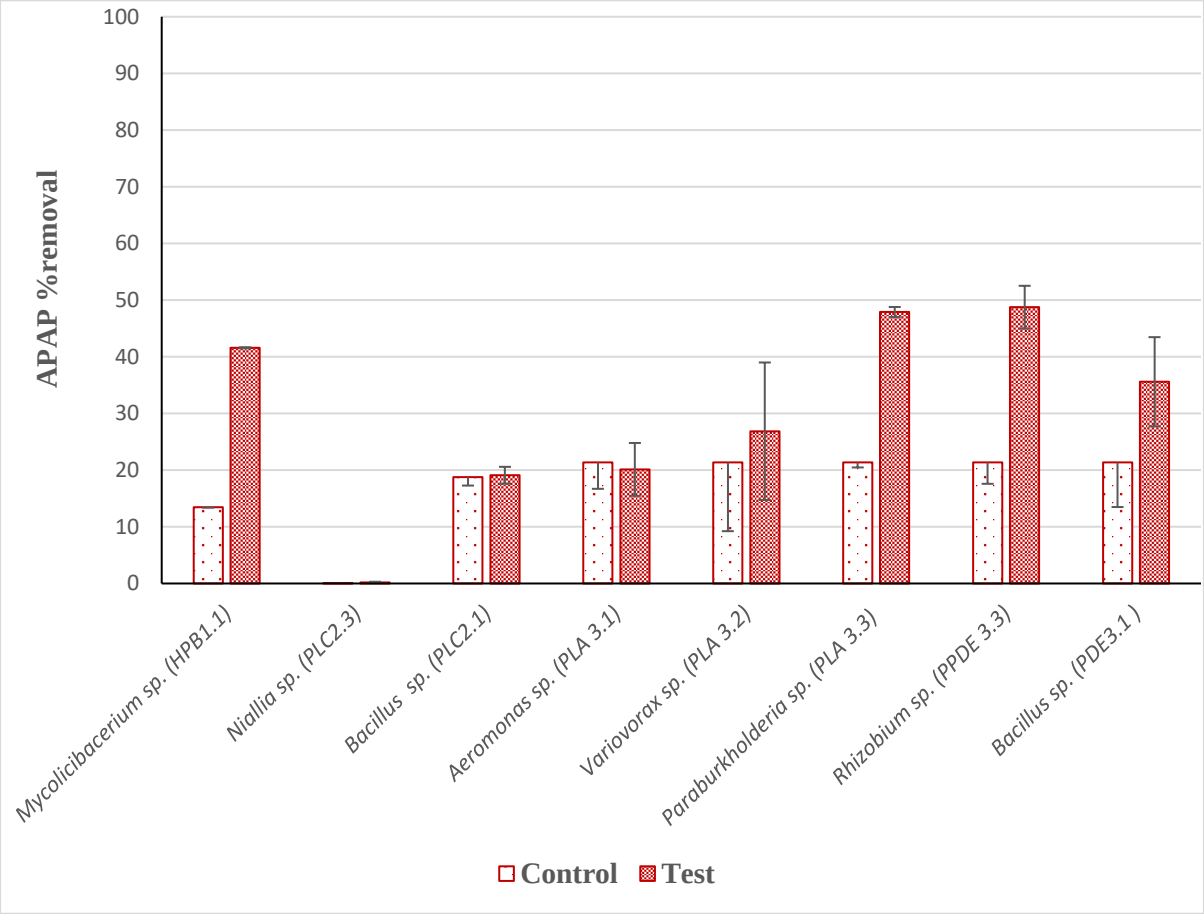


Figure 3.27. APAP %removal in MSM-APAP (50 mgL^{-1}) (Control) and in the presence of isolates (Test) at the end of the biodegradation experiment. The error bars represent the average deviation for two replicates. When error bars are not visible it means they are smaller than the data point markers.

3.5.4. APAP removal using the co-culture approach

It was aimed in this experiment to improve APAP removal using mixed cultures in comparison with pure isolate cultures. The co-culture of the three most promising isolates (*Rhizobium* sp. (PDE3.3), *Paraburkholderia* sp. (PLA3.3), and *Bacillus* sp. (PDE3.1)) was associated with brownish coloration (Figure 3.28) and showed APAP %removal of 39±2 compared with 16±2% in the negative control after 21 days (Figure 3.29 & Figure 3.30); hence, was considered superior to the co-culture representing all the seven potential APAP biodegrading isolates and for which APAP %removal of 12±2 was reported, which seemed almost same as it in the negative control where APAP %removal was 16±2. Therefore, joining the seven isolates in the same test somehow inhibited the biodegradation capacity that they had independently revealed, and even the mixed culture strategy with the three most promising isolates didn't improve APAP removal compared with using pure cultures of isolates; hence,

the combined isolates were not proven as an efficient bacterial consortium for APAP removal. According to Deng & Wang (2016), it is the complexity of the substrate that determines if the bacterial interaction in a mixed culture is synergistic or antagonistic; hence, a complex substrate can promote synergistic interactions, while a simple substrate such as glucose promotes negative interactions and competition.

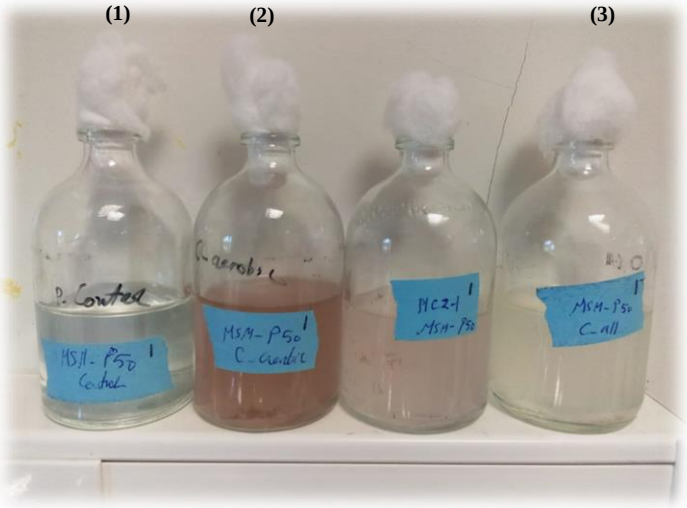


Figure 3.28. Co-culture of the most promising isolates (2) with more intense brownish coloration compared with the co-culture of the seven potential APAP biodegrading isolates (3), in MSM-APAP (50 mgL^{-1}), after 21 days. Negative control (1).

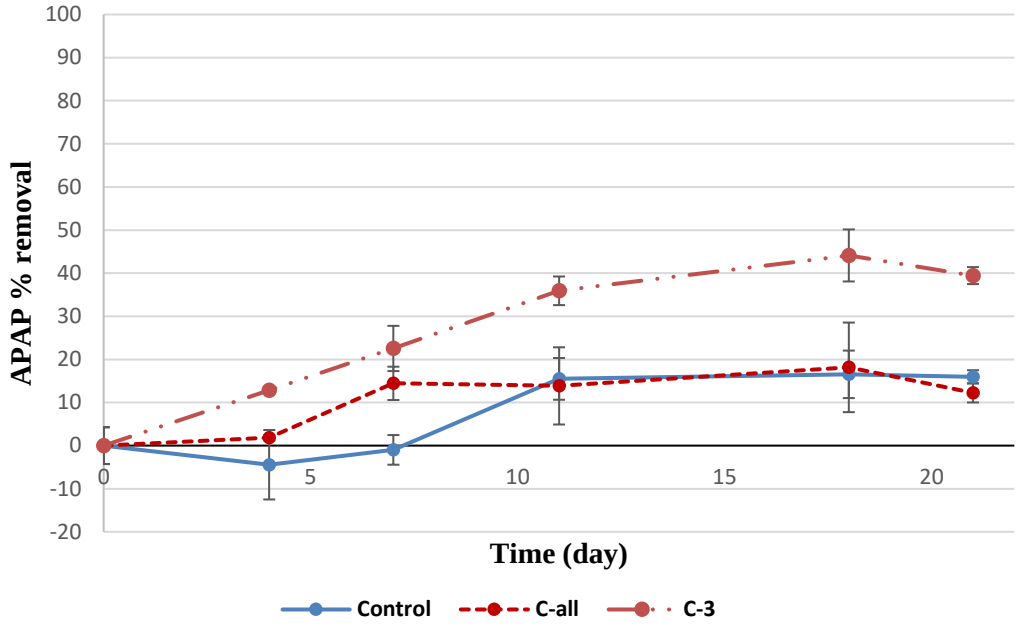


Figure 3.29. APAP % removal as a function of time in MSM-APAP (50 mgL^{-1}) co-cultures supplemented with 50 mgL^{-1} of APAP and in the negative control. C-all: co-culture of all isolates, C-3: co-culture of the three most promising isolates. The error bars represent the average deviation for two replicates. When error bars are not visible, it means they are smaller than the data point markers.

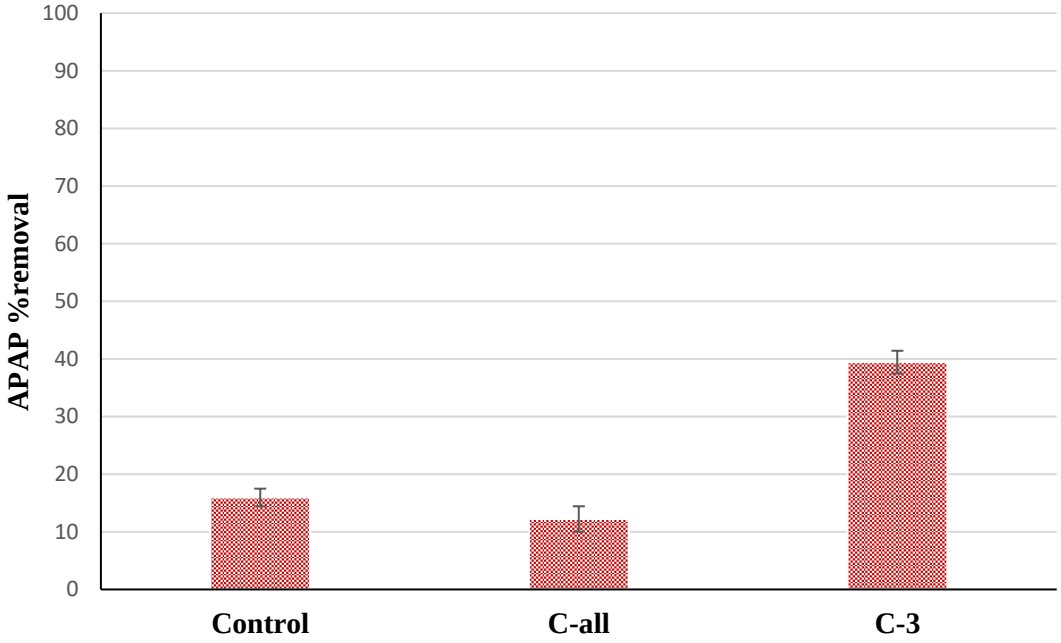


Figure 3.30. APAP %removal after 21 days in MSM-APAP (50 mgL^{-1}) using the co-culture approach. C-all: co-culture of the seven potential APAP biodegrading isolates. C-3: co-culture of the three most promising isolates. The error bars represent the average deviation for two replicates. When error bars are not visible, it means they are smaller than the data point markers.

3.5.5. HQ removal by Mycolicibacterium sp. (HPB1.1)

Poderosa mine isolate *Mycolicibacterium* sp. (HPB1.1) was the only HQ removing isolate in the current study. As shown in Figure 3.31, HQ removal from MSM supplemented with 50 mgL^{-1} of HQ was not associated with the brown coloration observed for the negative control. This brown coloration appeared very quickly, indicating that the medium's physical-chemical characteristics potentiated HQ oxidative polymerization. The zero-time samples revealed HQ concentration of 29 mgL^{-1} much lower than the amount initially added to the medium (50 mgL^{-1}). Interestingly, after four days at 25 °C under shaking conditions (160 rpm), no HPLC peak was reported for HQ in MSM culture of *Mycolicibacterium* sp. (HPB1.1), and considering that instrument LOD for HQ, previously reported in Section 3.1.3, was 3.6 mgL^{-1}; hence, *Mycolicibacterium* sp. (HPB1.1) realized at least 88% removal of HQ (i.e.,> 25 mgL^{-1}), while in the negative control the abiotic HQ %removal corresponded to 24±7% (Figure. 3.32). This observation coincides with the reports relating the single chain HQ 1,2-dioxygenases di-oxygenases to the genus *Mycobacterium* (Enguita & Leitão, 2013) as which the genus *Mycolicibacterium* was classified till 2018 (Gupta et al., 2018).

Poderosa mine *Bacillus* sp. isolate (PDE3.1), one of the APAP degrading isolates, was tested under the same conditions for HQ biodegradation using HPLC analysis; however, no degradation was observed after 21 days (data not shown).

Figure 3.31. MSM-HQ (50 mgL^{-1}) culture of *Mycolicibacterium* sp. **(**HPB1.1) (on the right) after seven days, showing no color change compared with the brownish coloration of the negative control medium (on the left).

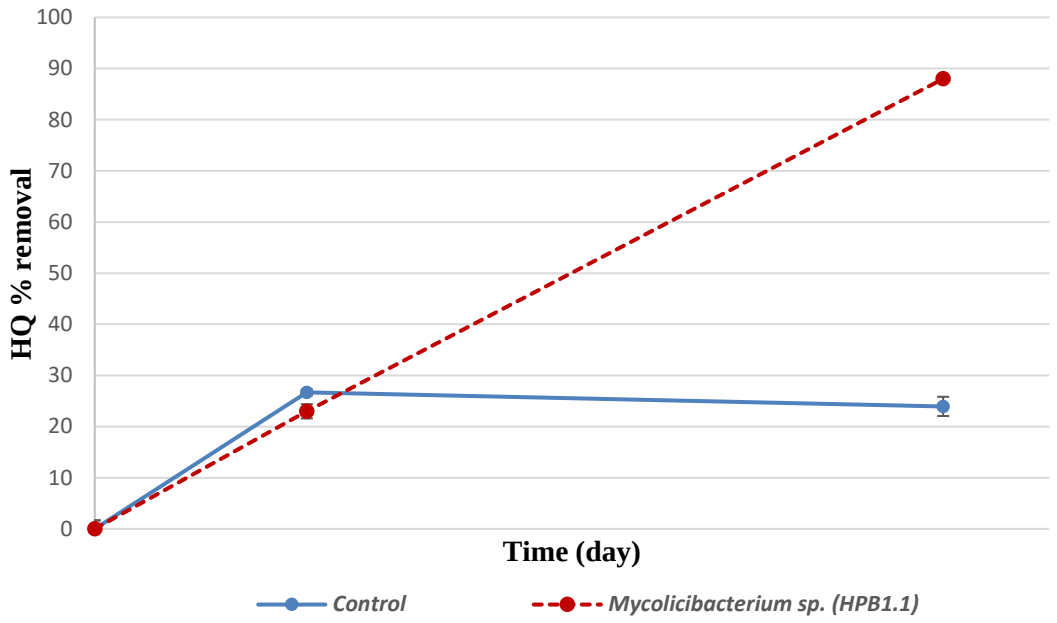


Figure 3.32. HQ %removal by Poderosa mine isolate *Mycolicibacterium* sp. (HPB1.1) after 4 days from MSM-HQ (50 mgL^{-1}), as monitored by HPLC analysis. The error bars represent the average deviation for two replicates. When error bars are not visible, it means they are smaller than the data point markers.

3.6. Growth kinetics of the selected study strain

The selected studied strain *Mycolicibacterium* sp. (HPB1.1) was grown on LB medium (pH 7) at 25 °C under shaking conditions (160 rpm) to study batch culture growth kinetics. Cells grew exponentially without an observable lag phase (Figure 3.33). Exponential growth, associated with the most rapid growth with determination coefficient R^2 of 0.99, extended till 36 h where maximal growth was attained (OD_{600} of 1.27). The specific growth rate (μ= 0.047 h^{-1}) during the exponential growth was calculated as the slope of the exponential phase line using MS-Excel 2010. Then, as observed in Figure 3.33, a deceleration or negative growth acceleration phase (where the growth rate is lower than it in the exponential phase) seemed to follow the exponential growth, and it extended almost from 36 h till 42 h where the stationary phase begins and extended till 68 h after which the OD_{600} showed decrease. This experiment is considered important for standardizing the inoculum cultures prepared before the biodegradation studies of the future work using this strain.

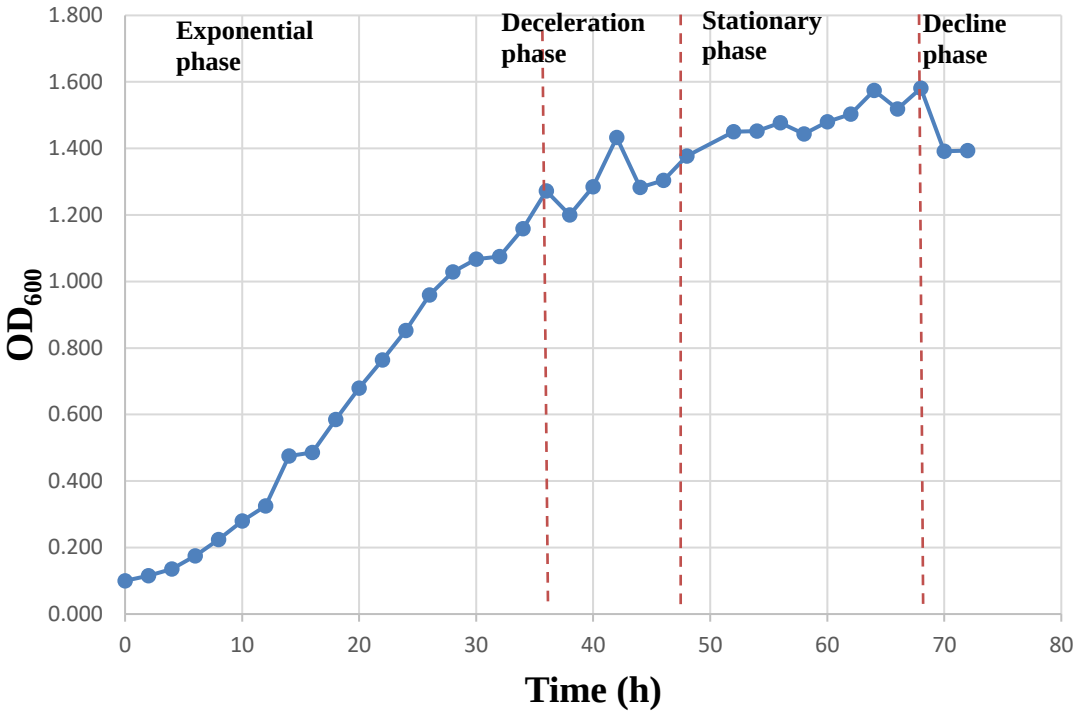


Figure 3.33. The Growth curve of the study strain *Mycolicibacterium* sp. (HPB1.1).

3.7. Conclusion

Based on the current study results, it can be inferred that the extreme environments of The Iberian Pyrite Belt mines should be further explored for microbes able to biodegrade emerging pollutants in general and pharmaceutical pollutants specifically, as implied by the 16S rRNA sequence analysis-based taxonomic classification of the study isolates, according to which it was found that most of the isolates had not been reported before in the literature for APAP biodegradation. This includes Lousal mine isolate *Paraburkholderia* sp. (PLA3.3), as well as Poderosa mine isolates *Rhizobium* sp. (PDE3.3), and *Mycolicibacterium* sp. (HPB1.1) which is also reported for the first time through the current study for HQ removal. Based on APAP %removal of almost 42 and HQ % removal of almost 88, *Mycolicibacterium* sp. (HPB1.1) was selected for the future work of probable plasmid-mediated bioaugmentation. Even Lousal mine isolates *Aeromonas* sp. (PLA3.1), *Niallia* sp. (PLC2.3), and *Variovorax* sp. (PLA3.2), which seemed able to utilize APAP as sole carbon source in MSM plates but didn't show considerable APAP removal in MSM broth, hadn't been reported earlier in the literature for APAP biodegradation or removal. Moreover, comparing APAP biodegradation experiments conducted separately using the Poderosa mine isolate *Bacillus* sp. (PDE3.1) in MSM at APAP initial concentrations of 500 mgL^{-1} and 50 mgL^{-1}, it was shown that APAP removal was higher

at the higher initial APAP concentration; hence, further optimization may be needed to enhance APAP biodegradation at environmentally relevant concentrations, and also to stimulate the biodegrading capacities in strains that showed this ability on MSM plates but not in MSM broth. In addition, for monitoring the intermediate metabolites of APAP biodegradation by the *Bacillus* sp. (PDE3.1), 4-aminophenol and HQ were successfully detected by GC-MS analysis. Accordingly, detection of 4-aminophenol or HQ in MSM culture of *Bacillus* sp. (PDE3.1) suggests that APAP biodegradation may have occurred through the initial decarboxylation of APAP into 4-aminophenol in which amino group is then replaced by the hydroxyl group; thus, producing HQ.

3.8. Future work based on the current study

The next step will be the genomic sequencing of the *Mycolicibacterium* sp. (HPB1.1), aiming to achieve complete coverage of the whole chromosome and any plasmid (if possible), and to search for genes with a potential role in the degradation of APAP and HQ. The presence of degradative genes in plasmids would allow the possibility of running plasmid-mediated genetic bioaugmentation of WWTP sludge, aiming to overcome the drawbacks of conventional bioaugmentation, which may fail due to the rapid decrease in the viability of the introduced microbial cells, by stimulating the spread of APAP and HQ degradation genes among the indigenous bacterial population of WWTP systems. In any case, even if the genes are not found in plasmidic DNA, revealing their sequences and the flanking genomic areas will contribute to the knowledge of metabolic pathways with a role in the biodegradation of these compounds. Furthermore, the genomic knowledge of species with biotechnological potential allows the development of genetic markers for their monitorization, for example in bioaugmentation assays, using easy-to-implement techniques such as PCR-based analyses.

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5. APPENDICES

Appendix 1: NCBI BLAST alignment hits

***Aeromonas* sp. (PLA3.1)**

>13_16S_Contig-PLA3-1

GTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAAT
GCCTGGGGATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGC
CCTACGGGGGAAAGGAGGGGACCTTCGGGCCTTTCGCGATTGGATGAACCCAGGTGGGATTAG
CTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGC
CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAA
TGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTT
TCAGCGAGGAGGAAAGGTTGGCGCCTAATACGTGTCAACTGTGACGTTACTCGCAGAAGAAGC
ACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACT
GGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGG
AATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGT
GAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACG
CTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG
ATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGC
CTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCTGGGATCCC
CTAGAGATACGGGAGTGCCTTCGGGAATCAGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGT
GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTA
ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGT
CATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGCGCGTACAGAGGGCTGCAAG
CTAGCGATAGTGAGCGAATCCCAAAAAGCGCGTCGTAGTCCGGATCGGAGTCTGCAACTCGACT
CCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCT
TGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGATAGCTTAACCTTCGG
G

Scientific Name	*Max Score*	*Total Score*	*Query Cover*	*E value*	*% Identity*
Aeromonas salmonicida	2566	2566	100%	0.0	99.79
A. salmonicida	2566	2566	100%	0.0	99.79
A. salmonicida	2566	2566	100%	0.0	99.79
A. salmonicida subsp. masoucida	2555	2555	100%	0.0	99.64
Haemophilus piscium	2555	2555	100%	0.0	99.64
A. salmonicida subsp. masoucida	2555	2555	100%	0.0	99.64
Aeromonas piscicola	2555	2555	100%	0.0	99.64
Aeromonas bestiarum	2555	2555	100%	0.0	99.64
A. salmonicida subsp. pectinolytica	2553	2553	100%	0.0	99.64
A. salmonicida subsp. achromogenes	2547	2547	100%	0.0	99.57%

***Bacillus* sp. (PDE3.1)**

>2_16S_Contig_PDE3-1

GTCGAGCGAATGGATTAGAGAGCTTGCTCTTAAGAAGTTAGCGGCGGACGGGTGAGTAACACG
TGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTT
TGAACTGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGC
ATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTG
ATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTT
CCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAA
AACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACC
AGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCG
GAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCA
ACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGT
GTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGT
AACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAA
GCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCT
CTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCC
ATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACG
TCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCT
GCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACT
CGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCG
GGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACC
TTTATGGAGCCAGCCGC

Scientific Name	***Max Score***	***Total Score***	***Query Cover***	***E value***	***% Identity***
Bacillus toyonensis	2603	2603	100%	0.0	99.86
Bacillus wiedmannii	2603	2603	100%	0.0	99.86
Bacillus sanguinis	2597	2597	100%	0.0	99.79
Bacillus proteolyticus	2597	2597	100%	0.0	99.79
Bacillus pacificus	2597	2597	100%	0.0	99.79
Bacillus mobilis	2597	2597	100%	0.0	99.79
Bacillus thuringiensis	2597	2597	100%	0.0	99.79
Bacillus fungorum	2597	2597	100%	0.0	99.79
Bacillus thuringiensis	2597	2597	100%	0.0	99.79
B. thuringiensis	2593	2593	100%	0.0	99.72

Bacillus sp. (PLC2.1)

>PLC_2-1_b_16S

TTGAGAGTTTTGATTCCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATG
GATTGAGAGCTTGCTCTCATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGAC
TGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATTTTGAACTGCATGGTTCGAAATTGAAAGGC
GGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGC
AACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGG
CTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTA
CCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCG
GAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGG
AGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATG
CGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAG
GGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAA
ACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAA
CCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGT
GGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCT
TAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACG
TCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGAC
CGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGC
TGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGTGCCTTGTACACACCGCCCGT
CACACCACGAGAGTTGCTCTCAGAGTTTGATCCTGGCTCAGAAGTCGAACAAGGATTCAGAGAGACATGCG
TAGTGTC

Scientific Name	*Max Score*	*Total Score*	*Query Cover*	*E value*	*% Identity*
B. mobilis	2603	2603	95%	0.0	99.65
B. toyonensis	2595	2595	95%	0.0	99.65
B. sanguinis	2593	2593	95%	0.0	99.51
B. thuringiensis	2593	2593	94%	0.0	99.79
B. proteolyticus	2591	2591	95%	0.0	99.51
B. pacificus	2591	2591	95%	0.0	99.51
Bacillus paranthracis	2591	2591	95%	0.0	99.51
B. wiedmannii	2591	2591	95%	0.0	99.51
B. cereus ATCC 14579	2588	2588	95%	0.0	99.44
B. fungorum	2588	2588	95%	0.0	99.44

***Mycolicibacterium* sp. (HPB1.1)**

>HPB_1-1_16S_clean

TCGAACGGAAAGGCCCTTCGGGGTACTCGAGTGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGCA
CTTTGGGATAAGCCTGGGAAACTGGGTCTAATACCGAATAGGACTACGCTCTTCATGGGGTGTGGTGGAAA
GCTTTTGCGGTGTGGGATGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGA
CGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTC
GGGTTGTAAACCTCTTTCACCAGGGACGAAGCGCAAGTGACGGTACCTGGAGAAGAAGCACCGGCCAACT
ACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTA
GGTGGTTTGTCGCGTTGTTCGTGAAAACTCACAACTCAATTGTGGGCGTGCGGGCGATACGGGCAGACTAG
AGTACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGGTG
GCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGGTGGGTACTAGGTGTGGGTTCCTTCCTTGGGATCCGTGCCGTAGCTAA
CGCATTAAGTACCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCA
CAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATGCACAGGAC
GCTGGTAGAGATATCAGTTCCCTTGTGGCCTGTGTGCAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGA
GATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTATGTTGCCAGCGGGTTATGCCGGGGACTCG
TAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGTCCAGG
GCTTCACACATGCTACAATGGCCGGTACAAAGGGCTGCGATGCCGTGAGGTGGAGCGAATCCTTTCAAAGC
CGGTCTCAGTTCGGATCGGGGTCTGCAACTCGACCCCGTGAAGTCGGAGTCGCTAGTAATCGCAGATCAGC
AACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGTAACACCCG
AAGCCGGTGGCCTAACCCTTGTGGAGGGAGC

Scientific Name	***Max Score***	***Total Score***	***Query Cover***	***E value***	***% Identity***
Mycolicibacterium aubagnense	2542	2542	100%	0.0	100.00
Mycolicibacterium phocaicum	2464	2464	100%	0.0	98.98
Mycolicibacterium mucogenicum	2464	2464	100%	0.0	98.98
M. mucogenicum	2464	2464	100%	0.0	98.98
Mycolicibacterium canariasense	2433	2433	100%	0.0	98.55
Mycolicibacterium llatzerense	2431	2431	99%	0.0	98.69
Mycobacterium diernhoferi	2427	2427	100%	0.0	98.48%
Mycolicibacterium neoaurum ATCC 25795	2425	2425	100%	0.0	98.48%
M. neoaurum ATCC 25795	2425	2425	100%	0.0	98.48%
Mycolicibacterium cosmeticum	2416	2416	100%	0.0	98.33%

***Niallia* sp. (PLC2.3)**

>**PLC2-3**_contig~clean

TTAAAAAGCTTGCTTTTTAAGTTAGCGGCGGACGGGGTGAGTAACACGTGGGCAACCTGCCTGT
AAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAATCCTTTTCTACTCATGTAGAA
AAGCTGAAAGACGGTTTACGCTGTCACTTACAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGA
GGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGA
CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAG
TCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGA
AGAACAAGTACGAGAGTAACTGCTCGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAG
CGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGG
AAACTGGGGGACTTGAGTGCAGAAGAGAAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTA
GAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGTCTGTAACTGACGCTGAGGCGCG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTA
AGTGTTAGAGGGTTTCCGCCCTTTAGTGC

Scientific Name	*Max Score*	*Total Score*	*Query Cover*	*E value*	*% Identity*
Niallia circulans subsp. circulans	348	348	100%	8e-96	98.00
N. circulans subsp. circulans	348	348	100%	8e-96	98.00
N. circulans	348	348	100%	8e-96	98.00
N. circulans	348	348	100%	8e-96	98.00
Cytobacillus solani	342	342	100%	4e-94	97.50
Cytobacillus praedii	342	342	100%	4e-94	97.50
N. circulans	342	342	100%	4e-94	97.50
Neobacillus ginsengisoli	342	342	100%	4e-94	97.50
Niallia nealsonii	339	339	100%	5e-93	97.03
Cytobacillus ciccensis	337	337	100%	2e-92	97.00

***Paraburkholderia* sp. (PLA3.3)**

>12_16S_Contig_PLA3-3

GTCGAACGGCAGCACGGGGGCAACCCTGGTGGCGAGTGGCGAACGGGTGAGTAATACATCGGA
ACGTGTCCTGTAGTGGGGGATAGCCCGGCGAAAGCCGGATTAATACCGCATACGCTCTACGGAG
GAAAGGGGGGGATCTTAGGACCTCCCGCTACAGGGGCGGCCGATGGCAGATTAGCTAGTTGGT
GGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCTGGTCTGAGAGGACGACCAGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGCA
ACCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCCGG

APPENDICES

AAAGAAAACGCCGTGGTTAATACCCGTGGCGGATGACGGTACCGGAAGAATAAGCACCGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAA
AGCGTGCGCAGGCGGTCCGCTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATT
TGTGACTGGCGGGCTAGAGTATGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCG
TAGAGATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGCCAATACTGACGCTCATGCA
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAAC
TAGTTGTCGGGTCTTCATTGACTTGGTAACGTAGCTAACGCGTGAAGTTGACCGCCTGGGGAGT
ACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGG
ATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGTATGGAATCCTGCTGAGAGG
TGGGAGTGCCCGAAAGGGAGCCATAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGA
GATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCTAGTTGCTACGCAAGAGCACTCT
AGGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTA
TGGGTAGGGCTTCACACGTCATACAATGGTCGGAACAGAGGGTCGCCAACCCGCGAGGGGGAG
CCAATCCCAGAAAACCGATCGTAGTCCGGATCGCACTCTGCAACTCGAGTGCGTGAAGCTGGAA
TCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCC
GTCACACCATGGGAGTGGGTTTTACCAGAAGTGGCTAGTCTAACCGCAAGGAGGACGGT

Scientific Name	***Max Score***	***Total Score***	***Query Cover***	***E value***	***% Identity***
Paraburkholderia fungorum	2571	2571	100%	0.0	100.00
P. fungorum	2564	2564	100%	0.0	99.86
Paraburkholderia phytofirmans PsJN	2494	2494	100%	0.0	98.99
P. phytofirmans PsJN	2483	2483	100%	0.0	98.85
Paraburkholderia phenazinium	2460	2460	100%	0.0	98.56
Paraburkholderia aromaticivorans	2457	2457	100%	0.0	98.49
Paraburkholderia megapolitana	2455	2455	100%	0.0	98.49
Paraburkholderia dioscoreae	2455	2455	100%	0.0	98.49
Paraburkholderia strydomiana	2449	2449	99%	0.0	98.63
Paraburkholderia rhynchosiae	2446	2446	100%	0.0	98.35

***Rhizobium* sp. (PDE3.3)**

>10_16S_Contig_PDE3-3

GTCGAGCGCCCCGCAAGGGGAGCGGCAGACGGGTGAGTAACGCGTGGGAATCTACCCTTTTCT
ACGGAATAACGCAGGGAAACTTGTGCTAATACCGTATGTGTCCTTCGGGAGAAAGATTTATCGG
GAAAGGATGAGCCCGCGTTGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATC
CATAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
GAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTG
ATGAAGGCCCTAGGGTTGTAAAGCTCTTTCACCGGAGAAGATAATGACGGTATCCGGAGAAGA
AGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATT
ACTGGGCGTAAAGCGCACGTAGGCGGATCGATCAGTCAGGGGTGAAATCCCAGGGCTCAACCC
TGGAACTGCCTTTGATACTGTCGATCTGGAGTATGGAAGAGGTGAGTGGAATTCCGAGTGTAGA
GGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGTCCATTACTGA
CGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGAATGTTAGCCGTCGGGCAGTATACTGTTCGGTGGCGCAGCTAACGCATTAAACATTCCG
CCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTG

GAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCCCTTGACATCCTGTGTTACC
TCTAGAGATAGGGGGTCCACTTCGGTGGCGCAGAGACAGGTGCTGCATGGCTGTCGTCAGCTCG
TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCTTAGTTGCCAGCATTT
AGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGCCGAGAGGAAGGTGGGGATGACGTCAAGT
CCTCATGGCCCTTACGGGCTGGGCTACACACGTGCTACAATGGTGGTGACAGTGGGCAGCGAGC
ACGCGAGTGTGAGCTAATCTCCAAAAGCCATCTCAGTTCGGATTGCACTCTGCAACTCGAGTGC
ATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTG
TACACACCGCCCGTCACACCATGGGAGTTGGTTTTACCCGAAGGTAGTGCGCTAACCGCAAG

Scientific Name	*Max Score*	*Total Score*	*Query Cover*	*E value*	*% Identity*
Rhizobium calliandrae	2451	2451	100%	0.0	99.85
Agrobacterium rhizogenes	2440	2440	100%	0.0	99.70
Ag. rhizogenes	2440	2440	100%	0.0	99.70
Rhizobium lusitanum	2438	2438	100%	0.0	99.70
Rhizobium multihospitium	2435	2435	100%	0.0	99.62
Rhizobium mayense	2435	2435	100%	0.0	99.62
Rhizobium jaguaris	2435	2435	100%	0.0	99.62
Rhizobium tropici	2429	2429	100%	0.0	99.55
R. tropici CIAT 899	2429	2429	100%	0.0	99.55
R. tropici CIAT 899	2429	2429	100%	0.0	99.55

***Variovorax* sp. (PLA3.2)**

>9_16S_Contig_PLA3-2

GTCGAACGGCAGCGCGGGAGCAATCCTGGCGGCGAGTGGCGAACGGGTGAGTAATACATCGGA
ACGTGCCCAATCGTGGGGGATAACGCAGCGAAAGCTGTGCTAATACCGCATACGATCTACGGAT
GAAAGCAGGGGATCGCAAGACCTTGCGCGAATGGAGCGGCCGATGGCAGATTAGGTAGTTGGT
GAGGTAAAGGCTCACCAAGCCTTCGATCTGTAGCTGGTCTGAGAGGACGACCAGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAA
AGCCTGATCCAGCCATGCCGCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGG
AACGAAACGGCCTTTTCTAATAAAGAGGGCTAATGACGGTACCGTAAGAATAAGCACCGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAA
AGCGTGCGCAGGCGGTTATGTAAGACAGTTGTGAAATCCCCGGGCTCAACCTGGGAACTGCATC
TGTGACTGCATAGCTAGAGTACGGTAGAGGGGGATGGAATTCCGCGTGTAGCAGTGAAATGCG
TAGATATGCAGAGGAACACCGATGGCGAAGGCAATCCCCTGGACCTGTACTGACGCTCATGCA
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAAC
TGGTTGTTGGGTCTTCACTGACTCAGTAACGAAGCTAACGCGTGAAGTTGACCGCCTGGGGAGT
ACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGG
TTTAATTCGATGCAACGCGAAAAACCTTACCCACCTTTGACATGTACGGAATTCGCCAGAGATG
GCTTAGTGCTCGAAAGAGAGCCGTAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGA
GATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGCTACATTCAGTTGGGCA
CTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCC

APPENDICES

CTTATAGGTGGGGCTACACACGTCATACAATGGCTGGTACAAAGGGTTGCCAACCCGCGAGGG
GGAGCTAATCCCATAAAACCAGTCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTC
GGAATCGCTAGTAATCGTGGATCAGAATGTCACGGTGAATACGTTCCCGGGTCTTGTACACACC
GCCCGTCACACCATGGGAGCGGGTTCTGCCAGAAGTAGTTAGCTTAACCG

Scientific Name	***Max Score***	***Total Score***	***Query Cover***	***E value***	***% Identity***
Variovorax boronicumulans NBRC 103145	2527	2527	100%	0.0	99.64
V. boronicumulans NBRC 103145	2521	2521	100%	0.0	99.57
Variovorax paradoxus NBRC 15149	2510	2510	100%	0.0	99.42
Variovorax guangxiensis	2492	2492	100%	0.0	99.20
Variovorax ginsengisoli	2483	2483	100%	0.0	99.06
V. paradoxus	2481	2481	100%	0.0	98.99
V. paradoxus	2481	2481	100%	0.0	98.99
Variovorax robiniae	2477	2477	100%	0.0	98.99
Variovorax ureilyticus	2433	2433	100%	0.0	98.41
Variovorax soli	2405	2405	100%	0.0	98.05

Appendix 2: The removal of APAP in MSM by Poderosa and Lousal microbial consortia

Table 5.1. APAP removal in MSM enrichment cultures of the microbial consortia of Poderosa and Lousal mines, supplemented with 500 mgL^{-1} of APAP, using UV-vis molecular spectroscopy analysis.

	Residual APAP concentration (mgL^{-1})	***%Removal***
Zero-Time control	576	0
LA 1	541	6
LA 2	2	100
LA 3	92	84
LC 1	491	15
LC 2	533	7
LC 3	500	13
PDE 1	450	22
PDE 2	456	21
PDE 3	495	14
PF 1	501	13
PF 2	489	15
PF 3	489	15
PB 1	510	11
PB 2	451	22
PB 3	455	21

1,2,3: first, second, and third enrichment cultures.

Appendix 3: Tables describing the removal of APAP and HQ in MSM by the study isolates separately

Table 5.2. APAP removal as a function of time in MSM cultures of *Bacillus*. sp. (PDE3.1), supplemented with 500 mgL^{-1} of APAP and in the negative control using HPLC analysis.

	***Bacillus* sp. (PDE3.1)**				**Control**			
Time (day)	**Averaged residual APAP (mgL^{-1})**	**Averaged deviation (mgL^{-1})**	**Removed APAP (mgL^{-1})**	**%Removal**	**Averaged residual APAP (mgL^{-1})**	**Average deviation (mgL^{-1})**	**Removed APAP (mgL^{-1})**	**%Removal**
0	412	12.03	BD	BD	412	12	BD	BD
2	400	8.21	12	3	379	0.7	34	8
4	305	6.30	107	26	386	82	26	6
7	250	5.20	162	39	360	47	53	13
14	279	5.78	133	32	424	0.2	-12	-3
18	154	3.28	258	63	315	13	97	24

BD: below the limit of detection

Table 5.3. APAP removal as a function of time in MSM cultures of *Bacillus*. sp. (PLC2.1), supplemented with 500 mgL^{-1} of APAP and in the negative control using HPLC analysis.

	***Bacillus* sp. (PLC2.1)**				**Control**			
Time (day)	**Averaged residual APAP (mgL^{-1})**	**Average deviation (mgL^{-1})**	**Removed APAP (mgL^{-1})**	**%Removal**	**Averaged residual APAP (mgL^{-1})**	**Average deviation (mgL^{-1})**	**Removed APAP (mgL^{-1})**	**%Removal**
0	485	17.92	BD	BD	485	BD	BD	BD
2	463	93.74	23	5	450	68	36	7
7	470	13.36	15	3	508	38	-23	-5
14	440	23.02	46	9	499	6	-13	-3
21	447	3.31	39	8	569	0.00	-84	-17

BD: below the limit of detection

Table 5.4. The concentration of 4-aminophenol and HQ, the metabolic intermediates of APAP biodegradation, in MSM culture of *Bacillus* sp. (PDE3.1), supplemented with 500 mgL^{-1} of APAP as a function of time as monitored by GC-MS analysis.

Time (day)	**4-Aminophenol concentration (mgL^{-1})**	**HQ concentration (mgL^{-1})**
0	BD	BD
4	6	6×10^{-7}
7	1	5×10^{-11}
14	BD	6×10^{-5}
18	0.6	0.3

BD: below the limit of detection

Table 5.5. The residual APAP concentration in MSM cultures of five Poderosa and Lousal mines isolates, supplemented with 50 mgL^{-1} of APAP as a function of time as monitored by HPLC analysis.

	Residual APAP (mgL^{-1})					
Time (day)	***Aeromonas* sp. (PLA 3.1)**	***Variovorax* sp. (PLA 3.2)**	***Paraburkholderia* sp. (PLA 3.3)**	***Rhizhobium* sp. (PDE3.3)**	***Bacillus* sp. (PDE3.1)**	**Control**
0	65	65	65	65	65	65
5	60	66	65	63	64	84
10	68	66	66	59	70	70
12	56	56	48	43	49	63
14	47	47	43	35	48	54
17	47	47	43	35	48	54
23	52	48	34	34	42	51

Table 5.6. The removed APAP concentration in MSM cultures of five Poderosa and Lousal mine strains, supplemented with 50 mgL^{-1} of APAP as a function of time as monitored by HPLC analysis.

	Removed (mgL^{-1})					
Time (day)	***Aeromonas* sp. (PLA 3.1)**	***Variovorax* sp. (PLA 3.2)**	***Paraburkholderia* sp. (PLA 3.3)**	***Rhizhobium* sp. (PDE3.3)**	***Bacillus* sp. (PDE3.1)**	**Control**
0	BD	BD	BD	BD	BD	BD
5	6	-0.01	0.3	3	2	-19
10	-2	-0.7	-1	7	-5	-5
12	9	9	18	22	16	3
14	19	18	23	32	17	11
17	19	18	23	32	17	11
23	13	18	31	32	23	14

BD: below the limit of detection

Table 5.7. The %removal of APAP in MSM cultures of five Poderosa and Lousal mines isolates, supplemented with 50 mgL^{-1} of APAP as a function of time as monitored by HPLC analysis.

	%Removal					
Time (day)	***Aeromonas* sp. (PLA 3.1)**	***Variovorax* sp. (PLA 3.2)**	***Paraburkholderia* sp. (PLA 3.3)**	***Rhizhobium* sp. (PDE3.3)**	***Bacillus* sp. (PDE3.1)**	**Control**
0	BD	BD	BD	BD	BD	BD
5	8	-0.1	0.4	4	3	-29
10	-3	-1	-2	10	8	-7
12	14	14	27	35	24	4
14	29	28	35	47	26	17
17	29	28	35	47	26	17
23	20	27	48	49	36	21

BD: below the limit of detection

Table 5.8. The average deviation values of APAP residual concentration in MSM-APAP (50 mgL^{-1}) removal cultures of five Poderosa and Lousal mines isolates (Table 5.4).

Time (day)	***Aeromonas* sp. (PLA 3.1)**	***Variovorax* sp. (PLA 3.2)**	***Paraburkholderia* sp. (PLA 3.3)**	***Rhizhobium* sp. (PDE3.3)**	***Bacillus* sp. (PDE3.1)**	**Control**
0	12	11.52	11.52	11.52	11.52	11.52
5	6.49	2.23	7.70	0.58	5.14	6.67
10	3.24	5.89	7.93	4.86	1.71	2.56
12	2.21	2.66	1.65	1.80	5.22	0.96
14	3.46	1.75	3.77	6.52	0.44	1.85
17	3.46	1.75	3.77	6.52	0.44	1.85
23	3.05	7.94	0.58	2.47	5.15	0.83

Table 5.9. APAP removal as a function of time in MSM cultures of *Bacillus* sp. (PLC2.1), supplemented with 50 mgL^{-1} of APAP, and in the negative control using HPLC analysis.

	***Bacillus sp*. (PLC2.1)**				**Control**			
Time (days)	**Averaged residual APAP (mgL^{-1})**	**Average deviation (mgL^{-1})**	**Removed APAP (mgL^{-1})**	**%Removal**	**Averaged residual APAP (mgL^{-1})**	**Average deviation (mgL^{-1})**	**Removed APAP (mgL^{-1})**	**%Removal**
0	46	5.11	BD	BD	46	5.11	BD	BD
1	38	5.10	8	17	37	11.61	9	19
4	8	4.37	38	83	30	0.00	16	34
7	39	1.09	7	16	38	3.01	8	18
11	36	6.84	10	21	38	4.00	8	18
18	43	1.64	3	7	37	4.56	9	19
21	37	0.56	9	19	37	1.26	9	19

BD: below the limit of detection

Table 5.10. APAP removal as a function of time in MSM cultures of *Niallia* sp. (PLC2.3), supplemented with 50 mgL^{-1} of APAP, and in the negative control using HPLC analysis.

	***Niallia* sp. (PLC2.3)**				**Control**			
Time (day)	**Averaged residual APAP (mgL^{-1})**	**Average deviation (mgL^{-1})**	**Removed APAP (mgL^{-1})**	**%Removal**	**Averaged residual APAP (mgL^{-1})**	**Average deviation (mgL^{-1})**	**Removed APAP (mgL^{-1})**	**%Removal**
0	44	0.18	BD	BD	44	0.32	BD	BD
1	44	0.15	0.1	0.3	45	0.09	-1	-2
4	44	0.36	0.3	1	45	0.17	-1	-2
7	44	0.11	0.5	1	46	0.12	-1	-3
14	44	0.09	BD	0.01	46	0.03	-1	-3

BD: below the limit of detection

Table 5.11. APAP removal as a function of time in MSM culture of *Mycolicibacterium* sp. (HPB1.1), supplemented with 50 mgL^{-1} of APAP, and in the negative control using HPLC analysis.

	***Mycolicibacterium* sp. (HPB1.1)**				**Control**			
Time (day)	**Averaged residual APAP (mgL^{-1})**	**Average deviation (mgL^{-1})**	**Removed APAP (mgL^{-1})**	**%Removal**	**Averaged residual APAP (mgL^{-1})**	**Average deviation (mgL^{-1})**	**Removed APAP (mgL^{-1})**	**%Removal**
0	50	0.63	BD	BD	50	0.90	BD	BD
1	33	0.45	17	34	40	2.89	10	20
4	33	1.67	18	35	37	5.06	14	27
7	32	3.15	18	36	37	5.46	13	26
14	27	3.58	23	47	36	4.62	15	29
21	29	0.05	21	42	43	0.66	7	13

ND: below the limit of detection

Table 5.12. The effect of applying the co-culture approach on APAP residual concentration in MSM supplemented with 50 mgL^{-1} of APAP using HPLC analysis.

	Residual concentration (mgL^{-1})					
	C-all		**C-3**		**Control**	
Time (day)	**Residual APAP (mgL^{-1})**	**Average deviation (mgL^{-1})**	**Residual APAP (mgL^{-1})**	**Average deviation (mgL^{-1})**	**Residual APAP (mgL^{-1})**	**Average deviation (mgL^{-1})**
0	54	2.31	54	2.31	54	2.31
4	53	0.34	47	0.17	56	4.36
7	46	2.09	42	2.84	55	1.86
11	38	4.84	27	1.79	38	2.62
18	36	5.62	22	3.26	37	2.98
21	39	1.20	25	1.07	37	0.83

C-all: co-culture of the seven potential biodegrading isolates, C-3: co-culture of the three most promising isolates.

Table 5.13. The effect of applying the co-culture approach on APAP removed concentration from MSM supplemented with 50 mgL^{-1} of APAP, in terms of removal and %removal, using HPLC analysis.

	Removed concentration (mgL^{-1})			***%Removal***		
Time (day)	**C-all**	**C-3**	**Control**	**C-all**	**C-3**	**Control**
0	BD	BD	BD	BD	BD	BD
4	1	7	-2	2	13	-4
7	8	12	-0.5	14	23	-1
11	7	19	8	14	36	15
18	10	24	9	18	44	17
21	7	21	9	12	39	16

BD: below the limit of detection, C-all: co-culture of the seven potential APAP biodegrading isolates, C-3: co-culture of the three most promising isolates.

Table 5.14. HQ removal as a function of time in MSM cultures of Poderosa mine *Mycolicibacterium* sp. isolate (HPB1.1), supplemented with 50 mgL^{-1} of HQ, as monitored by HPLC analysis.

	***Mycolicibacterium* sp. (HPB1.1)**				**Control**			
Time (day)	**Averaged residual APAP (mgL^{-1})**	**Average deviation (mgL^{-1})**	**Removed APAP (mgL^{-1})**	**%Removal**	**Averaged residual APAP (mgL^{-1})**	**Average deviation (mgL^{-1})**	**Removed APAP (mgL^{-1})**	**%Removal**
0	29	1.67	BD	BD	29	1.67	BD	BD
1	22	1.39	7	23	21	0.57	8	27
4	BD	BD	29	100	22	1.88	7	24

ND: below the limit of detection