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SOFIA PIMENTEL ARAÚJO

BIODEGRADATION OF NITRO AND CHLORINATED AROMATIC COMPOUNDS FOR BIOREMEDIATION OF A CONTAMINATED INDUSTRIAL SITE

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Thesis presented to the Graduate Program in Civil Engineering at the Universidade Federal de Pernambuco, as a partial requirement for obtaining the title of Doctor in Civil Engineering.

Concentration Area: Environmental Technology and Water Resources.

Supervisor: Prof. Sávia Gavazza dos Santos Pessôa. Ph.D.

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Para mainha, tudo e sempre.

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ABSTRACT

When attempting to remediate contaminated sites it is important to have a good understanding of the sites' physical, chemical, and hydrogeological properties, so that flow of contaminants can be predicted and evaluated. The contribution of biological processes is also of significance and needs to be better understood to improve the remediation strategy and process. Aerobic biotransformations of contaminants are often observed in the shallow layers, while anaerobic biotransformations processes are established in the deeper, more anoxic layers, including groundwater. Aromatic compounds, such as pesticides, dyes, resins, and solvents, are a major group of contaminants originating from a variety of industries. Aiming to combine academic research with remediation projects carried out by the industry partner, this Ph.D. thesis intended to contribute to bioremediation projects considering complex pollutants, such as halogenated aromatic compounds. To evaluate the biodegradability of dichloronitrobenzene (DCNB), dichloroaniline (DCA), monochloroaniline (MCA) and aniline by native microbes from a contaminated site, we first enriched cultures inoculated with soil and groundwater from a contaminated site, under aerobic and anaerobic conditions, amended with the target compounds individually, in the range of 10 and 20 mg/L. In addition to the inoculum and the contaminants, the enrichments contained mineral medium and ethanol and lactate as the electron donors (the anaerobic one). The cultures were kept in Boston glass bottles of 250 mL (150 mL of headspace). By enriching the cultures through multiple re-feedings, we could start to describe the mechanisms of biotransformation and identify organisms potentially involved in the observed reactions. Under aerobic conditions, 2,3-DCA, 3,4-DCA, 2-MCA, 3-MCA, and 4-MCA were potentially mineralized by members of the *Pandoraea* and *Burkholderia-Caballero-Paraburkholderia* genera, while chloride and ammonium ions were released. The anaerobic enrichments revealed nitro reduction of the 2,3-, 3,4-, and 2,5-DCNB by fermentative bacteria through a likely co-metabolic process, forming DCA isomers. *Desulfitobacterium* may be implicated in nitro group reduction in these enrichments, however, further investigation is needed for confirmation. The 2,3- and 3,4-DCA were reductively dehalogenated by organohalide-respiring bacteria, specifically *Dehalobacter* and *Anaeromyxobacter* genera. The well-established and active anaerobic community is composed of fermentative bacteria, organohalide-respiring bacteria, sulphate-reducing bacteria, and archaea. In general, from our lab experiments, we can affirm that

bioremediation with native microbes from the site is a promising strategy to be applied in the contaminated area. Bioaugmentation seems not to be necessary, but biostimulation through addition of oxygen should be considered since natural attenuation under anaerobic conditions did not result in complete dechlorination and mineralize the contaminants.

Keywords: Dichloronitrobenzene; Dichloroaniline; Monochloroaniline; aerobic biodegradation; anaerobic biotransformation; bioremediation.

RESUMO

Para remediação de áreas contaminadas, o entendimento das propriedades físicas, químicas e hidrogeológicas dos locais é essencial para que o fluxo de contaminantes possa ser previsto e avaliado. Além disso, a contribuição dos processos biológicos é também significativamente importante e precisa ser compreendida para impulsionar a estratégia e o processo de remediação. A biotransformação aeróbia de contaminantes é frequentemente observada nas camadas mais superficiais do solo, enquanto os processos de biotransformação anaeróbia são estabelecidos nas camadas mais profundas e anóxicas, incluindo as águas subterrâneas. Compostos aromáticos, como pesticidas, corantes, resinas e solventes, são um grande grupo de contaminantes originários de uma variedade de indústrias. Com o objetivo de alinhar pesquisas acadêmicas com projetos de remediação realizados pela indústria parceira desta pesquisa, este trabalho pretende contribuir para projetos de biorremediação considerando poluentes complexos, como compostos aromáticos halogenados. Para avaliar a biodegradabilidade de dicloronitrobenzeno (DCNB), dicloroanilina (DCA), monocloroanilina (MCA) e anilina por microrganismos nativos de uma área industrial contaminada, culturas inoculadas com solo e água subterrânea do local em estudo foram enriquecidas sob condições aeróbias e anaeróbias, alimentadas com os compostos alvo individualmente, na faixa de 10 e 20 mg/L. Além do inóculo e dos contaminantes, os enriquecimentos continham meio mineral e etanol e lactato como doadores de elétrons (somente os anaeróbios). As culturas foram mantidas em frascos de vidro do tipo Boston de 250 mL (150 mL de headspace). Ao enriquecer as culturas através de múltiplas realimentações, foi possível descrever os possíveis mecanismos de biotransformação e identificar organismos potencialmente envolvidos nas reações observadas. Sob condições aeróbias, houve provável mineralização dos contaminantes 2,3-DCA, 3,4-DCA, 2-MCA, 3-MCA e 4-MCA por membros dos gêneros *Pandoraea* e *Burkholderia-Caballero-Paraburkholderia*, enquanto íons cloreto e amônio foram liberados no meio. Os enriquecimentos anaeróbios revelaram redução do grupo nitro dos isômeros de dichloronitrobenzeno (2,3-, 3,4- e 2,5-DCNB) por bactérias fermentadoras através de um provável processo co-metabólico, formando isômeros de DCA. É possível que organismos do gênero *Desulfitobacterium* estejam envolvidos na redução do grupo nitro nestes enriquecimentos; no entanto, mais investigações são necessárias para confirmação. A remoção do primeiro cloro dos isômeros 2,3- e 3,4-DCA se deu através da respiração de bactérias dos gêneros *Dehalobacter* e

Anaeromyxobacter. A comunidade anaeróbia bem estabelecida e ativa é composta por bactérias fermentativas, bactérias que respiram organohaletos, bactérias redutoras de sulfato e arqueias. Em geral, a partir de nossos experimentos de laboratório, pode-se afirmar que a biorremediação com microrganismos nativos do solo e da água subterrânea é uma estratégia promissora a ser aplicada na área industrial contaminada. Dessa forma, a bioaumentação pode não ser necessária, mas a bioestimulação através da adição de oxigênio deve ser considerada, uma vez que a atenuação natural em condições anaeróbias não houve mineralização dos contaminantes.

Palavras-chave: Dicloronitrobenzeno; Dicloroanilina; Monocloroanilina; biodegradação aeróbia; biotransformação anaeróbia; biorremediação.

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LIST OF ABBREVIATIONS

AC	Abiotic control for the experiments
ASV	Amplicon sequence variant
BLAST	Basic local alignment search tool
COI	Contaminants of interest
CONAMA	Conselho Nacional do Meio Ambiente
DCNB	Dichloronitrobenzene
DCA	Dichloroaniline
DCB	Dichlorobenzene
GC	Gas chromatography
HPLC	High-performance liquid chromatography
IC	Ion Chromatography
KC	Heat killed control for the anaerobic experiment
MC	Microcosm(s)
MCA	Monochloroaniline
MM	Mineral medium
NCBI	National Center for Biotechnology Information
OM	Original microcosm
OHRB	Organohalide-respiring bacteria
qPCR	Quantitative polymerase chain reaction
SRB	Sulphate-reducing bacteria
STE	Sterile control for the pre-experiment
T	Transfer
UFPE	Universidade Federal de Pernambuco
UofT	University of Toronto
UWF	University of West Florida
ΔG_o’	Gibbs free energy

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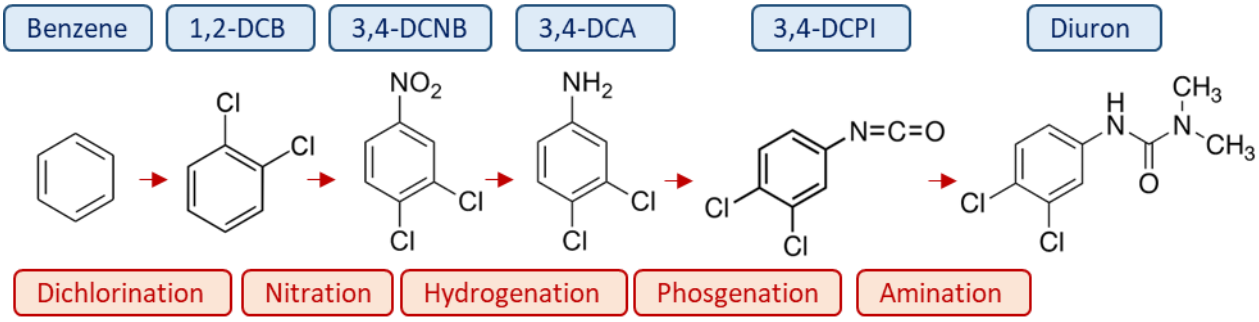
1 INTRODUCTION

Since benzene and toluene were first isolated from coal tar in 1845, by the chemist von Hofmann (ENCYCLOPAEDIA BRITANNICA, 2018), many industrial chemical processes have been developed to produce a huge variety of end-products. Aromatic compounds became the basic structure for industrial products, such as dyes, paint, solvents, resins, adhesives, ink, pesticides, and even simple plastic. Unfortunately, this chemical revolution was followed by cases of environmental pollution throughout the world. The stability of the benzene ring renders certain aromatic compounds very persistent to degradation, and many aromatic compounds have therefore become the major environmental pollutants at industrial sites. Most of the contaminants from wastes dumped in the mid-'90s are still in the environment and require remediation as they are toxic and/or carcinogenic and mutagenic (FAGIN, 2013; GIGER, 2009; MANI *et al.*, 2015; ORSANCO - OHIO RIVER VALLEY WATER SANITATION COMMISSION, 2020; SKIPPER *et al.*, 2009).

The interest in remediating contaminated sites has led to the development of new technologies that emphasize the destruction of pollutants. Bioremediation is technology in the field of environmental science and engineering and has become a hot field of research because of its low energy consumption, high efficiency, and environmental safety. This remediation strategy uses biological processes performed by microorganisms such as bacteria, fungi, or plants to aid in degrading and removing hazardous substances from a contaminated area (QIU; GUERIF; ZHANG, R. D., 2012).

The contaminated site target of our research is in an industrial complex in the state of Bahia, Brazil. One of the many products from the chemical industry was the herbicide diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea), an herbicide belonging to the phenylamide family and phenylurea subclass (GIACOMAZZI; COCHET, 2004). Its production starts from dichlorobenzene (1,2-DCB), which is formed through chlorination of benzene. The DCB is then nitrated to form dichloronitrobenzene (3,4-DCNB), which is hydrogenated, resulting in dichloroaniline (3,4-DCA). When submitted to phosgenation and amination, DCA forms dichlorophenylisocyanate (3,4-DCPI) and finally the diuron as the end-product (Figure 1).

Figure 1 - Production chain of the herbicide diuron



Source: elaborated by the author with the help of Lígia Carvalho.

Diuron has a very slow rate of natural hydrolysis in a neutral solution, at 25°C. When hydrolysis occurs, dichloroaniline (DCA) is formed as the only by-product (SALVESTRINI; CERBO; CAPASSO, 2002). DCA is largely used as intermediated in the industrial synthesis of pesticides, plastics, and dyes (STRUIJS; ROGERS, 1989; TRAVKIN *et al.*, 2002).

Apart from 3,4-dichloroaniline (3,4-DCA) and 2,3-dichloroaniline (2,3-DCA), a wide variety of other chemical compounds is found in the groundwater and soil of the site, including 2,3- and 3,4-dichloronitrobenzene (DCNB), 2-, 3-, and 4-monochloroaniline (MCA), 1,2-dichlorobenzene, and aniline.

The site operations were shut down in 2014; since then, through a demolition and remediation project, efforts have been put in place to decontaminate the soil and the groundwater of the site. The industry has brought together a team of experts, nicknamed “Bioteam”, with members from the site (industry and local laboratory workers), several consulting firms, and universities in Canada (University of Toronto - UofT), the U.S. (Clemson University, University of West Florida), and Brazil (Federal Universities of Pernambuco - UFPE - and Bahia - UFBA), who are working collaboratively to understand natural processes occurring in the site and to identify, design, implement, and evaluate the proper remediation options for this contaminated industrial site. In addition to evaluating and monitoring natural attenuation, several pilot tests have been performed or are in progress, including tests to evaluate biosparging, constructed wetland systems, and more recently thermal treatment.

As part of this large long-term collaborative research program, aiming to combine academic research with the remediation projects carried out by the industry, we intended to broaden the understanding of processes involved in the biodegradation of complex aromatic compounds, and

by doing this contribute to a more successful bioremediation strategy implementation at the site. The contaminants of interest (COI) of this project are related to the production chain of diuron, thus, they are aromatic compounds containing nitro or amino group and/or chlorine, such as DCNB, DCA, and MCA isomers, and anilines.

Considering the environmental problems raised by the contamination of aromatic compounds when they are released in soil and groundwater and the scarce information on the biodegradation of nitro and chlorinated aromatic compounds, this research is proven to be relevant to the field of bioremediation. Through this program, we aimed to elucidate the biodegradability of COI and their biodegradation pathways or biotransformation, seeking to understand the reactions that can occur in natural environments and identify organisms responsible for the activity observed under aerobic and anaerobic environments.

1.1 OVERALL OBJECTIVE

The overall objective of this research was to broaden the understanding of biodegradation of nitro- and chlorinated aromatic compounds under aerobic and anaerobic conditions and pH relevant to the study site, through the identification of rates of transformation, pathways and microbes responsible for observed biotransformation.

1.2 SPECIFIC OBJECTIVES

- To evaluate the potential for aerobic and anaerobic biodegradation of dichloronitrobenzenes (2,3-, 2,5- and 3,4-DCNB), dichloroanilines (2,3-, and 3,4-DCA), monochloroanilines (2-, 3- and 4-MCA), and aniline by native microbes from the contaminated site.
- To contribute to the understanding of the steps and mechanisms of biotransformation for each of the contaminants using batch bottle enrichment cultures.
- To identify the microbial community composition present in enrichment cultures during active contaminant biotransformation and identify putative microbes involved in the biodegradation process.

- To share experiences and to suggest or recommend strategies (such as biostimulation and bioaugmentation) to enhance bioremediation and improve remediation outcomes and understanding of natural attenuation at this complex contaminated industrial site.

1.3 STRUCTURE OF THE THESIS

The structure of this thesis is presented in Table 1.

Table 1 - Outline of the thesis

Sections	Topics
2 BACKGROUND AND LITERATURE REVIEW	ORIGIN OF CONTAMINATION AND LEGISLATION; BIOREMEDIATION; TREATABILITY STUDIES – LABORATORY SCALE; BIOTRANSFORMATION OF THE CONTAMINANTS OF INTEREST (COI): Chronological understanding of reductive dehalogenation in chloroaromatic compounds, Aerobic degradation of COI, Anaerobic transformations of COI
3 MATERIAL AND METHODS	EVALUATING THE BIODEGRADABILITY OF COI – ENRICHMENT CULTURES: Aerobic microcosms (Microcosms setup and maintenance, Monitoring the experiment and analytical procedures), Anaerobic microcosms (Microcosms setup and maintenance, Monitoring the experiment and analytical procedures), Stock solutions of COI. *Related to the specific objective 1.*
	UNDERSTANDING BIOTRANSFORMATIONS - ANALYTICAL METHODS: Liquid chromatography (HPLC), Gas chromatography (GC), Ion Chromatography (IC). *Related to the specific objective 2*.
	MICROBIAL COMMUNITY ANALYSIS AND QUANTITATIVE PCR: DNA extraction and 16S sequencing, Quantitative polymerase chain reaction (qPCR), Viable culture preservation. *Related to specific objective 3.*
	LEARNING AND SHARING EXPERIENCES. Related to the specific objective 4.
4 ANALYSIS AND DISCUSSION OF RESULTS	AEROBIC BIODEGRADATION OF THE CONTAMINANTS. Biodegradation of monochloroanilines and dichloroanilines. Ion analysis (chloride and N-compounds). Analysis of microbial community. Crossing the data obtained from aerobic tests. Learnings from aerobic tests. *Related to the specific objectives 1 to 3.* ANAEROBIC BIOTRANSFORMATIONS OF THE CONTAMINANTS. Negative controls and inactive microcosms. Biotransformations of contaminants and microbial community. Anion analysis (chloride, acetate, sulphate). Crossing the data from anaerobic tests. Learnings from anaerobic tests. *Related to the specific objectives 1 to 3.* LEARNINGS AND SHARING EXPERIENCES. Related to the specific objective 4.
5 FINAL CONSIDERATIONS	Final conclusions, future studies, improvements, applicability
REFERENCES	
APPENDIX A - SUPPLEMENTARY INFORMATION FOR METHODOLOGY (SECTION 3)	Figures to support analytical procedures
APPENDIX B - SUPPLEMENTARY INFORMATION FOR ANALYSIS AND DISCUSSIONS OF RESULTS (SECTION 4)	B1. Supplementary information - Aerobic biodegradation of contaminants; B2. Supplementary information - Anaerobic biotransformations of contaminants
APPENDIX C - PRE-EXPERIMENTS PERFORMED AT THE SITE (2017-2019)	Aerobic test with soil – microcosms containing 10 g of soil, Aerobic teste with groundwater – microcosms containing 100 mL of groundwater, Enrichment cultures, Aerobic test with soil (bioaugmentation) – microcosms containing 10 g of soil, Aerobic test with soil (bioaugmentation) – mesocosms containing 1 Kg of soil, Anaerobic versus aerobic tests in water with active enrichments – microcosms containing 100 mL of water and enrichment, Aerobic pH test with enrichment – microcosms containing 100 mL of water and enrichment, Microbial community from 16S sequencing.

2 LITERATURE REVIEW

2.1 ORIGIN OF CONTAMINATION AND LEGISLATION

The report published this year by The United Nations Educational, Scientific and Cultural Organization (UNESCO) highlighted the importance of the groundwater for the global population, as it represents half of the volume of water withdrawn for domestic use and around 25% of the resource used for irrigation. It is also mentioned that insecticides, herbicides, and fungicides, when improperly applied or disposed of, can pollute groundwater with carcinogens and other toxic substances (THE UNITED NATIONS WORLD WATER DEVELOPMENT REPORT, 2022).

By scanning Brazilian regulations for soil and groundwater quality, we only see a few chlorinated aromatic compounds included in the National Council for the Environment resolution (CONAMA - CONSELHO NACIONAL DO MEIO AMBIENTE, 2008) which lists contaminants most likely to occur in groundwater and their respective "maximum allowed values" for the considered uses. In the cited resolution, 1,4-dichlorobenzene is listed as allowed to be found in water for human consumption at a concentration up to 300 µg/L. The same level is found in the resolution CONAMA nº 420 (CONAMA - CONSELHO NACIONAL DO MEIO AMBIENTE, 2009), which provides values for contaminants in soil and groundwater and establishes some guiding criteria for the management of contaminated areas. None of the chloroanilines are considered in those legislations. Fortunately, the Brazilian National Environmental Policy (Law 6.938/81) obliges the industries to maintain the environment free of pollution as well as requires them to clean up the site where they are located in case of contamination (BRASIL, 1981).

In this case study, wastes and aromatic compounds stored at the site were released into the soil and groundwater from rain leaching through equipment where solvents were stored, mainly, before the construction of the industrial complex wastewater treatment facility. Contamination of the aromatic compounds is in the order of 10^4 µg/L in groundwater and in the range of 10^1 to 10^3 mg/Kg in soil.

Aerobic biotransformations of contaminants is often observed in the shallow layers where oxygen can permeate from the atmosphere, while anaerobic biotransformations processes are established in slightly deeper, or more organic rich layers, where oxygen rapidly becomes depleted but is not replenished by diffusion. The removal of pollutants from the environment via natural

physicochemical and biological processes (natural attenuation) is, in general, a slow and unpredictable way of counteracting anthropogenic pollution (HARWOOD; PARALES, 1996). This encourages the responsible for the contaminated site managers to seek strategies to solve this problem; that is how the bioremediation technology emerges.

2.2 BIOREMEDIATION

The need to remediate contaminated sites has led to the development of new technologies that emphasize the mineralization of pollutants. Bioremediation is a very useful technology in the field of environmental science and engineering and has various applications for decontaminating water, soils, and waste streams. The technique frequently must address multiphasic environments in which the contaminant is usually associated with particles, dissolved in liquids and sorbed to soil. This complexity leads to a dependence on an interdisciplinary approach involving microbiology, engineering, ecology, geology, and chemistry (BOOPATHY, 2000; QIU; GUERIF; ZHANG, R. D., 2012).

Bioremediation has become a technology of choice and a hot spot of research because of its low energy consumption, high efficiency, and environmental safety. This remediation strategy uses biological processes performed by organisms such as bacteria, fungi, or even plants to aid in degrading and removing hazardous substances from a contaminated area (QIU; GUERIF; ZHANG, R. D., 2012). Some examples of bioremediation methods with bacteria playing the main role are given in Table 2.

Table 2 - Bioremediation methods and treatments

Method	Treatment application
Bioreactors (ex-situ)	Biodegradation in a container or reactor; may be used to treat liquids or slurries. The bioreactor provides the ideal growing conditions for the microorganisms. Nutrients and oxygen can also be added into the system for the degradation of waste.
Bioventing (in-situ)	Method of treating contaminated soils by drawing oxygen through the unsaturated zones to stimulate microbial activity.
Biosparging (in-situ)	Consists of adding oxygen to the saturated zone to stimulate biodegradation in groundwater.
Bioaugmentation (in-situ or ex-situ)	Addition of microbes to a contaminated area or medium.
Biostimulation (in-situ or ex-situ)	Stimulation of native microbial populations in soil or groundwater by providing necessary conditions, such as pH adjustment, nutrients addition, or other substances.

Source: Adapted from other studies (BOOPATHY, 2000; CAPOZZI *et al.*, 2018; GONÇALVES; DELABONA, 2022; JABBAR *et al.*, 2022; PATEL *et al.*, 2022; SHARMA, B.; DANGI; SHUKLA, 2018).

Biochemical processes applied to decontamination also have limitations. Although some compounds are metabolized relatively quick by soil and aquatic microorganisms, others are slowly degraded and some chemical compounds are difficult to biodegrade, for instance, some heavily chlorinated or fluorinated compounds. Metals are only modified by microbial action (oxidized, reduced or complexed) resulting in enhanced or decreased mobility or volatility. It may take microorganisms a long time to acquire the ability to degrade all the new synthetic chemicals introduced into the environment by modern technology.

To overcome the challenges, many studies in the field of bioremediation of contaminated sites have reported a wide variety of strategies tested to degrade herbicides such as diuron (ATTAWAY; CAMPER; PAYNTER, 1982; RUBIO-BELLIDO; MORILLO; VILLAVERDE, 2018; VILLAVERDE *et al.*, 2017) and other pollutants for instance petroleum hydrocarbons (CHRISTOPHER *et al.*, 2021; DORST, VAN *et al.*, 2020; LUO, F. *et al.*, 2014; LUO, F.; DEVINE; EDWARDS, E. A., 2016; MANCINI *et al.*, 2003; ROY *et al.*, 2018; ULRICH; EDWARDS, E. A., 2003), aniline (KAHNG; KUKOR; OH, 2000; SEHNELL; SEHINK, 1991;

SUN, W. *et al*., 2015; ZHANG, Q. *et al*., 2020), and nitro compounds (JUGNIA *et al.*, 2019; MADEIRA *et al.*, 2021; PALATUCCI *et al.*, 2019).

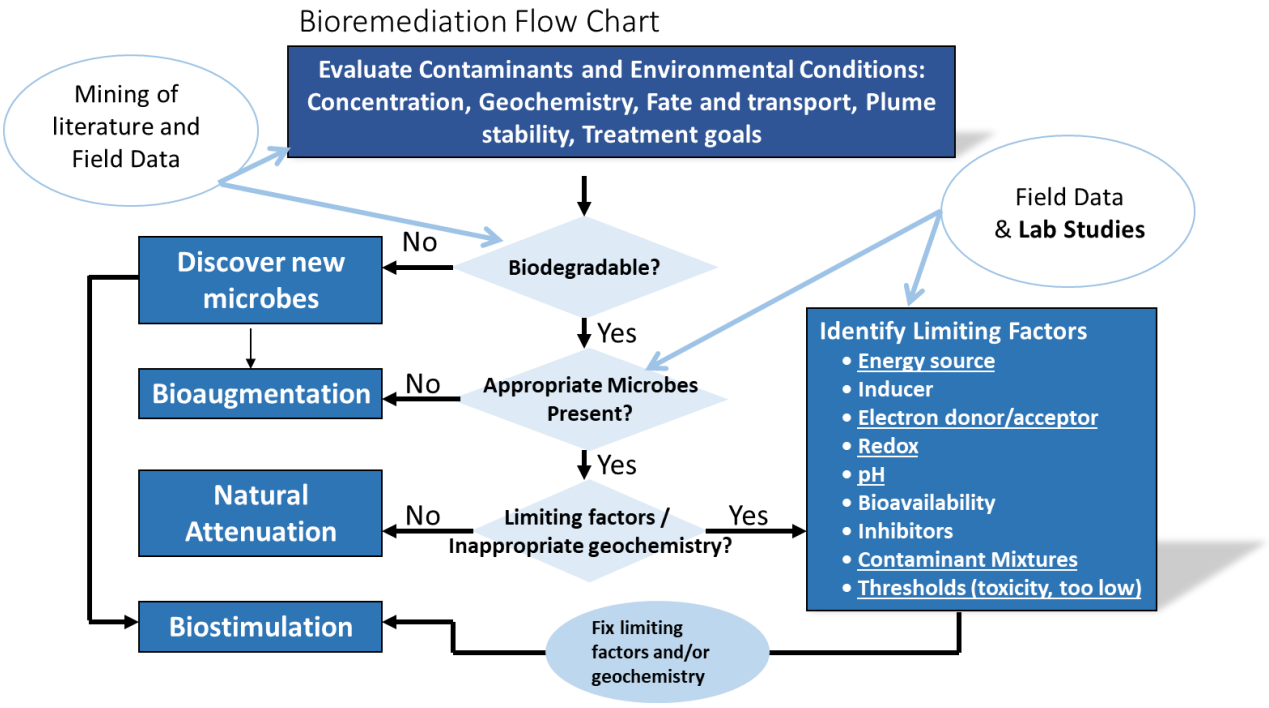
Chlorinated aromatic compounds, often released in substantial quantities, are major environmental pollutants due to their toxicity and resistance to biodegradation, leading to accumulation in sediment and organisms. In some cases, microbial metabolism may produce toxic metabolites, which can be a disadvantage of bioremediation if these new metabolites are not biodegradable (BOOPATHY, 2000; CHAUDHRY; CHAPALAMADUGU, 1991; MAJONE *et al.*, 2015). Dechlorination of aromatic compounds has been investigated extensively (CAPOZZI *et al.*, 2018; DINGLASAN-PANLILIO *et al.*, 2006; GILEVSKA *et al.*, 2021; GROSTERN; EDWARDS, E. A., 2006; JUSTICIA-LEON *et al.*, 2014; LIANG *et al.*, 2013; LÖFFLER; EDWARDS, E. A., 2006; LOMHEIM *et al.*, 2020; PÉREZ-DE-MORA *et al.*, 2014; QIAO, W. *et al.*, 2018; STEPP; CAMPER; PAYNTER, 1985; STRUIJS; ROGERS, 1989).

In Brazil specifically, some bioremediation projects were implemented already, aiming to clean up areas mainly contaminated with 2,4-Dichlorophenoxyacetic acid (2,4-D), glyphosate, and atrazine (GONÇALVES; DELABONA, 2022).

2.3 TREATABILITY STUDIES – LABORATORY SCALE

Some in-situ bioremediation techniques were considered at the studied site, such as bioventing, bioaugmentation, and biostimulation. Natural attenuation is also the focus of the Bioteam program. That said, some important questions regarding bioactivity and the biochemical processes need to be addressed: Are the contaminants of interest (COI) biodegradable under specific conditions? Is natural attenuation occurring? Is the native microbial community capable of degrading the COI? What other specific characteristics need to be evaluated to improve bioremediation applicability? What are the limiting factors inhibiting bioactivity and what should be done to promote or improve it? Figure 2 provides a flowchart that situates our team in this big remediation project and the specific questions we aimed to answer.

Figure 2 - Specific questions we collaboratively aimed to answer as part of our contribution to the remediation project



Source: Elaborated by prof. Dr. Jim Spain (UWF).

The success of bioremediation depends on having the right microbes and favourable conditions. A feasible remediation process requires microorganisms capable of using the pollutants as growth substrates or perhaps as co-substrates not supporting growth. Key aspects of the degradation process include microbial characteristics (metabolic ability, biomass concentration, population diversity, and enzyme activities), substrate (physiochemical characteristics, molecular structure, and concentration), and environmental factors (pH, temperature, moisture content, oxygen content, availability of electron acceptors and carbon and energy sources). Therefore, identifying the right conditions, catabolic pathways, mechanisms, and responsible enzymes, is essential to define important factors for efficient pollutants removal (BOOPATHY, 2000; KURT; MACK, E. E.; SPAIN, 2016; SEO; KEUM; LI, Q. X., 2009; SHARMA, B.; DANGI; SHUKLA, 2018).

In this context, studying biodegradation processes has become essential to improve bioremediation technologies implemented at contaminated sites. Treatability studies – lab scale experiments performed in bottles called microcosms - have been performed to evaluate and

understand compound biodegradability under specific conditions before applying the technology at full scale. When biodegradation is achieved, the identification of organisms, genes, and expressed enzymes involved in the processes is also a strategy of interest for a bioremediation project. Those biomolecules can be used as biomarkers indicating that specific reactions or processes are taking place in the studied area (HEAVNER *et al.*, 2019).

When this team project began, there were large knowledge gaps concerning the degradation of the problematic nitro- and chloro-aromatic compounds driving remediation at the study site. Efforts were needed to elucidate complete routes, aiming to understand the reactions that occur in natural environments, where mixtures of contaminants co-occur and can be both electron acceptors or donors, and to improve bioactivity, considering parameters that have been recognized as influencing biodegradation under aerobic and anaerobic environments.

2.4 BIOTRANSFORMATION OF THE CONTAMINANTS OF INTEREST (COI)

Microorganisms can adapt to remove many toxic substances; however, the great variety of xenobiotics used today disrupted the balance of the ecosystem. When microorganisms encounter a new organic chemical in their environment, they may obtain the new catabolic genes needed for the degradation of that compound from other microorganisms through conjugational or transformational events or they may modify existing genes through mutational processes (CHAUDHRY; CHAPALAMADUGU, 1991).

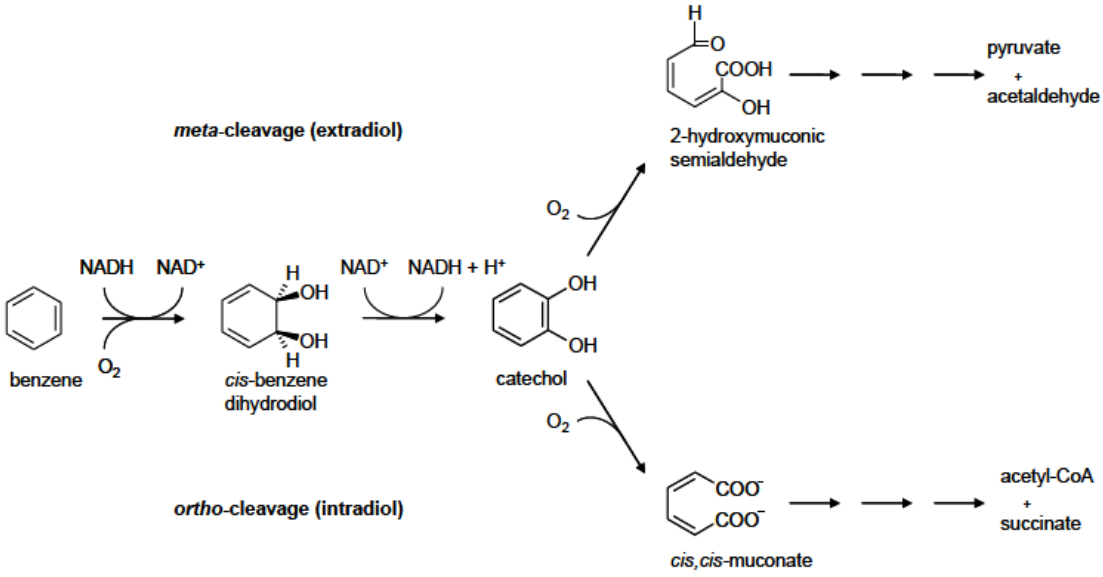
Human-made xenobiotic compounds have been in contact with the microbiota only for about 100 years; therefore, some of them are still poorly degraded (DÍAZ, 2004). Even though chlorinated hydrocarbons have been synthesized and released into the environment in vast quantities only during the past few decades, microorganisms in nature have already developed the ability to degrade many of them, perhaps because there are also many naturally-occurring chlorinated organic molecules (CHAUDHRY; CHAPALAMADUGU, 1991).

Organisms that use aromatic compounds as growth substrates need to overcome the stabilizing resonance energy of the aromatic ring system. Thus, some biochemical strategies are employed by microbes to destabilize the aromatic ring in preparation for their degradation (FUCHS; BOLL; HEIDER, 2011).

Microbes deal with the aromatic compounds by transforming them into a few key central intermediates such as catechol and protocatechuate, under aerobic conditions, and benzoyl-CoA, under anaerobic conditions. Subsequent steps cleave the aromatic ring, converting the central intermediates into metabolites such as acetyl-CoA, succinyl-CoA and pyruvate, which can finally be used for microbial growth (FUCHS; BOLL; HEIDER, 2011).

Under aerobic conditions, in most cases, the first steps in the catabolism of aromatic compounds involve the oxidation of the substrate by oxygenase enzymes, resulting in dihydroxy aromatic compounds (catechols). Then, the organic compounds serve as substrates for ring-cleavage enzymes (*ortho* or *meta* cleavage) that use molecular oxygen, leading to the formation of Krebs cycle intermediates (Figure 3) (DÍAZ, 2004; FUCHS; BOLL; HEIDER, 2011; LEAHY; COLWELL, 1990; SALEH *et al.*, 2017; SINGH; MISHRA; RAMANTHAN, 2015; WEELINK, 2008). These steps are also reported to happen with the nitroaromatic compounds and aniline (AOKI *et al.*, 1984; SPAIN, 1995). When considering the chlorinated compounds, in general, chlorinated catechol isomers are key metabolites of their degradation under aerobic conditions (NEILSON, 1990).

Figure 3 - Aerobic degradation pathway of the benzene



Source: reproduced from Weelink (2008).

In the absence of oxygen, substituted aromatic compounds can serve as either electron donors or electron acceptors. Aniline was first reported to be anaerobically degraded by the *Desulfobacterium anilini* through a carboxylation to 4-aminobenzoate, followed by activation of 4-aminobenzoate to 4-aminobenzoyl-CoA. Then, 4-amino-benzoyl-CoA is reductively deaminated to benzoyl-CoA which enters the normal benzoate pathway, leading to three acetyl-CoA (SCHNELL; BAK; PFENNIG, 1989; SCHNELL; SCHINK, 1991b).

The nitro group substituent of aromatic compounds is expected to be reduced into an amino group by the addition of pairs of electrons. Many living organisms can fortuitously catalyze the transformation of the nitro group by using a wide variety of redox enzymes that can serve as nitroreductases. The reaction pathway includes the toxic and carcinogenic nitroso and hydroxylamine intermediates, which present high reactivity and instability (BUNCE; MERRICK; CORKE, 1983; SPAIN, 1995). Studies also reported nitroaromatic reduction as a respiratory process (ESTEVE-NUNEZ *et al.*, 2000; HUANG, J. *et al.*, 2015).

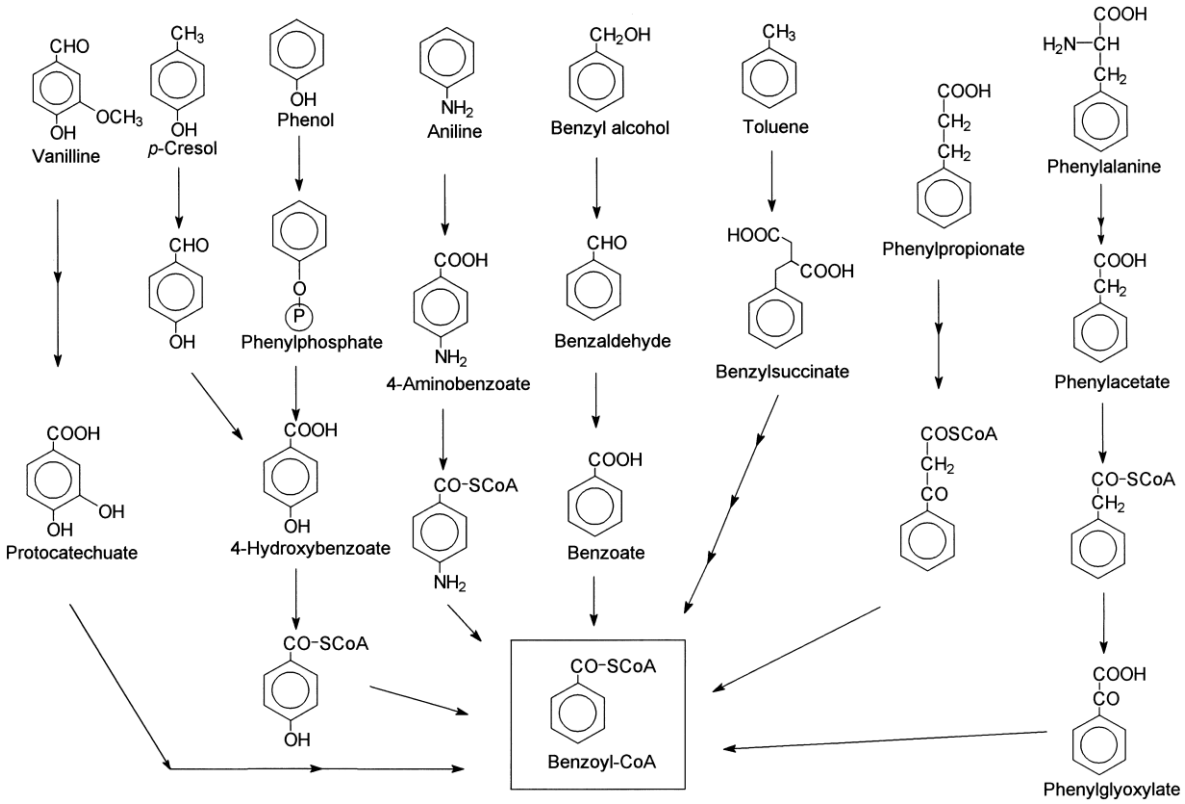
When serving as electron acceptors, chlorinated aromatic compounds are transformed through reductive dehalogenation by many different groups of strictly anaerobic and facultative anaerobic microbes, which remove chlorine substituents from contaminants (as chloride) in hydrogenolysis reactions. Reductive dehalogenation is considered to occur mainly under anaerobic conditions, and it most commonly consists of the halogen substituent removal through its replacement by a hydrogen atom, with no alteration of the aromatic ring. This process requires a source of electron and releases halide anion, being the initial degradation step of haloaromatic compounds, leading afterwards to their eventual mineralization (EVANS; FUCHS, 1988; GIBSON; HARWOOD, 2002; KAMESWARAN *et al.*, 2021; MOHN, W. W.; TIEDJE, J. M., 1992; REINEKE; KNACKMUSS, 1988; SUFLITA *et al.*, 1982).

In metabolic and some co-metabolic dehalogenation reactions, the key enzymes involved are reductive dehalogenases (RDase). Co-metabolic reductive dechlorination can also be catalyzed by reduced metals such as cobalt or iron or coordinated metals in vitamins like B12. However, when dehalogenation is a metabolic process, energy is gained via an anaerobic respiratory, in which the chlorinated compound serves as the terminal electron acceptor (KAMESWARAN *et al.*, 2021).

Back to the aromatic compounds, after the substituent removal, the aromatic compounds are, in general, transformed into benzoyl-CoA which will be finally cleaved, forming intermediary

metabolites easily used by the microbes (Figure 4) (Fuchs *et al*., 2011; Gibson & Harwood, 2002; Harwood *et al*., 1999; Weelink, 2008).

Figure 4 - Anaerobic pathways for degradation of aromatic compounds



Source: reproduced from Harwood *et al*. (1999).

2.4.1 Chronological understanding of reductive dehalogenation in chloroaromatic compounds

At the end of the 1980's, dechlorination of 2,4- and 3,4-dichloroaniline was reported and referred to as reductive dehalogenation. The process led to the formation and accumulation of 3- and 4-monochloroaniline isomers, which appeared to be more resistant to further dechlorination (STRUIJS; ROGERS, 1989). At this time, chlorine removal was largely thought of as a co-metabolism process, in which the microorganisms were unable to grow on halogenated compounds but degraded them while using other compounds as their source of energy for growth (COMMANDEUR; PARSONS, 1990).

However, during the same period, other authors provided evidence that reductive dehalogenation might occur as a respiratory process. From a thermodynamical point of view, the authors sustained the possibility that chlorinated aromatic compounds may act as terminal electron acceptors and that the energy generated from this reaction might then become useful for cell synthesis (BOSMA *et al.*, 1988).

The first unequivocal demonstration of growth-associated reductive dechlorination came from research with a defined 3-chlorobenzoate degrading consortium. The authors demonstrated that 3-chlorobenzoate provided biologically useful energy after performing growth yield and ATP experiments (DOLFING; TIEDJE, J M, 1987). The 3-chlorobenzoate (3-CB) degrading consortium consisted of DCB-1 as the dechlorinator strain (*Desulfomonile tiedjei*), plus a benzoate degrader, strain BZ-2, and a methanogen, *Methanospirillum* strain PM-I which were previously studied (SHELTON; TIEDJEI, 1984).

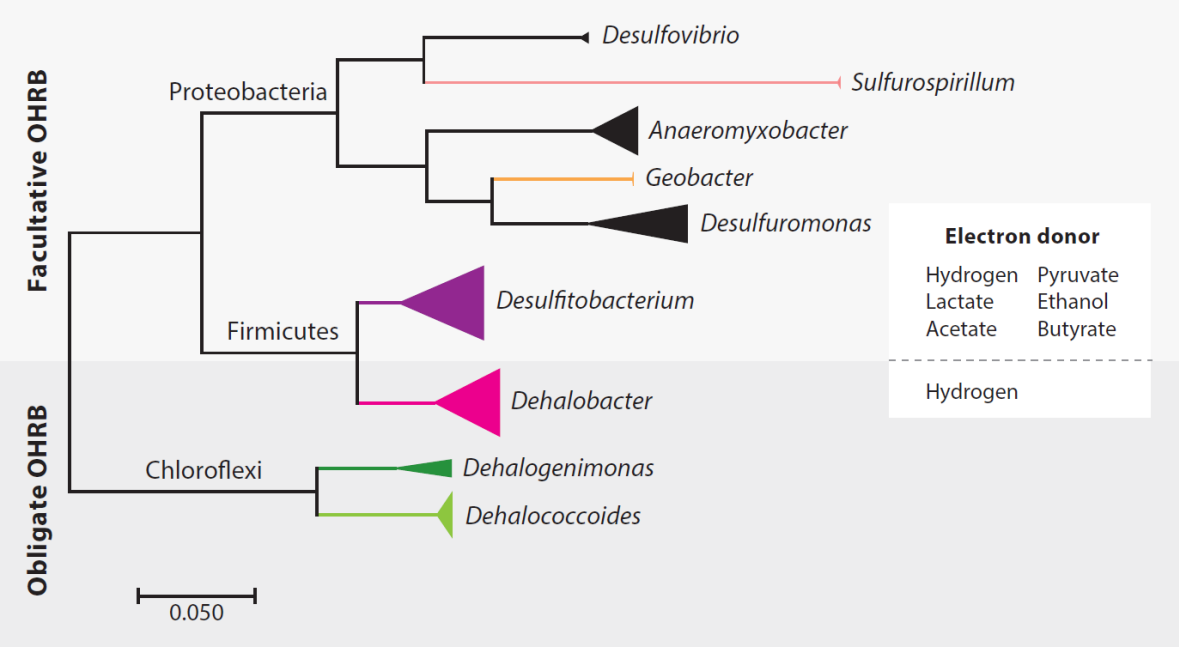
The strain DCB-1 could conserve energy for growth from reductive dechlorination of 3-CB and 3,5-dichlorobenzoate coupled to formate and hydrogen oxidation. It was suggested that neither electron donor supported phosphorylation at the substrate level, thus, it was defended that phosphorylation happens via electron transport with the chlorinated compounds acting as the terminal electron acceptor (MOHN, William W; TIEDJE, James M, 1990).

Considering all these revolutionary findings at the time, Mohn & Tiedje (1992) summarized what was already understood. They came up with some exciting questions such as: Which microbes perform dehalogenation activity? Which enzymes and cofactors are responsible for the activity? What are the chemical mechanisms involved in this activity? How do various factors directly affect activity? How does the activity benefit or harm the responsible microorganisms? Many research groups have been answering these questions and much more were found since these authors first raised them in 1992.

In the ensuing 30 years, many bacteria have been identified as what are now called organohalide-respiring bacteria (OHRB). These organisms are categorized as non-obligate (facultative) or obligate organohalide-respiring (Figure 5). For the first group of organisms, organohalide compounds are just one of many acceptors they can use for respiration; other acceptors include nitrate and sulphate. Facultative OHRB often can also use a variety of different electron donors. The second group can only use halide compounds for respiration and H_2 (and sometimes formate) as the electron donor. It is also known that these microbes have better growth

conditions if they are part of a complex community, with fermenting and methane-producing microbes. Other members of the community provide vitamins and a carbon source for the dechlorinators (Adrian & Loffler, 2016; Edwards, 2014; Fincker & Spormann, 2017).

Figure 5 - Phylogenetic tree with some organohalide respiring bacteria



Source: reproduced from Fincker and Spormann (2017).

Enzymatic studies have been improving the understanding of the critical enzymes for dechloriniation called reductive dehalogenases. These enzymes involved in reductive dechlorination are now known to be periplasmic membrane-associated iron–sulphur and corrinoid-containing proteins, which require cofactors such as vitamin B_{12}. Studies on genome sequencing and reductive dehalogenase genes (RdhA) permitted phylogenetic comparisons and provided information on the diversity of the protein family. The specific electron flow in dehalogenases, inside the cell, has also been studied (EDWARDS, E. A., 2014; FINCKER; SPORMANN, 2017; HUG *et al.*, 2013; JUGDER *et al.*, 2016; LEYS; ADRIAN; SMIDT, 2013; MOLENDA *et al.*, 2020).

The OHRB have been applied in bioremediation projects. For instance, for many years, the University of Toronto team, led by prof. Dr. Edwards, has been studying a mixed community, named KB-1, capable of dechlorinating diverse chlorinated compounds. The community was

enriched from the soil and groundwater of a contaminated site and is composed of methanogens, acetogens and dechlorinating organisms, particularly *Dehalococcoides* (DUHAMEL *et al.*, 2002; MOLENDA *et al.*, 2020). KB-1 has been deployed for remediation at over 700 sites worldwide (SIREM, 2022).

2.4.2 Aerobic biodegradation of COI

Table 3 shows some of the studies reporting aerobic degradation of the target DCNB, DCA, and MCA isomers, and aniline. It includes the intermediates observed and the microbes responsible for the activity described in each of the mentioned references. Apart from these published papers, Edwards' group, at the UofT, also observed biodegradation of 2,3-DCA and 3,4-DCA, 2-, 3-, and 4-MCA and aniline by a mixed microbial community enriched from soil and groundwater of the same contaminated site of the one studied in this research (KRAUS, 2018).

Table 3 - Studies reporting aerobic biodegradation of COI

Contaminants	Intermediates	Microbes	References
2,3-DCNB	3,4-dichlorocatechol	*Diaphorobacter* sp. cepa JS3051	Palatucci *et al*., 2019; Li *et al*., 2021
3,4-DCNB	3,4-dichlorocatechol, 4,5-dichlorocatechol	*Diaphorobacter* sp. cepa JS3050; *Diaphorobacter* sp. cepa JS3052	Palatucci *et al*., 2019; Gao *et al*., 2021
2,3-DCA; 3,4-DCA	Chlorocatechol	*Delftia tsuruhatensis* H1	Zhang L. *et al*., 2010
2,3-DCA	Isomers of dichloroaminophenol	*Bacillus megaterium* IMT21	Yao *et al*., 2011
3,4-DCA	3,4-dichloroacetanilide	*Bacillus megaterium* IMT21	Yao *et al*., 2011
3,4-DCA; 4-MCA	4-MCA, aniline, catechol or 4-chlorocatechol	*Acinetobacter baylyi* cepa GFJ2	Hongsawat and Vangnai, 2011
2-, 3-, 4-MCA	4-Chlorocatechol	*Delftia tsuruhatensis* H1	Zhang L. *et al*., 2010
4-MCA	4-chlorocatechol	*Diaphorobacter* sp. PCA039	Zhang T. *et al*., 2010
3-MCA	4-chlorocatechol	*Comamonas testosterone* cepa; A; *Delftia acidovorans* cepa B; *Delftia acidovorans* cepa C	Shah, 2014
2-, 3-, 4-MCA and aniline	4-chlorocatechol and catechol	*Acinetobacter baumannii* CA2; *Pseudomonas putida* CA16; *Klebsiella* sp. CA17	Vangnai and Petchkroh, 2007
4-MCA and aniline	4-chlorocatechol and catechol	*Comamonas testosterone*; *Pseudomonas stutzeri*; *Pseudomonas putida*; *Stenotrophomonas maltophilia*	Shah, 2015
Aniline	Catechol*	Many (Review)	Arora, 2015

*Catechol was reported as intermediate in all studies included in the referred review.

Source: cited authors.

Recently, the first report on biodegradation of 2,3- and 3,4-DCNB isomers by isolated strains of *Diaphorobacter* was published by members of our bioteam (PALATUCCI *et al.*, 2019). The authors reported that nitrite was released from the contaminants before they are transformed into the dichlorocatechol isomers. The complete genome of the strains JS3051 and JS3050 were sequenced and more details of 2,3- and 3,4-DCNB degradation pathways, respectively, were published (GAO *et al.*, 2021; LI, T. *et al.*, 2021). As far as we know, the biodegradation pathway for 2,5-DCNB has not yet been investigated.

Literature on biodegradation of dichloroaniline isomers is more available. Through chlorinated catechol pathway, *Delftia tsuruhatensis* H1 was able to grow on 3,4-DCA as the sole

carbon and energy source but was only able to grow on 2,3-DCA as a co-substrate, in the presence of 3- and 4-MCA (ZHANG, L. *et al.*, 2010).

A strain of *Bacillus megaterium* was reported to grow on 2,3-, 3,4-, 2,4-, 2,5-, and 3,5-DCA isomers as sole sources of carbon and energy (YAO *et al.*, 2011). The 2,3-DCA was degraded via dichloroaminophenol, which had not been previously described as an intermediate. The authors suggested that hydroxylation of the aromatic ring might have taken place before the deamination or dechlorination step. However, they discussed the possibility of the new intermediate being further degraded via catechol. The intermediate for 3,4-DCA isomer biodegradation was interpreted as 3,4-dichloroacetanilide, which was, surprisingly, observed as an intermediate in an anaerobic pathway in previous studies (TRAVKIN *et al*., 2002; ZHANG, L. Li *et al*., 2010).

Another surprising finding was obtained with *Acinetobacter baylyi* strain able to grow on 3,4-DCA and 4-MCA. Before forming catechol or chlorocatechol, the authors observed two reduction steps in which 3,4-DCA was transformed into 4-MCA, and then 4-MCA was reduced into aniline and further oxidized to catechol or 4-MCA was directly oxidized to 4-chlorocatechol, releasing ammonia. The formation of dichlorocatechol was not observed (HONGSAWAT; VANGNAI, 2011).

For the monochloroaniline isomers, *Delftia tsuruhatensis* H1 was able to grow on the 2-, 3-, and 4-MCA isomers, but only co-metabolically on 2-MCA (ZHANG, L. *et al.*, 2010).

The strain *Diaphorobacter* sp. PCA039 was reported to utilize 4-MCA as sole carbon, nitrogen and energy source for growth (ZHANG, T *et al.*, 2010). The strains *Comamonas testosterone* cepa A, *Delftia acidovorans* cepa B, *Delftia acidovorans* cepa C were able to grow on 3-MCA (Shah, 2014). The isolates *Acinetobacter baumannii* CA2, *Pseudomonas putida* CA16, and *Klebsiella* sp. CA17 could grow on 2-, 3-, 4-MCA, and aniline, and co-metabolically on 3,4-DCA (VANGNAI; PETCHKROH, 2007). *Comamonas testosterone*, *Pseudomonas stutzeri*, *Pseudomonas putida*, and *Stenotrophomonas maltophilia* grew on 4-MCA and aniline as sole carbon and nitrogen source (SHAH, 2015). In all the cited studies, MCA isomers were degraded via 4-chlorocatechol and aniline via catechol.

Biodegradation of many monocyclic aromatic amines was extensively studied, including aniline being degraded through catechol as an intermediate produced by many cited organisms (ARORA, P K, 2015).

2.4.3 Anaerobic biotransformations of COI

Table 4 shows some studies reporting biotransformations of DCNB, DCA, MCA and aniline under anaerobic conditions, describing the steps and the mechanisms observed and the microbes responsible for the activity of each reference. In addition to these cited studies, Kraus (2018), at the UofT, described transformations of 3,4-DCNB (forming 3,4-DCA, 3- and 4-MCA), 3,4-DCA (forming 3- and 4-MCA), and 2,3-DCA (forming 3-MCA) by a microbial community enriched from soil and groundwater of the same contaminated site of the research reported in this document. At the UFPE, biodegradation of aniline under sulphate reducing condition and without the contribution of sulphate was also studied (CARVALHO, 2020).

Table 4 - Studies reporting anaerobic biotransformations of COI

Contaminant ⟶ products	Mechanisms/conditions	Microbes	References
3,4-DCNB ⟶ 3,4-DCA, tetrachloroazoxybenzene and tetrachloroazobenzene	Presence of $NaNO_3$	*Escherichia coli*	Bunce *et al*. (1983)
3,4-DCA ⟶ 3-MCA, 4-MCA	Anaerobic environment (atmosphere N_2 + H_2)	Not identified	Struijs and Rogers (1989)
2,3-DCA ⟶ 3-MCA, 2-MCA 3,4-DCA ⟶ 3-MCA	Acetate as carbon source and hydrogen as electron donor (OHR)	*Dehalococcoides mccartyi* strain CBDB1; *Dehalobacter* sp. strain 14DCB1	S. Zhang *et al*., 2017
2,3-DCA ⟶ 3-MCA, aniline, 4-aminobenzoate 3,4-DCA ⟶ 3 + 4-MCA, aniline, 4-aminobenzoate	Iron-reducing conditions - Fe (III)	*Geobacter* sp. KT5	Duc and Oanh, 2019
2,3-DCA ⟶ 3-CA, Aniline 2 and 4-MCA⟶ Aniline	Reductive dehalogenation / Sulphate reducing condition	Not specified	Susarla *et al*., 1997 Susarla *et al*., 1998
3,4-DCA ⟶ 3,4-di-chloroacetanilide, 3,4- dichloro-N-(3,4-dichlorophenyl)benzamide, 1,2-DCB	Reductive deamination - Nitrate reducing conditions	*Rhodococcus* sp. strain 2	Travkin *et al*., 2002
2-MCA ⟶ aniline ⟶ hexanoic acid	2-aminoanthraquinone-graphene oxide (electron mediator), pyruvate (donor)	Firmicutes; *Oscillospira*; *Lactobacillales*; *Veillonellaceae*; *Ruminococcus*	Lu *et al*., 2019
Aniline ⟶ 4-aminobenzoate	Sulphate as acceptor	*Desulfobacterium anilini*	Schnell *et al*., 1989, Schnell and Schink, 1991
Aniline ⟶ 4-aminobenzoate	Nitrate reducing conditions	strain HY99, similar to *Delftia acidovorans*	Kahng *et al*., 2000
Aniline	Nitrate and sulphate reducing and methanogenic conditions	*Ignavibacterium album*; *Anaerolineaceae*; *Acidovorax* spp.	Sun *et al*., 2015

Source: cited authors.

Literature on anaerobic biodegradation of DCNB, DCA, MCA and aniline is less available than that under aerobic conditions. Although the reduction of the nitro group in aromatic compounds is reported to be a simple reaction (discussed earlier), among the three DCNB isomers considered in this study (2,3-, 3,4-, and 2,5-), we could only find one study describing anaerobic

3,4-DCNB transformation to 3,4-DCA, tetrachloroazoxybenzene, and tetrachloroazobenzene (BUNCE; MERRICK; CORKE, 1983). The product 3,4-DCA agrees with the expected nitro-reduction pathway based on the literature for reduction of the nitro group (discussed earlier).

Anaerobic biotransformation steps of 2,3- and 3,4-DCA has been reported in a few studies. Struijs and Rogers (1989) reported reductive 3,4-DCA dechlorination to monochlorinated compounds 3-MCA and 4-MCA, but aniline formation was not observed, and organisms were not identified. Many years later, *Dehalococcoides mccartyi* strain CBDB1 and *Dehalobacter* sp. strain 14DCB1, both reported as OHRB (discussed earlier), were able to dechlorinate 2,3-DCA into 2- and 3-MCA and 3,4-DCA into 3-MCA, using acetate as carbon source and hydrogen as electron donor (ZHANG, S. *et al.*, 2017).

Under iron-reducing conditions, the isolate *Geobacter* sp. KT5 was able to go further in the anaerobic transformation of the DCA isomers. The strain formed 3-MCA from 2,3-DCA and 3- and 4-MCA from 3,4-DCA, with subsequent mineralization of the subproducts via aniline and 4-aminobenzoate (DUC; OANH, 2019).

Different pathways were observed by Travkin *et al*. (2002). In their study, 3,4-DCA deamination occurred, forming three subproducts as a result of anaerobic transformation by a *Rhodococcus* strain: dichlorobenzene, 3,4-dichloroacetanilide and 3,4-dichloro-N(3,4-dichlorophenyl)-benzamide, with a prevalence of dichlorobenzene.

Significant dechlorination of monochlorinated compounds has not yet been observed under anaerobic conditions. The same isolated strain of *Geobacter* mentioned before was able to grow on 2-, 3-, and 4-MCA as sole organic carbon, nitrogen, and energy source under iron-reducing conditions.

In the presence of an electron mediator and pyruvate as electron donor, an enriched community was able to dechlorinate 2-MCA, degrading it via aniline and hexanoic acid. Organisms from *Oscillospira*, *Lactobacillales*, *Veillonellaceae*, and *Ruminococcus* groups were positively correlated with the constant rate of 2-MCA degradation and the dehydrogenase activity (LU *et al.*, 2019).

Aniline was reported to be degraded by *Desulfobacterium anilini* under sulphate-reducing conditions (SCHNELL; BAK; PFENNIG, 1989; SCHNELL; SCHINK, 1991b) by the strain HY99, and by *Delftia acidovorans* under nitrate-reducing conditions (KAHNG; KUKOR; OH, 2000), both via 4-aminobenzoate.

More recently, novel putative aniline degraders were proposed. In microcosms inoculated with material from an industrial site heavily contaminated with aniline, nitrate-reducing, sulphate-reducing and methanogenic conditions have promoted degradation of this compound. Carbon labelling indicated that a phylotype similar to *Ignavibacterium album*, bacterial phylotype within the family *Anaerolineaceae* and the *Acidovorax* spp are related to its degradation (SUN, W *et al.*, 2015).

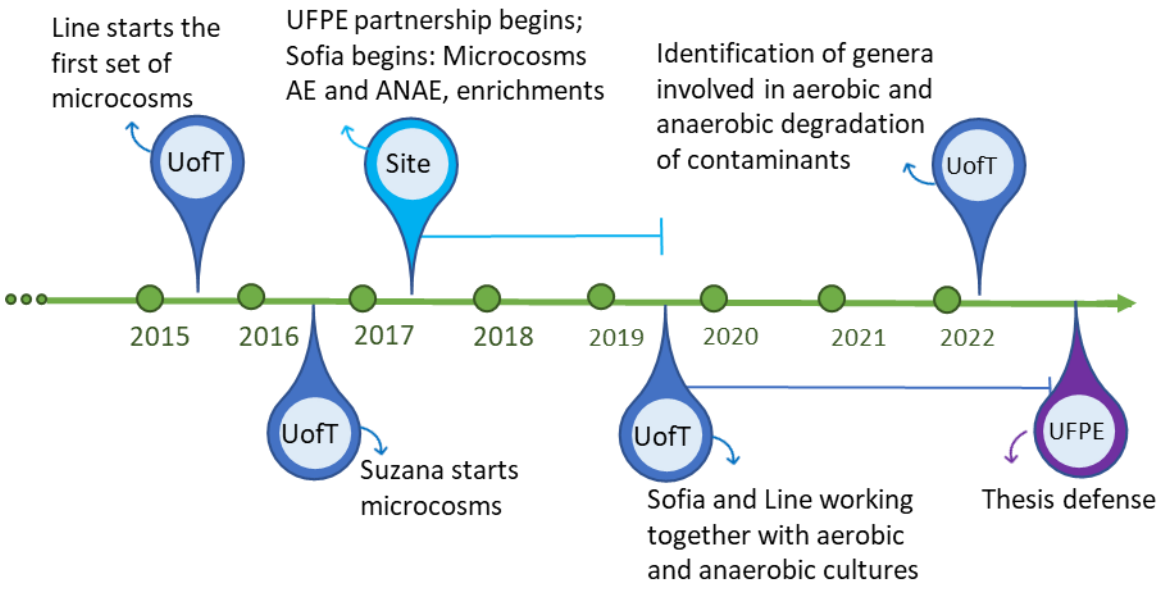
3 METHODOLOGY

Figure 6 presents a timeline of my Ph.D. journey. The Federal University of Pernambuco team, led by professor Gavazza, started the collaboration in 2017, when we started to be part of the Bioteam, working at the site for almost 2 years. At that moment, bioremediation was a new topic for our research group in UFPE. Thus, the research in a new field and place were a big challenge. This challenge is reflected in the results we obtained at the site: I am aware that many questions were not answered and that some questions were not asked at the right moment. On another hand, it was during these two years that we could improve our knowledge in a way that now we are able to discuss bioremediation with experts and professors. The results from this valuable period are included as Appendix C of this document.

I then went to the University of Toronto, where I stayed from October 2019 to April 2022 as a visiting student. The University of Toronto team (where I performed the experiments discussed in this thesis) became involved in this project in 2015. Under Edwards' supervision, our research aims to characterize aerobic and anaerobic biodegradation activity in site samples, and to determine associated microbial community composition in relation to site geochemistry, informed by data from a series of laboratory microcosms. The first microcosms were started by Line Lomheim, who trained and helped me in the use of equipment and methodologies that were applied during this research.

The experiments discussed in the main text of this document (section 4) were all performed at the BioZone (Centre for Applied Bioscience and Bioengineering Research at the University of Toronto's Faculty of Applied Science and Engineering). They were conducted by Sofia Pimentel and Line Lomheim, between October 2019 and April 2022.

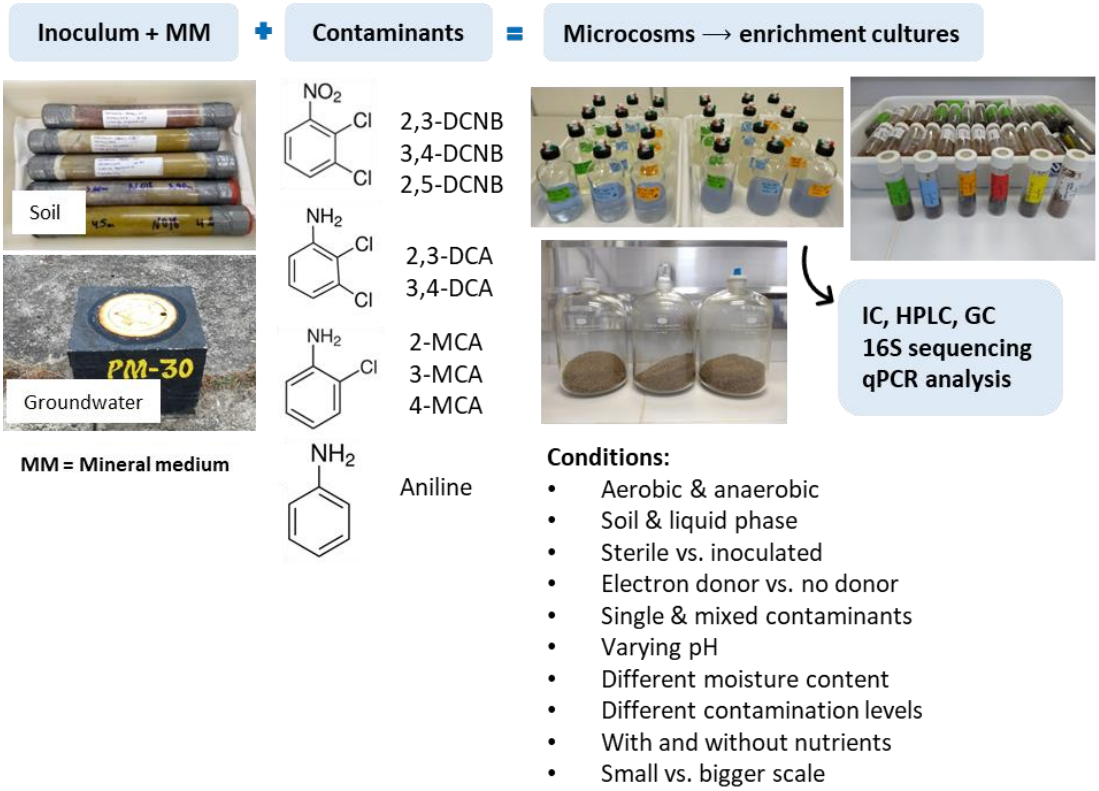
Figure 6 - Timeline of my Ph.D. journey



Source: author (2022)

Setting up microcosms was the starting point of each pre-experiment and experiment performed in this study. Microcosms were set up in glass bottles containing a volume corresponding to the inoculum and mineral medium, and a volume of headspace. As inoculum, we used a source of bacteria which could be either soil or groundwater from the site or sludge from a wastewater treatment plant (for the pre-experiments performed at the contaminated industrial site). The inoculum was added to a bottle containing the target compounds and mineral medium (MM), then we have what we call “microcosm”. Contaminants used in this study were: 2,3-, 3,4-, and 2,5-dichloronitrobenzene (DCNB), 2,3- and 3,4-dichloroaniline (DCA), 2-, 3-, and 4-monochloroaniline (MCA), and aniline. Microcosms were monitored by ion chromatography (IC), high-performance liquid chromatography (HPLC), gas chromatography (GC), and DNA sequencing of the 16S rRNA gene and its quantification by quantitative polymerase chain reaction (qPCR) (Figure 7).

Figure 7 - General experiments and methods applied in this research



Source: author (2022)

3.1 EVALUATING THE BIODEGRADABILITY OF COI – ENRICHMENT CULTURES

The first objective of our research was achieved by enriching active cultures able to transform or degrade each of the contaminants of interest. We monitored contaminants transformation products and microbial composition over a period of almost 1 year in aerobic microcosms, and for almost 3 years in anaerobic microcosms as described below.

3.1.1 Aerobic microcosms

3.1.1.1 Microcosms setup and maintenance

Microcosms were set up in sterilized 250 mL boston clear glass bottles with mininert caps, containing mineral medium (MM) free of ammonium (adapted from Bruhn *et al.*, 1987), the target

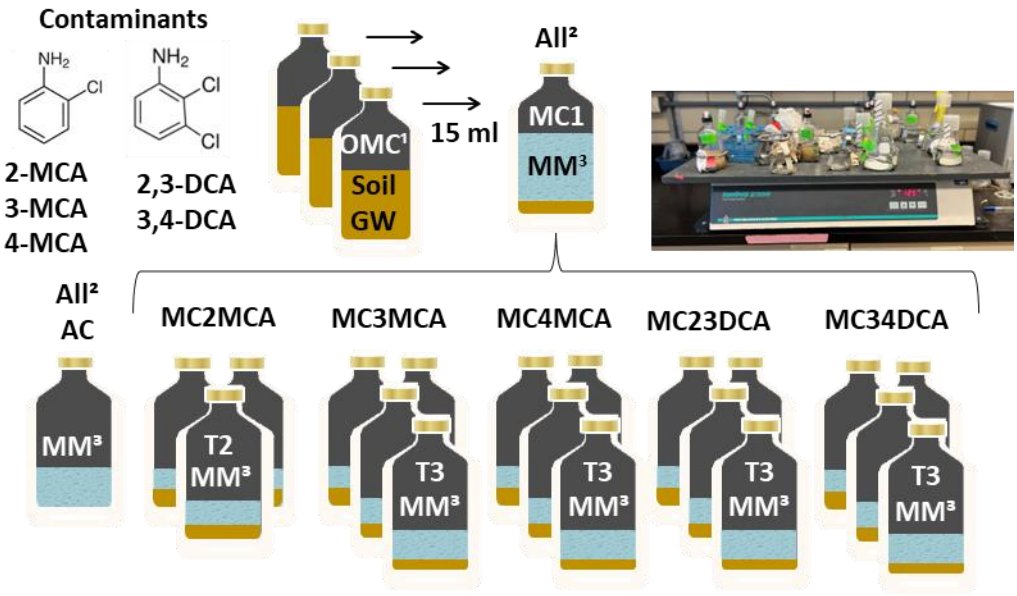
compounds and the inoculum. To prepare 2 L of MM in ddH_2O, the following constitutes were added: $Na_2HPO_4.12H_2O$ (7 g), KH_2PO_4 (1 g), and $CaCl_2$. $2H_2O$ (10 mg), ferric citrate (2 mg), $MgSO_4$. $7H_2O$ (20 mg), and 2 mL of a solution of trace minerals (EDWARDS, E. A.; GRBIC-GALIC, 1994). The medium solution was then autoclaved, and its pH was measured and adjusted if necessary to 7.

During the experimental period, all bottles were kept sealed in a shaker at 150 rpm, at 24 ± 1°C. At least once a week, the bottles were open in the fumehood where they stayed for around 20 minutes while they were refed and the atmosphere refreshed to replenish oxygen to ambient conditions. Contaminants tested in this experiment were 2-, 3-, and 4-monochloroaniline, and 2,3- and 3,4-dichloroaniline.

Figure 8 shows a scheme of our experimental setup. We started our first microcosm (MC1) with all 5 target chloroanilines mixed together (2-, 3-, and 4-MCA and 2,3 and 3,4-DCA), the initial feeding concentration was 10 mg/L for each contaminant (feeding procedure is explained later in this section). As inoculum of MC1, we used three original microcosms (OMC) that were previously set up in 2017 at the University of Toronto (by Line Lomheim and Suzana Kraus), which contained soil and groundwater from the contaminated site and had degraded a mixture of chloroanilines before (KRAUS, 2018). From each of those OMC, we took a homogenized sample of 5 mL, totalling 15 mL as inoculum in MC1. Then, we topped MC1 up with medium to 100 mL. To verify abiotic losses, we also set an abiotic control (AC) with the same contaminants and medium only (total volume of 100 mL).

After showing activity for all 5 chloroanilines, 10 new microcosms were set up, 2 replicates for each specific compound: MC2MCA (A and B); MC3MCA (A and B); MC4MCA (A and B); MC23DCA (A and B); MC34DCA (A and B). Each of the new microcosms was inoculated with 5 mL from MC1 and received 45 mL of MM. Cultures were enriched by feeding them once a week with the specific compound. The initial target feeding concentration was 10 mg/L for all the 10 microcosms and to the AC (corresponding to around 78 and 62 μM for MCA and DCA isomers, respectively). After that, we increased the concentration to 20 mg/L for each compound (157 and 123 μM for MCA and DCA isomers, respectively). After many feedings, 1:10 transfers (T1, T2, and T3) to new bottles were done.

Figure 8 - Experimental scheme of the aerobic experiment



[1]Original microcosms from previous studies set with a mixture of contaminants (KRAUS, 2018). [2]All compounds together (2-, 3-, and 4-MCA, 2,3- and 3,4-DCA). [3]Mineral medium (MM) free of NH_4^+.
Source: author (2022)

3.1.1.2 Monitoring the experiment and analytical procedures

Activity in microcosms was monitored by using a Hewlett-Packard/Agilent 1050 series high-performance liquid chromatography (HPLC) system and calibration curves built with standard solutions prepared with the chemicals in methanol. Contaminant biodegradation was verified by the decrease or the disappearance of the peak in the specific retention time for each contaminant (2-MCA, 3-MCA, 4-MCA, 2,3-DCA, and 3,4-DCA). The concentration of ions (ammonium, nitrite, nitrate, and chloride) was measured by using a DionexTM ICS-2100 pump (Ion Chromatography System). Changes in the microbial community abundance were monitored by sequencing and quantifying by qPCR the 16S rRNA gene aiming to infer which microorganisms are likely to be responsible for the enzymatic reactions taking place in the microcosms. The pH value was monitored using a pH Spear meter (Oakton Instruments). Specific methods are described in section 4.1.

3.1.2 Anaerobic microcosms

3.1.2.1 Microcosms setup and maintenance

Anaerobic microcosms were set in sterilized 250 mL boston round bottles with mininert caps, containing the inoculum, the target individual compound, electron donor (ethanol and lactate) and an anaerobic mineral medium (MM) free of ammonium (adapted from EDWARDS & GRBIC-GALIC, 1994) (available in the Appendix B2) with the pH in the range of 6.60 and 6.95. We decided not to provide ammonium in the medium expecting to see the cation being released from biotransformation of the contaminants and used by the organisms as nitrogen source. Electron acceptor as nitrate or sulphate was not intended to be provided, tiny amount of sulphate (around 30 μM) was part of the medium composition.

Contaminants tested in this experiment were 2,3-, 3,4-, and 2,5-dichloronitrobenzene (DCNB), 2,3- and 3,4-dichloroaniline (DCA), 2-monochloroaniline (MCA), and aniline. The microcosms were continually amended when activity was verified, in other words, if the concentration of the contaminants was decreased aligned to the detection of other compounds (indicating the COI were biotransformed into by-products), individual contaminants were re-amended.

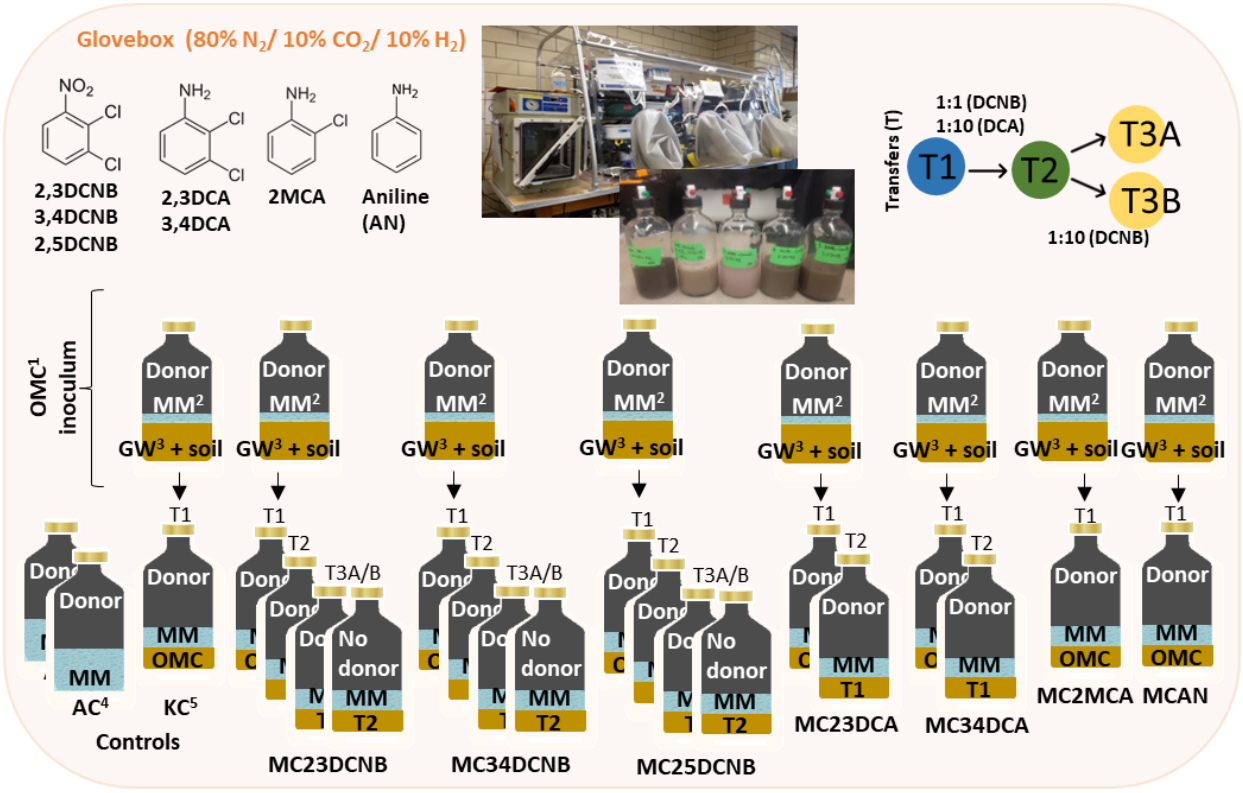
All bottles were kept inside a Vinyl Anaerobic Chamber glove box (Coy Lab Products), in which the atmosphere is free of molecular oxygen, composed of 80% of N_2, 10% of CO_2, and 10 % H_2, at 24 ± 1°C. The solutions used in this experiment (mineral medium, neat ethanol and sodium lactate 0.7M) were brought into the glovebox after being purged with N_2. Additionally, all the bottles, caps, and feeding vials are kept inside the glovebox for at least 3 days before being used, to ensure oxygen is not being provided to the cultures. To avoid contamination of the glovebox with methane, every time we needed to open the anaerobic microcosms, we had to purge their headspace with a mix of N_2 and CO_2 (80% and 20%) to get rid of any gasses formed inside the bottles.

An overview of the experimental setup is shown in Figure 9. The experiment started with transfer 1 (T1) of microcosms (MC), consisting of 9 bottles with a total of 100 mL of liquid and 150 mL of headspace each. One of the bottles was not inoculated and contained only mineral medium with a mix of contaminants, which was our abiotic control (AC).

Each of the other 8 bottles were inoculated with the pellet of 40 mL of homogenized material (centrifuged at 12,000 g for 15 min) from 8 original anaerobic microcosms (OMC) set up by Line Lomheim at Edwards Lab (UofT) previously. Those original microcosms contained soil and groundwater from the contaminated site where the remediation project is taking place, they have been amended with a mixture of contaminants and showed activity for the specific contaminants of this research under anaerobic conditions.

After being inoculated, one of these 8 bottles was three times autoclaved in three consecutive days, to ensure all organisms from the OMC used for this bottle were inactivated. This bottle was amended with a mixture of contaminants and was named as our heat killed control (KC). The other 7 bottles corresponded to our microcosms (MC), amended with 2,3-DCNB (MC23DCNB-T1), 3,4-DCNB (MC34DCNB-T1), 2,5-DCNB (MC25DCNB-T1), 2,3-DCA (MC23DCA-T1), 3,4-DCA (MC34DCA-T1), 2-MCA (MC2MCA-T1), and aniline (MCAN-T1). Transfers of active microcosms (T2 and T3) were made during the experimental period.

Figure 9 - Experimental scheme of the anaerobic experiment



[1]8 original microcosms (OMC), each one used as inoculum to the 8 subsequent microcosms. [2]Mineral medium (MM) free of NH_4^+. [3]Groundwater. [4]Abiotic control (AC) with mineral medium and 3,4-DCNB, 2,3-DCNB, 2,3-DCA, 3,4-DCA, 2-MCA, and aniline. [5]Heat killed control (KC) with autoclaved soil from OMC, MM, and 3,4-DCNB, 2,5-DCNB, 2,3-DCA, 3,4-DCA, 2-MCA, and aniline.
Source: author (2022)

The active microcosms from T1 (MC-T1) were used as inocula to transfer 2 (MC-T2). The microcosms amended with DCNB were transferred in 1:1 dilution, while for the DCA microcosms the dilution was 1:10. Thus, 50 mL of the MCDCNB-T1 were merged with 50 mL of medium in new sterile bottles, while 10 mL of MCDCA-T1 were merged with 90 mL of medium in the new bottles. The microcosms with 2-MCA and aniline were not transferred since they did not show any activity.

The microcosms amended with DCNB isomers from transfer 2 (MCDCNB-T2) were used as inocula for the next group of microcosms, transfer 3 (T3). This new transfer was done in 1:10 dilution into two new bottles for each DCNB culture, thus, 10 mL of each microcosm were added into two new bottles with 90 mL of medium each. One new bottle for each DNCB culture had donor addition, as in the previous transfers, the second bottle had no donor added. The objective

of the third transfer was to observe differences in COI biotransformation when there was limited availability of electrons. The last group of microcosms were called MC-T3A, with donor, and MC-T3B, without added donor.

Fresh mineral medium was added to the bottles that served as inocula to a new transfer and/or when the volume of the enrichments was close to 50 mL. The addition of fresh mineral medium aims to replace the amount of liquid taken out for analyses and to give the community more favourable conditions by diluting the products accumulating in the microcosms and by giving them more medium constituents. Another reason for adding more medium was to gradually increase the volume of the enrichment and thus be able to perform analyses for future studies that may require bigger volume of sample. For that, at certain point of the research, medium was added to the active microcosms for a final volume of 150 mL.

The target feeding concentration also changed along the experiment period. The initial concentration was 10 mg/L of each contaminant, corresponding to 52.08 μM of DCNB, 61.72 μM of DCA, 78.39 μM of MCA, and 107.38 μM of aniline. Concentration was then adjusted to 13.3 mg/L and then to 20 mg/L of each compound if activity was observed. Similarly, the amount of donor added was increased from 514 μM and 213 μM for ethanol and lactate, respectively, to 685 μM and 308 μM of the corresponding values. The ratio between available electrons and electrons needed for the contaminant to be converted into aniline was aimed to be always higher than 5 for each source. This ratio was chosen as a balance to ensure there was no electron limitation and to avoid the fermenters to take over the microbial community (based on previous tests performed by the group at the UofT, not included in this document). The initial pH of the microcosms and controls was around 6.60 and stayed in the range of 6.56 and 7.09 in active microcosms.

3.1.2.2 Monitoring the experiment and analytical procedures

Activity in microcosms was monitored by using a Hewlett-Packard/Agilent 1050 series high-performance liquid chromatography (HPLC) system for contaminants and their by-products measurement (DCNB isomers, DCA isomers, MCA isomers, and aniline). The calibration curves were built with standard solutions prepared with the chemicals in methanol. Contaminant biotransformation was verified by the decrease or the disappearance of the peak in the specific retention time for each contaminant, aligned to a possible increase or appearance of a peak in the

retention time of their correspondent by-products. As the contaminants 2,3 and 2,5-DCNB eluted at the same retention time, the control bottles with a mixture of contaminants had either one or the other isomer.

Ion analysis of lactate, acetate, chloride, and sulphate was performed by using a DionexTM ICS-2100 pump (Ion Chromatography System - IC). Lactate was just qualitatively verified, as we did not have standard curve for that anion.

The microbial community was monitored by 16S rRNA sequencing and quantified by qPCR with the objective to infer which microorganisms are involved in the activity taking place in the microcosms. The pH value was monitored using a pH Spear meter (Oakton Instruments). Specific methods are described in section 4.2.

3.1.3 Stock solutions of COI

Stock solutions for feeding the microcosms with the individual contaminants were prepared in acetone for chlorinated compounds and in water for aniline at the concentration of 5 g/L. Compounds were purchased from Sigma Aldrich (Table 5). To avoid adding acetone into the microcosms when feeding the cultures, stock solutions were not added directly into the bottles. Instead, inside the fumehood, the needed volume was completely evaporated in a 2 mL sterile vial which was then dropped inside the microcosms. For instance, for 10 mg/L in 100 mL of liquid, 200 μL of the stock solution (5 g/L) were evaporated. The corresponding volume of the stock solution of aniline was added directly.

Table 5 - Chemical compounds used for feeding in aerobic and anaerobic experiments

Contaminant	Molecular weight (g/mol)	Purity	Solubility in water (g/L)	Form	Density
Aniline	93.13	99.5%	Soluble	Liquid	1.022 g/cm^3 at 25 °C
2-MCA	127.57	99.0%	5.13 at 20 °C	Liquid	1.213 g/mL at 25 °C
3-MCA	127.57	99.0%	20 at 30 °C	Liquid	1.206 g/mL at 25 °C
4-MCA	127.57	98.0%	2	Crystals	1.14 g/cm^3 at 100°C
2,3-DCA	162.02	99.0%	No data available	Liquid	1.37 g/mL at 20 °C
3,4-DCA	162.02	99.9%	0.58 at 20 °C	Crystals	1.57 g/cm^3 at 20 °C
2,3-DCNB	192.00	99.9%	No data available	Crystals	No data available
2,5-DCNB	192.00	99.0%	0.083 at 20 °C	Crystals	1.6 g/cm^3 at 20 °C
3,4-DCNB	192.00	99.0%	No data available	Crystals	No data available

Source: adapted from information available in Sigma-Aldrich catalogue.

3.2 UNDERSTANDING BIOTRANSFORMATIONS - ANALYTICAL METHODS

Regarding the second specific objective, the steps for biodegradation of contaminants were studied through the observation of the contaminant consumption/concentration decrease and the formation of other compounds and/or final products.

To observe any abiotic reactions or any physical losses that might take place, control bottles were prepared for each experiment. For example, a sterile bottle, with mineral medium only and without inoculum, or a sterile bottle with medium and autoclaved inoculum. The pH value was monitored using a pH Spear meter (Oakton Instruments). Other specific analyses performed during experiments will be mentioned in the following sections.

3.2.1 Liquid chromatography (HPLC)

For both aerobic and anaerobic experiments, contaminant concentrations were measured using a Hewlett-Packard/Agilent 1050 series high-performance liquid chromatography (HPLC) system (Acclaim™ 120 C18 column, Thermo Scientific). Calibration curves were built with standard solutions prepared with the chemicals in methanol. Standards were a mix of contaminants: Aniline, 4-MCA, 3-MCA, 2-MCA, 3,4-DCA, 2,3-DCA, 2,5-DCA, 2,5-DCNB, 2,3-DCNB, 3,4-DCNB in concentrations of 0.1, 0.2 ,1.0, 2.0, 5.0, 10.0 mg/L. Retention times were 4.3 min for

aniline, 5.4 min for 4-MCA, 5.8 min for 3-MCA, 6.2 min for 2-MCA, 8.3 min for 3,4-DCA, 9.6 min for 2,3-DCA, 10.9 for 2,5-DCA, 14.9 min for 2,3- and 2,5-DCNB together, and 17.4 min for 3,4-DCNB (a chromatogram of the 10 mg/L standard and the calibration curves from one run are included in Appendix A, Figures 39A and 40A). The UV detector set was 254 nm, and the mobile phase was composed of 50% acetonitrile and 50% Milli-Q™ (Millipore Sigma) at a flow rate of 1 mL/min, in an isocratic flow for 25 min.

For this analysis, samples were collected by using a 2 mL Gastight® glass syringe and then filtered through a 0.22 μm Chromspec UV Syringe filter 13 mm pre-washed with methanol. A volume of 1.5 mL was sampled from each bottle, from which 1 mL was used for cleaning the filter and flushing the methanol. The remaining 0.5 mL of sample was analyzed in the HPLC. Details of this method, developed by Line Lomheim (UofT), can be read in Kraus (2018). Contaminant biodegradation was verified by the decreased or the total disappearance of the peak in the specific retention time for each compound.

3.2.2 Gas chromatography (GC)

For the anaerobic microcosms, methane was measured through gas chromatography (GC), using an Agilent 7890A gas chromatograph with a GSQ-Plot column (Agilent Technologies) and a flame ionization detector (FID). Methane measurement was not included in our routine analysis. Few samples were analyzed by Line Lomheim (UofT). More details of this methodology can be found in Lomheim *et al*. (2020) and Kraus (2018), both studies were performed at the UofT.

3.2.3 Ion Chromatography (IC)

The concentration of ions was measured using a DionexTM ICS-2100 pump (Ion Chromatography System), with an AS18 analytical column for the anions and a CS19 analytical column for the cations. Our standards were in the range of 0.005 and 1 mM. The volume of samples collected from each microcosms was 1.5 mL.

For the aerobic enrichments, we were mainly interested in measuring ammonium (cation) and chloride, nitrite, and nitrate (anions). Considering the anaerobic experiments, lactate, acetate,

chloride, and sulphate (anions) were the ions of interest. Samples were filtered through 0.2 μm nylon filters (Fisher Scientific).

The method for cations consists of a non-isocratic run: for the first 20 min the eluent is held as 2 mM of methanesulfonic acid (MSA), after that, it ramps up to 12 mM in 2 min, staying isocratic for 8 more minutes and then going back to 2 mM of MSA in 2 min, the run finishes at 38 minutes. The flow was 1 mL/min. An example of a chromatogram of the method applied for our samples and the calibration curve for ammonium are included in Appendix A (Figures 41A and 42A). The method described here was an adaptation of an existing isocratic method used at the UofT BioZone lab (1 mL/min, 8 mM for 15 min), however, with the existing method, we were not able to see a satisfactory separation between the sodium and the ammonium peaks in our samples.

For the anions, two different existing methods were applied (eluent was potassium hydroxide and its flow was 0.25 mL/min in both). For the aerobic experiment, we used an isocratic method (23 mM) for 20 min. This gives us peaks of chloride (4.7 min), nitrite (5.6 min), sulphate (7.5 min), nitrate (8.7 min), and phosphate (16.4 min) (a chromatogram of the standard 1 mM and the calibration curves for chloride, nitrite and nitrate are included in Appendix A, Figures 43A and 44A).

For the anaerobic samples, we applied a longer and non-isocratic method. It starts with the eluent concentration of 0.5 mM and reaches 2.5 mM in 10 min and 30 mM in the minute 29. The concentration is kept at 30 mM until the minute 35, then it is decreased to 0.5 mM when time is 37.1 min and the run finishes at minute 45.

By using the non-isocratic method, we obtained concentrations of acetate (12.6 min), chloride (17.8 min), nitrite (19.5 min), sulphate (23.6 min), nitrate (26.6 min), and phosphate (32.1 min). This method also gives us peaks for lactate and organic acids, such as propionate and butyrate. The lactate peak shows up before the acetate peak (~12 min), thus, we could easily detect it in some of the anaerobic samples, even though we did not quantify it. The organic acid peaks are expected to show up between chloride and nitrite peaks. We did see some tiny peaks in this range, however, we could not identify to which acid they would correspond, as we did not include them in our standards. Future tests will clear this up. A chromatogram of the standard 1 mM, a chromatogram showing the lactate peak, and the calibration curves for acetate, chloride, and sulphate are included in Appendix A (Figures 45A, 46A and 47A).

3.3 MICROBIAL COMMUNITY ANALYSIS AND QUANTITATIVE PCR

Samples for DNA sequencing and quantification were collected from each enrichment culture (aerobic and anaerobic) to evaluate the microbial composition in active microcosm across the experimental period. Genera found in microbial communities were analyzed at the amplicon sequence variant (ASV) level. Details are provided below.

3.3.1 DNA extraction and 16S sequencing

From the samples collected from the cultures, at the UofT, the DNA was extracted using DNeasy PowerSoil Kit (Qiagen), and DNA concentrations were quantified using a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific). The extracted DNA was sent for Illumina MiSeq PE300 16S rRNA amplicon sequencing at Genome Québec, using forward primer 926f modified (5'-AAACTYAAAKGAATWGRCGG-3') and reverse primer 1392r modified (5'-ACGGGCGGTGWGTRC-3'), targeting the V6-V8 variable region of the 16SrRNA. The sequencing results were processed using Qiime2 by Line Lomheim and Courtney Toth (also from UofT) using the Silva database. After that, output files were manipulated using Excel software. Results of organisms found in the microbial community were obtained as relative abundance.

Most abundant genera found in microbial communities were analyzed at the amplicon sequence variant (ASV) level; thus, many ASV of interest found in the cultures were compared to other microorganisms submitted to the National Center for Biotechnology Information (NCBI). For this comparison, we used the Basic Local Alignment Search Tool (BLAST), prioritizing similarities of 100% (considering query coverage and percent identity), completely sequenced genome reported and published studies.

3.3.2 Quantitative polymerase chain reaction (qPCR)

We proceeded to the quantification of the total copies of bacteria in each DNA sample by quantitative polymerase chain reaction (qPCR), using a CFX96TM real-time PCR detection system, with a C1000 thermocycler (Bio-Rad Laboratories Inc.). Each qPCR sample was composed of SsoFastTM EvaGreen® SuperMix (Bio-Rad Laboratories Inc.), the forward and the reverse 16S

rRNA primers GenBac1055f (5'-ATGGYTGTCGTCAGCT-3') and GenBac1392r (5'-ACGGGCGGTGTGTAC-3'), UltraPureTM Distilled water (Invitrogen), and the DNA extracted or the standards. Samples of DNA were diluted in 1:10 (sample:water) to avoid interferences of the matrix. The standards were in the range of 10^1 and 10^8 gene copies/mL, they were prepared by doing serial dilutions with buffer, to avoid adsorption in the plate. Samples and standards were arranged in triplicate and at least three blanks (with no sample or standard) were included at the end of the plate to check for any contamination that could occur during the run preparation.

The qPCR run consisted of cycles of 98°C for 2 min, 40 cycles of 98°C for 5 seconds and 55°C for 10 seconds, followed by an increase from 65°C to 95°C at 0.5°C increments over 10 seconds. Samples from the same microcosms were arranged in the same plate and quantified in the same run. The R^2 of the four plates were 0.998, 0.998, 0.999, and 0.997 while the efficiency values were 106.1%, 101.8%, 102.4%, and 101.6%. The amplification curves for the plasmid standards (Std), for the blanks (NTC), and for samples (Unknown) from each of the four plates are available in Appendix A, as Figures 48A to 50A (aerobic samples) and 51A to 59A (anaerobic samples).

Results of the qPCR runs were converted into copies/mL and used to turn the relative abundance obtained from the DNA sequencing into the absolute abundance of the microbial community found in each of the five microcosms.

3.3.3 Viable culture preservation

From the active enrichments, we took samples to be preserved in glycerol, under -80°C. The glycerol protects the cell when samples are frozen, avoiding cell lysis. The procedure steps are the following:

1. Take the sample of the cultures (4 mL from the aerobic, 20 mL from the anaerobic);
2. Centrifuge the sample at 7000 g for 20 min;
3. Resuspend the sample in 1 mL;
4. Transfer the sample to a screw o-ring cap micro tube;
5. Add 1 mL of an sterile glycerol 50% solution;
6. Let the sample sit for 30 minutes;
7. Flash the sample with liquid nitrogen;
8. Keep the sample under -80°C.

The glycerol solution used for the anaerobic cultures was anaerobic and the manipulation steps when dealing with the anaerobic cultures were performed inside the glovebox.

3.4 LEARNING AND SHARING EXPERIENCES

As mentioned before, this research is part of a broader remediation project carried out by the industry, in partnership with many experts, universities, and consultancy firms. Monthly meetings with the Bioteam were the strategy found to achieve the fourth objective of this study. During the meetings, all the knowledge acquired since the beginning of this study was shared with the members. By presenting results from research activities, it was possible to share and get valuable feedback from people involved in the project. Thus, through the group discussions, consultants and researchers from universities in Canada, the U.S., and Brazil, get together to discuss the different aspects related to site remediation and contribute to the bioremediation project.

In addition to the regular meetings, but still in the collaborative spirit, as part of the Bioteam, we also performed experiments on-demand and worked on side activities that were not the focus of the research described here, in this document. These activities were important to some aspects of the project itself (for instance to the pilot tests performance improvement or to the local laboratory workers) or to another academic research taking place and related to the project.

Apart from the submission of the final version of this thesis, the data on aromatic compounds biodegradation found and discussed here will be published in relevant and open-access peer-reviewed journals in the research field. Additionally, the results from this research are expected to contribute to the discussions of bioremediation projects that are being planned/performed by the industry and its research team, and in a more optimistic view, bring benefits to the environment and society in general.

4 ANALYSIS AND DISCUSSION OF RESULTS

4.1 AEROBIC BIODEGRADATION OF THE CONTAMINANTS

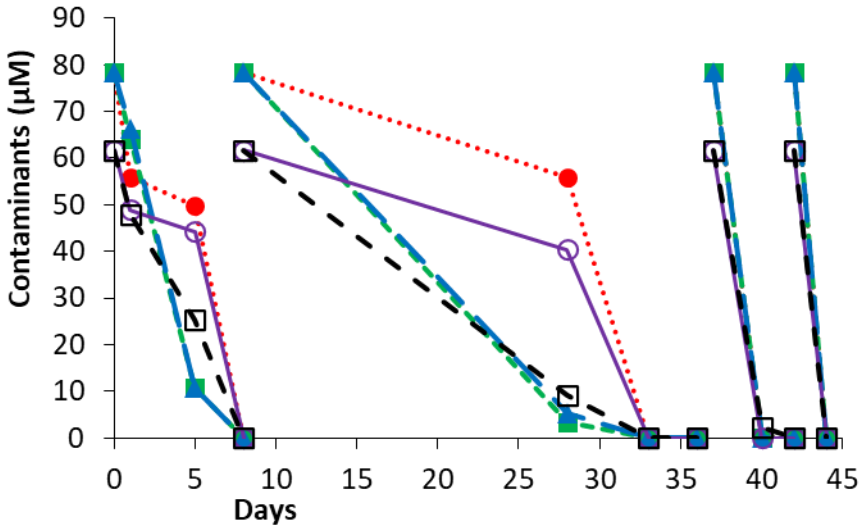
The objectives of this series of experiments were to verify natural attenuation at the site and the possibility of applying biostimulation or bioaugmentation at the site. Aiming that, we focused on finding and enriching native organisms from the contaminated site with ability to aerobically biodegrade monochloroaniline isomers (2-, 3- and 4-MCA) and dichloroaniline isomers (2,3- and 3,4-DCA) individually. Studying the mechanisms of biodegradation and the microbial community involved in the biodegradation observed and highlighting how the findings are related to our case study was what a matter of interest to show how the industry could improve site bioremediation. It is already known that the target contaminants are all biodegradable, and some organisms were identified as able to utilize them as sole carbon, nitrogen, and energy sources for growing (see section 2).

This experiment lasted for around 250 days. Even though the cultures are not maintained actively anymore, we have samples from each culture preserved in glycerol, under -80^{o}C, available for future studies (Edwards Lab, Barcode freezer, box A061 – details available in the inventory).

4.1.1 Biodegradation of monochloroanilines and dichloroanilines

Figure 10 shows the activity of MC1, amended with 2-, 3-, and 4-monochloroaniline and 2,3- and 3,4-dichloroaniline and inoculated with 15 mL of previous microcosms containing soil and groundwater from the contaminated site (OMC). MC1 was amended 4 times and all compounds rapidly degraded in the last two feedings. After that, we split part of the 100 mL culture into 10 new microcosms (1:10 dilutions): MC2MCA (A and B); MC3MCA (A and B); MC4MCA (A and B); MC23DCA (A and B); MC34DCA (A and B), as explained before.

Figure 10 - Concentrations of contaminants in MC1



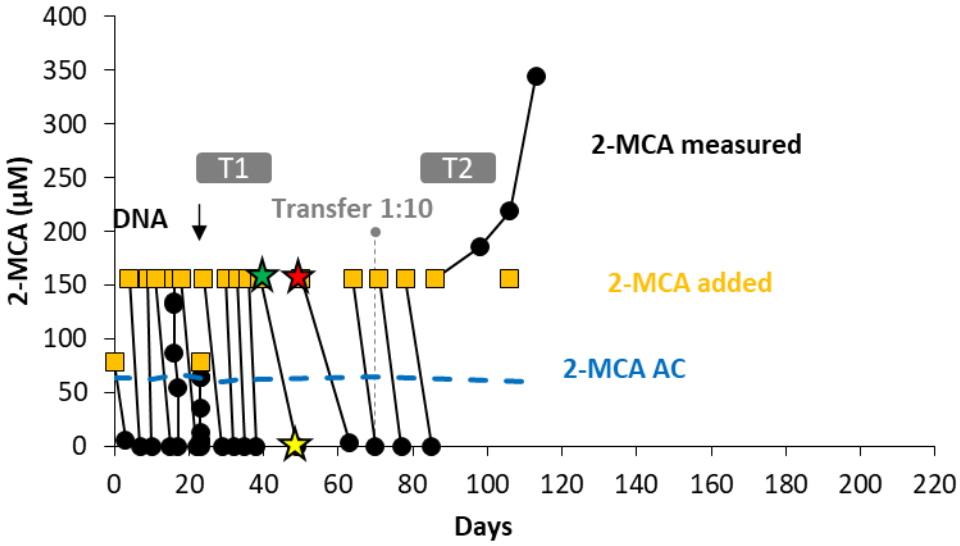
First microcosm (MC1) with all 5 target chloroanilines mixed together 2-MCA (···●···), 3-MCA (-■-), 4-MCA (▲), 2,3-DCA (○), and 3,4-DCA (□). As inoculum of MC1, we used three original microcosms (OMC) that were previously set in 2017 at the University of Toronto, which contained soil and groundwater from the contaminated site.

Source: author (2022)

The activity of the enrichments in terms of concentration of target contaminant is presented in Figures 11 to 15 for MC2MCA, MC3MCA, MC4MCA, MC23DCA, and MC34DCA.

The initial target feeding concentration was 10 mg/L of each contaminant in MC1 and this was the first feeding concentration for all the 10 microcosms and to the AC. After that, we increased the concentration to 20 mg/L for each compound (157 and 123 µM for MCA and DCA isomers, respectively). During the experiment, no subproducts were detected in the HPLC results from the active microcosms. The AC stayed inactive the whole period. The pH value measured for each microcosm was in the range of 6.9 and 7.1.

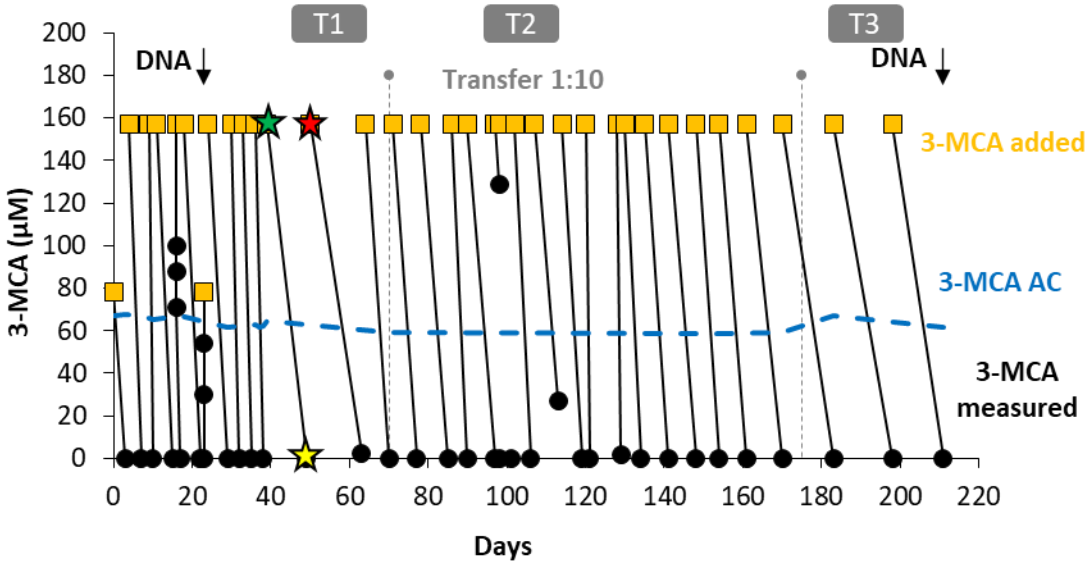
Figure 11 - Concentration profile over time in MC2MCA



Two bottles were set up for each compound, but as they performed the same, the contents were merged on day 23. Therefore, the data of this graphic represent one bottle only. Each transfer was 1:10 (transfer 2 starts on day 70). Frequency of sampling was once a week, usually contaminant was gone, and microcosm was refed. More frequent sampling was performed on days 16 and 23 (profiles of day 23 are shown on Figure 16). On days 86 and 106, the culture did not degrade the contaminant, which accumulated in the medium. Elements of graphic represent: transfer 1 (T1) and transfer 2 (T2) of the microcosm; calculated concentrations of target compound amended to the bottle ( ), measured concentrations of target compounds (—●—), and concentrations measured of target compound in abiotic control (AC) ( ); DNA sampling day (16S sequencing and qPCR) ( ); day when culture was transferred to a new bottle ( ); last feeding day before laboratory closure ( ), first day of HPLC analysis after laboratory closure ( ), and first feeding day after laboratory reopening ( ).

Source: author (2022)

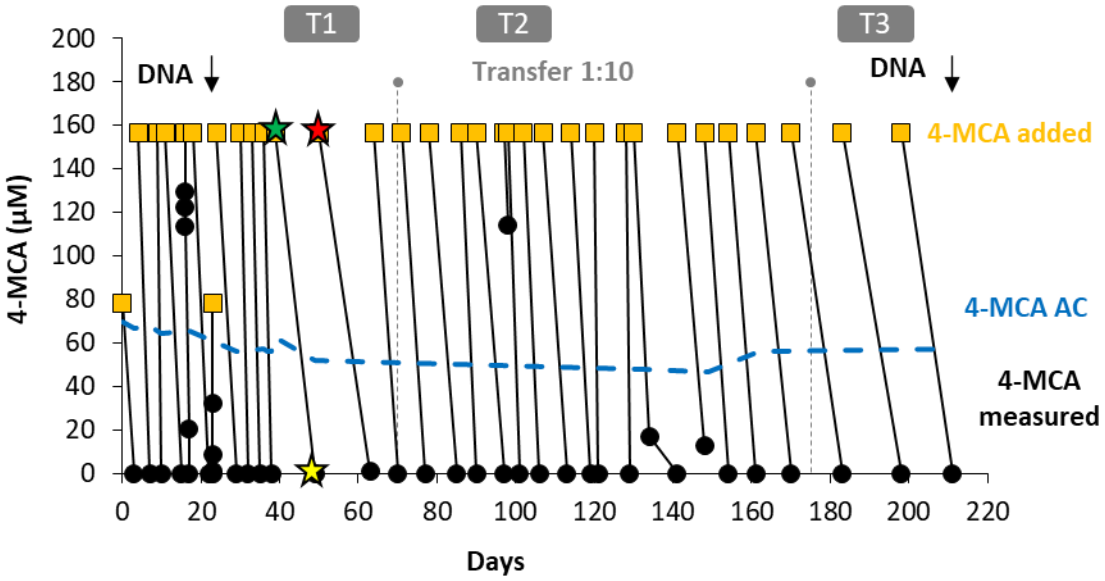
Figure 12 - Concentration profile over time in MC3MCA



Two bottles were set up for each compound, but as they performed the same, the contents were merged on day 23. Therefore, the data of this graphic represent one bottle only. Each transfer was 1:10 (transfer 2 starts on day 70, transfer 3 starts on day 175). Frequency of sampling was once a week, usually contaminant was gone, and microcosm was refed. More frequent sampling was performed on days 16 and 23 (profiles of day 23 are shown on Figure 16). Elements of graphic represent: transfer 1 (T1), transfer 2 (T2), transfer 3 (T3) of the microcosm; calculated concentrations of target compound amended to the bottle ( ), measured concentrations of target compounds (—●—), and concentrations measured of target compound in abiotic control (AC) (– – –); DNA sampling day (16S sequencing and qPCR) ( ); day when culture was transferred to a new bottle ( ); last feeding day before laboratory closure ( ), first day of HPLC analysis after laboratory closure ( ), and first feeding day after laboratory reopening ( ).

Source: author (2022)

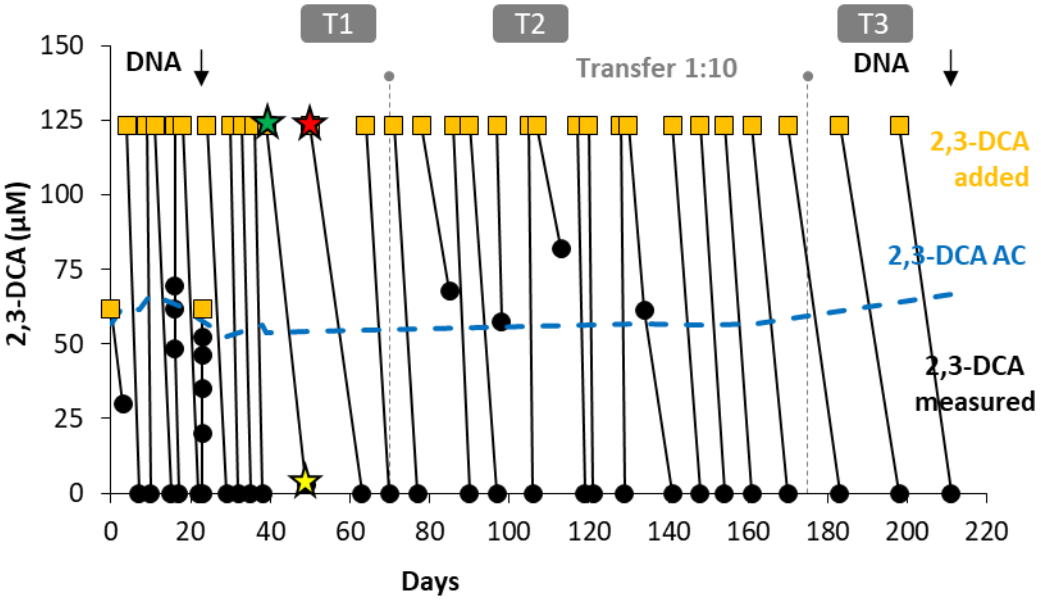
Figure 13 - Concentration profile over time in MC4MCA



Two bottles were set up for each compound, but as they performed the same, the contents were merged on day 23. Therefore, the data of this graphic represent one bottle only. Each transfer was 1:10 (transfer 2 starts on day 70, transfer 3 starts on day 175). Frequency of sampling was once a week, usually contaminant was gone, and microcosm was refed. More frequent sampling was performed on days 16 and 23 (profiles of day 23 are shown on Figure 16). Elements of graphic represent: transfer 1 (T1), transfer 2 (T2), transfer 3 (T3) of the microcosm; calculated concentrations of target compound amended to the bottle ( ), measured concentrations of target compounds (—●—), and concentrations measured of target compound in abiotic control (AC) (– – –); DNA sampling day (16S sequencing and qPCR) ( ); day when culture was transferred to a new bottle ( ); last feeding day before laboratory closure ( ), first day of HPLC analysis after laboratory closure ( ), and first feeding day after laboratory reopening ( ).

Source: author (2022)

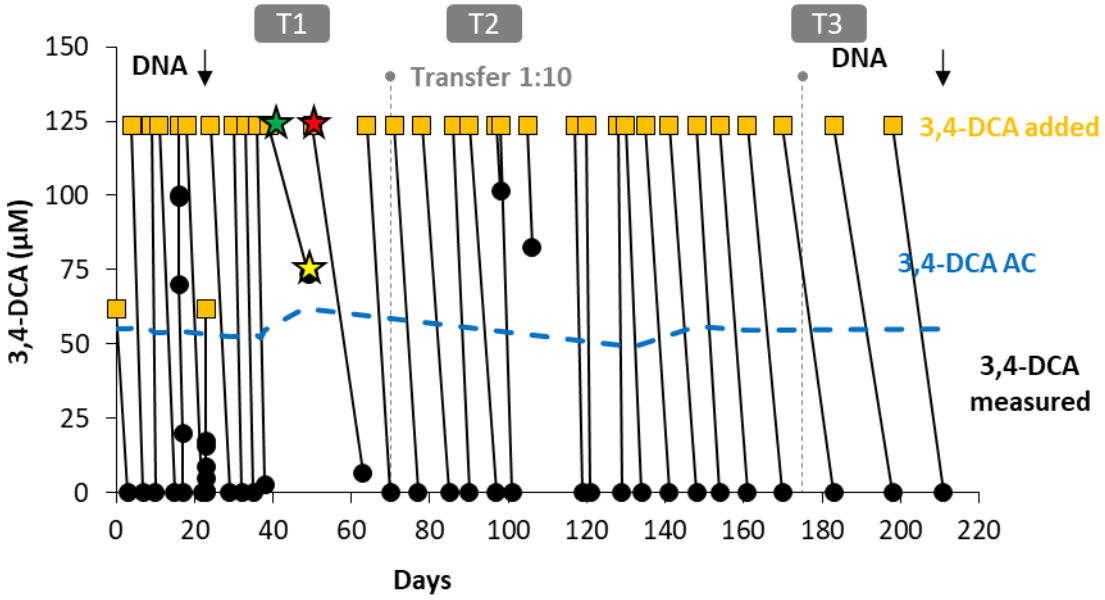
Figure 14 - Concentration profile over time in MC23DCA



Two bottles were set up for each compound, but as they performed the same, the contents were merged on day 23. Therefore, the data of this graphic represent one bottle only. Each transfer was 1:10 (transfer 2 starts on day 70, transfer 3 starts on day 175). Frequency of sampling was once a week, usually contaminant was gone, and microcosm was refed. More frequent sampling was performed on days 16 and 23 (profiles of day 23 are shown on Figure 16). Elements of graphic represent: transfer 1 (T1), transfer 2 (T2), transfer 3 (T3) of the microcosm; calculated concentrations of target compound amended to the bottle ( ), measured concentrations of target compounds (—●—), and concentrations measured of target compound in abiotic control (AC) (– – –); DNA sampling day (16S sequencing and qPCR) ( ); day when culture was transferred to a new bottle ( ); last feeding day before laboratory closure ( ), first day of HPLC analysis after laboratory closure ( ), and first feeding day after laboratory reopening ( ).

Source: author (2022)

Figure 15 - Concentration profile over time in MC34DCA

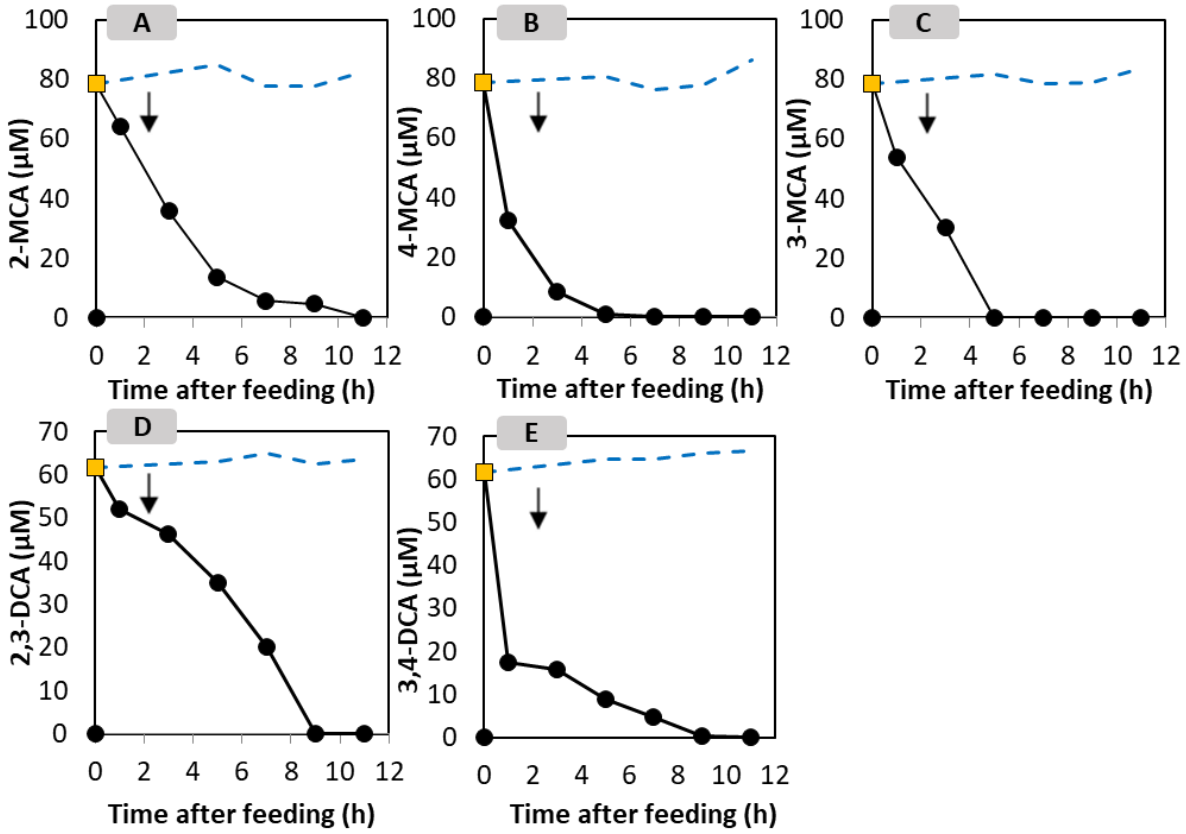


Two bottles were set up for each compound, but as they performed the same, the contents were merged on day 23. Therefore, the data of this graphic represent one bottle only. Each transfer was 1:10 (transfer 2 starts on day 70, transfer 3 starts on day 175). Frequency of sampling was once a week, usually contaminant was gone, and microcosm was refed. More frequent sampling was performed on days 16 and 23 (profiles of day 23 are shown on Figure 16). Elements of graphic represent: transfer 1 (T1), transfer 2 (T2), transfer 3 (T3) of the microcosm; calculated concentrations of target compound amended to the bottle ( ), measured concentrations of target compounds (—●—), and concentrations measured of target compound in abiotic control (AC) (– – –); DNA sampling day (16S sequencing and qPCR) ( ); day when culture was transferred to a new bottle ( ); last feeding day before laboratory closure ( ), first day of HPLC analysis after laboratory closure ( ), and first feeding day after laboratory reopening ( ).

Source: author (2022)

Since we had never observed remaining contaminants in any of the replicates until then, on days 16 and 23, after feeding, we performed two temporal profiles by taking a series of samples for HPLC analysis from each microcosm every two hours to try to see decay in the contaminant's concentration. On the second temporal profile, on the 23rd experimental day, we amended microcosms with 10 mg/L of each contaminant only to try to see complete degradation during one day of sequential sampling. To verify abiotic losses, we also set a new sterile bottle, with mineral medium only and a mix of all contaminants. We did see concentration of contaminants decreasing along the day in the active microcosms (days 16 and 23, Figures 11 to 15), but due to rapid degradation and few samples analyzed, it was not possible to have kinetics adjustment. The specific graphics for the second temporal profile (day 23) are presented in Figure 16.

Figure 16 - Concentration profile over one day of analysis in MC2MCA (a); MC3MCA (b); MC4MCA (c); MC23DCA (d), and MC34DCA (e)



Calculated concentrations of target compound amended to the bottle ( ), measured concentrations of target compounds (—●—), and concentrations measured of target compound in abiotic control (AC) (– –); DNA sampling on day 23 (16S sequencing and qPCR) (↓).

Source: author (2022)

Unfortunately, around day 30, the pandemic arrived, and labs were closed for 3 months. The microcosms were kept under 8°C until we could restart enriching the cultures. Day 39 was the last feeding before closure and the first day of analysis after lab reopening is represented by the day 49 (Figures 11 to 15). MC2MCA did not survive the transfer made after the period in the fridge without being fed. The culture did not present activity anymore; indicated by the contaminant being accumulated in the microcosm after two feedings (Figure 11, days 86 and 106). Thus, we continued the study with transfers 2 (T2) of MC3MCA, MC4MCA, MC23DCA, and MC43DCA.

4.1.2 Ion analysis (chloride and N-compounds)

For each mole of monochloroaniline added (2-MCA, 3-MCA, 4-MCA), 1 mole of chlorine anion should be released; while for the dichloroanilines, 2 moles are the theoretical value. Ammonium is also a cation that might be available when compounds are mineralized in the proportion of 1 mole of NH_4^+ to each mole of both compounds. However, considering the cell growth, around 66% of ammonium is expected to be released in the medium, according to the equations 1 and 2 below (reactions for the other isomers are available in Appendix B1). The equations were calculated using methods outlined by Rittmann and Mccarty (2020) and Susarla *et al*. (1997), the energy transfer efficiency of the reaction (coefficient epsilon) was 0.3, based on personal communication with Dr Elizabeth Edwards. To check the mole balance, concentrations of chloride, nitrite, nitrate, and ammonium were measured from samples collected from each microcosm.

From the MC2MCA we only sampled during the first transfer (T1, days 0 and 17), as the second (T2) stopped degrading the 2-MCA (Figure 11). For the MC23DCA we just have results from the second transfer, due to a sampling problem during the initial period (T1). The other microcosms (MC3MCA, MC4MCA, and MC34DCA) were sampled twice during the first transfer, on days 0 and 17, and twice during the second transfer (T2), on days 70 and 129.

Equation 1 for 2-MCA: $1.00C_6H_6ClN + 4.81O_2$

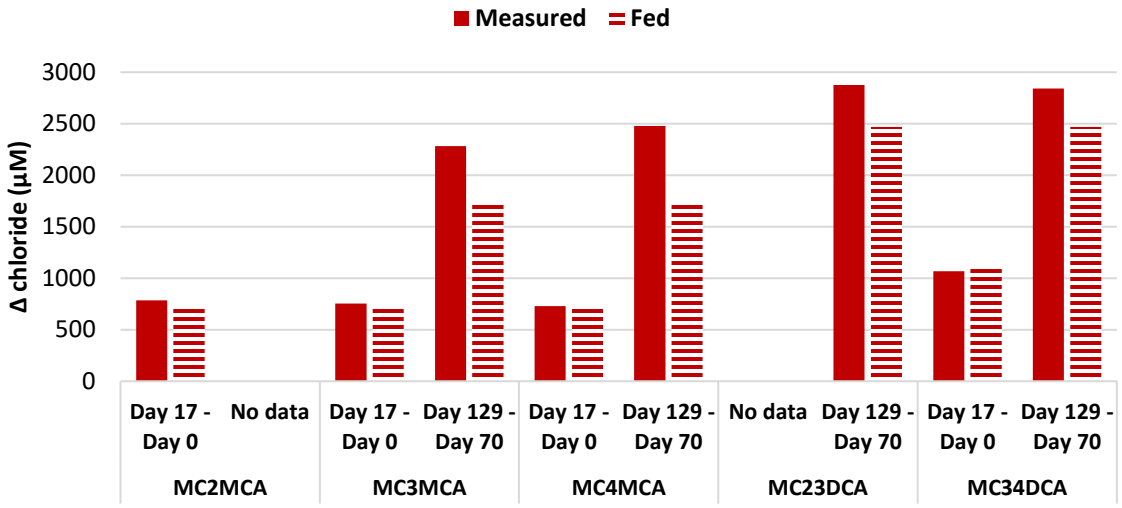
$$\rightarrow 4.31CO_2 + 0.66NH_4^+ + 1.00Cl^- + 0.32H_2O + 0.34C_5H_7O_2N + 0.34H^+ \quad (1)$$

Equation 2 for 2,3-DCA: $1.00C_6H_5Cl_2N + 4.15O_2 + 0.74H_2O$

$$\rightarrow 4.15CO_2 + 0.63NH_4^+ + 2.00Cl^- + 0.37C_5H_7O_2N + 1.37H^+ \quad (2)$$

Figure 17 shows the measured and expected difference of the chloride concentration between day 17 and day 0 and between day 129 and day 70 for each microcosm. In other words, it shows the chloride released from the biodegradation of the compounds and the amount we expect to see based on the biochemical reactions presented above.

Figure 17 - Chloride concentrations in aerobic microcosms



Values of chloride measured (■) and expected from feeding (〓) released from biodegradation of compounds between days 17 and 0 (the concentration of chloride on day 17 minus its concentration on day 0) and days 129 and 70 (the concentration of chloride on day 129 minus its concentration on day 70).
Source: author (2022)

Chloride accumulation measured in almost all the microcosms was close but a bit higher than the expected amount. That happened because the real volume of each microcosm was lower than 100 mL, since we were frequently sampling and did not top them up during the analyzed period. These chloride measurements confirm that MCA and DCA were biodegraded as the mole balance is so close. Also, the comparison indicates that no other chlorinated organic by-product was accumulating in the microcosms. The concentrations of chloride (measured and expected from biodegradation of contaminants) are available in Appendix B1, Table 8B1.

Concerning the other ions of interest (ammonium, nitrite, and nitrate), we saw changes in ammonium and nitrate concentrations, the nitrite was not detected in the analyzed samples. Table 6 summarizes the measurements and calculations for the N-compounds.

We did not expect to see similar values between measured and the theoretical values of ammonium added as part of the amended contaminant, as the cells use the contaminant as nitrogen source for growth. However, the sum of the N-compounds measured (nitrate and ammonium) stayed between 9 and 36% of the N-compounds expected to be released from biodegradation (based on the equations 1 and 2). This difference indicates that N-compounds might have been lost or that the percentage of N-compounds used for cell growth was higher and, consequently, the proportion

of N-compounds released in the medium was lower than the calculated values of equations 1 and 2 (discussed in the following topic).

Table 6 - Nitrogen balance in aerobic microcosms

Microcosms	Day	Cumulative substrate consumed (µM) (COI added)	N available from substrate (µM)	N incorporated into biomass (µM) (from eq 1 and 2)	Predicted NH_4^+-N released (µM) (from eq 1 and 2)	NO_3^--N measured (µM)	NH_4^+-N measured (µM)	Sum of Measured N-compounds produced (µM)	Sum of N-compounds measured / N theoretically released
MC2MCA	0	0	0			2.68	not analyzed	2.68	
	17	705.49	705.49	465.62	239.87	57.57	9.54	67.11	14%
MC3MCA	0	0	0			3.09	not analyzed	3.09	
	17	705.49	705.49	465.62	239.87	56.50	11.66	68.16	15%
	70	0	0			4.24	5.83	10.07	
	129	1567.75	1567.75	1034.72	533.04	43.01	333.09	376.10	36%
MC4MCA	0	0	0			2.48	not analyzed	2.48	
	17	705.49	705.49	465.62	239.87	57.45	9.89	67.34	14%
	70	0	0			1.17	4.94	6.12	
	129	1567.75	1567.75	1034.72	533.04	31.57	158.24	189.81	18%
MC23DCA	70	0	0			1.30	14.14	15.44	
	129	987.55	987.55	622.16	365.39	28.46	147.34	175.79	28%
MC34DCA	0	0	0			2.84	not analyzed	2.84	
	17	555.5	555.5	349.97	205.54	62.81	9.89	72.70	21%
	70	0	0			49.15	6.89	56.03	
	129	987.55	987.55	622.16	365.39	48.58	7.77	56.36	9%

Days 0 and 70 are the first day of transfer 1 (T1) and transfer 2 (T2). Ammonium was not measured on day 0, it was assumed that ammonium concentration was 0 at this day, since the mineral medium is free of ammonium).

Source: author (2022)

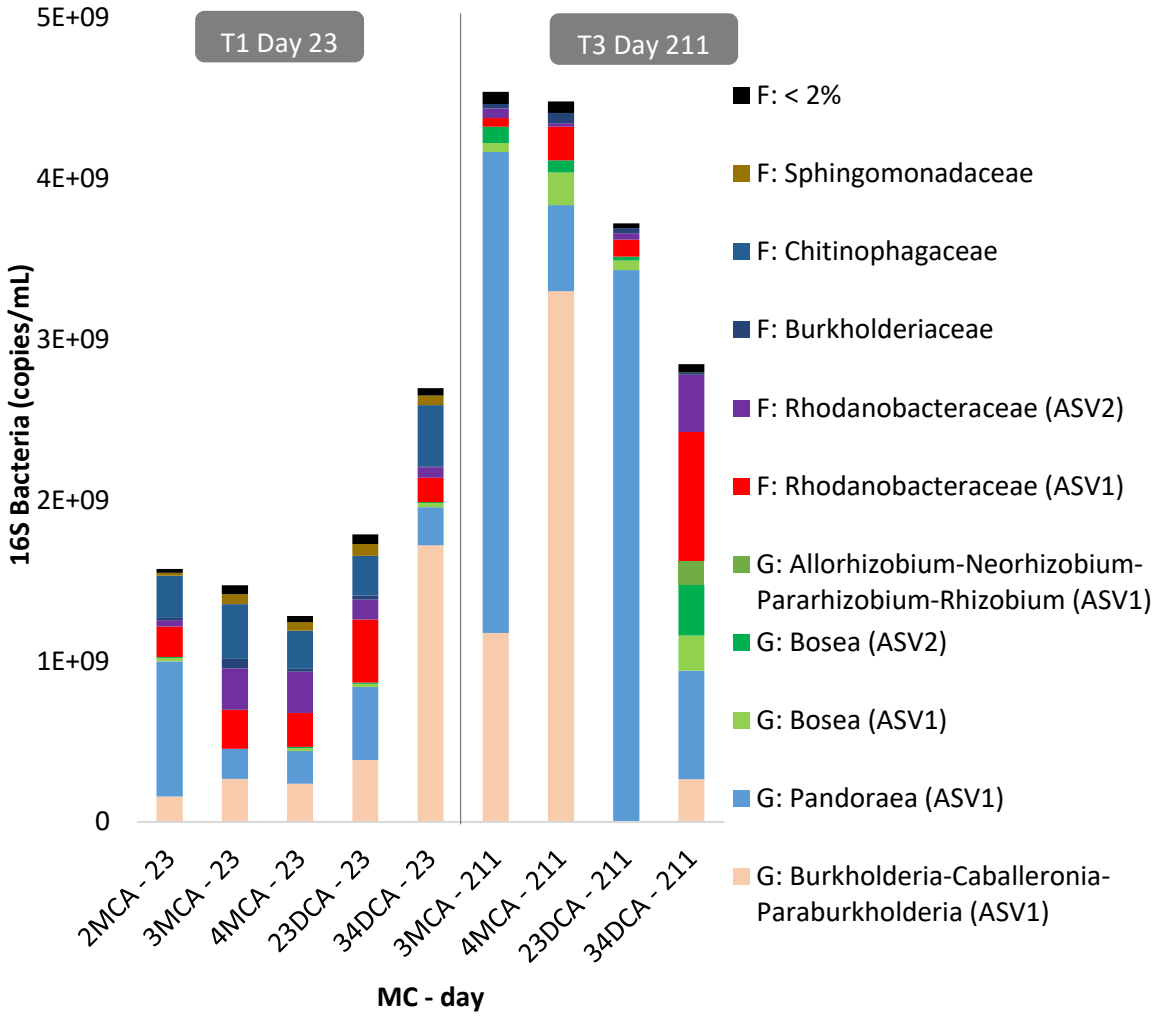
4.1.3 Analysis of microbial community

Samples for microbial community analyses were collected on day 23 (during the temporal profile of T1) from all five microcosms and on day 211 (T3) from MC3MCA, MC4MCA, MC23DCA, and MC34DCA. Figure 18 shows the absolute abundance of the microbial community found in the microcosms in these two sampling days. In this figure, the dilutions of the cultures when the two 1:10 transfers were made were not considered; thus, the absolute abundances of T3 presented are the concentrations quantified in each microcosm. All the relevant amplicon sequence variants (ASV) found in the communities are available in Appendix B1.

Considering all cultures, the most enriched genera were the *Pandoraea* (ASV1) and the group *Burkholderia-Caballero-Paraburkholderia* (ASV1). The first was present in all five communities at the beginning of the experiment, and it was enriched in all four microcosms: MC3MCA, MC4MCA, MC23DCA, and MC34DCA. In MC3MCA and MC23DCA *Pandoraea* was the predominant genus on day 211 of the experiment (enriched from 1.87E+08 to 2.99E+09 and from 4.54E+08 to 3.43E+09 copies/mL in the mentioned cultures, respectively). The latter group was also present in all five microcosms; however, its abundance was not significantly increased in DCA isomers communities; in MC23DCA we could not even find this genus on day 211 of DNA analysis. The sequence affiliated with the *Burkholderia-Caballero-Paraburkholderia group* (ASV1) was dominant in the microbial community of MC4MCA, being enriched from 2.38E+08 to 3.30E+09 copies/mL.

The *Bosea* genus, represented by 2 sequences (ASV1 and ASV2), was slightly enriched in all four cultures. The *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* genus group was observed in the MC34DCA community on day 211. At the family level, *Burkholderiaceae* was present in all enriched cultures. The presence of *Rhodanobactereaceae* is also relevant in the microbial community, since it appears on the second DNA analysis (day 211) in all communities, being more significantly enriched in MC34DCA. If considering the dilutions in each transfer, we can say that organisms from the two families were enriched, but not as expressively as the other organisms cited so far. Organisms from *Chitinophagaceae* and *Sphingomonadaceae* families were not found on the second day of DNA analysis (Day 211, Figure 18). No archaea were found in any of the samples sequenced.

Figure 18 - Absolute abundance of the microbial community in each of the five aerobic microcosms on days 23 and 211



G: for genus level; F: for family level.

Source: author (2022)

4.1.4 Crossing the data obtained from aerobic tests

Based on results revealed by the DNA analyses and the activity observed in the microcosms, we can infer that some genera are directly involved in the degradation of the target chloroanilines. *Pandoraea* (ASV1) and the ASV affiliated with the *Burkholderia-Caballero-Paraburkholderia group* (ASV1), the dominant genera in our study, seem to be able to grow on both MCA and DCA, but the *Pandoraea* (ASV1) was more successful in growing on DCA compared to the *Burkholderia-Caballero-Paraburkholderia* (ASV1) group. Growth yields of both

genera together were 0.33, 0.32, 0.29, and 0.07 gram of biomass per gram of 3-MCA, 4-MCA, 2,3-DCA, and 3,4-DCA, respectively, which are in accordance with the theoretical values from stoichiometric equations (detailed calculations for each microcosm are presented in the Table 9B1, in Appendix B1).

BLAST results showed a similarity of 100% (query coverage and percent identity) between the ASV of the *Pandoraea* genus found in our experiment and many complete genomes of *Pandoraea* genus sequenced so far. Among those, the *Pandoraea pnomenusa* MCB032 (NCBI CP015371.1) was described as able to degrade chlorobenzenes via 3-chlorocatechol, 2-chloromuconate and finally through Krebs Cycle intermediates, by encoding chlorobenzene dioxygenase, cis-chlorobenzene dihydrodiol dehydrogenase, chlorocatechol 1,2-dioxygenase and other enzymes (CHAO *et al.*, 2019; JIANG, X. W. *et al.*, 2009).

When the 16S fragment of the *Burkholderia-Caballero-Paraburkholderia* group was submitted to the BLAST tool, no match with 100% similarity (query coverage and percent identity) was found considering the complete genomes sequenced of microbe reported as chloroaromatic degrader. However, at least the first 100 complete genomes with similarity >99% corresponded to the *Burkholderia* genus. This genus has been reported as chloroaromatic degrader in studies related to environmental decontamination (ARORA, P K; BAE, 2014; ARORA, P K; JAIN, R K, 2012; HAIGLER; PETTIGREW; SPAIN, 1992; MIN; WANG, B.; HU, X., 2017; PONCE *et al.*, 2011; URGUN-DEMIRTAS *et al.*, 2003; YOSHIDA *et al.*, 2009).

For instance, the *Burkholderia* sp. RKJ 800 was reported as a suitable candidate for bioremediation of sites contaminated with 2-chloro-4-nitrophenol and 4-chloro-2-aminophenol, as it was able to degrade both contaminants via hydroquinone and 4-chlorocatechol, respectively, releasing chloride, nitrite and ammonium ions (ARORA, P K; BAE, 2014; ARORA, P K; JAIN, R K, 2012). The RKJ 800 was partially sequenced (NCBI HM585226.1) and presented a query cover of 100% and a percent identity of 96.76% compared to the ASV found in our study. Similarly, the strain SJ98 was also reported as 2-chloro-4-nitrophenol and other chloronitroaromatic compounds degrader (KUMAR; VIKRAM; RAGHAVA, 2012; MIN; WANG, B.; HU, X., 2017; MIN; ZHANG, J.-J.; ZHOU, N.-Y., 2014; PANDEY, J. *et al.*, 2011, 2012). The whole genome shotgun sequence of this strain (NCBI AJHK02000001.1) presented 100% query cover and 97.61% of percent identity compared to the ASV found in the microcosms of this research.

Other organisms were less significantly enriched in the microcosms. The two ASV classified as the genus *Bosea*, reported as putative N_2-fixing bacteria (JARAMILLO *et al.*, 2013; RILLING *et al.*, 2018), were detected at an absolute abundance magnitude of E+08 in the enriched cultures. The same function is observed to be performed by the *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* group (TENG *et al.*, 2015; YANG, L. L. *et al.*, 2020; YOU *et al.*, 2021), which was enriched from "not detected" to E+08 in MC34DCA. Those genera are likely to be involved in a symbiosis associated with the nitrogen cycle (side process), possibly fixing N_2 from the atmosphere.

In addition to the *Allorhizobium-Pararhizobium-Rhizobium* genus group and the *Bosea* genus, the family *Rhodanobactereaceae* appeared in higher abundance in MC34DCA compared to the other cultures. As far as we know, there is no studies correlating this family to the dechlorination process. The two sequences of *Rhodanobactereaceae* found (ASV1 and ASV2) have high similarity (100% for query cover and ~99% for percent identity) to the complete genomes of *Rhodanobacter denitrificans* strains (NCBI NC_020541.1). The organism was reported as facultative anaerobic bacteria, able to use nitrate (NO_3^-), nitrite (NO_2^-) and nitrous oxide (N_2O) as sole electron acceptors under anoxic conditions, or when the available free oxygen in the medium is low (PENG *et al.*, 2022; PRAKASH, O. *et al.*, 2012). Even though the comparison at the family level is not precise, this makes sense considering the low concentrations of N-compounds measured in relation to the expected amount calculated for the microcosms (Table 6).

Although the microcosms were kept in a shaker to ensure aeration, low concentration of O_2 might have taken place. In MC34DCA, we did not observe any accumulation of the ammonium or nitrate ions between day 70 and day 129 of transfer 3, indicating that N-compounds were possibly being converted to N_2. The reason why these organisms from the family *Rhodanobactereaceae* have grown more expressively in MC34DCA was not investigated.

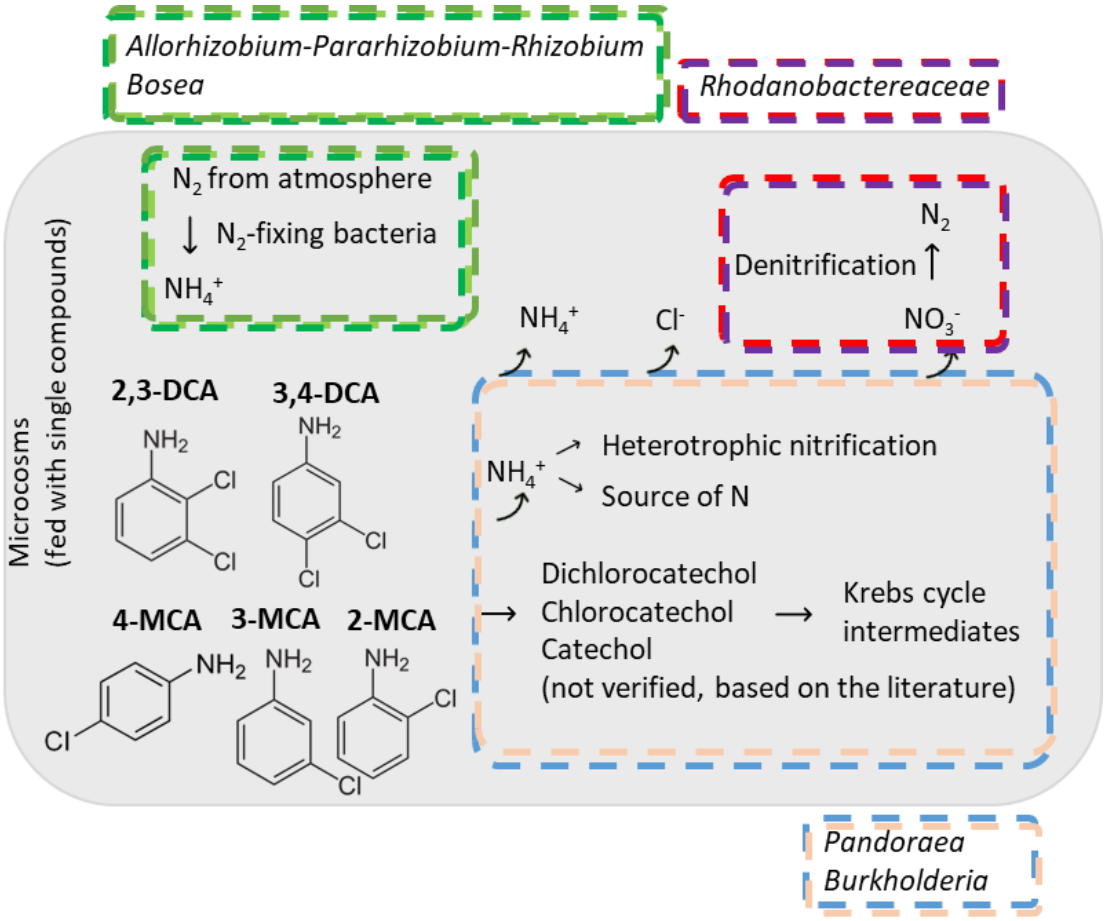
Yet regarding the intriguing N-compounds dynamics, even though organisms related to autotrophic nitrification were not identified in the microbial communities, nitrate was detected in samples from microcosms, suggesting that part of the ammonium released from biodegradation was oxidized to nitrate (Table 6). It may be attributed to heterotrophic nitrification, in which the ammonium is converted into nitrate by organisms using an organic carbon source for their growth (BASKARAN *et al.*, 2020; FITZGERALD *et al.*, 2015; PAPEN *et al.*, 1989).

Many microbes were identified as able to aerobically oxidize ammonium through the heterotrophic pathway, including organisms from the *Burkholderia* and the *Bosea* genus, and some were even able to denitrify the nitrate into N_2 (BASKARAN *et al.*, 2020; FITZGERALD *et al.*, 2015; JOONG *et al.*, 2005; PAPEN *et al.*, 1989; QIAO, Z. *et al.*, 2020; XIA *et al.*, 2020; ZHANG, Jibin *et al.*, 2011; ZHANG, Q. *et al.*, 2020). Those nitrifier microbes can use the ammonium from an inorganic source and carbon from an organic source or even from an amino organic compound, serving also as a source of carbon (ZHANG, Jinbo *et al.*, 2014).

Simultaneous removal of aniline and inorganic ammonium was observed by Qiao *et al*. (2020) by a mixed culture with no presence of autotrophic nitrifiers, and as likely in our study, they described aniline mineralization followed by heterotrophic nitrification of the ammonium released and further denitrification to N_2. Considering the enriched microbial communities of this study, it is not clear which organisms would be responsible for these side reactions. If *Pandoraea* or *Burkholderia* can gain energy from heterotrophic nitrification, ammonium from contaminants itself would be both N and one of the electron sources for their growth, while using the contaminants as carbon source. If other genera were able to oxidize ammonium, they possibly used dead biomass or remaining organic matter from the soil as the carbon source, or even the CO_2 produced from biodegradation in case of autotrophic nitrification.

Figure 19 represents the dynamics considered to happen in the microcosms. It shows the *Pandoraea* and the *Burkholderia* genera as the main actor of contaminants biodegradation, possibly using the ammonium as nitrogen source and nitrifying it through heterotrophic process. N_2-fixing organisms are suggested to be the *Allorhizobium-Pararhizobium-Rhizobium* and *Bosea* genera, while organisms from the *Rhodanobactereaceae* might be involved in denitrification. The relation between the organisms discussed is presented in the phylogenetic tree (Figure 20).

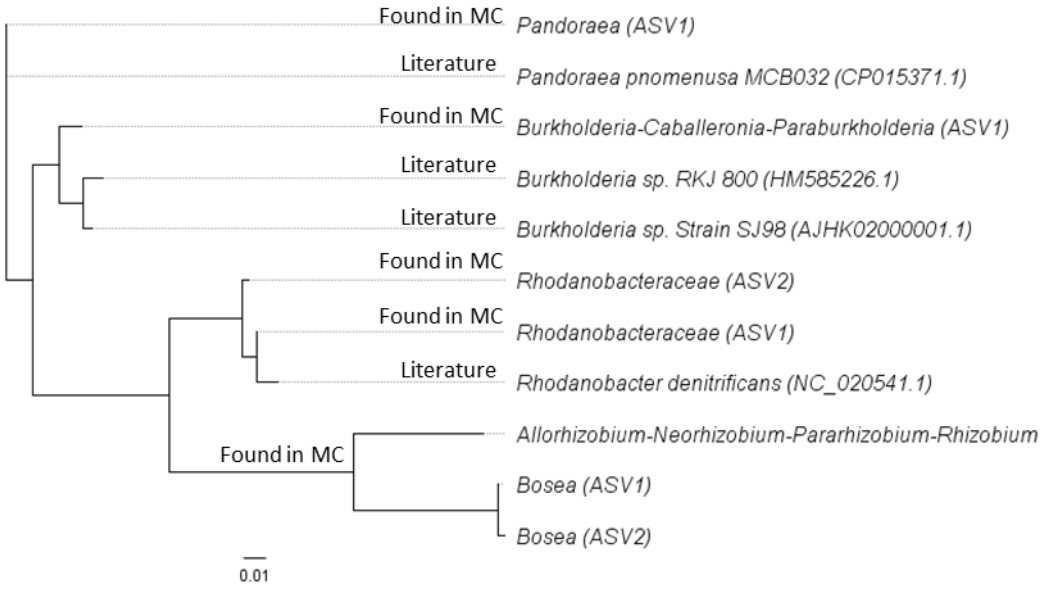
Figure 19 - Overview of the dynamics and microbial community observed in the aerobic microcosms



Pandoraea (ASV1) and *Burkholderia* (ASV1) genera represented as COI degraders, the family *Rhodanobacteraceae* (ASV1 and ASV2) involved in denitrification and the genera *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* and *Bosea* as N2-fixing organisms.

Source: author (2022)

Figure 20 - Phylogenetic tree of organisms found in aerobic microcosms and organisms from literature



Pandoraea (ASV1) (COI degrader, genus) found in microcosms was 100% identical to *Pandoraea pnomenusa* MCB032 (chlorobenzene degrader – full genome). *Burkholderia-Caballeronia-Paraburkholderia* (ASV1) (COI degrader, genus) found in microcosms was 96.76% identical to *Burkholderia* sp. RKJ 800 (2-chloro-4-nitrophenol and 4-chloro-2-aminophenol degrader – partially sequenced) and 97.61% identical to *Burkholderia* sp. SJ98 (2 chloronitroaromatic compounds degrader - whole genome shotgun sequence). *Rhodanobacteraceae* (ASV1 and ASV2) (denitrifier, family) found in microcosms presented percent identity of around 99% with *Rhodanobacter denitrificans* (able to use nitrate as electron acceptor). Good match for *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* and *Bosea* was not found. Tree was made with the function MUSCLE alignment and Geneious Tree Builder (default algorithms) on Geneious 8.1.9. Matrix is available in Figure 60B1 of Appendix B1.

Source: author (2022)

4.1.5 Learnings from aerobic tests

Our study provided insights on the biodegradation of monochloroanilines and dichloroanilines by aerobic native microbes enriched from soil and groundwater collected at the contaminated site where bioremediation strategies have been implemented. Based on the activity observed in each microcosm and DNA analyses (sequencing and quantification) performed during the experimental period, we could infer that 1 ASV from the genus *Pandoraea* and 1 ASV from the *Burkholderia-Caballero-Paraburkholderia* group were likely responsible for the degradation of the chloroanilines. The second group was more enriched when DCA isomers were the growth substrate. Microorganisms associated with nitrogen metabolism were less significantly enriched in

the microbiomes but might play a side role in sourcing nitrogen through the fixation of N_2 to ammonium and/or converting N-compounds to N_2.

The amount of chloride released from the chloroanilines was close to the theoretical, supporting the biodegradation process. Contrastively, the sum of nitrogenated compounds measured as ammonium and nitrate was way below the added amount, suggesting that cells were using the released ammonium as the nitrogen source for growth.

As we have these cultures preserved in glycerol under -80^{o}C, they are all available for possible future studies to better describe the pathway by which contaminants are biodegraded. Enzymatic essays can be performed to find out which intermediates are formed: catechol, dichlorocatechol or chlorocatechol isomers, for instance, as it was done already in previous studies. Further isolation of the organisms responsible for the main activity can be the next step of this research and complete genome sequencing would improve the learnings from this experiment.

In an applicability context, the biodegradability of the chloroanilines by native microbes means that natural attenuation might be happening when oxygen is available for the native microbes. Also, it indicates that bioaugmentation might not be necessary, but injecting oxygen is a promising strategy to mineralize the chloroanilines (biostimulation) found in the contaminated site. In addition, the genera identified to grow on monochloroanilines and dichloroanilines can be used as biomarkers in the project, when analyzing data from the field. Further studies with the isolated organisms can give better evidence of the active biodegradation, based on expressed enzymes involved in the process of interest.

4.2 ANAEROBIC BIOTRANSFORMATIONS OF THE CONTAMINANTS

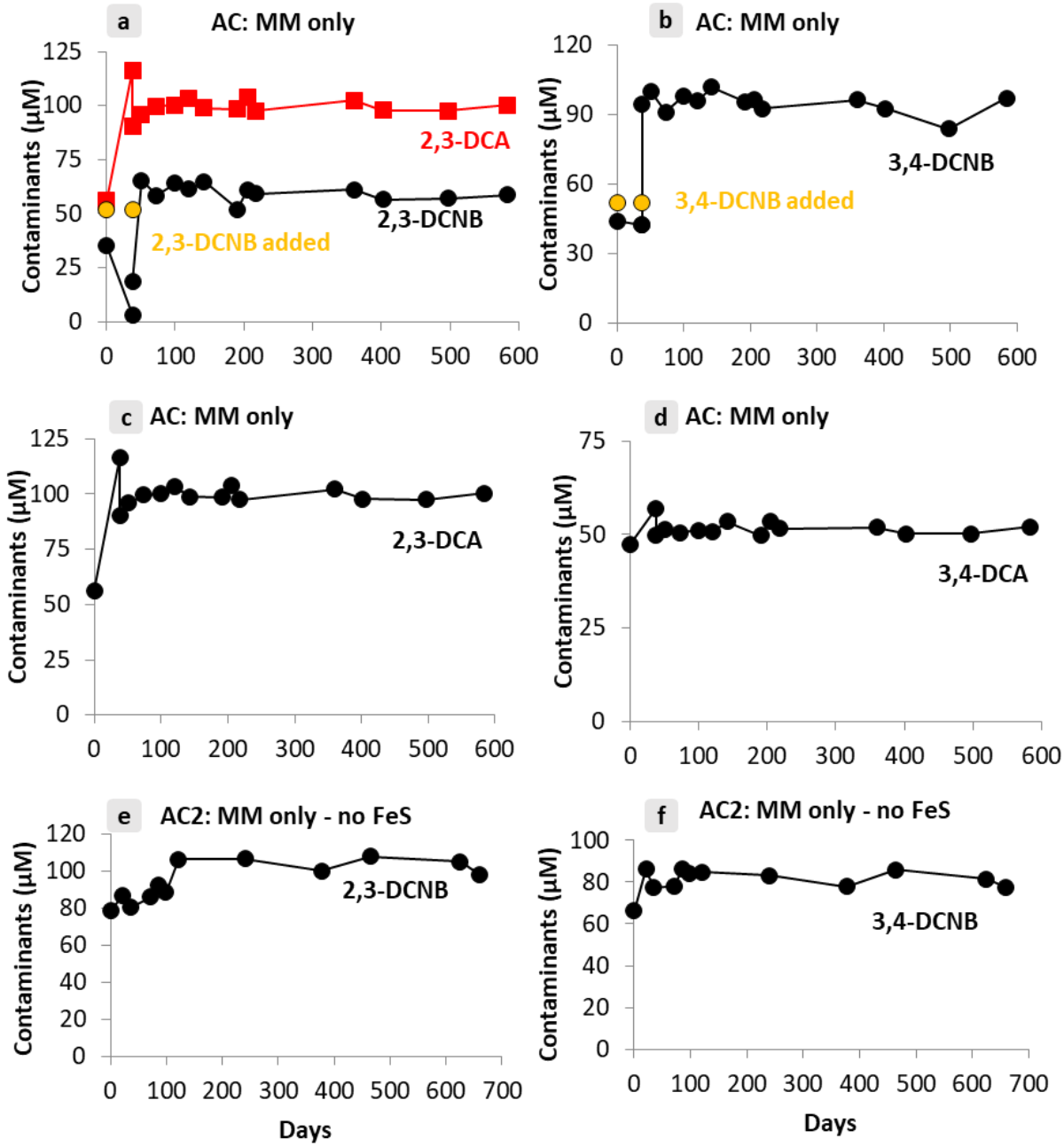
The objectives of this experiment were to verify natural attenuation at the site and the possibility of applying biostimulation or bioaugmentation at the anaerobic zones of the site, where free oxygen is not available. Aiming that, we focused on finding and enriching native organisms from the contaminated site with ability to anaerobically biotransform or biodegrade dichloronitrobenzene isomers (2,3-, 3,4-, and 2,5-DCNB), dichloroaniline isomers (2,3- and 3,4-DCA), 2-monochloroaniline (2-MCA), and aniline, individually, and to evaluate the mechanisms and the microbial community involved in the transformation observed. We then suggest how findings can improve natural attenuation understanding at the industrial contaminated site.

Biotransformation of some compounds was already verified (3,4-DCNB, 2,3- and 3,4-DCA, MCA, aniline), some compounds are still to be studied (see section 2). The anaerobic experiment is still ongoing, as the cultures are active and being maintained by Line Lomheim at the Edwards Lab (UofT). The cultures are also preserved in glycerol, under -80ºC, available for future studies (Edwards Lab, Barcode freezer, box A061 – details available in the inventory).

4.2.1 Negative controls and inactive microcosms

The first set of bottles had 2 negative controls. The first, named abiotic control (AC), contained only the anaerobic mineral medium and was amended with 3,4-DCNB, 2,3-DCNB, 2,3-DCA, 3,4-DCA, 2-MCA, and aniline (Figure 21a-d). Surprisingly, the 2,3-DCNB was reduced into 2,3-DCA at the beginning of the experimental period. After the second feeding with 2,3-DCNB, this contaminant stayed stable, as did the others (Figure 21a). We first hypothesized that the FeS from the mineral medium could had driven an abiotic reduction in the surface of the precipitated material. Based on that, we set a new abiotic control (AC2) without FeS in the medium (Figure 21e and f). As both showed no changes in the concentration of DCNB and DCA isomers (Figure 21), we could conclude that the anaerobic mineral medium was not promoting any abiotic reduction in our microcosms.

Figure 21 - Concentration of 2,3-DCNB (a), 3,4-DCNB (b), 2,3-DCA (c), and 3,4-DCA (d) in the abiotic control with FeS (AC), and 2,3-DCNB (e) and 3,4-DCNB (f) in the abiotic control with no FeS (AC2)



Source: author (2022)

The second negative control was the heat killed control (KC) made with autoclaved soil, mineral medium, and the following compounds of concern were added: 3,4-DCNB, 2,5-DCNB, 2,3-DCA, 3,4-DCA, 2-MCA, and aniline (Figure 22). Unfortunately, this control did not work as

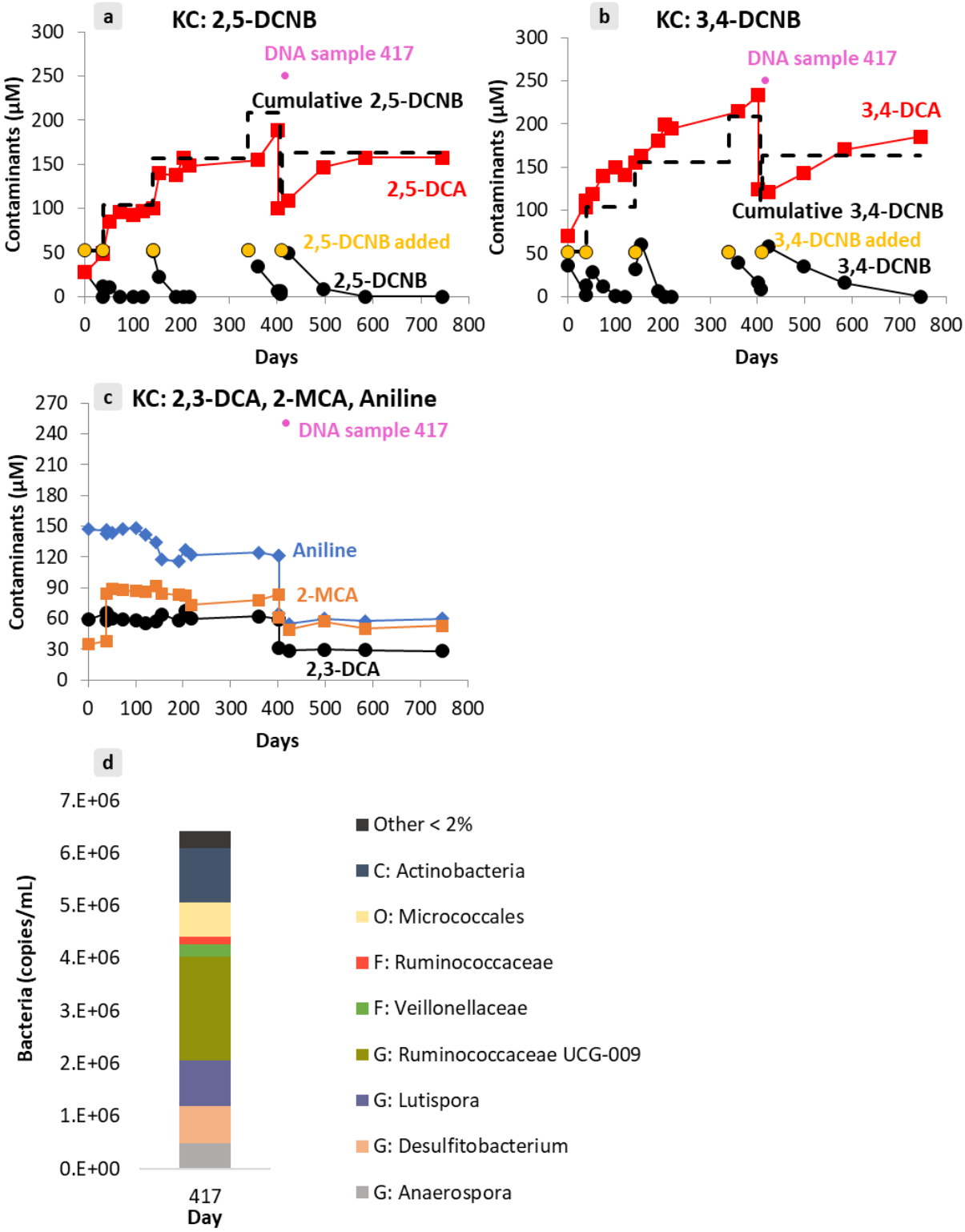
expected. In KC, both 2,5- and 3,4-DCNB were reduced into their respective dichloroaniline isomers (2,5- and 3,4-DCA) (Figure 22a and b). We amended the bottles with the DCNB isomers four more times, and the behaviour was the same. Because of this, this bottle was not considered as negative control for 2,5-DCNB and 3,4-DCNB. On day 417 sample for DNA sequencing was collected and mineral medium was added to it, diluting all the contaminants remaining in the bottle.

Although the inoculum was autoclaved three times in three consecutive days to inactivate the microbes, bacteria, including some spore-forming, survived and were likely responsible for the activity observed in this bottle (Figure 22d).

Seeking to have better evidence of biotic DCNB reduction in the active microcosms, the transfer 3 (T3) of bottles amended with DCNB isomers was performed. We expected to see differences in the reduction rate between microcosms with the addition of the electron source (T3A) and the microcosms with electron limitation (T3B), since the control with the autoclaved soil (KC) did not work as expected for the DCNB isomers. The amount of electron added versus needed for reduction of DCNB into aniline is presented in Table 10B2 of Appendix B2.

Regarding the other contaminants, the KC worked well for 2,3-DCA, 3,4-DCA, 2,5-DCA, 2-MCA and aniline, indicating that there was no reaction happening between the autoclaved soil or the survivor cells and those contaminants (Figure 22a-c).

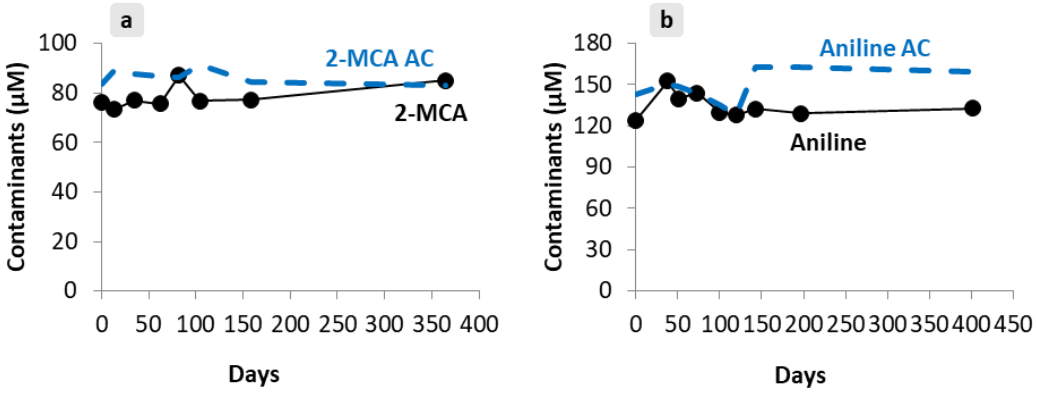
Figure 22 - Heat killed control (KC; one bottle) showing data for 2,5-DCNB (a), 3,4-DCNB (b), 2,3-DCA, 2-MCA, and aniline (c), and bacterial community on day 417 KC (d)



Source: author (2022)

No degradation of 2-MCA and aniline was observed in this study in abiotic bottles. The microcosms amended with those compounds (MC2MCA-T1 and MCAN-T1) were monitored for 400 and 450 days, respectively, and no changes in contaminants concentration were observed (Figure 23).

Figure 23 - Results from MC2MCA-T1 (a) and MCAN-T1 (b), including the respective contaminants from the abiotic control (AC) bottle



Source: author (2022)

4.2.2 Biotransformation of contaminants and microbial community

Biotransformation of contaminants was observed in all microcosms from transfer 1 (MC-T1) amended with DCA and DCNB (concentration of contaminants over time and bacterial community observed in each MC-T1 are included in Figures 61B2, 62B2, 63B2 and 64B2, in Appendix B2). The microcosms amended with DCA (MC23DCA-T1 and MC34DCA-T1) were used as inocula for MCDCA-T2 after a few feedings (1:10 transfer). Because DCNB reduction was observed in the KC and quickly happened in transfer 1 of the microcosms as well (MC23DCNB-T1, MC34DCNB-T1, and MC25DCNB-T1), we decided to dilute the microcosms to get rid of some soil that was possibly reducing the nitro group via abiotic reaction. For this reason, we started the MCDCNB-T2 with 1:1 dilution.

Figures 24, 25, 26, 27 and 28 show the activity of transfer 2 of the five microcosms (MC-T2) (a) and the bacterial community (b) in terms of absolute abundance (copies/mL) in samples collected from the microcosms along the experimental period. Both MC23DCA-T2 and MC34DCA-T2 produced monochloroaniline isomers from the DCA reduction; however, when 2,3-

DCA was the contaminant, 3-MCA was the only compound produced (Figures 24a and 25a). All DCNB microcosms (MC23DCNB-T2, MC34DCNB-T2, and MC25DCNB-T2) produced the respective DCA isomers (Figures 26a, 27a, and 28a). The MC34DCNB-T2 was the only bottle that degraded further and dechlorinated 3,4-DCA to 3- and 4-MCA (Figure 26a).

In MC34DCNB-T2 and MC25DCNB-T2, the target compound (DCNB) measured was always close to zero, as it was quickly reduced into 3,4-DCA and 2,5-DCA, respectively (Figures 26a and 27a); thus, the theoretical amount of the target contaminants added in each microcosm was included in the graphics, represented by yellow dots. In MC23DCNB-T2 (Figure 28a), reduction was faster in the first 100 days, but it was impaired possibly after medium was added to replace the volume used for the third transfer (T3A and T3B), on day 127, when the microbial community was diluted.

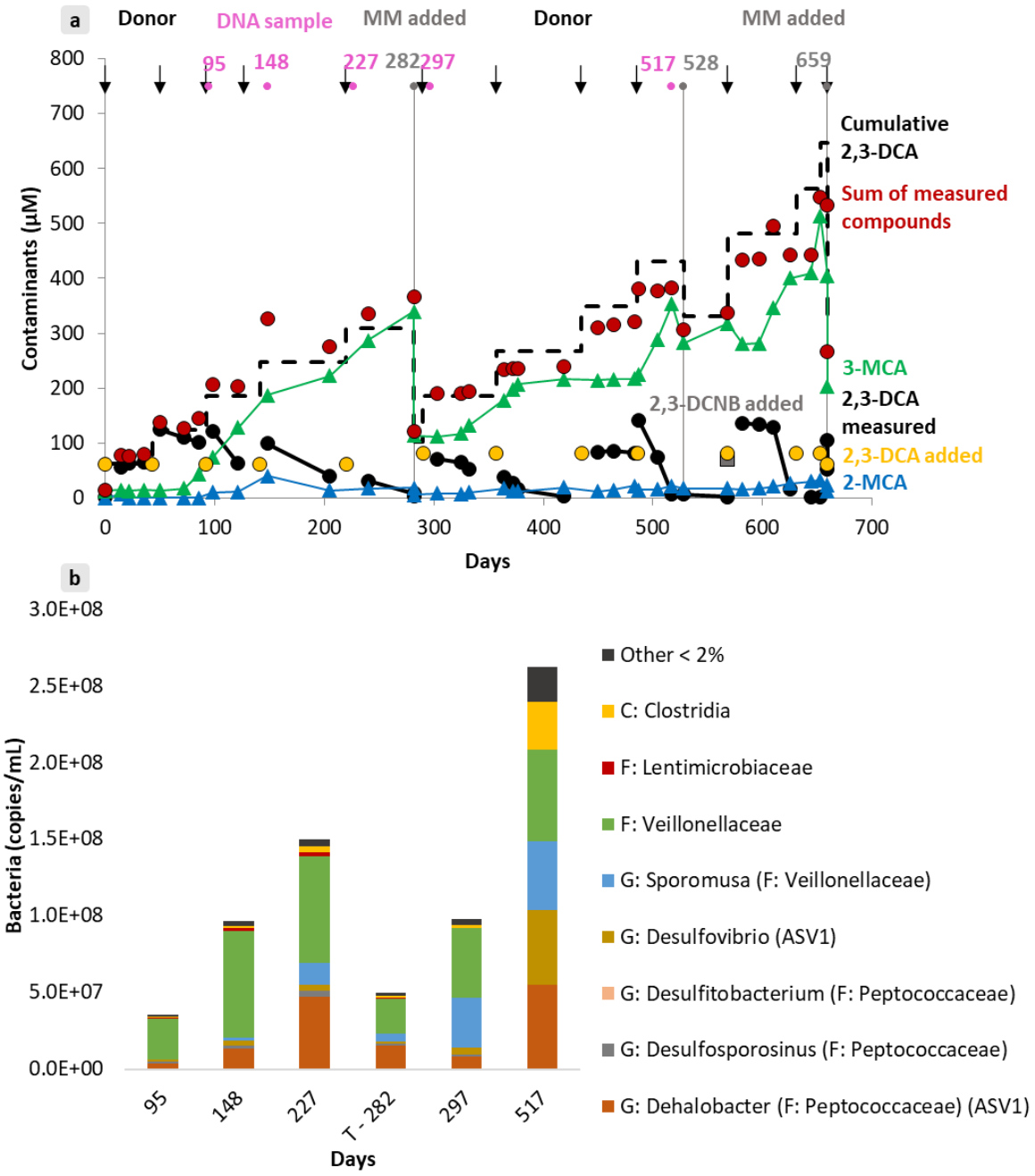
The cumulative concentration of the contaminant added (represented by the dashed black line in the graphics) and the sum of the contaminants measured (the target contaminants added and compounds formed from their transformation) are also included in the graphics as mass balance between what was added as feeding contaminant and what was measured by the HPLC. In all five MC-T2, the line for the cumulative contaminants added close to the line representing the sum of measured compounds indicates that there were no undetected compounds being formed, nor losses of compounds. On every feeding day, the electron donors were also added; the addition of ethanol and lactate is represented by the vertical arrows in graphics “a” of the Figures 24 to 28.

In general, the microbial community found in the active microcosms was composed of archaea in higher abundance and bacteria reported as fermentative, sulphate-reducing (SRB) and organohalide-respiring (OHRB) (when dechlorination occurs) (Figures 24b, 25b, 26b, 27b, and 28b for bacterial community). Several samples were collected from each microcosm during the experimental period; those days are indicated as pink dots in Figures 24a to 28a, corresponding to the days of the graphics “b” in the cited Figures. Likewise, the days when fresh mineral medium was added into the microcosms are indicated in both graphics; as vertical lines, in “a”, and as theoretical days in “b”.

The Appendix B2 includes the relative abundance (percentage) of archaea versus bacteria (Table 11B2) and the archaea genera found during the experimental period in DNA samples of all microcosms (Table 12B2). Some CH_4 measurements in microcosms are included in Appendix B2,

Table 13B2. The ASV of the organisms of interest found in the communities is also available in Appendix B2.

Figure 24 - Concentrations of contaminants (a) and bacterial community (b) of MC23DCA-T2 during the experimental period

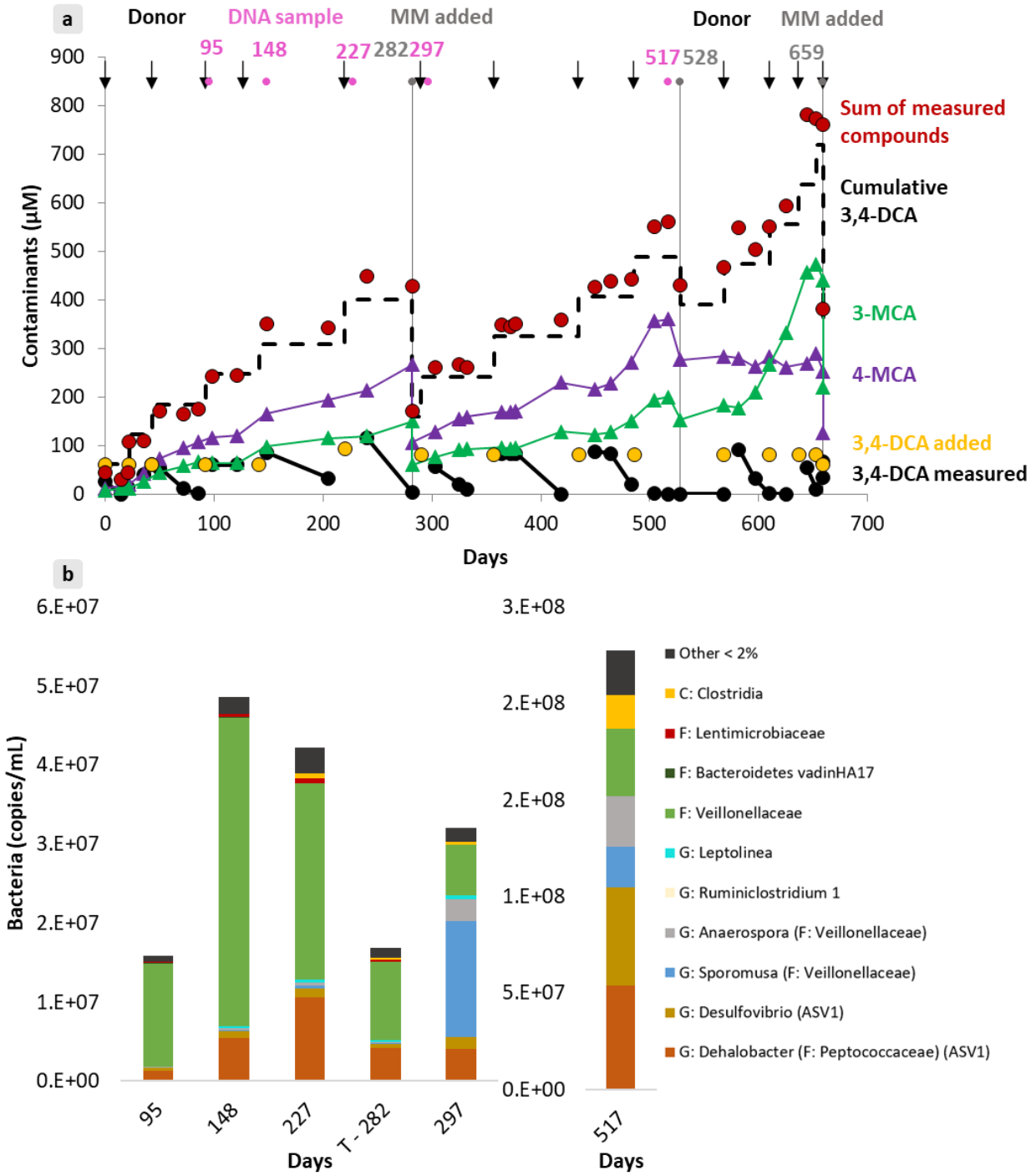


Indicated in the right side of the graphic, the panel "a" presents the concentration of contaminants measured, the cumulative concentration of the target contaminant added, the sum of the measured compounds, and the theoretical concentration of the target contaminant (2,3-DCA added). In this microcosm amended with 2,3-DCA, the 2,3-DCNB was added as substrate on day 568 to check the culture activity for the reduction of the nitro group. In addition, on top of the graphic, the panel indicates the days when donor was added to the microcosm, when DNA samples was collected and when fresh medium was added and microcosm was diluted. Starting volume of this microcosm was 100 mL in 250 mL Boston bottle. On day 282, volume was increased to 150 mL; the dilution factor considering the fresh medium added was 0.3. On day 528, dilution

factor due to medium addition was 0.8. On day 659, volume was increased to 200 mL and culture was transfer to a 1 L bottle; the dilution factor was 0.5. In the panel “b”, the absolute abundance of the bacterial community was obtained by multiplying its relative abundance (composition as percentage) from the sequencing results and the total bacteria quantified by qPCR. The microbial community after the dilution on day 282 is theoretically represented by the T-282 bar on the panel “b” (calculation was the absolute abundance of day 277 multiplied by the dilution factor 0.3). A more detailed view of days 0-300 is presented in Figure 30.

Source: author (2022)

Figure 25 - Concentrations of contaminants (a) and bacterial community (b) of MC34DCA-T2 during the experimental period

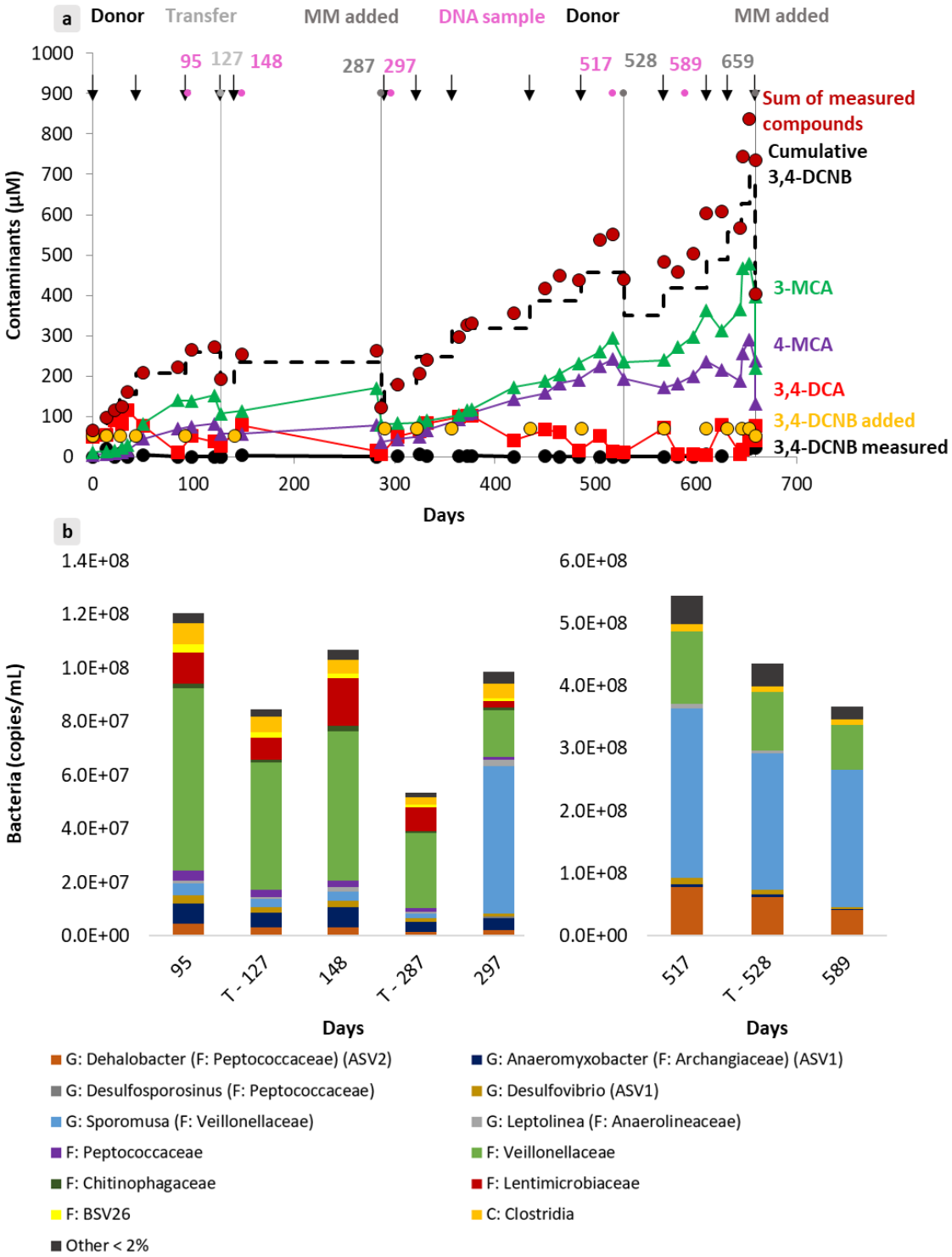


Indicated in the right side of the graphic, the panel "a" presents the concentration of contaminants measured, the cumulative concentration of the target contaminant added, the sum of the measured compounds, and the theoretical concentration of the target contaminant (3,4-DCA added). In addition, on top of the graphic, the panel indicates the days when donor was added to the microcosm, when DNA samples was collected and when fresh medium was added and microcosm was diluted. Starting volume of this microcosm was 100 mL

in 250 mL Boston bottle. On day 282, volume was increased to 150 mL; the dilution factor considering the fresh medium added was 0.4. On day 528, dilution factor due to medium addition was 0.8. On day 659, volume was increased to 200 mL and culture was transfer to a 1 L bottle; the dilution factor was 0.5. In the panel “b”, the absolute abundance of the bacterial community was obtained by multiplying its relative abundance (composition as percentage) from the sequencing results and the total bacteria quantified by qPCR. The microbial community after the dilution on day 282 is theoretically represented by the T-282 bar on the panel “b” (calculation was the absolute abundance of day 277 multiplied by the dilution factor 0.4. A more detailed view between days 0-300 is presented in Figure 30.

Source: author (2022)

Figure 26 - Concentrations of contaminants (a) and bacterial community (b) of MC34DCNB-T2 during the experimental period

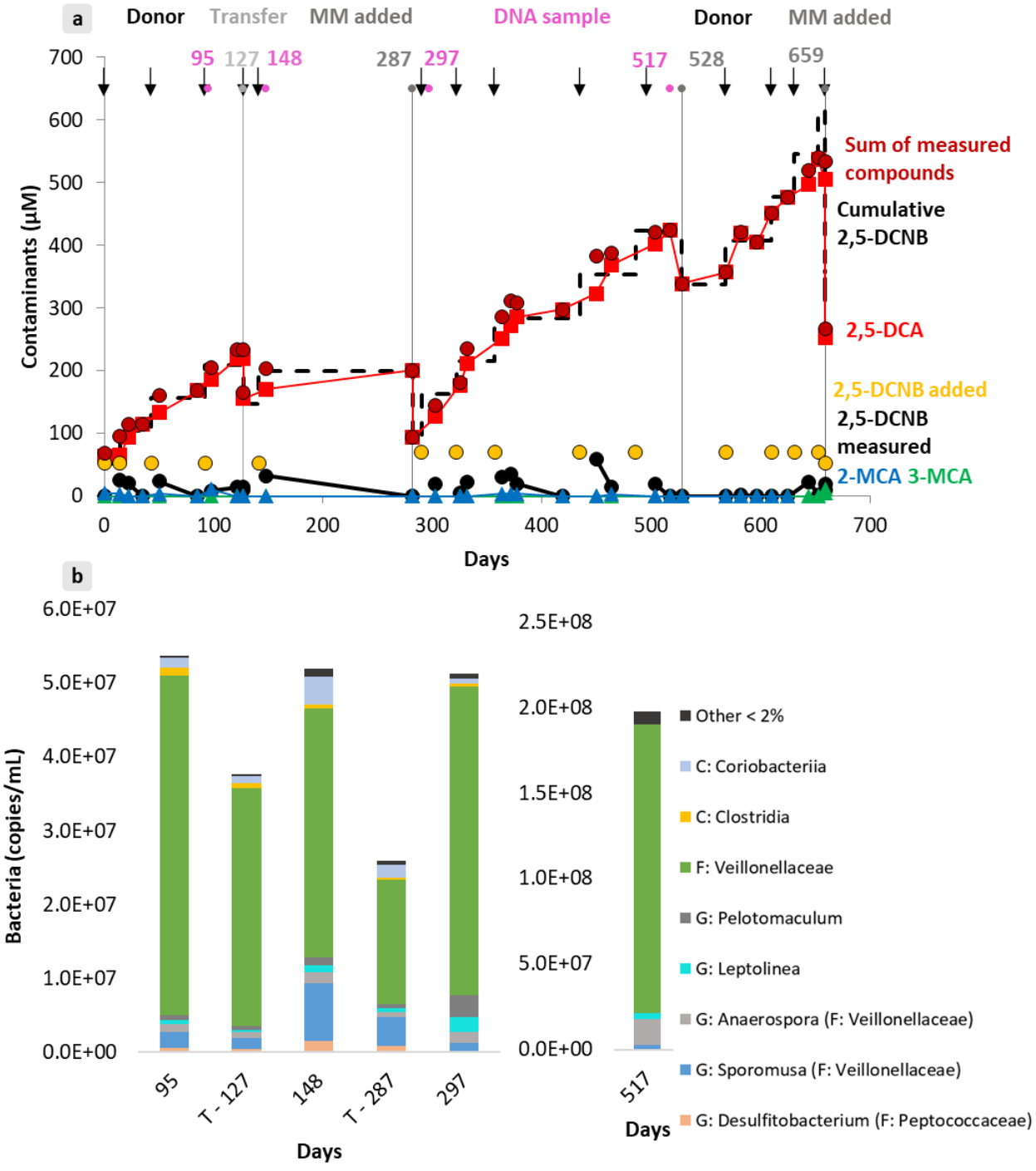


Indicated in the right side of the graphic, the panel "a" presents the concentration of contaminants measured, the cumulative concentration of the target contaminant added, the sum of the measured compounds, and the theoretical concentration of the target contaminant (3,4-DCNB added). In addition, on top of the graphic,

the panel indicates the days when donor was added to the microcosm, when DNA samples was collected and when fresh medium was added and microcosm was diluted. Starting volume of this microcosm was 100 mL in 250 mL Boston bottle. On day 127, 20 mL were taken to use as inocula for group 3 of microcosms (Appendix B2, Figure 65B2), and more medium was added to top up the volume of 100 mL (dilution factor was 0.7). On day 287, volume of the microcosm was increased to 150 mL; the dilution factor considering the fresh medium added was 0.5. On day 528, dilution factor due to medium addition was 0.8. On day 659, volume was increased to 200 mL and culture was transfer to a 1 L bottle; the dilution factor was 0.5. In the panel "b", the absolute abundance of the bacterial community was obtained by multiplying its relative abundance (composition as percentage) from the sequencing results and the total bacteria quantified by qPCR. The microbial community after the dilution on days 127, 287, and 528 is theoretically represented by the T-127, T-287, and T-528 bars, respectively, on the panel "b" (calculation was the absolute abundance of the previous DNA quantification multiplied by the dilution factors). A detailed view between days 0-300 of T3 of this culture (with and without donor) is presented in Figure 31.

Source: author (2022)

Figure 27 - Concentrations of contaminants (a) and bacterial community (b) of MC25DCNB-T2 during the experimental period

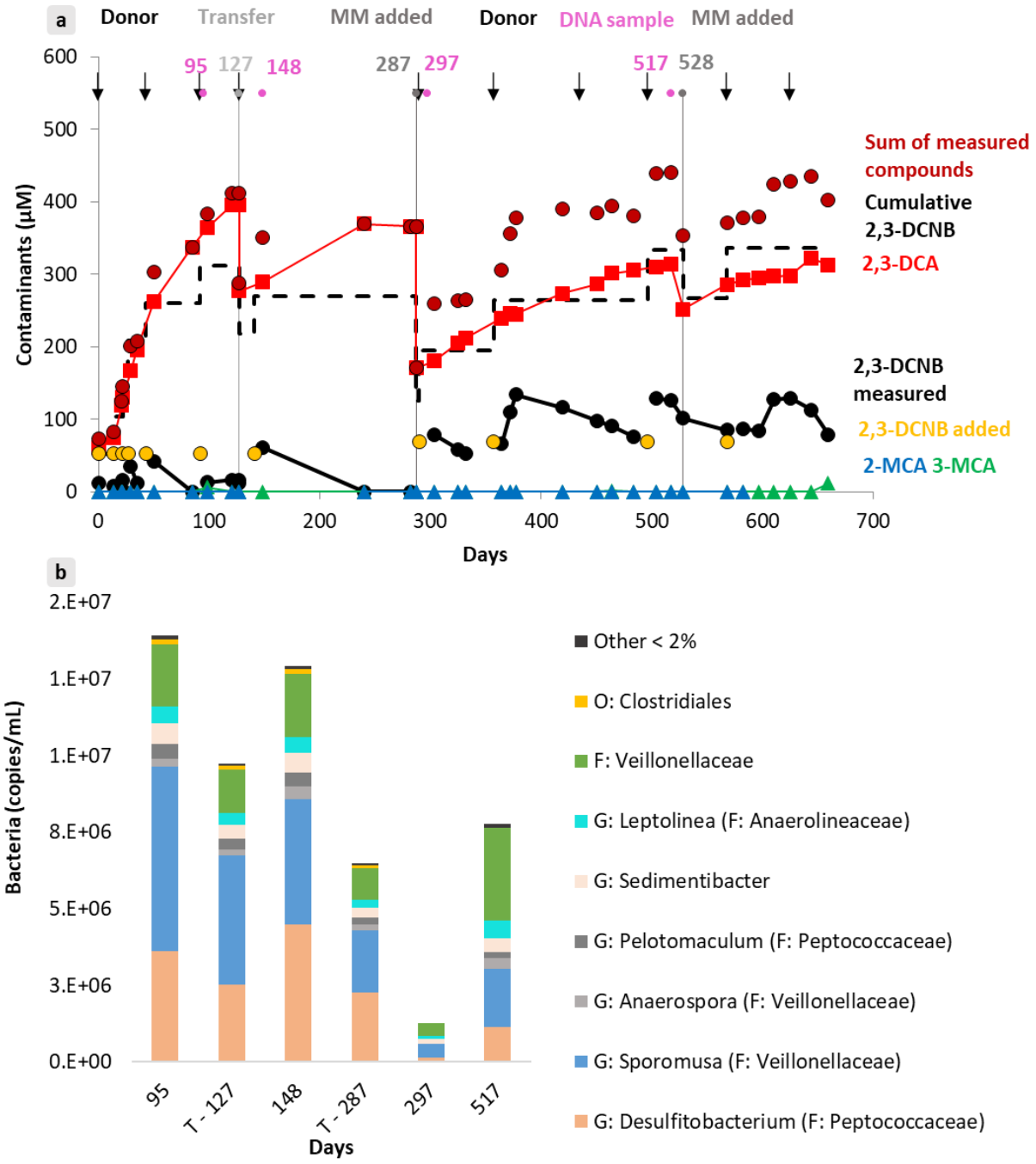


Indicated in the right side of the graphic, the panel "a" presents the concentration of contaminants measured, the cumulative concentration of the target contaminant added, the sum of the measured compounds, and the theoretical concentration of the target contaminant (2,5-DCNB added). In addition, on top of the graphic, the panel indicates the days when donor was added to the microcosm, when DNA samples was collected

and when fresh medium was added and microcosm was diluted. Starting volume of this microcosm was 100 mL in 250 mL Boston bottle. On day 127, 20 mL were taken to use as inocula for group 3 of microcosms (Appendix B2, Figure 66B2), and more medium was added to top up the volume of 100 mL (dilution factor was 0.7). On day 287, volume of the microcosm was increased to 150 mL; the dilution factor considering the fresh medium added was 0.5. On day 528, dilution factor due to medium addition was 0.8. On day 659, volume was increased to 200 mL and culture was transfer to a 1 L bottle; the dilution factor was 0.5. In the panel "b", the absolute abundance of the bacterial community was obtained by multiplying its relative abundance (composition as percentage) from the sequencing results and the total bacteria quantified by qPCR. The microbial community after the dilution on days 127 and 287 is theoretically represented by the T-127 and T-287 bars, respectively, on the panel "b" (calculation was the absolute abundance of the previous DNA quantification multiplied by the dilution factors). A more detailed view of days 0-200 is presented in Figure 32.

Source: author (2022)

Figure 28 - Concentrations of contaminants (a) and bacterial community (b) of MC23DCNB-T2 during the experimental period



Indicated in the right side of the graphic, the panel "a" presents the concentration of contaminants measured, the cumulative concentration of the target contaminant added, the sum of the measured compounds, and the theoretical concentration of the target contaminant (2,3-DCNB added). In addition, on top of the graphic, the panel indicates the days when donor was added to the microcosm, when DNA samples was collected and when fresh medium was added and microcosm was diluted. Starting volume of this microcosm was 100 mL in 250 mL Boston bottle. On day 127, 20 mL were taken to use as inocula for group 3 of microcosms (Appendix B2, Figure 67B2), and more medium was added to top up the volume of 100 mL (dilution factor

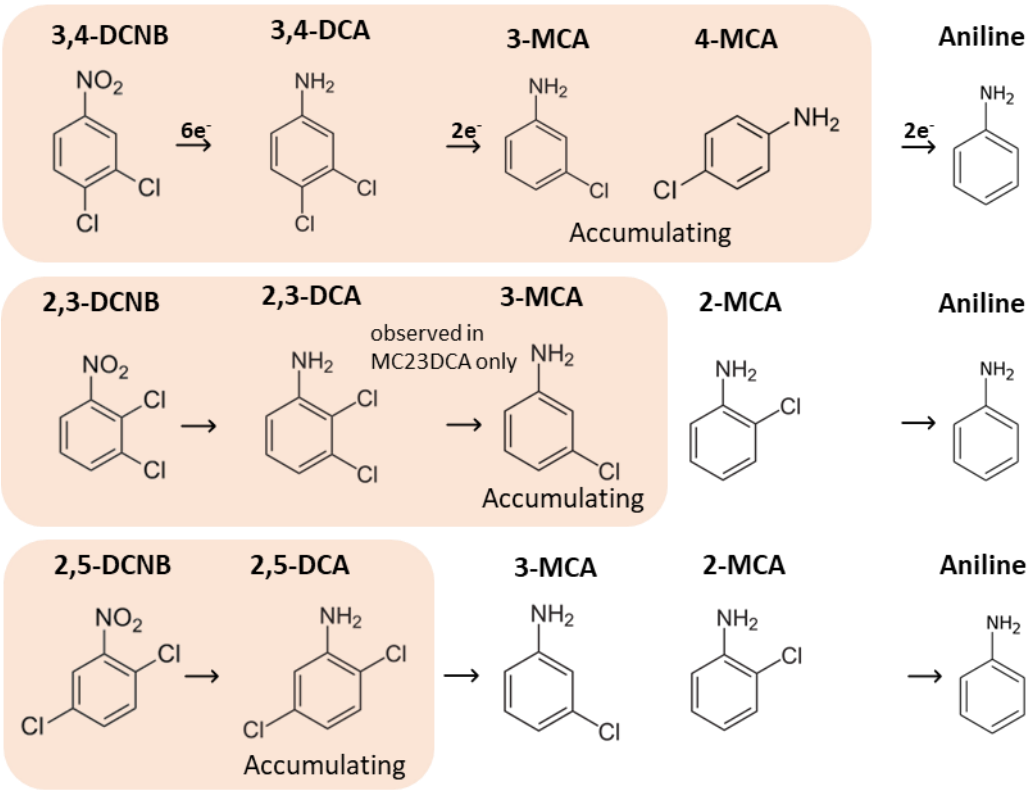
was 0.7). On day 287, volume of the microcosm was increased to 150 mL; the dilution factor considering the fresh medium added was 0.5. On day 528, dilution factor due to medium addition was 0.8. In the panel "b", the absolute abundance of the bacterial community was obtained by multiplying its relative abundance (composition as percentage) from the sequencing results and the total bacteria quantified by qPCR. The microbial community after the dilution on days 127 and 287 is theoretically represented by the T-127 and T-287 bars, respectively, on the panel "b" (calculation was the absolute abundance of the previous DNA quantification multiplied by the dilution factors). A more detailed view of days 0-200 is presented in Figure 33.

Source: author (2022)

The results obtained from the MC34DCNB-T3A (with donor), MC34DCNB-T3B (without donor), MC25DCNB-T3A (with donor), MC25DCNB-T3B (without donor), MC23DCNB-T3A (with donor), and MC23DCNB-T3B (without donor) are included in the Appendix B2, as Figures 65B2, 66B2, and 67B2. Aligned to the consistent reduction of the nitro group by the active bacterial community indicated in the DNA analysis of the killed control (KC), the differences in the rate of the DCBN reduction observed between the microcosms transfer 3 (MC-T3A - with donor and MC-T3B - without donor) for all three isomers also suggested that the reduction of the nitro group was driven by a biotic reaction.

The results discussed in this section are mainly based on findings from MC-T2 (Figures 24 to 28) and MC-T3A and MC-T3B (Appendix B2, Figures 65B2, 66B2, and 67B2). Certain periods of interest and relevant data from each microcosm will be highlighted and discussed in the following topics, aiming to compare active and non-active moments, when different processes were happening, in a tentative to identify the microbes involved in the activity observed in microcosms that led to the biotransformation of the individual contaminants. A summary of the biotransformation steps observed in the active microcosms (highlighted in boxes) and the expected products to be formed based on findings in the literature is presented in Figure 29.

Figure 29 - Biotransformations observed in microcosms (highlighted in boxes) and biotransformations expected to happen based on literature



Source: author (2022)

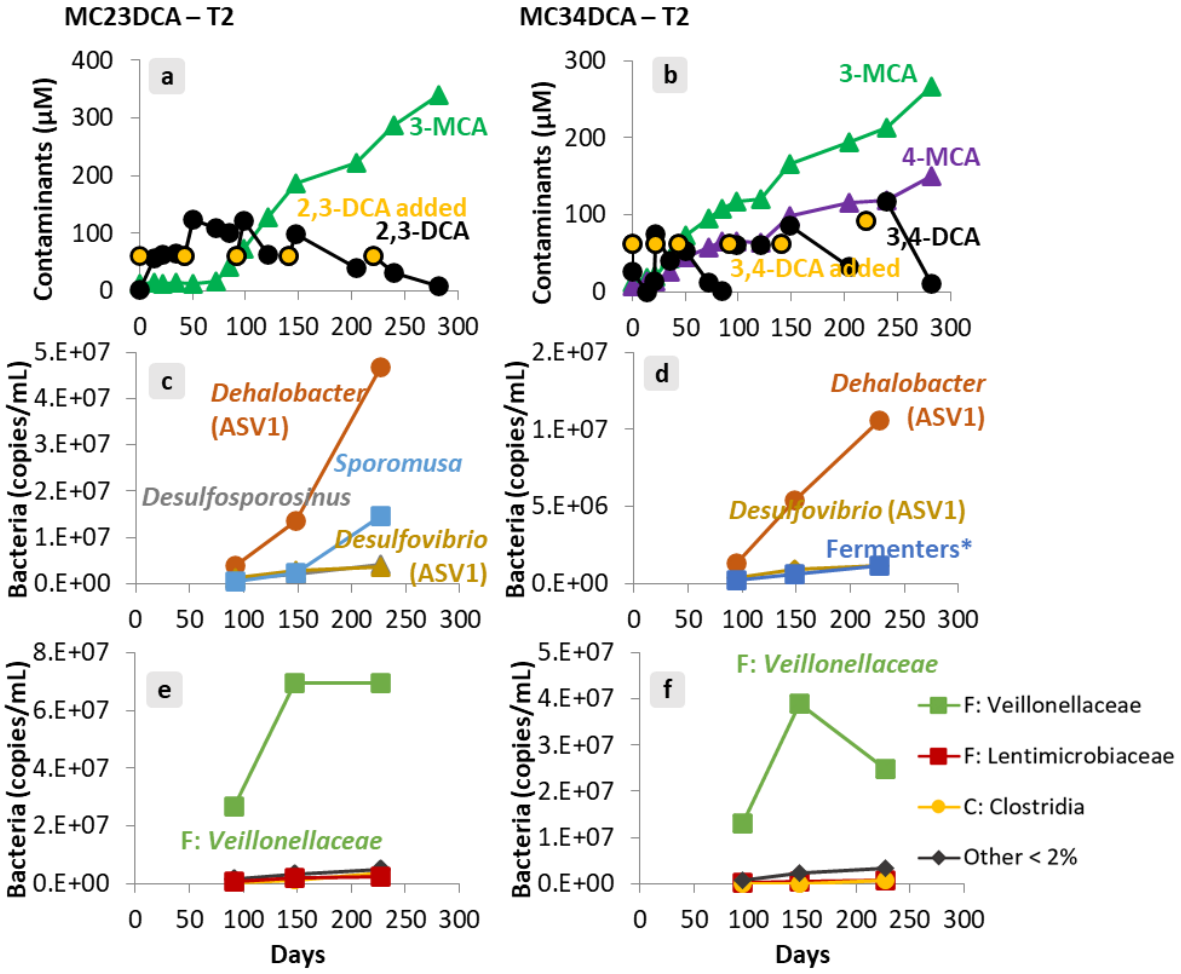
4.2.2.1 Detailed activity in microcosms amended with 2,3- and 3,4-DCA (MC23DCA and MC34DCA)

To evaluate the DCA biotransformation, results between the days 0 and 300 from MC23DCA-T2 (Figure 24a) and MC34DCA-T2 (Figure 25a) are presented now in Figure 30. The microcosms amended with the DCA isomers were always active, meaning that the target contaminants were biotransformed. The same activity and microbial composition are observed if we consider the last monitoring days and DNA samples analyzed.

In the case of MC23DCA-T2 (Figure 30a), 2,3-DCA was reduced into 3-MCA, while the 3,4-DCA was reduced into 3-MCA and 4-MCA in MC34DCA-T2 (Figure 30b). Regarding microbial community, an expressive growth of one ASV of the *Dehalobacter* genus was observed in both microcosms for the same period. Some other genera were also present but in lower abundance, such as *Sporomusa*, *Desulfosporosinus* and one ASV of *Desulfovibrio* in MC23DCA-

T2 (Figure 30c) and *Sporomusa*, *Anaerospora*, *Leptolinea*, and the same ASV of *Desulfovibrio* in MC34DCA-T2 (Figure 30d). Organisms of the *Veillonellaceae* family were highly abundant in both microcosms, represented by many ASV (Figures 30e and 30f).

Figure 30 - Biotransformation, the most abundant genera, and other organisms observed in MC23DCA-T2 (a, c, and e) between days 0 to 300 and in MC34DCA-T2 (b, d, and f) between days 0 to 300



In "a" and "b", 2,3-DCA added and 3,4-DCA added represent the theoretical amount of the specific contaminants added to the cultures, on feeding days. In "d", *Fermenters in MC34DCA – T2 are *Sporomusa*, *Anaerospora*, and *Leptolinea*. Data from the whole experimental period can be found in Figures 24 (MC23DCA-T2) and 25 (MC34DCA-T2).

Source: author (2022)

4.2.2.2 Detailed activity in microcosms amended with 3,4-DCNB (MC34DCNB)

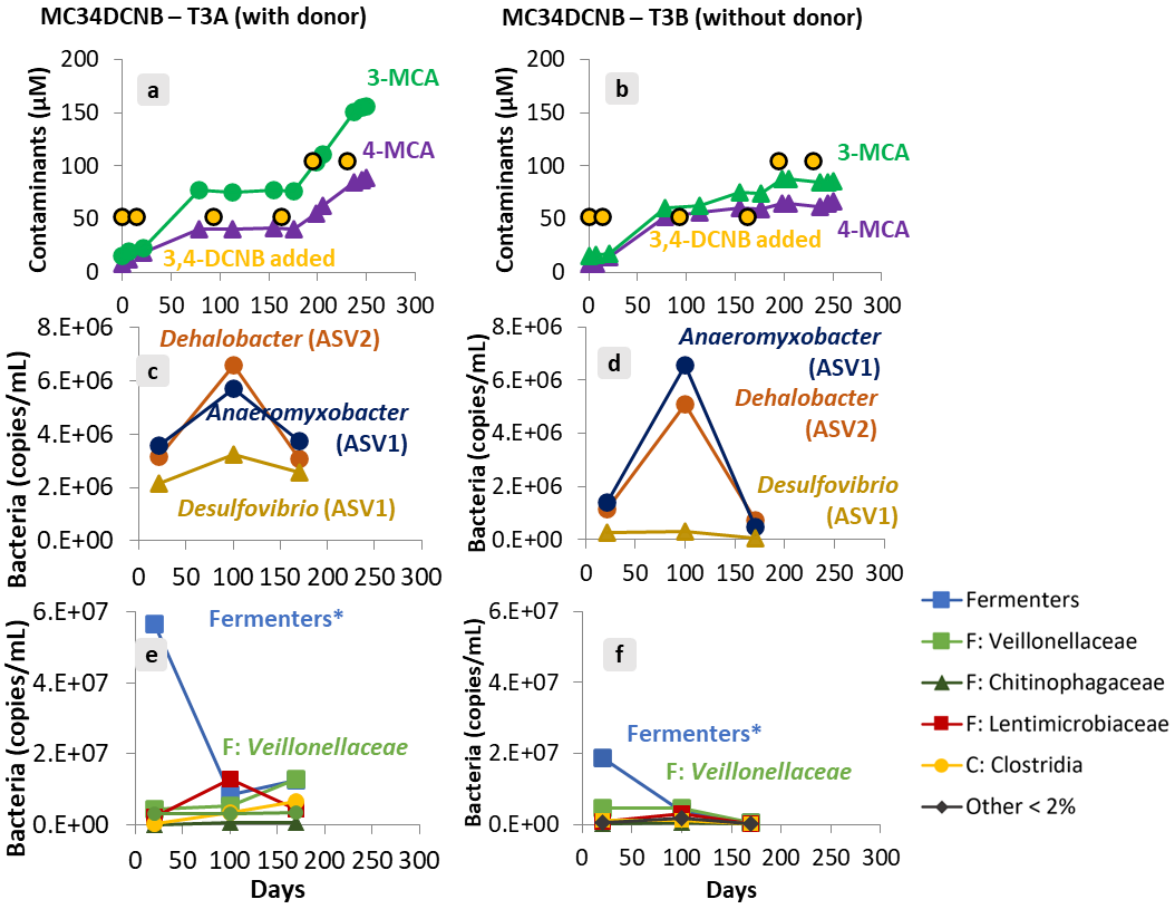
The microcosms amended with 3,4-DCNB (MC34DCNB-T1, MC34DCNB-T2, and MC34DCNB-T3A and MC34DCNB-T3B) presented high activity considering the reduction of the nitro group. As mentioned before, the 3,4-DCNB added was hardly detected on samples extracted from the microcosms due to fast reduction of the nitro group. After 3,4-DCNB is reduced into 3,4-DCA, the isomers 3-MCA and 4-MCA are formed (Figure 26).

The MC34DCNB-T3A (with donor) started to show slight faster reduction of the nitro group rate compared MC34DCNB-T3B (without donor) (Appendix B2, Figure 65B2), but it was not possible to use the specific data from 34DCNB microcosms to infer microbes responsible for this step so far. A comparison between the behaviour of MC34DCNB-T3A and MC34DCNB-T3B at the first 300 days provides good evidence of microbes performing the dechlorination step observed. Figures 31a and 31b show the 3,4-DCNB added, and the MCA isomers being formed after the reduction of the 3,4-DCA. The 3,4-DCA produced is shown in Appendix B2, Figure 65B2, but not included in the Figures 31a and 31b to avoid having much information in these two graphics.

Although there was a plateau in MCA concentration around days 80 and 180 in MC34DCNB-T3A (Figure 31a), the formation of MCA continued to happen until day 300. While in MC34DCNB-T3B (Figure 31b), MCA formation only started after around day 100. This agrees with the bacterial community composition (Figures 31c and 31d), which shows that the absolute abundance of the three most relevant genera increased significantly in MC34DCNB-T3B until the detection of the MCA isomers (day 100), and then decreased sharply.

The genera found in the cultures amended with 3,4-DCNB were the *Dehalobacter* (different ASV from the one found in microcosms amended with the DCA isomers – MC23DCA and MC34DCA - with 100% query coverage and 97.01% of percent identity), *Anaeromyxobacter* (1 ASV), and *Desulfovibrio* (same ASV found in microcosms amended with DCA isomers). The abundance of these three genera was higher in MC34DCNB-T3A than in MC34DCNB-T3B. The genera *Sporomusa* and *Leptolinea*, grouped as fermenters, the families *Veillonellaceae*, *Chitinophagaceae*, and *Lentimicrobiaceae*, and the class of Clostridia decreased in abundance when the source of electron was not added, in MC34DCNB-T3B, compared to MC34DCNB-T3A, in which most of these organisms were enriched (Figures 31e and 31f).

Figure 31 - Biotransformation, the most abundant genera, and other organisms observed in MC34DCNB-T3A between days 0 to 300 (a, c, and e) and in MC34DCNB-T3B between days 0 to 300 (b, d, and f)



In "a" and "b", 3,4-DCNB added represents the theoretical amount of the specific contaminant added to the cultures, on feeding days. The 3,4-DCA formed from the 3,4-DCNB reduction was detected, but it is not included in this figure to avoid having much information in these two graphics. The monochloroanilines in the graphics are formed from the 3,4-DCA. In "e" and "f", *Fermenters are *Sporomusa* and *Leptolinea*. Data from the whole experimental period can be found in Appendix B2, Figure 65B2.

Source: author (2022)

4.2.2.3 Detailed activity in microcosms amended with 2,5-DCNB (MC25DCNB)

The transfers of MC25DCBN were active since the beginning of the experiment. The T2 (Figure 27) and T3A (Appendix B2, Figure 66B2) results were compared in a tentative to identify possible organisms responsible for nitro group reduction.

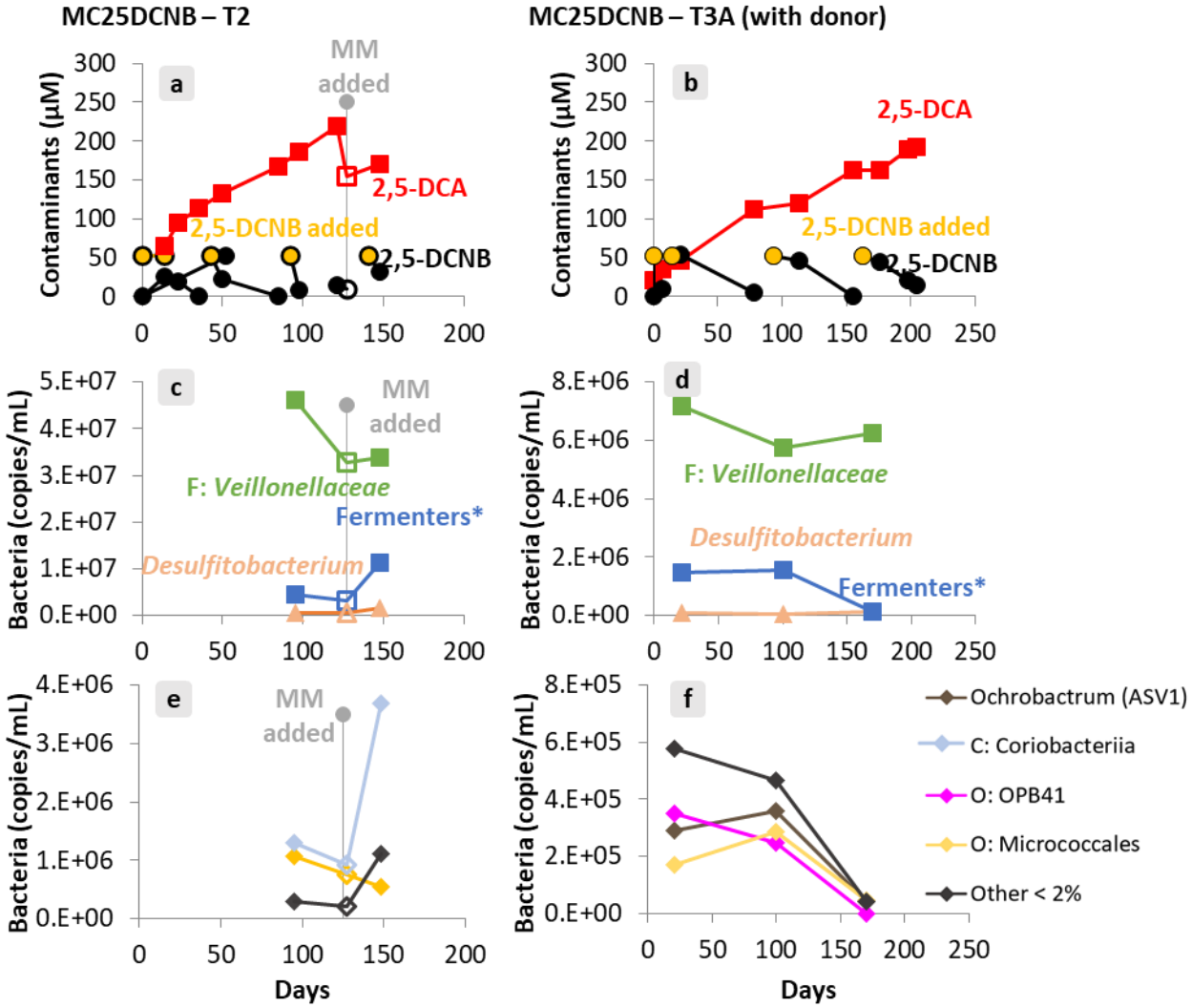
Figures 32a and 32b show the 2,5-DCNB being reduced into 2,5-DCA in both transfers. In MC25DCNB-T2, there is a theoretical point, on day 127, due to a dilution in this microcosm after

adding fresh MM. The dilution was calculated based on the volume of liquid remaining in the microcosm before the addition of medium and the volume of fresh medium added, considering the previous DNA quantification by qPCR.

The microbial community seems to be stable in microcosms of group 2 (T2) and group 3 with donor (T3A). The most abundant organisms found in both microcosms are part of the *Veillonellaceae* family, followed by the *Sporomusa*, *Leptolinea, Anaerospora*, and *Pelotomaculum* genera (grouped as fermenters), and the *Desulfitobacterium* in lower absolute abundance (Figures 32c and 32d). Other organisms less abundant are presented in Figures 32e and 32f.

The DNA data from MC25DCNB-T3A (with donor added) reducing the nitro group against the data from MC25DCNB-T3B (without donor added) during a period of less activity (day 390) (Appendix B2, Figure 66B2) shows again the *Veillonellaceae* family as the most abundant and enriched organism. It consisted of more than 90% of the growing community in the active MC25DCNB-T3A (with donor), against a significant decay of the bacterial abundance and the nitro reduction rate observed in MC25DCNB-T3B (with no donor).

Figure 32 - Biotransformation, the most abundant genera, and other organisms observed in MC25DCNB-T2 between days 0 to 148 (a, c, and e) and in MC25DCNB-T3A between days 0 to 205 (b, d, and f)



In all panels “a”, “c”, and “e”, the empty elements on day 127 indicate a theoretical data calculated after the dilution of the microcosm when fresh mineral medium was added (dilution factor was 0.7). In “a” and “b”, 2,5-DCNB added represents the theoretical amount of the specific contaminant added to the cultures, on feeding days. *Fermenters are *Sporomusa*, *Leptolinea*, *Anaerospora*, and *Pelotomaculum*. Data from the whole experimental period can be found in Figure 27 and Appendix B2, Figure 66B2.

Source: author (2022)

4.2.2.4 Detailed activity in microcosms amended with 2,3-DCNB (MC23DCNB)

The microcosms amended with 2,3-DCNB were the most fragile among all microcosms. A period of consistent activity in MC23DCNB-T2 was observed only in the first 100 days (Figure

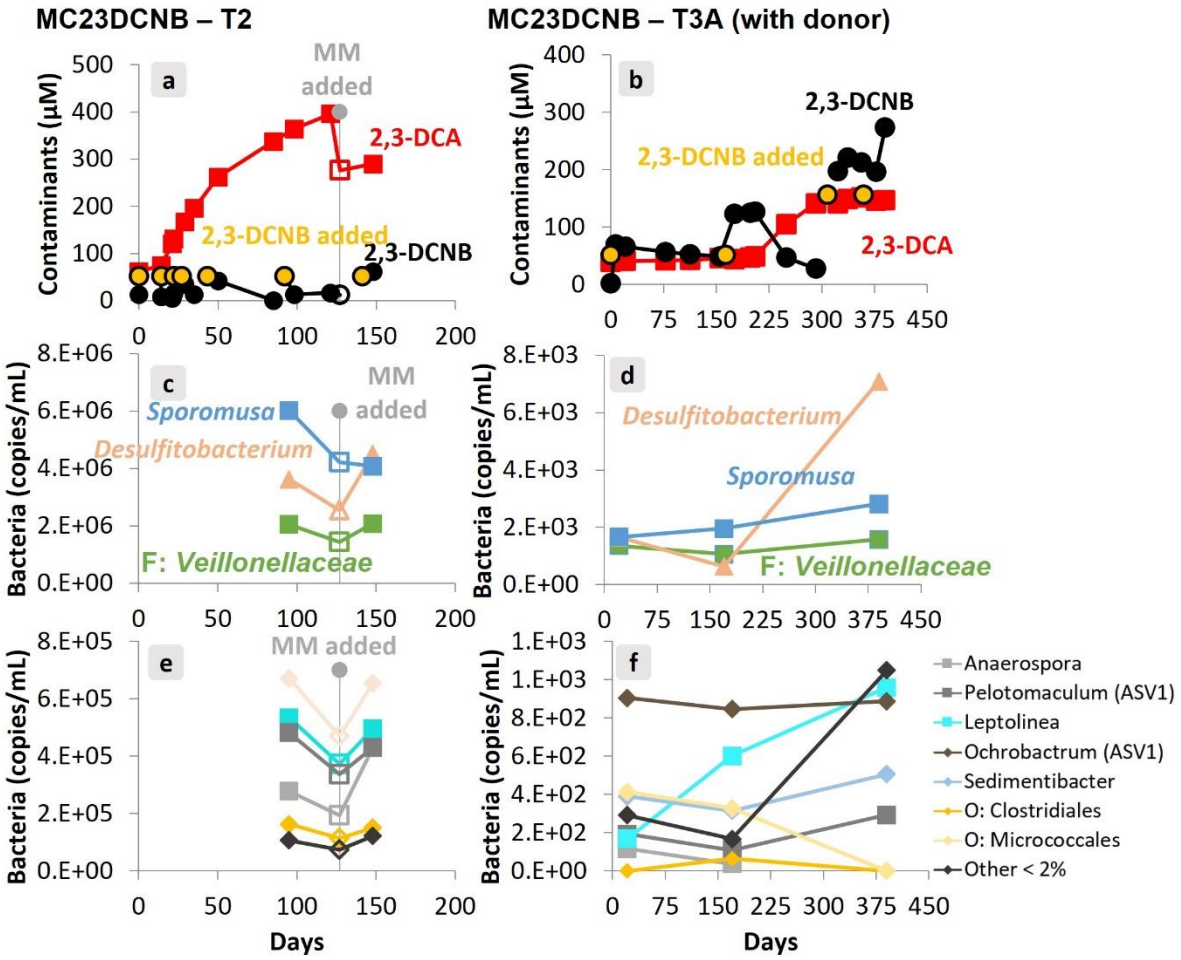
28a). MC23DCNB-T3A (with donor) and MC23DCNB-T3B (without donor) were not efficient in reducing the nitro group but based on the last days of operation reported here, the MC23DCNB-T3A seems to be recovering its activity (Appendix B2, Figure 67B2).

In this topic, the most active moment of MC23DCNB-T2 (from day 0 to around day 150 from Figure 28a) is compared to MC23DCNB-T3A (from day 0 to around day 400 from Appendix B2, Figure 67B2a), when 2,3-DCNB was not being reduced and thus not forming 2,3-DCA followed by a more promising period (Figures 33a and 33b). In MC23DCNB-T2 there is a theoretical point represented by empty markers which are the day when more medium was added, diluting contaminants and the microbial community.

It is known that MC23DCNB-T3A does not represent the best scenario of non-active microcosm since its microbial community copies are in the magnitude of 10^3 copies/mL against the 10^6 copies/mL found in MC23DCNB-T2. However, this comparison gives us some tips on what to expect if it recovers activity.

Considering the most abundant organisms in both transfers, in MC23DCNB-T2, while the 2,3-DCNB was reduced to 2,3-DCA, the *Sporomusa* genus, the *Desulfitobacterium* genus, and *Veillonellaceae* family seemed to be enriched. In MC23DCNB-T3A, interestingly, during the first 150 days, a decrease of the *Desulfitobacterium* genus when the 2,3-DCNB was not being reduced into 2,3-DCA was observed, while the *Sporomusa* genus was increased and the *Veillonellaceae* stayed close to what it was in the first DNA result. After that, in T3A, 2,3-DCNB was reduced to 2,3-DCA and the DNA result showed the growth of the same three organisms mentioned, being the *Desulfitobacterium* the most enriched among them (Figures 33c and 33d). Other organisms in lower absolute abundance were present (Figures 33e and 33f).

Figure 33 - Biotransformation, the most abundant genera, and other organisms observed in MC23DCNB-T2 between days 0 to 148 (a, c, and e) and in MC23DCNB-T3A between days 0 to 390 (b, d, and f)



In all panels “a”, “c”, and “e”, the empty elements on day 127 indicate a theoretical data calculated after the dilution of the microcosm when fresh mineral medium was added (dilution factor was 0.7). In “a” and “b”, 2,3-DCNB added represents the theoretical amount of the specific contaminant added to the cultures, on feeding days. Data from the whole experimental period can be found in Figure 28 and Appendix B2, Figure 67B2.

Source: author (2022)

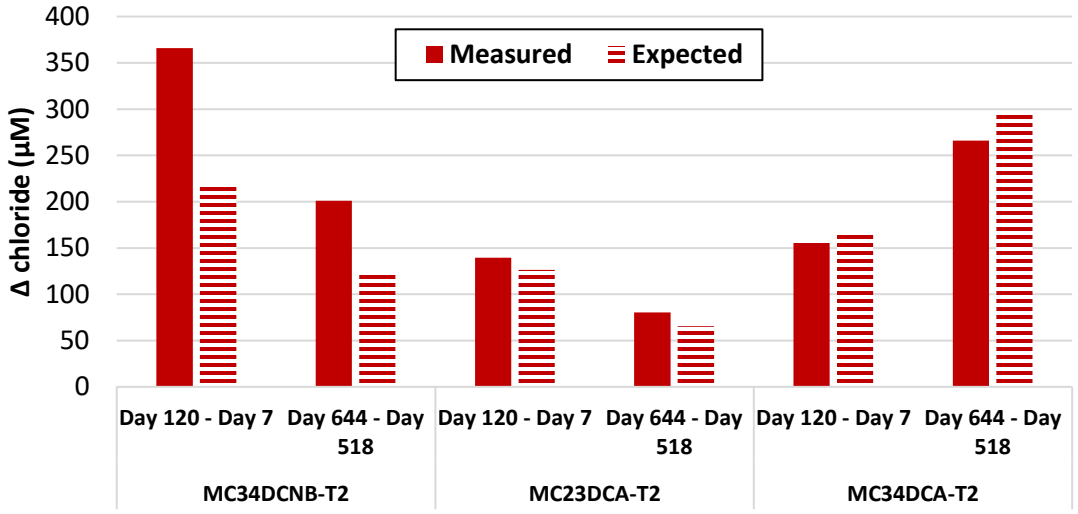
4.2.3 Anion analysis (chloride, acetate, sulphate)

Figure 34 shows the difference in the amount of chloride measured between days 120 and 7, and between days 644 and 518 compared to the amount of chloride released from the target contaminant when it is biotransformed in transfer 2 of the microcosms amended with 2,3-DCA, 3,4-DCA, and 3,4-DCNB (Figures 24 to 26). The microcosms amended with 2,3- and 2,5-DCNB

did not accumulate chloride, which is consistent with the transformation stopping at the respective DCA. In these two microcosms, only the nitro group was reduced.

It is expected that each mol of DCNB or DCA releases 2 moles of chloride, if complete dechlorination happens. However, as monochloroanilines accumulated in the medium, the expected amount of chloride released was assumed to be equivalent to the amount of monochloroanilines measured (in micromolar). The chloride expected to be released based on contaminant transformation was close to the amount measured but did not match perfectly. A relevant fact to be considered is that samples for IC analysis were not collected on the same day as those samples for HPLC analysis, so the expected amount of chloride was calculated based on sample collected on the closest day. The actual concentrations of chloride (measured and expected from feeding) are available in Appendix B2, Table 14B2.

Figure 34 - Changes in chloride concentrations in MC34DCNB-T2, MC23DCA-T2, and MC34DCA-T2 compared to expected values

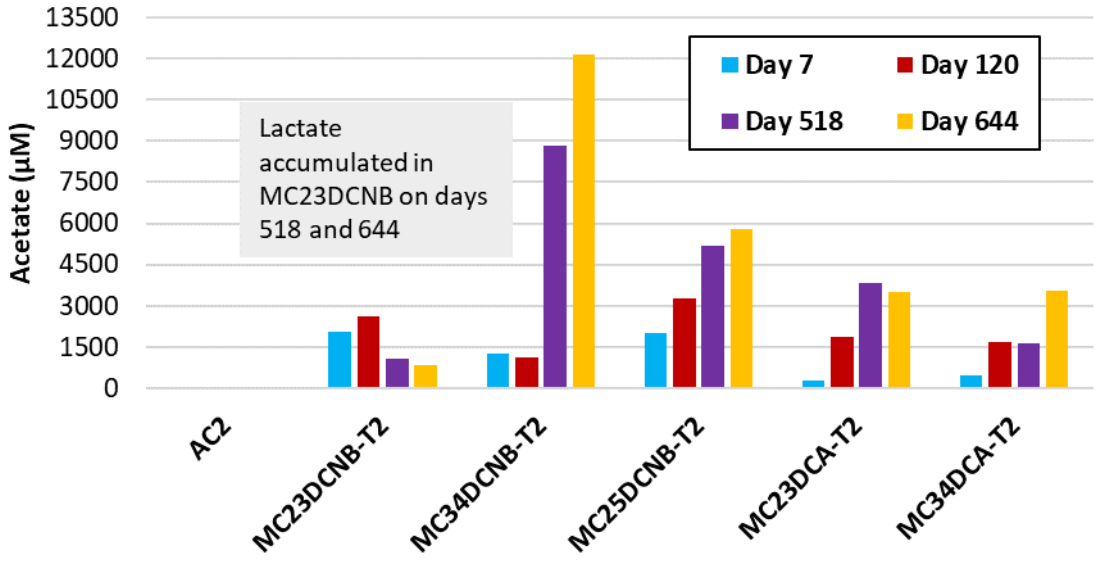


Values of chloride measured (■) and expected from feeding (≡) released from biotransformation of compounds between days 120 and 7 (the concentration of chloride on day 120 minus its concentration on day 7) and days 644 and 518 (the concentration of chloride on day 644 minus its concentration on day 518). Source: author (2022)

Figure 35 shows the amount of acetate measured on different days in the five MC-T2 (Figures 24 to 28) and AC2 (Figure 21). Acetate was not detected in any sample collected from the

AC2, while the detection of the ion in the microcosms indicates that it is produced as a fermentation product by the microbial communities. In MC34DCNB-T2, MC25DCNB-T2, MC23DCA-T2, and MC34DCA-T2, the acetate accumulated on days 518 and 644 was much higher than the expected amount based on the donor added; considering the maximum amount of donor added (685 µM for ethanol and 308 µM for lactate) would form 993 µM (FORESTI, 1994). It may be formed by acetogenic microbes using H_2 and CO_2 from the headspace (discussed later). In MC23DCNB-T2 lactate was detected, but not quantified, in samples from days 518 and 644.

Figure 35 - Concentration of acetate measured in AC2, MC23DCNB-T2, MC34DCNB-T2, MC25DCNB-T2, MC23DCA-T2, and MC34DCA-T2



Sampling days were days 7 (■), 120 (■), 518 (■) and 644 (■). A peak correspondent to lactate was detected on days 518 and 644 of MC23DCNB-T2.

Source: author (2022)

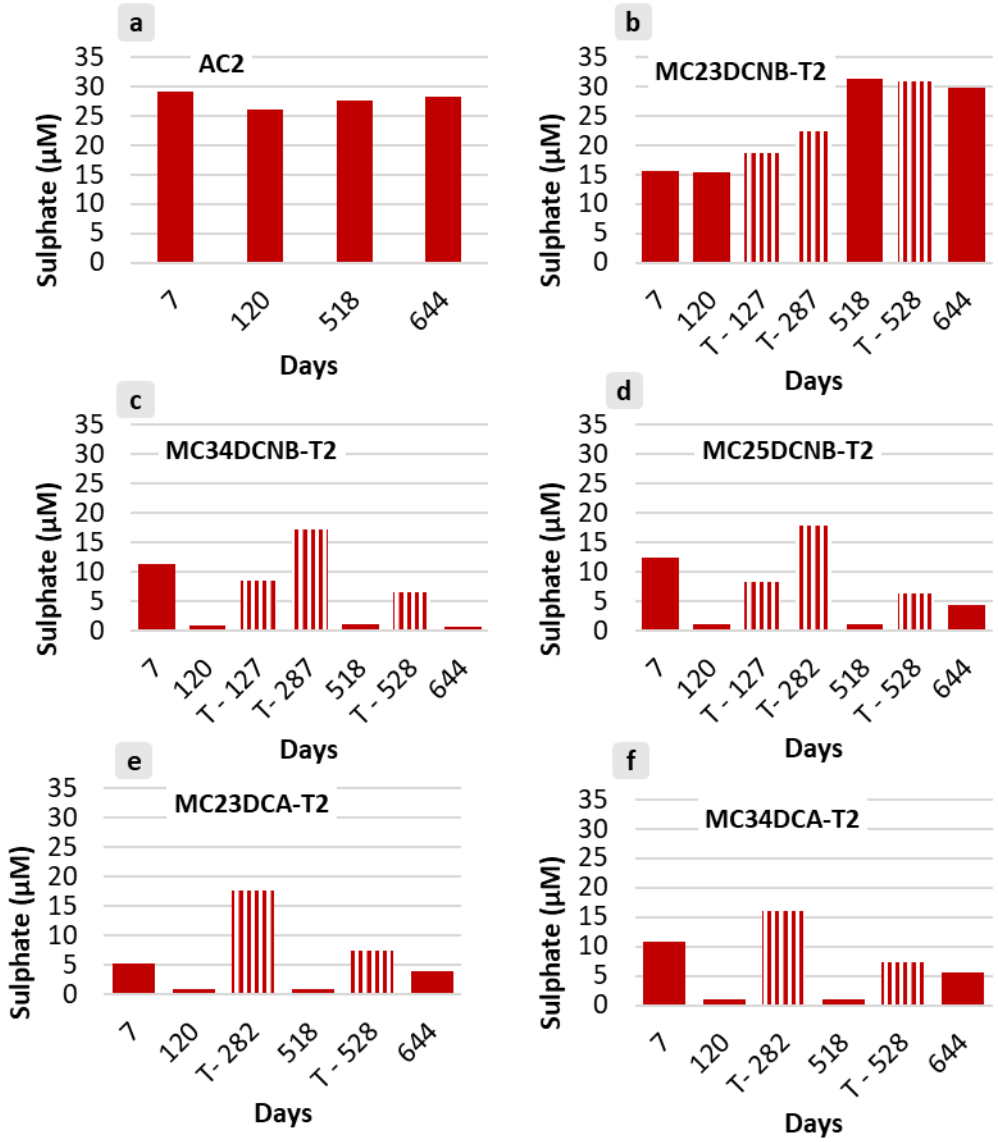
Figure 36 shows the amount of sulphate measured in AC2 (Figure 36a) and in each of the five MC-T2 (Figure 36b-f) from 4 samples (days 7, 120, 518, and 644). Based on the sulphate content in the anaerobic mineral medium (around 30 µM), some calculated values of the ion for the days when fresh medium was added are represented in the graphics as theoretical values (T-127, T-282, T-287, or T-528).

The negative control with mineral medium only, AC2, did not show any change in sulphate concentration. On day 7, all five microcosms had lower than 50% of the sulphate concentration

from the mineral medium already. After that, in MC23DCNB-T2, there was no decrease in the sulphate content, on the contrary, it was accumulated when more medium was added into the microcosm (Figure 36b). The sulphate accumulation in MC23DCNB-T2 is aligned to the slower activity observed in this microcosm (Figure 28a).

However, in MC34DCNB-T2 (Figure 36c), MC25DCNB-T2 (Figure 36d), MC23DCA-T2 (Figure 36e), and MC34DCA-T2 (Figure 36f), the sulphate content decayed every time when medium (containing sulphate) was added. Thus, sulphate reduction is likely to be happening in the active cultures (MC34DCNB-T2, MC25DCNB-T2, MC23DCA-T2, and MC34DCA-T2) but not anymore in MC23DCNB-T2.

Figure 36 - Sulphate concentrations in AC2 (a), MC23DCNB-T2 (b), MC34DCNB-T2 (c), MC25DCNB-T2 (d), MC23DCA-T2 (e), and MC34DCA-T2 (f)



Values of sulphate measured (■) in samples analyzed through IC and theoretical (≡) calculated based on the dilution when fresh mineral medium was added to the bottles and the concentration of the anion previously measured. Sulphate content of the mineral medium is around 30 µM. Theoretical values of sulphate are represented by T-127, T-282, T-287 and T-528, calculated based on the dilutions of the microcosms after mineral medium added on the respective days (dilution factors for are described in legends of Figures 24 to 28). There was sulphate reduction in the bottles, except for bottles represented by panels “a” and “b”, which are the abiotic control 2 (AC2) and the inactive microcosm amended with 2,3-DCNB.

Source: author (2022)

4.2.4 Crossing the data obtained from anaerobic tests

Based on the comparison between periods of activity and non-activity and the microbial community observed in the microcosms (MC-T2, MC-T3A – with donor, MC-T3B – without donor) during the experimental period, some organisms responsible for the biotransformation of the contaminants can be inferred. In a tentative to schematize all the findings together, Table 7 is presented. It brings MC-T2 information regarding the activity of the cultures, the final pH value, the contaminants formed as products of biotransformation, whether acetate is detected and sulphate is reduced, and a summary of which groups of organisms were mainly observed in each of the microcosms.

The MC23DCNB-T2 (Figure 28) ended up being a negative control as its activity got slower along the period of the experiment. Activity in this microcosm was observed only for the first 100 days, when 2,3-DCA was formed at a rate comparable to the rates observed in the other microcosms. On day 644, the pH value of MC23DCNB-T2 was 7.42, acetate was still formed, but lactate was accumulated (Figure 35). Sulphate was not reduced (Figure 36b), although some ASV of the *Desulfitobacterium* genus, known as SRB, were present in the community (Figure 28b).

Contrastively, MC23DCA-T2 (Figure 24), MC34DCA-T2 (Figure 25), MC34DCNB-T2 (Figures 26), and MC25DCNB-T2 (Figure 27) had a pH value closer to 7.0, or even lower, in the case of MC34DCNB-T2. In the samples analyzed from these four microcosms with great activity throughout the experimental period, we did not detect lactate, and we did see consistent acetate formation and sulphate reduction, aligned to a microbial community composed of archaea, fermentative bacteria, *Desulfovibrio* (SRB), and *Dehalobacter* and/or *Anaeromyxobacter* (OHRB) in dechlorinating microcosms.

Table 7 – Overview of parameters in monitored MC-T2

Microcosms	Active?	pH* day 644	Products	Acetate formed?	SO_4^{2-} reduced?	Community
MC23DCNB	For the first 100 days	7.42	2,3-DCA	Yes Lactate accumulating	No	Fermenters + *Desulfitobacterium* (many ASV)
MC34DCNB	Yes	6.56	3,4-DCA, 3-MCA, 4-MCA	Yes	Yes	Fermenters + *Desulfovibrio* (ASV1) + *Dehalobacter* (ASV2) + *Anaeromyxobacter* (ASV1) + Archaea
MC25DCNB	Yes	7.02	2,5-DCA	Yes	Yes	Fermenters + *Desulfitobacterium* (many ASV) + Archaea
MC23DCA	Yes	7.09	3-MCA	Yes	Yes	Fermenters + *Desulfovibrio* (ASV1) + *Dehalobacter* (ASV1) + Archaea
MC34DCA	Yes	7.05	3-MCA, 4-MCA	Yes	Yes	Fermenters + *Desulfovibrio* (ASV1) + *Dehalobacter* (ASV1) + Archaea

*Initial pH of all microcosms = 6.61

Source: author (2022)

The first group of microbes listed includes the fermentative bacteria. The *Veillonellaceae* family is reported to be, in general, fermenting microbes. Some genera from this family can oxidize lactate producing CO_2, H_2, and various volatile fatty acids (C_2 to C_6 atoms) (MARCHANDIN; JUMAS-BILAK, 2014). The *Veillonellaceae* family was also reported as able to produce propionate as a fermentation product (LOUIS; FLINT, 2017; SASAKI, K. *et al.*, 2018; STAMS *et al.*, 1998). Although there were some tiny peaks in the range of propionate and butyrate of the IC analysis, it can not be affirmed that these products were formed or not, since few samples from each microcosm were analyzed and standards of the products were not included in the runs.

The genera *Sporomusa* and *Anaerospora* are both included in the *Veillonellaceae* family and are also abundant in the microcosms. *Sporomusa* organisms have been described to produce acetate from many substrates, such as pyruvate, lactate, ethanol, methanol, formate, and H_2-CO_2 (KUHNER *et al.*, 1997; LIU, Y; WHITMAN, 2008). The ability that *Sporomusa* species have to

grow autotrophically with H_2 and CO_2 through the Wood-Ljungdahl pathway (DIEKERT; WOHLFARTH, 1994; HUMPHREYS; DANIEL; POEHLEIN, 2017; KREMP; ROTH; MÜLLER, 2020; TREMBLAY *et al.*, 2015; VISSER *et al.*, 2016) might be the explanation of the high concentration of acetate in MC34DCNB-T2 and MC25DCNB-T2, considering that H_2 and CO_2 are both constituents of the headspace in the microcosms, as they are constituents of the glovebox atmosphere.

Fermentative organisms are enriched in all communities supplemented with donor (ethanol and lactate) and are more prevalent in microcosms that have the nitro group reduction as the only step observed (MC23DCNB-T2 and MC25DCNB-T2). They are possibly responsible for a co-metabolic reduction of the nitro group in microcosms amended with DCNB, via fortuitous reactions, as it is reported to be performed by many living organisms (SPAIN, 1995).

The *Desulfitobacterium*, in MC23DCNB-T2, is also a promising organism involved in the nitro group reduction, as it was enriched when this microcosm showed activity for the DCNB reduction. *Desulfitobacterium* genus was described as a strictly anaerobic bacteria able to use many compounds as the electron acceptor, including sulphite, nitrate, and halogenated compounds (VILLEMUR *et al.*, 2006).

To investigate the involvement of this genus in the reduction of the nitro group, as it was performed by Madeira *et al*. (2021), it would be valuable to assess the relationship between the electrons added and the reduction of the nitro group, as well as between cell growth and ATP production. The cited study inspired me to come up with the following questions:

Is the electron donor used with and without the nitro group reduction? If the donor is only used when the nitro group is reduced, it might be evidence of respiratory process. In case the donor is used without the presence or the reduction of the nitro group, it is probably a co-metabolic mechanism. Similarly, co-metabolism is likely to be happening if cell growth occurs independently of the nitro group reduction.

Other questions are raised related to the electron balance: Do all the available electrons added go to the nitro group reduction? Where does the donor go? If a huge amount of electrons is needed for the nitro reduction to happen in our cultures, co-metabolism might be happening. If the amount of electrons needed is just a bit more than the chemical stoichiometric, considering nitro reduction and cell growth, it is likely to be a respiratory process.

We did not perform any test to solve these questions; however, a relevant fact to be considered so far is that the microcosms evaluated in our research have a mixed culture, differently from the pure culture used by Madeira *et al*. (2021). Thus, some organisms can be reducing the nitro group through co-metabolism (fermentative organisms), but some can be respiring it (*Desulfitobacterium*). Another fact is that in the communities of this study, more than 5 times of electrons were added compared to the amount needed for DNCB reduction, thus, evidence of nitro reduction mechanisms can be masked. Therefore, some adjustments to the experimental design should be planned to answer some of the raised questions.

The oxidation of ethanol and lactate to acetate was verified in all active microcosms (Figure 35). Those redox half oxidation reactions (electron donating reactions) are not expected to happen spontaneously, based on their thermodynamic (AG_o of +9.6 KJ and -4.2 KJ, respectively) (HARPER; POHLAND, 1986). However, the production of acetate, H_2 and HCO_3^- is more favourable when methanogenesis is active in the community. Methane produced by archaea is relevant because hydrogen must be continually removed to drive the fermentation reactions to the formation of acetate (HARPER; POHLAND, 1986; LIU, Y; WHITMAN, 2008). This explains why archaea are essential in the active communities.

The hydrogen content in our microcosms was not measured; however, it can be inferred that it was produced based on the literature discussed above and because the *Dehalobacter* present in dechlorinating communities is a strictly OHRB that is reported to use H_2 as the only source of electron for its respiration (FINCKER; SPORMANN, 2017).

Considering the 16S fragment sequenced, the two ASV found in the microcosms, ASV1 in MC23DCA-T2 and MC34DCA-T2 and ASV2 in MC34DCNB-T2, had 100% of query cover with the *Dehalobacter* restrictus DSM 9455 (CP007033), the *Dehalobacter* sp. CF (CP003870), and the *Dehalobacter* sp. DCA (CP003869). The ASV1 was 97.01% identical to them, while the ASV2 was 100%. The three strains were related to dehalogenation; the first to dechlorination of tetrachloroethene and trichloroethene (KRUSE *et al.*, 2013), while the two latest were involved in dehalogenation of chloroform, trichloroethane, dichloroethane (TANG *et al.*, 2016; TANG; GONG; EDWARDS, E. A., 2012). *Dehalobacter* sp. strain 14DCB1 dechlorinated 2,3-DCA forming 2- and 3-MCA, and 3,4-DCA forming 3-MCA only, using acetate as carbon source and hydrogen as the electron donor. Like in this study, the authors did not observe dechlorination of the MCA (ZHANG, S. *et al.*, 2017).

The growth yields for the two affiliated amplicon sequence variants enriched in the three microbial communities (MC23DCA-T2, MC34DCA-T2, and MC34DCNB-T2) varied between 6.79E+07 and 1.95E+08 copies per μmol of Cl^- released (calculations are provided in Appendix B2, Table 15B2.

The *Anaeromyxobacter* genus, also present in MC34DCNB-T2, is a facultative OHRB (FINCKER; SPORMANN, 2017). When comparing it to complete sequenced genomes available in the NCBI bank, the unique ASV found in the community had 100% of query cover with two strains of *Anaeromyxobacter dehalogenans* and a percent identity of 99.57% with the 2CP-C (CP000251) and 99.36% with the 2CP-1 (CP001359), these were the most similar findings from BLAST. Both strains were isolated from soil and can gain energy from reductive dechlorination of chlorophenol and contain putative dehalogenases (COLE, James R *et al.*, 1994; SANFORD; COLE, J R.; TIEDJE, J M., 2002; THOMAS *et al.*, 2008). Thus, it is likely that the *Dehalobacter* and *Anaeromyxobacter* enriched in the community are involved in reductive dehalogenation of the DCA through a respiratory process.

Desulfovibrio is known as SRB and a facultative OHRB (FINCKER; SPORMANN, 2017; QIAN *et al.*, 2021). When reducing sulphate into sulphide, SRB can use lactate and form acetate and CO_2 or convert acetate into CO_2 and HCO_3^- (CHEN, Y; CHENG, J. J.; CREAMER, 2008; MUYZER; STAMS, 2008). The quantity of electrons needed for the reduction of the 30 μM of sulphate from the medium is 240 μM of electrons (8 mol of electrons for each mol of sulphate). This sulphate demand is quite small compared to the total amount of electron donor added to the microcosms: at least 514 μM and 231 μM of ethanol and lactate, respectively, which correspond to 6,156 μM and 2,772 μM of electrons available for each donor spike (1 mol of each donor provides 12 mol of electron assuming complete oxidation to CO_2).

Comparison between the free energy provided by the reductive dehalogenation of 2,3- and 3,4-DCA with H_2 as the donor (in the range of -135.0 KJ and -137.7 KJ) (SUSARLA *et al.*, 1997) and the lower free energy provided by the reduction of sulphate when lactate is converted into acetate (-80.2 KJ) or when acetate is completely oxidized to CO_2 (-47.6 KJ) explains why the growth observed for the *Desulfovibrio*, as a SRB, was not as significant as it was for the *Dehalobacter* and the *Anaeromyxobacter* genera in MC23DCA-T2, MC34DCA-T2, and MC34DCNB-T, as OHRB.

In addition, although there were enough electrons for simultaneous reductive dehalogenation by the *Dehalobacter* and the *Anaeromyxobacter* and sulphate reduction by the *Desulfovibrio*, sulphate was not added in the same proportion and frequency (as constituent of the MM) as the donor and contaminants were. If sulphate content was in excess in the medium, the SRB could compete for the hydrogen and benefit from -151.9 KJ as free energy from this reaction (MUYZER; STAMS, 2008), which was not our case.

Thus, the decrease of sulphate content observed in MC34DCNB-T2, MC23DCA-T2, and MC34DCA-T2 is thought to be related to the enrichment of the unique *Desulfovibrio* ASV found in all these communities, which was not similar to any complete genome of *Desulfovibrio* involved in dechlorination.

Although the *Desulfovibrio* is not discarded as dechlorinator in the communities, since it is only present in microcosms dehalogenating DCA isomers (MC23DCA, MC34DCA, and MC34DCNB), it is more likely to be performing a parallel process happening in the active communities, being relevant to the flow of electrons and the health of the communities. In MC25DCNB-T2 *Desulfovibrio* was not present, but we did see the *Desulfitobacterium* genus that can be related to the changes in sulphate content of the microcosm amended with 2,5-DCNB and not removing the first chlorine from the 2,5-DCA.

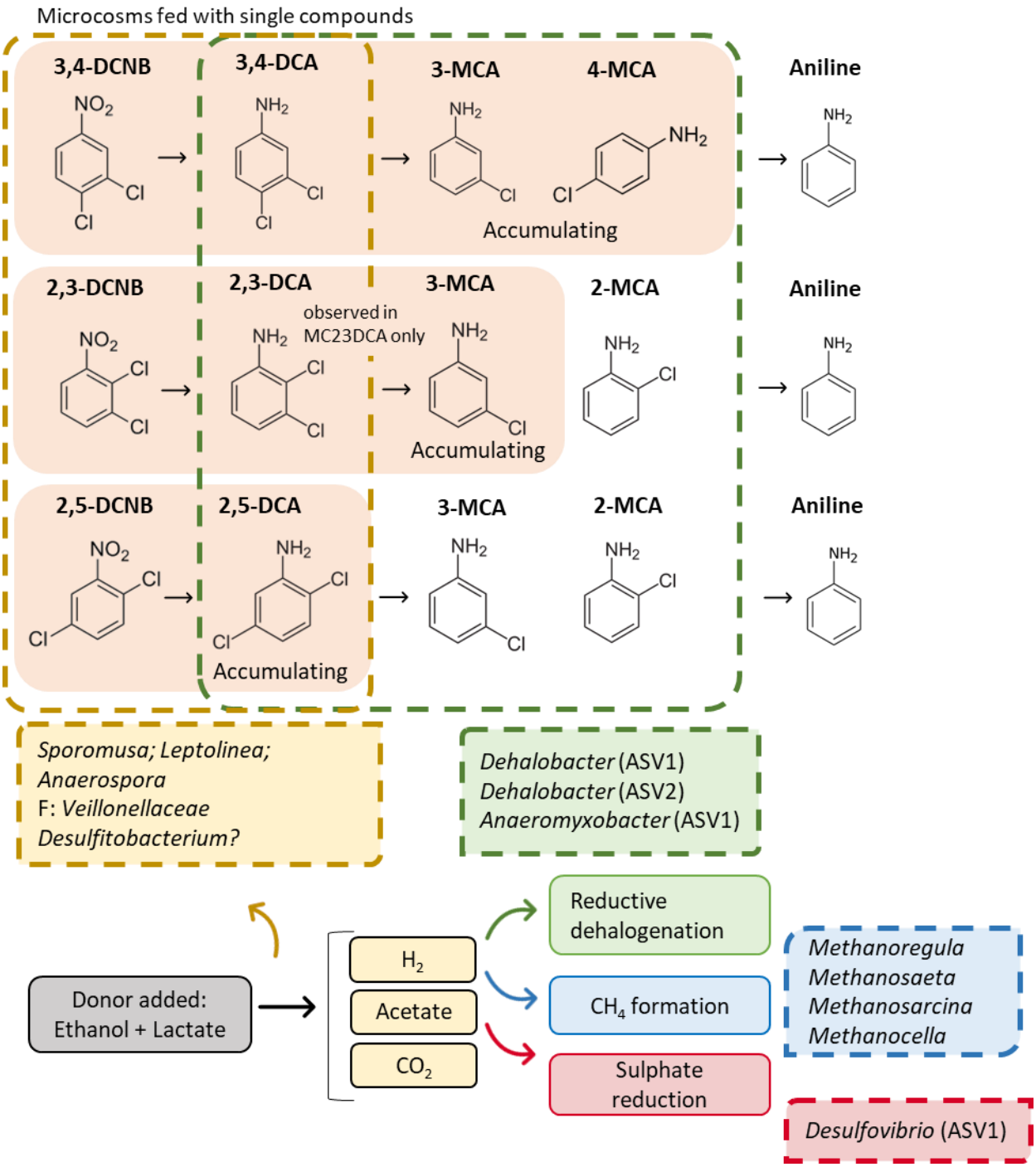
Based on what we have found so far, Figure 37 shows a scheme of the activity observed in our microcosms aligned to the microbial community. The steps observed in the microcosms are again highlighted in boxes. The relation between the organisms discussed is presented in the phylogenetic tree (Figure 38).

The reduction of the nitro group is related to a co-metabolic process, fortuitously performed by fermentative organisms. A respiratory process performed by the *Desulfitobacterium* is not discarded, but this still needs to be verified. The H_2 is used as the electron donor by the OHRB to breathe the 2,3- and 3,4-DCA isomers, removing the first chlorine from the contaminants. However, the 2,5-DCA is accumulating in the medium. Archaea are responsible for methane production and *Desulfovibrio* for sulphate reduction, both groups of organisms are involved in the stability of the communities.

In MC23DCA-T1 (Appendix B2, Figure 63B2) and MC23DCA-T2 (Figure 24a), 2,3-DCNB was added as feeding contaminant instead of 2,3-DCA on days 606 and 751 and on day 568, respectively, to check the activity of nitro reduction and it was successfully reduced. Thus,

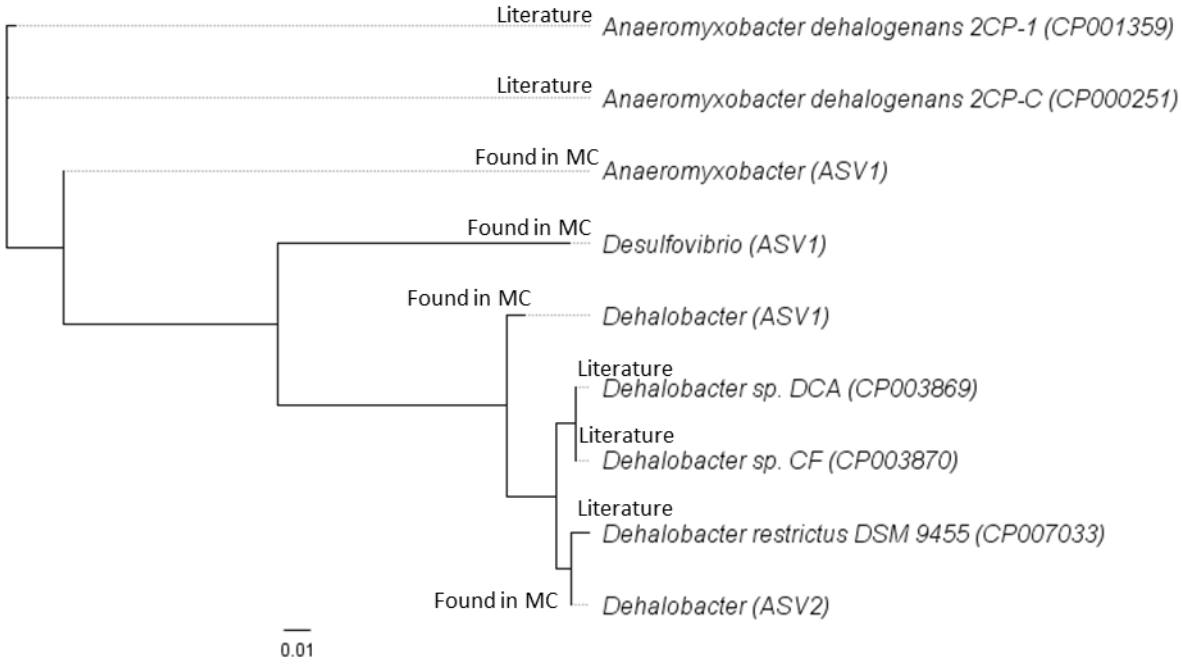
the research group ended up with active communities able to reduce the nitro group from 2,3-, 3,4-, and 2,5-DCNB, and to dechlorinate 2,3- and 3,4-DCA into 3-MCA, and 3-MCA and 4-MCA, respectively.

Figure 37 - Overview of biotransformations and microbial community observed in the anaerobic microcosms



Source: author (2022)

Figure 38 - Phylogenetic tree of organisms found in anaerobic microcosms and organisms from literature



The two ASV affiliated to the *Dehalobacter* genus found in the microcosms, ASV1 in MC23DCA-T2 and MC34DCA-T2 and ASV2 in MC34DCNB-T2, are compared to the *Dehalobacter restrictus* DSM 9455 (CP007033), the *Dehalobacter* sp. CF (CP003870), and the *Dehalobacter* sp. DCA (CP003869). The three strains brought from literature were related to dehalogenation. The ASV1 was 97.1% identical to all of them, while the ASV2 was 100%. The only ASV of *Anaeromyxobacter* found in MC34DCNB was compared to two strains of *Anaeromyxobacter dehalogenans*, being 99.57% identical to the 2CP-P (CP000251) and 99.36% to the 2CP-1 (CP001359). Tree was made with the function MUSCLE alignment and Geneious Tree Builder (default algorithms) on Geneious 8.1.9. Matrix is available in Figure 68B2 of Appendix B2.
Source: author (2022)

Despite the activity found in the microcosms so far, the dechlorination of monochloroaniline isomers was not observed. Literature on the dechlorination of MCA isomers is still scarce. The first report found on dechlorination of monochloroaniline isomers described the dechlorination of 2,3-DCA into 3-MCA and then to aniline through reductive dehalogenation (SUSARLA *et al.*, 1997). After that, dechlorination of 2-MCA and 4-MCA was observed under sulphate-reducing conditions, again forming aniline (SUSARLA; YONEZAWA; MASUNAGA, S., 1998). However, the authors of those papers did not specify the microbes responsible for that.

More recently, 2-MCA was dechlorinated into aniline by a community composed of organisms from *Oscillospira*, *Lactobacillales*, *Veillonellaceae*, and *Ruminococcus*, with the help of 2-aminoanthraquinone-graphene oxide as electron mediator to enhance the reaction, with

pyruvate as donor (LU *et al.*, 2019). As an isolated microbe, the *Geobacter* sp. KT5 was able to dehalogenate 2-MCA, 3-MCA, and 4-MCA, forming aniline and 4-aminobenzoic acid under iron-reducing conditions (DUC; OANH, 2019).

In the case considered in the present study, the MCA are exposed to a microbial community working with no supply of an electron mediator, the opposite conditions that were reported so far. Although there were available electrons for that reaction, the dechlorinator organisms from *Dehalobacter* and *Anaeromyxobacter* genera might not have found favourable conditions to perform MCA dehalogenation.

The Gibbs free energy (ΔG_o') for the reductive dehalogenation of 2,3- and 3,4-DCA into the respective MCA are in the range of -135.0 KJ and -137.7 KJ, depending on the MCA formed, while the values for the dehalogenation of 2-MCA, 3-MCA, and 4-MCA into aniline increase to between -122.7 KJ and -126.8 KJ (SUSARLA *et al.*, 1997). Based on that, in a microbial community with both DCA and MCA available, the dehalogenation of DCA will predominate. The aniline was reported to be oxidized under sulphate-reducing, nitrate-reducing or methanogenic conditions (KAHNG; KUKOR; OH, 2000; SCHNELL; BAK; PFENNIG, 1989; SCHNELL; SCHINK, 1991b; SUN, W *et al.*, 2015), as mentioned before. In this study, we did not focus on that.

4.2.5 Learnings from anaerobic tests

The findings presented here demonstrated that the DCNB and DCA isomers can be biotransformed into other compounds by native organisms found in the soil and groundwater from the contaminated site, indicating the expected pathway that might be happening under anaerobic conditions at the site, where natural attenuation is taking place. The most active microcosm was amended with 3,4-dichloronitrobenzene (DCNB) and was able to reduce the nitro group and dechlorinate the by-product 3,4-dichloroaniline (DCA), forming 3- and 4-monochloroaniline (MCA), with the 3-MCA prevailing in all microcosms amended with 3,4-DCA or 3,4-DCNB. The other microcosms were able to reduce the 2,5-DCNB and the 2,3-DCNB into the 2,5- and the 2,3-DCA, respectively. The 2,3-DCA was dehalogenated, forming 3-MCA. Some important aspects for satisfactory activity were the pH in the range of 6.5 and 7.1, and the electron donor availability for nitro reduction. Active microbial community is composed of fermentative bacteria, OHRB,

SRB and organisms from the archaea domain; however, despite the well-established syntrophic community, monochloroaniline isomers and aniline were more resistant to biodegradation.

Results suggest some microbes can be used as biomarkers for the bioremediation projects that are being implemented by the industry, as the *Dehalobacter* and the *Anaeromyxobacter* are involved in the dechlorination of the DCA isomers. As biomarkers, enzymatic studies of these genera's activity can be performed in the future. The understanding of the nitro reduction step needs to be improved, but a first hypothesis is that nitro group reduction may be catalyzed by *Desulfitobacterium*.

Although many questions were raised by this first insight on the anaerobic biotransformations of the target contaminants, the data explored here will be useful not only to predict new contaminants in the soil and groundwater, but to start considering strategies that consider anaerobic and aerobic mechanisms to decontaminate the site. In this context, injection of O_2 may be needed in areas where microbes biotransform the DCNB isomers into DCA and then to the respective MCA isomers.

4.3 LEARNINGS AND SHARING EXPERIENCES

The last specific objective of this project was to share learnings and experiences and to link the findings to the strategies implemented in the industry, aiming to contribute to a successful project.

In general, from the lab experiments, we can affirm that bioremediation with native microbes from the site is a promising strategy to be applied in the contaminated area. Bioaugmentation seems not to be necessary, but biostimulation can be considered since the natural attenuation was not able to completely dechlorinate and mineralize the contaminants. Based on previous findings from the Bioteam work and the research presented in this thesis, oxygen is already being injected into the soil and groundwater, as a biosparging technique. The strategy has been successful in mineralizing the halogenated and nitro aromatic compounds.

The findings from this research have been extensively discussed by the Bioteam, mainly by the UofT, UFPE, and UWF professors and students. In addition to the monthly meetings with the Bioteam, during this Ph.D. journey, countless meetings were realized when needed to discuss any new topics or results. The "on-demand" activities were mainly designed during these meetings.

The pre-experiments performed at the site were also designed by demand. The partnership between the industry and UFPE was consolidated with the beginning of the pre-experiments, all performed at the industrial site, between September 2017 and October 2019. The pre-experiments are all included in Appendix C.

In addition to the experiments presented here, we have been working collaboratively with other Bioteam projects. At the UofT, under the supervision of the professor Dr. Elodie Passport, Compound Specific Isotope Analysis (CSIA) research has been performed by Dr. Suchana Shamsunnahar and student Shuping Wang using the aerobic and the anaerobic enriched cultures mentioned in this document.

Recently, the lab team working at the site started to perform qPCR analysis with samples from the site, collected from the biosparging pilot. At the beginning of 2022, I was trained by Line Lomheim on this analysis at the UofT and since then I have been helping the lab team with the implementation of this new technique.

Apart from the "take-home" messages, we have raised some "take to the lab" messages. When crossing results from the field with results from the lab, we identify gaps that need to be solved. The 2,3-DCNB and MCA isomers seem to be persistent in the soil at the site, where the natural attenuation is monitored. Similar results were obtained from the anaerobic microcosms amended with 2,3-DCNB, which showed slower reduction rate to 2,3-DCA compared to other microcosms amended with 3,4- and 2,5-DCNB, and from microcosms accumulating MCA isomers. Experiments on that can be designed to investigate which limiting factors are lacking in the lab experiments and in the field, aiming to find out the favourable conditions to mineralize the persistent contaminants under anaerobic conditions. On the other hand, the mineralization of contaminants when O_2 is injected into the soil has been achieved by the biosparging pilot and observed in our microcosms.

Regarding the limitations of our study, it is important to mention that using microcosms to evaluate the biodegradability of contaminants has also disadvantages. Although setting up microcosms is helpful to analyze certain reactions and provide insights about the microbes involved in them, enriching the culture under a specific condition might not represent all the dynamics that occur simultaneously in a complex environmental media.

The initial microcosms from our experiments might be the closest figure we had from the field, as the soil and groundwater contained in them were less diluted and modified, in terms of

chemical and biological composition. The maintenance procedures applied to the microcosms – transfers and mineral medium added (dilutions of the inoculum) and successive feedings with target contaminants and addition of electron donors - moved the studied condition away from the real field conditions, while providing answers to specific processes we are interested in (the biotic transformations mediated by the enriched organisms from the field).

Considering the reduction of nitro group, for example, in our microcosms, this biotic reaction was driven by native organisms responsible for the dichloroaniline formation using ethanol and lactate as electron sources. In the field, however, if the DCNB isomers are removed, this is possibly enhanced by other organic or mineral sources of electrons, through biotic or even abiotic reactions.

Some natural organic matter (NOM) in their reduced state can donate electrons to the nitroaromatic compounds through abiotic reactions, forming the corresponding amines (MENEZES *et al.*, 2022; MURILLO-GELVEZ *et al.*, 2021; NIEDŹWIECKA *et al.*, 2017; NIEDŹWIECKA; MCGEE; FINNERAN, 2020; RATASUK; NANNY, 2007; UCHIMIYA; STONE, 2009). Similarly, naturally occurring iron-containing minerals, such as mackinawite, goethite, sulphate green rust, and hematite, have been reported as electron mediators for the abiotic reduction of a nitro group from aromatic compounds (CÁRDENAS-HERNÁNDEZ *et al.*, 2020; CHENG, J.; SUIDAN; VENOSA, 1996; ELSNER; SCHWARZENBACH; HADERLEIN, 2004; KHATIWADA *et al.*, 2018; KLUPINSKI, 2004; MENEZES *et al.*, 2021; NIEDŹWIECKA *et al.*, 2017; NIEDŹWIECKA; MCGEE; FINNERAN, 2020).

At the beginning of our lab experiments, reactions between organic matter and our target contaminants were likely to occur as the microcosms contained groundwater and soil as inoculum (and probably NOM and minerals). In the abiotic control, without inoculum, we have seen some reactions between the DCNB and the precipitated FeS (from the mineral medium). Even though we consider that abiotic reactions may occur in the field, there were no further efforts to check those reactions during the experimental period.

Given that, further investigation with the data from the field would provide a better understanding of the fate of the compounds in the subsurface of the site. Organisms can reduce the nitro group from the DCNB isomers, at the same time that abiotic reactions might happen, all leading to the removal of the compound and promoting natural attenuation.

Additionally, regarding the dichloroaniline compound, an increase in its concentration in groundwater samples can be a consequence of the activity of the organisms and/or the abiotic reactions, as well as caused by the original contamination, considering the groundwater flow and the release of contaminants by the soil highly contaminated. Thus, new tests with fresh samples from the field can be planned and carried out aiming to verify the real processes occurring in the field.

Overall, based on what it was observed considering findings obtained in this study, both strategies aerobic and anaerobic seem to be valuable for bioremediation. The understanding of how the reactions occur under these two conditions can mean lower costs and higher effectiveness for the project. As future studies, enzymatic essays can help monitor the bioremediation through genes and expressed enzymes involved in the biotic processes. All cultures are preserved in glycerol, under -80^{o}C, at the University of Toronto, available for next studies. The active anaerobic ones were scaled up to 200 mL in 2L bottles and are being maintained by Line Lomheim. As a written document, the data discussed in this thesis will be published as open access papers.

5 FINAL CONSIDERATIONS

Our research provided some initial insights on the biodegradation/biotransformation of dichloronitrobenzene (DCNB), dichloroaniline (DCA) and monochloroaniline (MCA) isomers through aerobic and anaerobic reactions led by native bacteria from a contaminated site in Brazil.

Through the enrichment cultures inoculated with soil and groundwater from the site and available at the UofT we could evaluate biodegradability of the compounds (first specific objective), verify aerobic mineralization of DCA and MCA isomers and evaluate some steps of the anaerobic biotransformation of DCNB and DCA isomers (second specific objective) and suggest possible microbes responsible for the biotransformations of contaminants (third specific objective).

Under aerobic conditions, the 2,3-DCA, 3,4-DCA, 2-MCA, 3-MCA, and 4-MCA were mineralized by organisms from the *Pandoraea* (1 ASV) and *Burkholderia-Caballero-Paraburkholderia* (1 ASV) genera, while chloride and ammonium ions were released. Future studies can determine which intermediates are formed during biodegradation (isomers of dichlorocatechol, chlorocatechol, or catechol) through enzymatic essays. Microbes can be isolated, complete genomes sequenced and expressed genes will be useful for the aerobic projects performed by the industry.

The anaerobic experiments revealed the nitro reduction of the 2,3-, 3,4-, and 2,5-DCNB possibly by fermentative bacteria through a co-metabolic process, forming DCA isomers. The *Desulfitobacterium* is another candidate for nitro reduction, however, this step needs to be better investigated based on the coming results. The 2,3- and 3,4-DCA were reductively dehalogenated by organohalide-respiring bacteria from the *Dehalobacter* (2 ASV) and *Anaeromyxobacter* (1 ASV) genera. The *Desulfovibrio* is reported as facultatively respire chlorinated compounds, but in this study, this genus was more likely to be involved in sulphate reduction. The well-established and active anaerobic community is composed of fermentative bacteria, organohalide-respiring bacteria, sulphate-reducing bacteria, and organisms from the archaea domain, with a pH in the range of 6.5 and 7.1, and electron donor availability (ethanol and lactate were the donor sources added). The 2,5-DCA, the MCA isomers, and aniline accumulated, with no further biotransformation.

Considering the applicability of the findings, the results suggested that aerobic biodegradation of the chloroanilines (monochloroaniline and dichloroaniline isomers) by microbes

from the site might be happening, meaning that natural attenuation is likely to occur when oxygen is available. Bioaugmentation might be interesting where those native microbes are not present. In addition, findings indicated that anaerobic biotransformation of dichloronitrobenzene and dichloroaniline can occur driven by anaerobic organisms, but accumulation of monochloroanilines and aniline is relevant in regions where oxygen is not available.

Although the anaerobic process did not show complete transformation of the contaminants to harmless products, its products (DCA and MCA isomers) are proven to be degraded aerobically. Thus, biostimulation strategy through oxygen injection to mineralize those compounds is a promising strategy.

In addition to help interpret the data collected from the field, based on the findings and the discussions we continually have with the Bioteam regarding the demands for the bioremediation projects (last specific objective), we propose as future studies:

- Describe the mechanisms by which the nitro group is reduced into the amino group by the anaerobic community enriched and amended with DCNB isomers;
- Evaluate the anaerobic biodegradability of the 2,3-DCNB, the MCA isomers and aniline, aiming to achieve consistent biotransformation and hopefully mineralization;
- Develop biomarkers based on expressed genes from aerobic microbes and anaerobic cultures;
- Isolate the aerobic cultures;
- Perform enzymatic essays to describe intermediates.

The enriched cultures are available for future works at the UofT. The aerobic cultures are inactive, but samples are preserved in glycerol, under -80^{o}C. For future studies with these microbes, it is recommended to better control the oxygen content in the bottle and to replan the feeding routine and frequency, as the cultures were amended once a week during the experimental period presented in this document. Samples from the anaerobic cultures are also preserved in the same way, but microcosms are still being maintained by Line Lomheim. For them, the addition of ammonium in the medium is something to be considered. We decided not to add the compound expecting to see the ammonium being released from the contaminants, becoming available as N source for the cultures. However, the ammonium was not released. The lack of nitrogen source might have hindered the cultures to perform faster or more consistent biotransformations.

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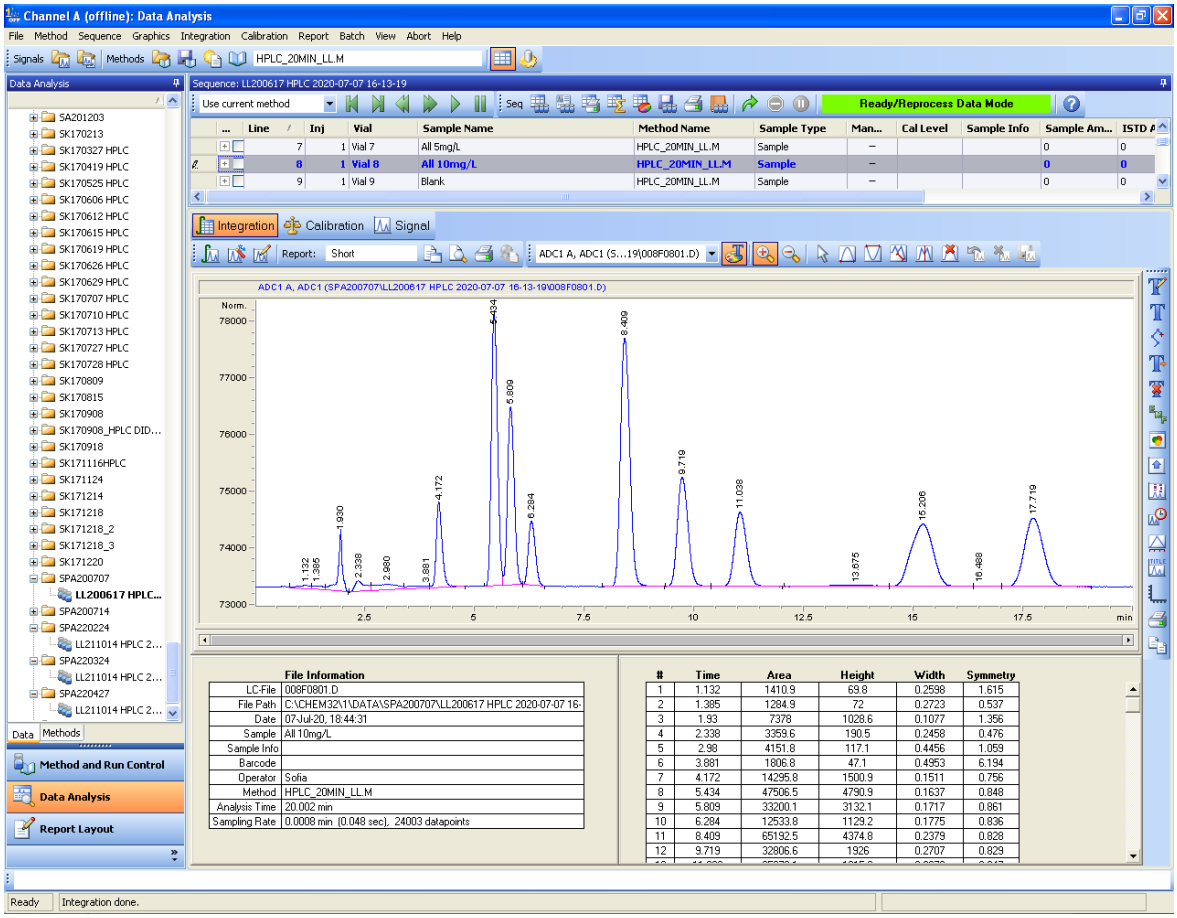
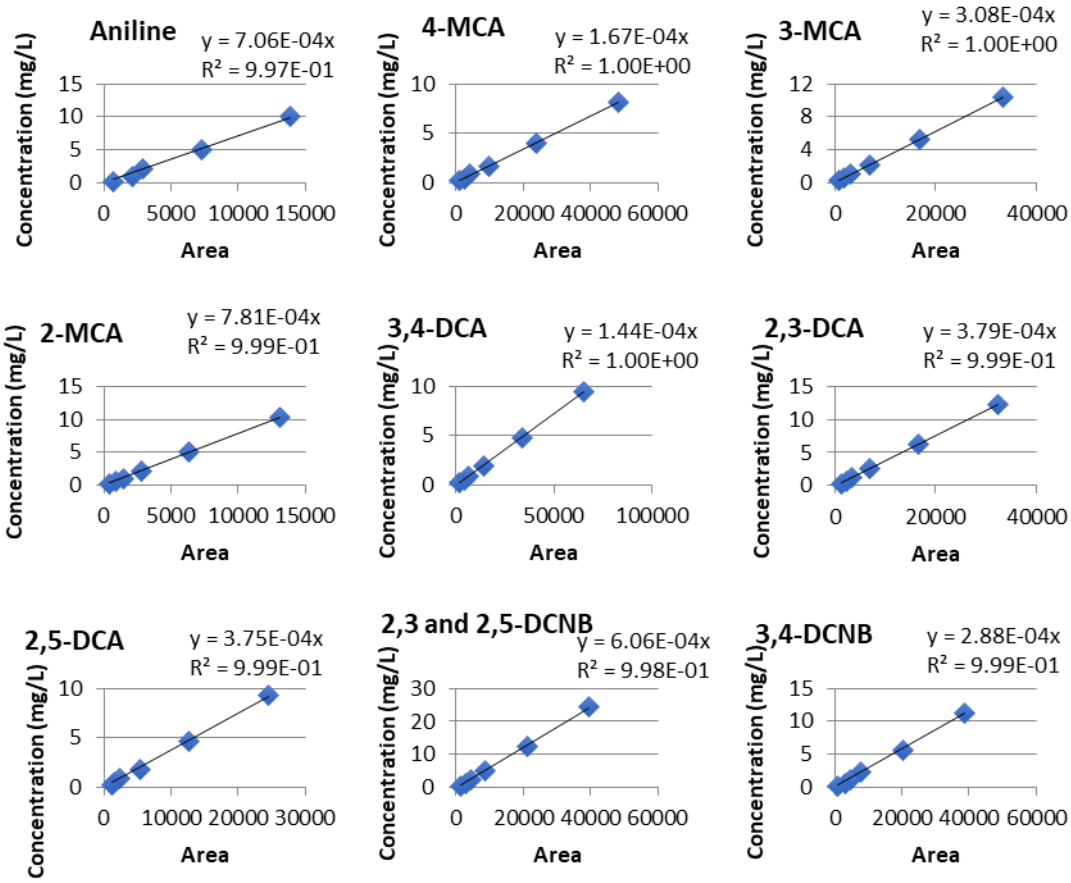
APPENDIX A - SUPPLEMENTARY INFORMATION FOR METHODOLOGY (SECTION 3)

Figure 39A - Example of a chromatogram from the HPLC (standard 10 mg/L)



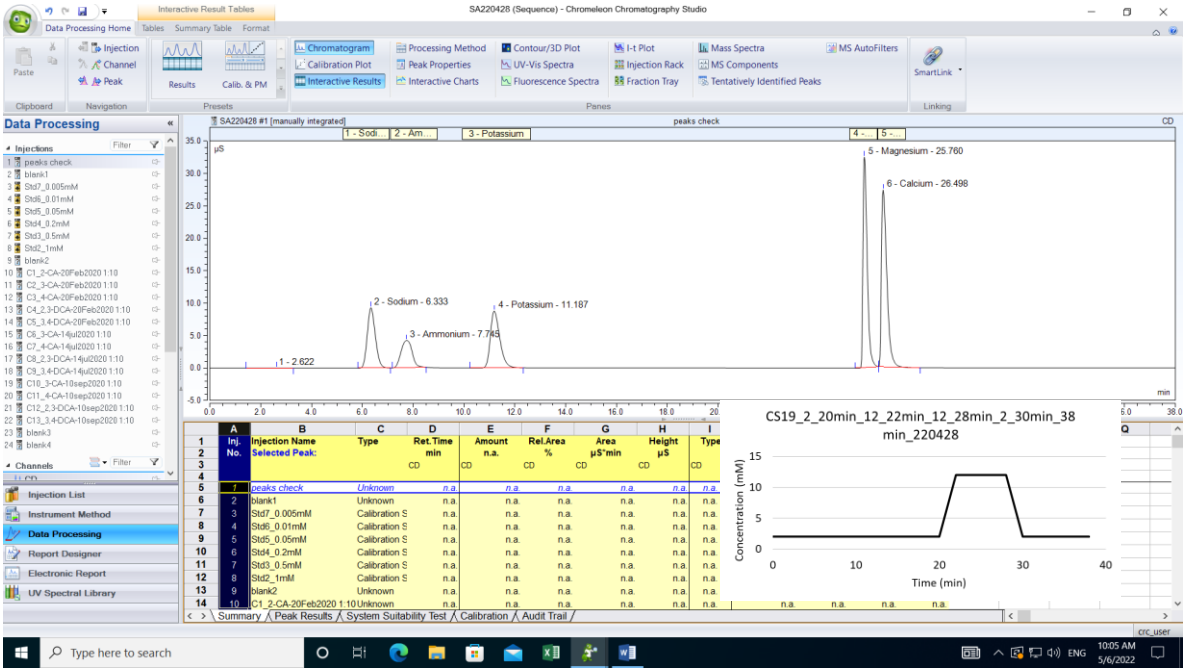
Source: author (2022)

Figure 40A - Calibration curves of HPLC run (21st July 2020)



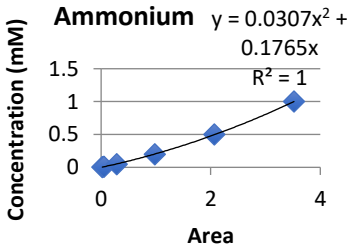
Source: author (2022)

Figure 41A - Example of a chromatogram from the IC-Cation method (standard 0.2 mM) applied for the aerobic experiments



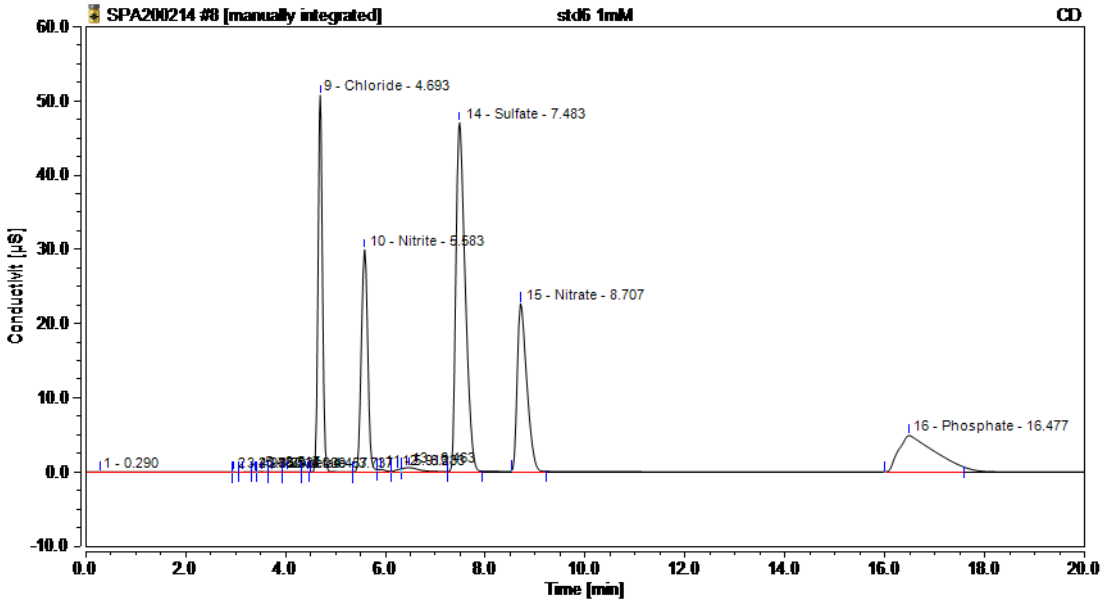
Source: author (2022)

Figure 42A - Calibration curve for ammonium measured in the IC-Cation (28th Apr 2022) applied for the aerobic experiment



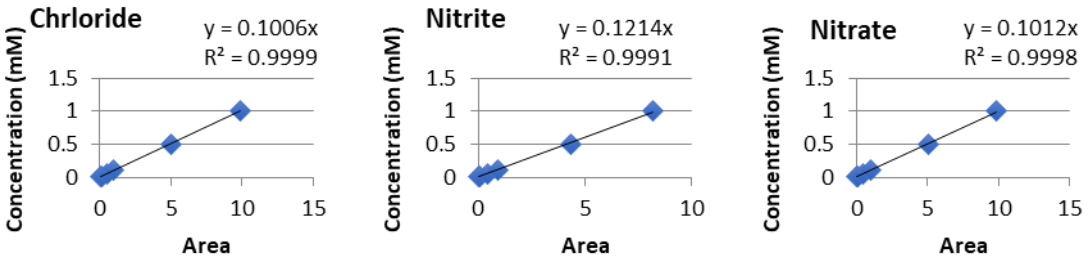
Source: author (2022)

Figure 43A - Example of a chromatogram from the IC-Anion isocratic method (23 mM for 20 min) applied for the aerobic experiment (standard 1 mM)



Source: author (2022)

Figure 44A - Calibration curve for chloride, nitrite and nitrate measured in the IC-Anion (27th Nov 2020) applied for the aerobic experiment



Source: author (2022)

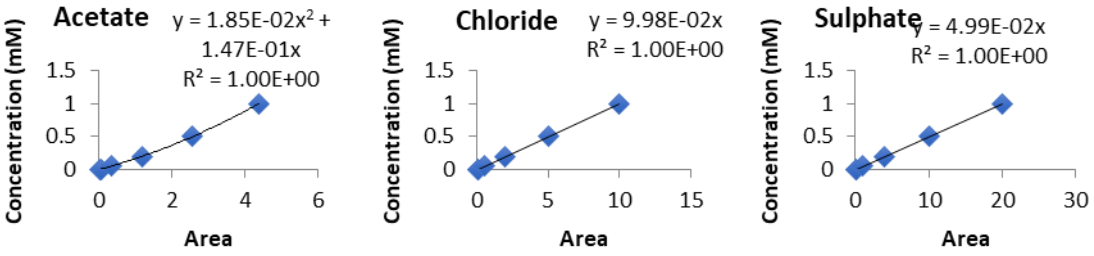
Figure 45A - Example of a chromatogram from the IC-Anion isocratic method (23 mM for 20 min) applied for the anaerobic experiment (standard 1 mM)

Source: author (2022)

Figure 46A - Example of a chromatogram from the IC-Anion isocratic method (23 mM for 20 min) applied for the anaerobic experiment showing the lactate peak

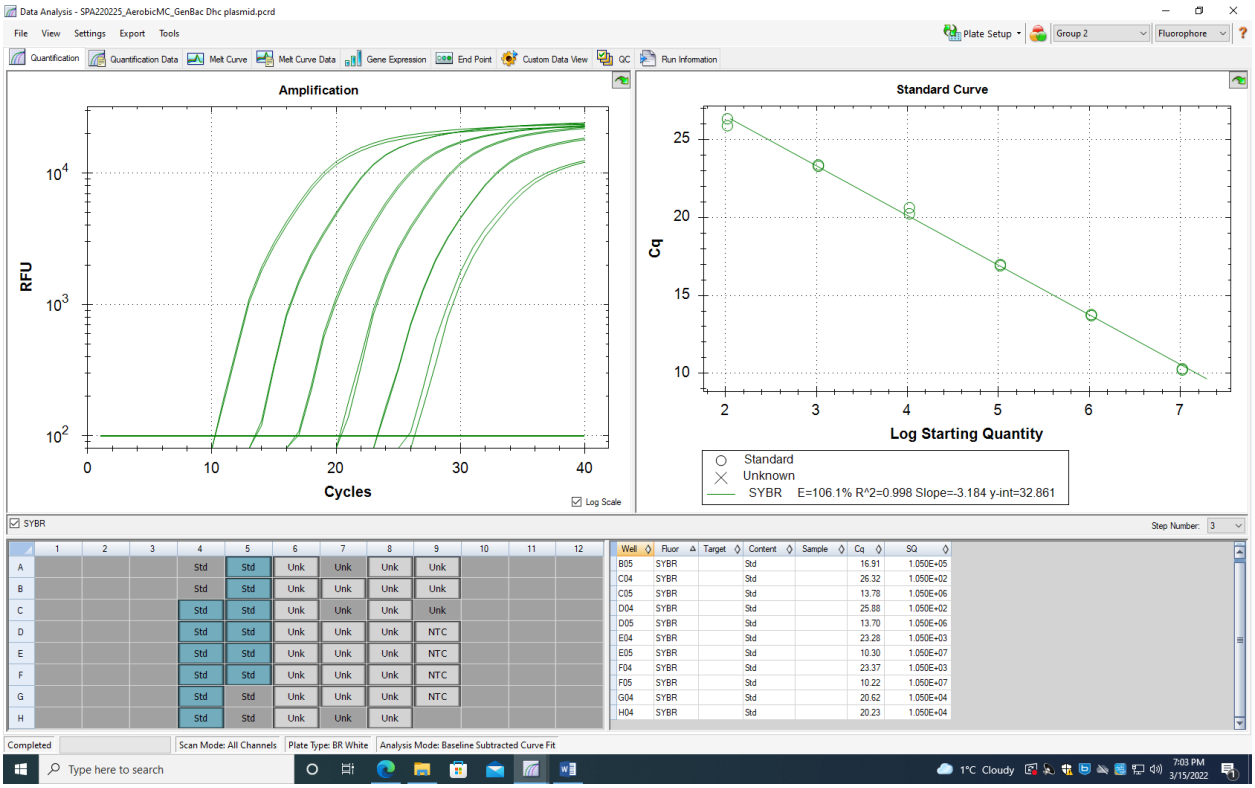
Source: author (2022)

Figure 47A - Calibration curves for acetate, chloride, and sulphate (29th Apr 2022) applied for the anaerobic experiment



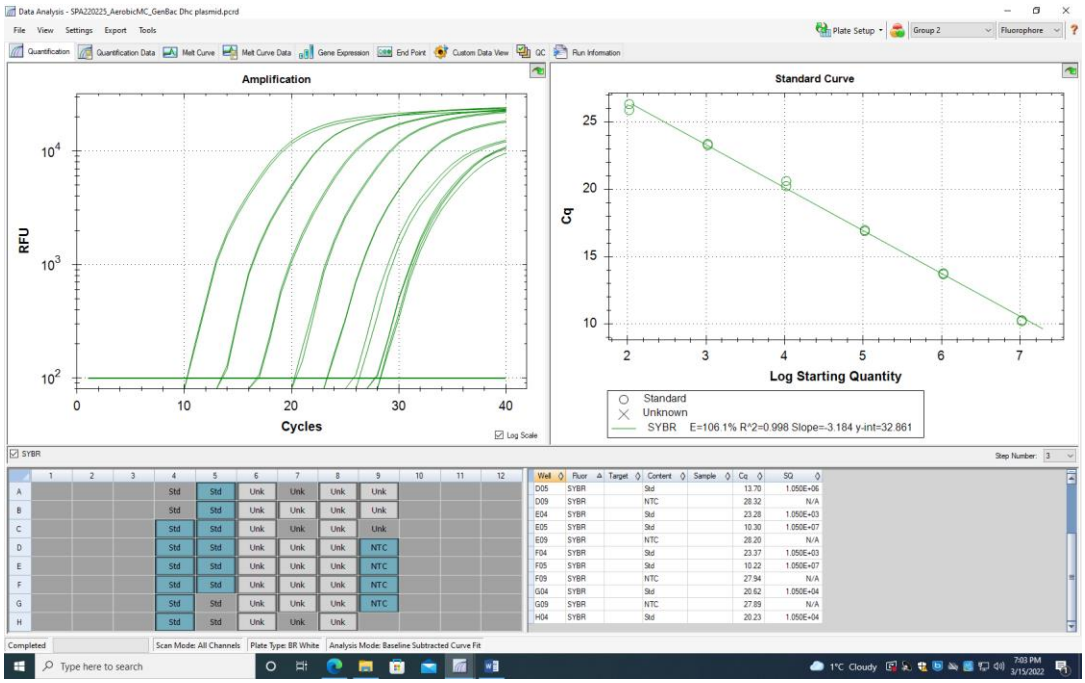
Source: author (2022)

Figure 48A - Amplification curves of plasmid standards (Std) from plate 1 (aerobic experiment)



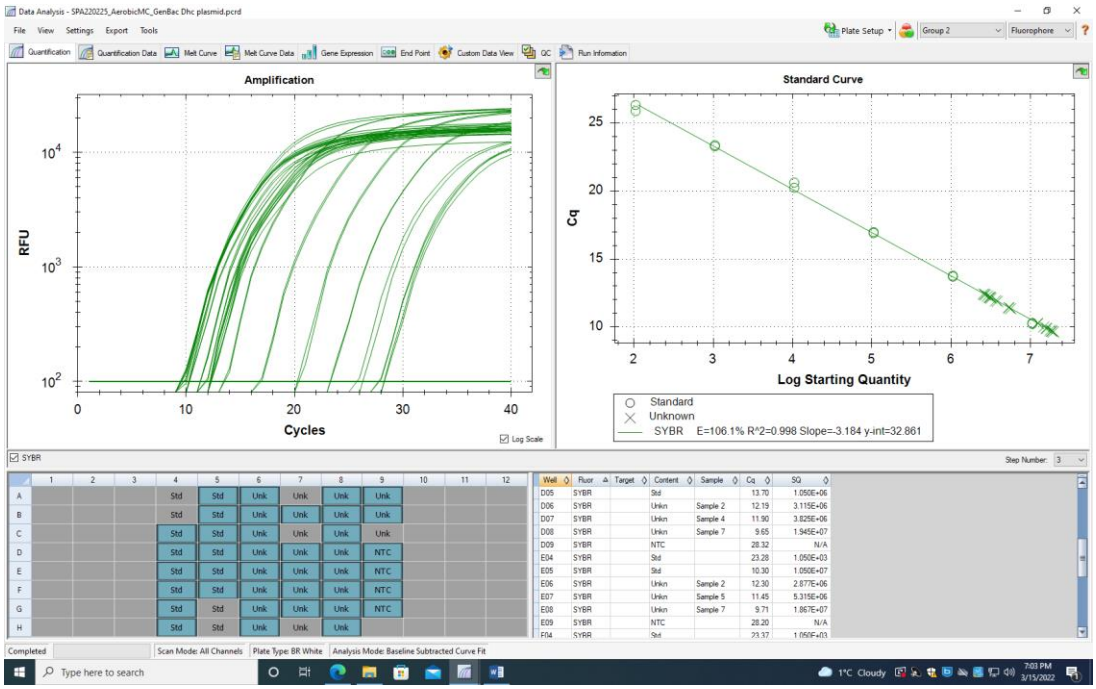
Source: author (2022)

Figure 49A - Amplification curves of plasmid standards (Std) and blanks (NTC) from plate 1 (aerobic experiment)



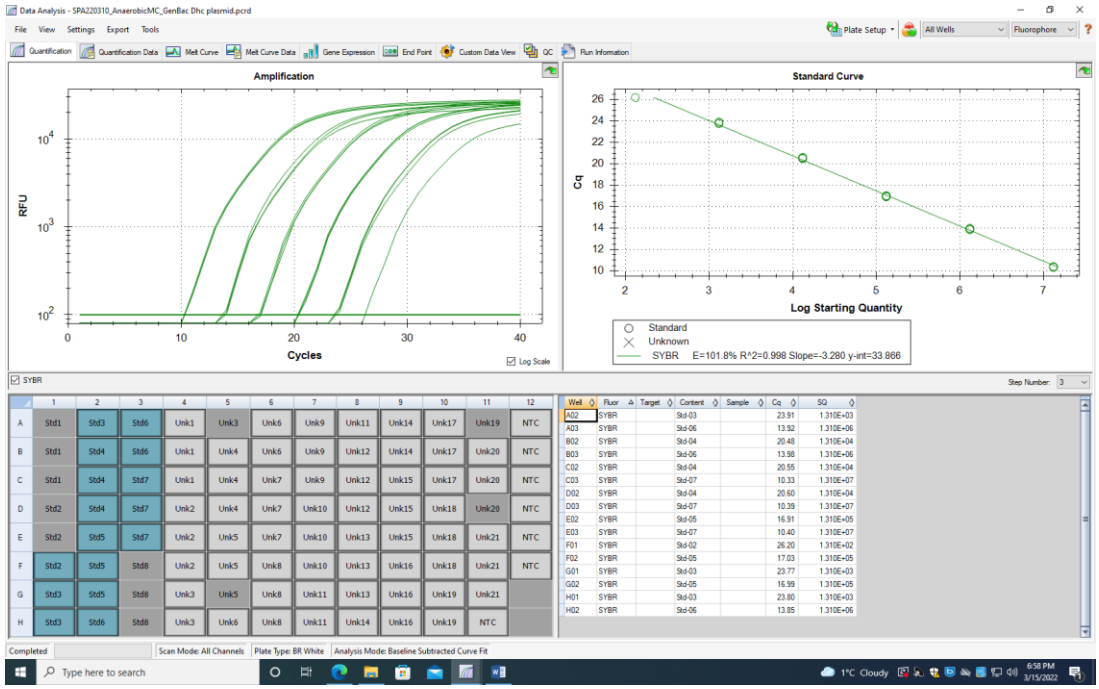
Source: author (2022)

Figure 50A - Amplification curves of plasmid standards (Std), blanks (NTC), and samples (Unk) from plate 1 (aerobic experiment)



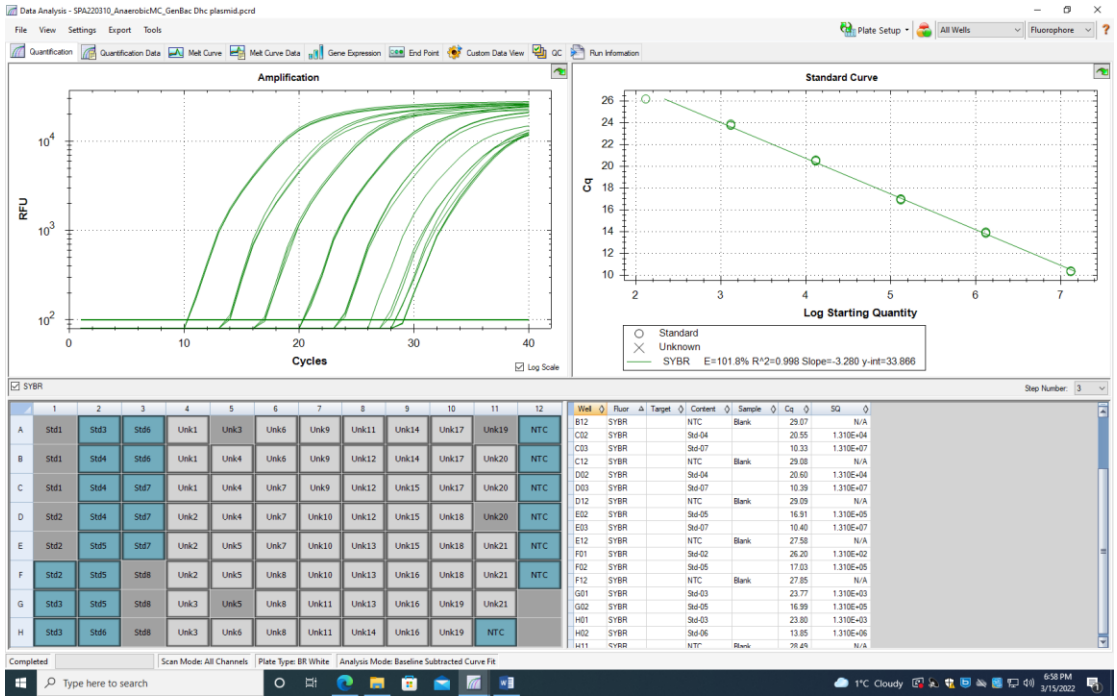
Source: author (2022)

Figure 51A - Amplification curves of plasmid standards (Std) from plate 2 (anaerobic experiment)



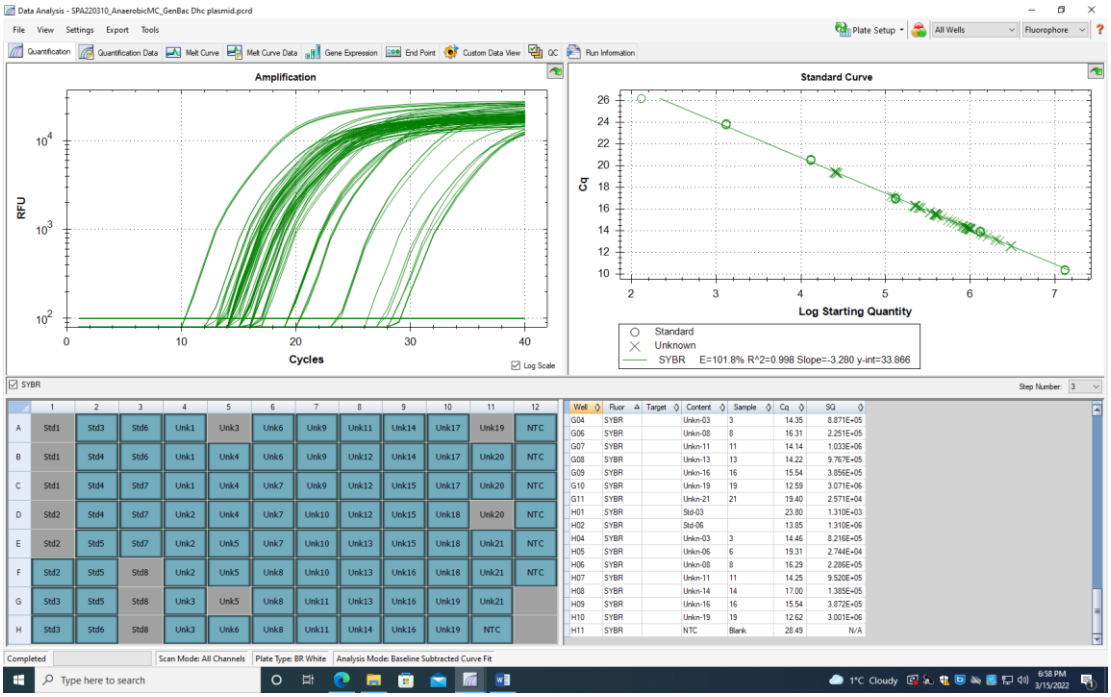
Source: author (2022)

Figure 52A - Amplification curves of plasmid standards (Std) and blanks (NTC) from plate 2 (anaerobic experiment)



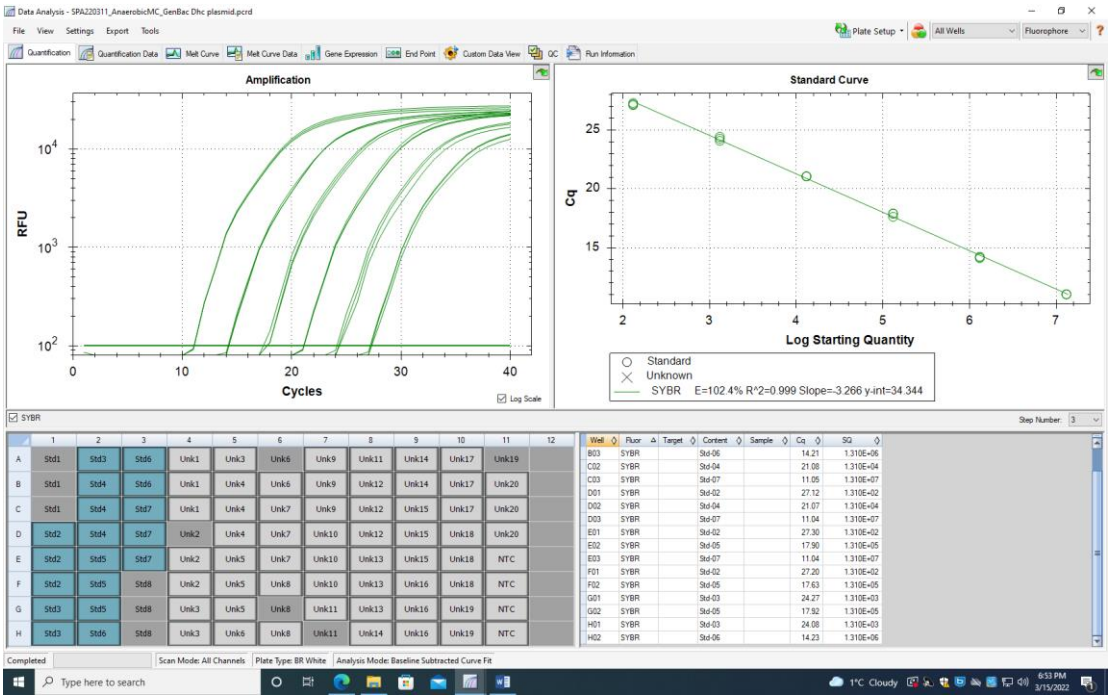
Source: author (2022)

Figure 53A - Amplification curves of plasmid standards (Std), blanks (NTC), and samples (Unk) from plate 2 (anaerobic experiment)



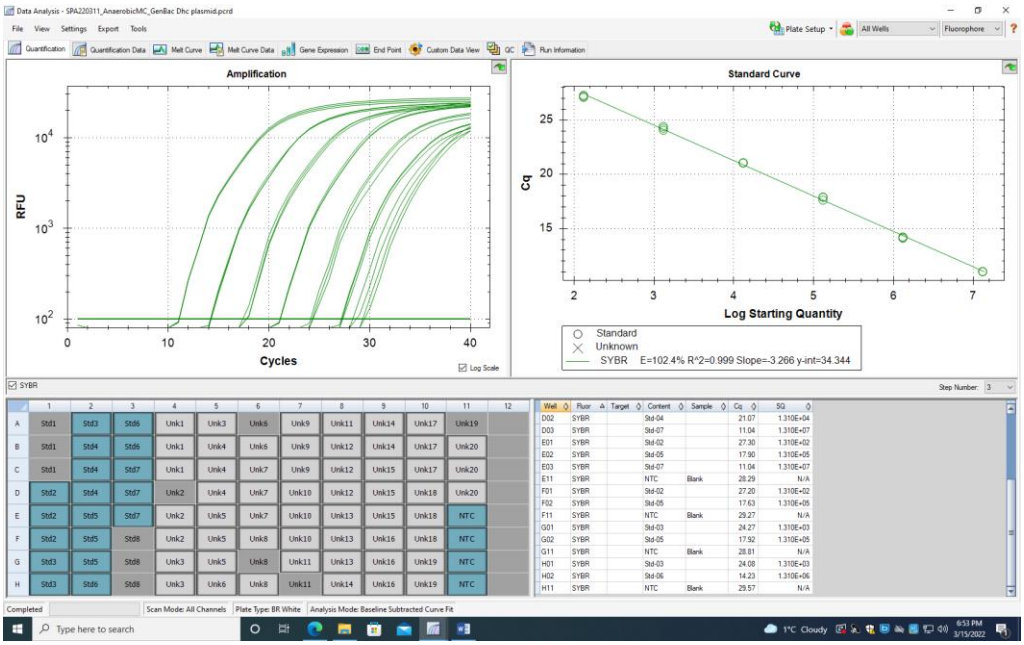
Source: author (2022)

Figure 54A - Amplification curves of plasmid standards (Std) from plate 3 (anaerobic experiment)



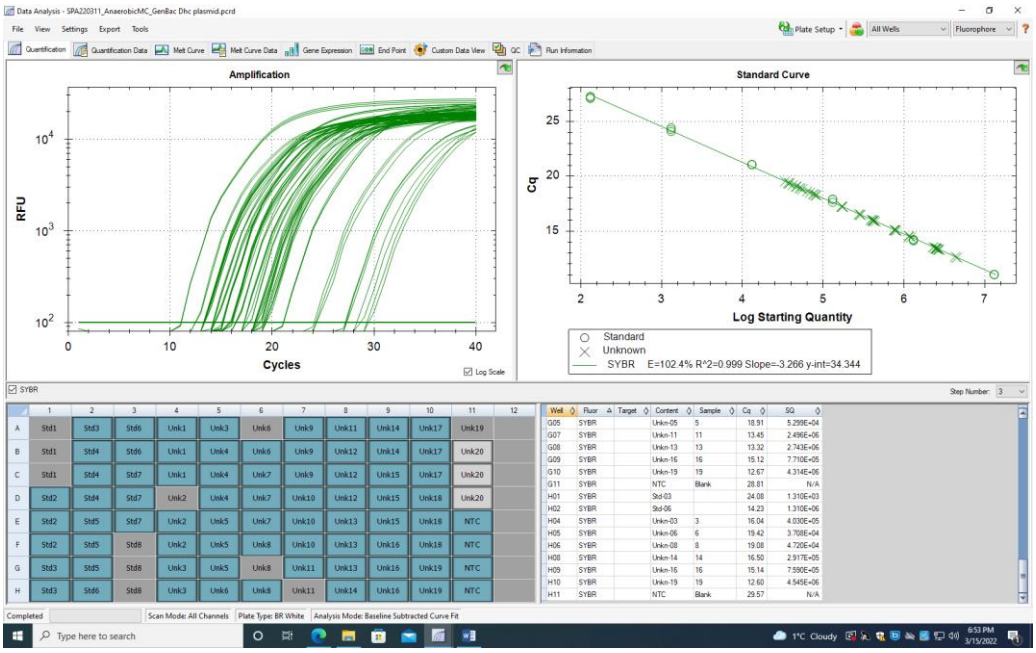
Source: author (2022)

Figure 55A - Amplification curves of plasmid standards (Std) and blanks (NTC) from plate 3 (anaerobic experiment)



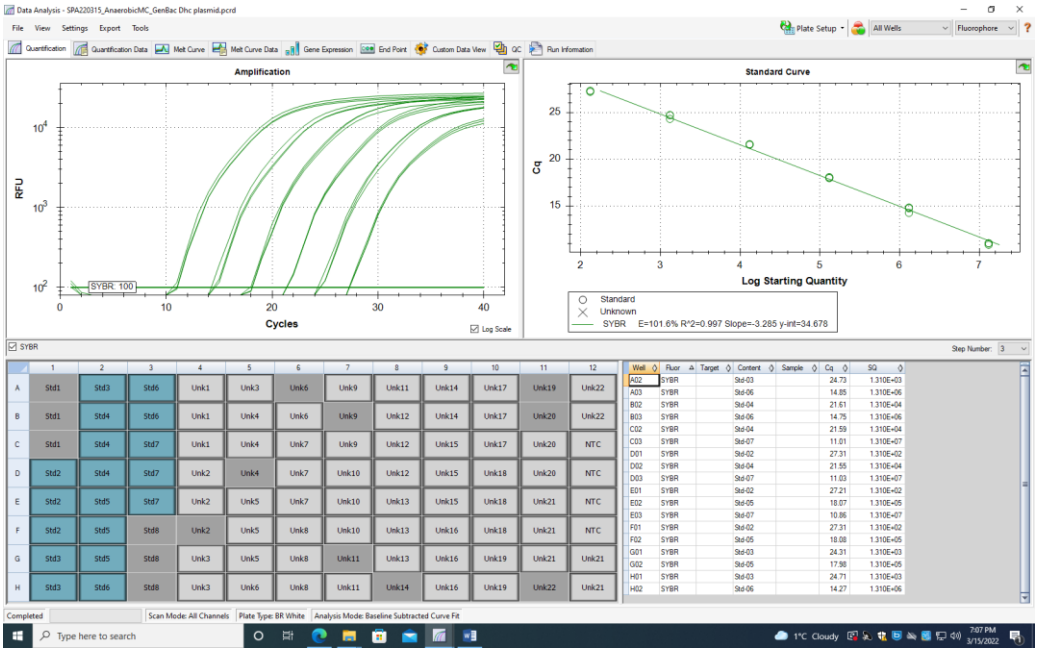
Source: author (2022)

Figure 56A – Amplification curves of plasmid standards (Std), blanks (NTC), and samples (Unk) from plate 3 (anaerobic experiment)



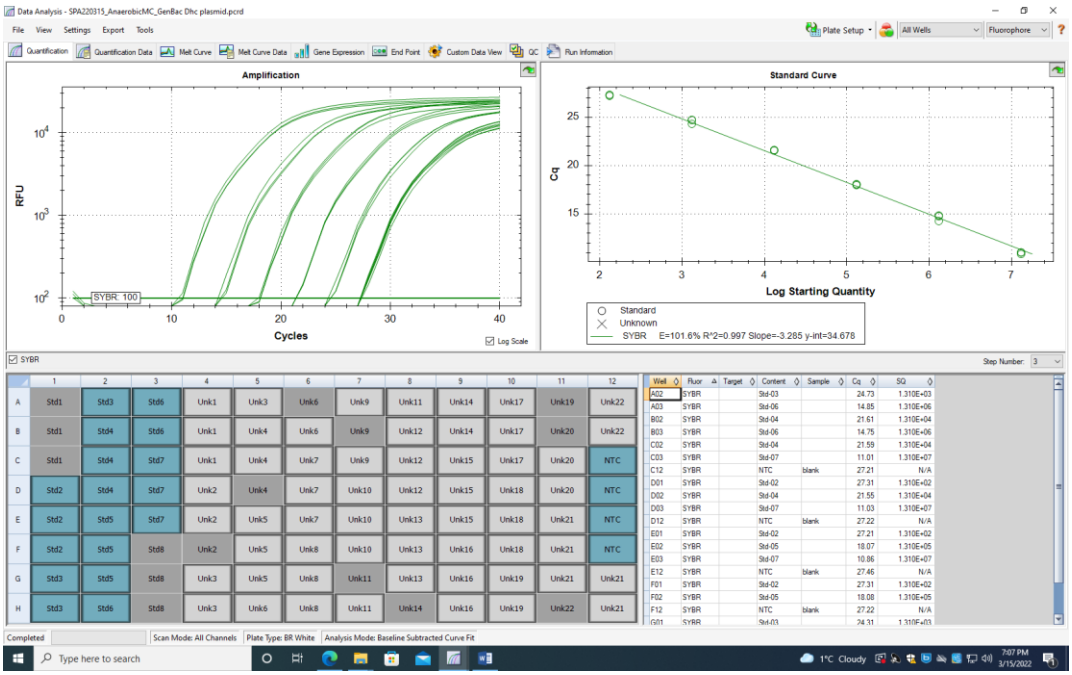
Source: author (2022)

Figure 57A – Amplification curves of plasmid standards (Std) from plate 4 (anaerobic experiment)



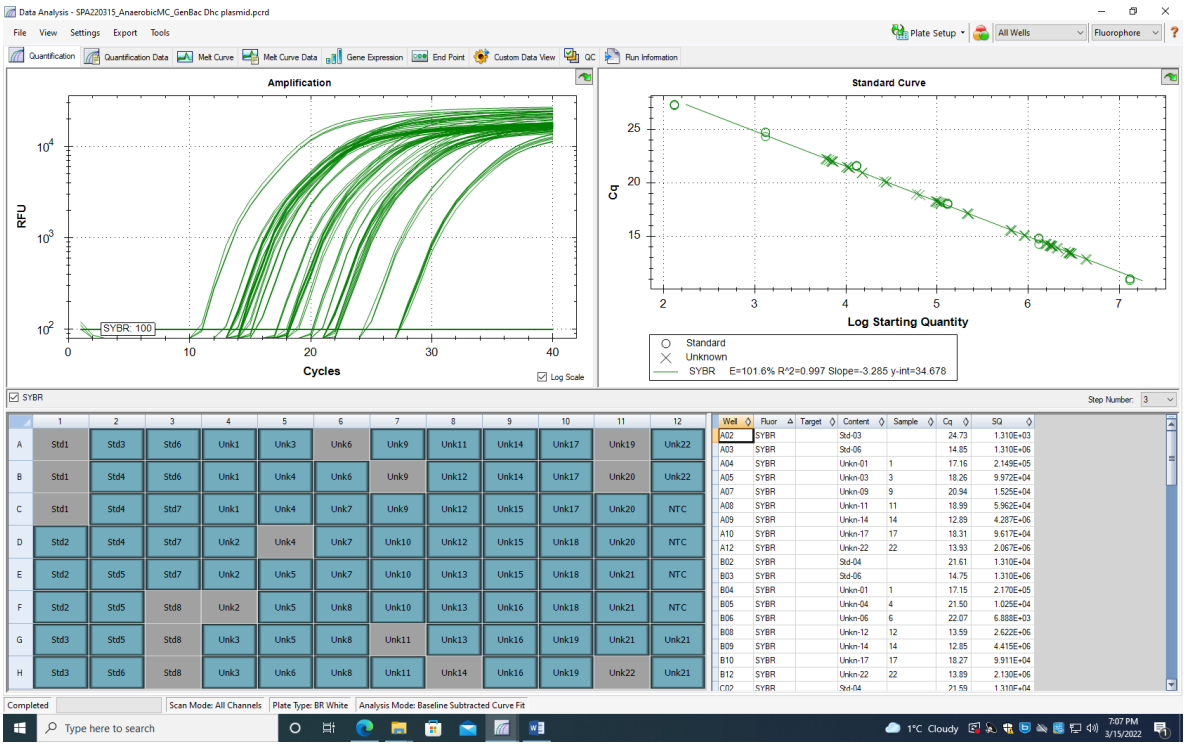
Source: author (2022)

Figure 58A - Amplification curves of plasmid standards (Std) and blanks (NTC) from plate 4 (anaerobic experiment)



Source: author (2022)

Figure 59A - Amplification curves of plasmid standards (Std), blanks (NTC), and samples (Unk) from plate 4 (anaerobic experiment)



Source: author (2022)

APPENDIX B - SUPPLEMENTARY INFORMATION FOR ANALYSIS AND DISCUSSION OF RESULTS (SECTION 4)

B1. SUPPLEMENTARY INFORMATION - AEROBIC DEGRADATION OF CONTAMINANTS

Equation 3 for 3-MCA: $1.00C_6H_6ClN + 4.81O_2$

$\rightarrow 4.31CO_2 + 0.66NH_4^+ + 1.00Cl^- + 0.33H_2O + 0.34C_5H_7O_2N + 0.34H^+$ (3)

Equation 4 for 4-MCA: $1.00C_6H_6ClN + 4.82O_2$

$\rightarrow 4.32CO_2 + 0.66NH_4^+ + 1.00Cl^- + 0.33H_2O + 0.34C_5H_7O_2N + 0.34H^+$ (4)

Equation 5 for 3,4-DCA: $1.00C_6H_5Cl_2N + 4.16O_2 + 0.74H_2O$

$\rightarrow 4.16CO_2 + 0.63NH_4^+ + 2.00Cl^- + 0.37C_5H_7O_2N + 1.37H^+$ (5)

Table 8B1 - Concentrations of cumulative chloride amended and measured on days 0, 17, 70 and 129 in aerobic microcosms

Microcosms	Days	Cumulative COI amended (µM)	Cumulative Cl^- amended (µM)	Cl^- measured (µM)	Difference of measured Cl^- (µM)
MC2MCA	0	0	0	3235.64	
	17	705.49	705.49	4021.76	786.12
MC3MCA	0	0.00	0.00	3286.01	
	17	705.49	705.49	4040.34	754.33
	70	0.00	0.00	4256.41	
	129	1724.52	1724.52	6539.16	2282.75
MC4MCA	0	0.00	0.00	3269.67	
	17	705.49	705.49	4000.46	730.79
	70	0.00	0.00	4246.19	
	129	1724.52	1724.52	6722.98	2476.79
MC23DCA	0	0.00	0.00	3259.57	
	17	555.50	1111.00	no data	
	70	0.00	0.00	4330.44	
	129	1234.44	2468.88	7206.49	2876.06
MC34DCA	0	0.00	0.00	3316.67	
	17	555.50	1111.00	4333.28	1016.61
	70	0.00	0.00	4315.32	
	129	1234.44	2468.88	7155.80	2840.48

Days 0 and 70 were the first days of Transfer 1 and Transfer 2; thus, the cumulative concentration of contaminants is 0. Difference of measured Cl^- is the concentration accumulated from day 0 to 17 and from day 70 to 129 (values are presented in Figure 17 of the main text).

Source: author (2022)

ASV found in the communities:

Bosea (genus level) (ASV1):

GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTT
ACCAGCTTTTGACATGTCCGGTTTGATCGGCAGAGATGCCTTTCTTCAGTTCGGCTGG
CCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTCGCCCCTAGTTGCCATCATTCAGTTGGGAACTCTA
GGGGGACTGCCGGTGATAAGCCGCGAGGAAGGTGGGGATGACGTCAAGTCCTCATG
GCCCTTACAGGCTGGGCTACACACGTGCTACAATGGCGGTGACAATGGGCAGCGAA
GGGGCGACCTGGAGCTAATCCCAAAAAGCCGTCTCAGTTCAGATTGTACTCTGCAAC

TCGAGTACATGAAGGTGGAATCGCTAGTAATCGTGGATCAGCATGCCACGGTGAATA
CGTTCCCGGGCCTT

Bosea (genus level) (ASV2):

GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTT
ACCAGCTTTTGACATGTCCGGTTTGATCGGCAGAGATGCCTTTCTTCAGTTCGGCTGG
CCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTCGCCCCTAGTTGCCATCATTCAGTTGGGAACTCTA
GGGGGACTGCCGGTGATAAGCCGCGAGGAAGGTGGGGATGACGTCAAGTCCTCATG
GCCCTTACAGGCTGGGCTACACACGTGCTACAATGGCGGTGACAATGGGCAGCGAA
GGGGTGACCTGGAGCTAATCCCAAAAAGCCGTCTCAGTTCAGATTGTACTCTGCAAC
TCGAGTACATGAAGGTGGAATCGCTAGTAATCGTGGATCAGCATGCCACGGTGAATA
CGTTCCCGGGCCTT

Burkholderia-Caballeronia-Paraburkholderia (genus level) (ASV1):

GGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTT
ACCTACCCTTGACATGGTCGGAATCCTGCTGAGAGGCGGGAGTGCTCGAAAGAGAA
CCGGCGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCTACGCAAGAGCACTCTAAGGAG
ACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTT
ATGGGTAGGGCTTCACACGTCATACAATGGTCGGAACAGAGGGTTGCCAACCCGCG
AGGGGGAGCTAATCCCAGAAAACCGATCGTAGTCCGGATTGCACTCTGCAACTCGA
GTGCATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTT
CCCGGGTCTT

Pandoraea (genus level) (ASV1):

GGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTT
ACCTACCCTTGACATGTACGGAATCCTGCTGAGAGGTGGGAGTGCTCGAAAGAGAA
CCGTAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCTACGCAAGAGCACTCTAAGGAG
ACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTT
ATGGGTAGGGCTTCACACGTCATACAATGGTCGGTACAGAGGGCTGCCAAACCGCG
AGGTGGAGCTAACCCCAGAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGA

CTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGAATACGTT
CCCGGGTCTT

Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium (genus level) (ASV1):

GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTT
ACCAGCTCTTGACATCTGGGTCGCGGACAGTGGAGACATTGTCCTTCAGTTAGGCTG
GACCCAAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTCGCCCTTAGTTGCCAGCATTTGGTTGGGCACTCT
AAGGGGACTGCCGGTGATAAGCCGAGAGGAAGGTGGGGATGACGTCAAGTCCTCAT
GGCCCTTACGGGCTGGGCTACACACGTGCTACAATGGTGGTGACAGTGGGCAGCGA
GACCGCGAGGTCGAGCTAATCTCCAAAAGCCATCTCAGTTCGGATTGCACTCTGCAA
CTCGAGTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAAT
ACGTTCCCGGGCCTT

Rhodanobacteraceae (family level) (ASV1):

GGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTA
CCTGGCCTTGACATGTCCGGAATCCTGCAGAGATGCGGGAGTGCCTTCGGGAATCGG
AACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC
CGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTCTAAG
GAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCC
CTTACGGCCAGGGCTACACACGTACTACAATGGTCGGTACAGAGGGTTGCCATACCG
CGAGGTGGAGCCAATCCCAGAAAGCCGATCCCAGTCCGGATTGGAGTCTGCAACTC
GACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCTATGCCGCGGTGAATAC
GTTCCCGGGCCTT

Rhodanobacteraceae (family level) (ASV2):

GGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTA
CCTGGCCTTGACATGTCCGGAATCCTGCAGAGATGCGGGAGTGCCTTCGGGAATCGG
AACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC
CGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCGAGTAATGTCGGGAACTCTAAG
GAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCC
CTTACGGCCAGGGCTACACACGTACTACAATGGTCGGTACAGAGGGTTGCGATACCG
CGAGGTGGAGCCAATCCCAGAAAGCCGATCCCAGTCCGGATTGGAGTCTGCAACTC

GACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCTATGCCGCGGTGAATAC
GTTCCCGGGCCTT

Table 9B1 - Bacterial growth yields calculations for *Pandoraea* (ASV1) and *Burkholderia-Caballero-Paraburkholderia* (ASV1) and for all bacterial community from day 23 to 211

Considering *Pandoraea* (ASV1) and *Burkholderia-Caballero-Paraburkholderia* (ASV1) only								
Substrate	Substrate consumed from day 23 to day 211 (g)	Both genera on day 23 (copies of 16S sequences)	Both genera after dilutions (copies of 16S sequences) - Theoretical values	Both genera on day 211 (copies of 16S sequences)	Growth (copies of 16S sequences)	Growth (g of biomass)	Growth yield (g of biomass / g of substrate)	Expected growth yields from Eq 1 and 2 (g of biomass / g of substrate)
MC3MCA	0.056	4.56E+10	4.56E+08	2.08E+11	2.08E+11	1.85E-02	0.33	0.30
MC4MCA	0.054	4.40E+10	4.40E+08	1.92E+11	1.91E+11	1.71E-02	0.32	0.30
MC23DCA	0.052	8.40E+10	8.40E+08	1.72E+11	1.71E+11	1.52E-02	0.29	0.26
MC34DCA	0.054	1.96E+11	1.96E+09	4.71E+10	4.51E+10	4.02E-03	0.07	0.26
Considering all bacterial community								
Substrate	Substrate consumed from day 23 to day 211 (g)	All community on day 23 (copies of 16S sequences)	All community after dilutions (copies of 16S sequences) - Theoretical values	All community on day 211 (copies of 16S sequences)	Growth (copies of 16S sequences)	Growth* (g of biomass)	Growth yield (g of biomass / g of substrate)	Expected growth yields from Eq 1 and 2 (g of biomass / g of substrate)
MC3MCA	0.056	1.47E+11	1.47E+09	2.27E+11	2.25E+11	2.01E-02	0.36	0.30
MC4MCA	0.054	1.28E+11	1.28E+09	2.24E+11	2.22E+11	1.98E-02	0.37	0.30
MC23DCA	0.052	1.79E+11	1.79E+09	1.86E+11	1.84E+11	1.64E-02	0.32	0.26
MC34DCA	0.054	2.70E+11	2.70E+09	1.42E+11	1.39E+11	1.24E-02	0.23	0.26

*Growth in grams of biomass was calculated by dividing the growth as copies of 16S sequences per 8.92E-14 gram per copy of 16s (LUO, F.; DEVINE; EDWARDS, E. A., 2016).

Source: author (2022)

Figure 60B1 - Matrix of the phylogenetic tree (aerobic microcosms)

	Rhodanob...	Rhodanob...	Rhodanob...	Pandoraea...	Pandoraea...	Burkholder...	Burkholder...	Burkholder...	Allorhizobi...	Bosea (AS...	Bosea (AS...
Rhodanobacteraceae (...		98.9%	98.9%	88.1%	88.1%	87.8%	86.6%	87.4%	82.8%	83.1%	82.8%
Rhodanobacteraceae (...	98.9%		99.8%	88.5%	88.5%	88.3%	87.0%	87.8%	82.8%	83.1%	82.8%
Rhodanobacter denitrif...	98.9%	99.8%		88.3%	88.3%	88.1%	83.5%	84.6%	82.8%	83.1%	82.8%
Pandoraea (ASV1)	88.1%	88.5%	88.3%		100%	96.5%	96.1%	97.0%	80.4%	79.5%	79.3%
Pandoraea pnomenus...	88.1%	88.5%	88.3%	100%		96.5%	96.1%	97.0%	80.4%	79.5%	79.3%
Burkholderia-Caballero...	87.8%	88.3%	88.1%	96.5%	96.5%		96.8%	97.6%	80.2%	80.2%	80.0%
Burkholderia sp. RKJ 8...	86.6%	87.0%	83.5%	96.1%	96.1%	96.8%		98.1%	79.0%	78.3%	78.1%
Burkholderia sp. Strain...	87.4%	87.8%	84.6%	97.0%	97.0%	97.6%	98.1%		79.5%	78.9%	78.7%
Allorhizobium-Neorhiz...	82.8%	82.8%	82.8%	80.4%	80.4%	80.2%	79.0%	79.5%		89.3%	89.1%
Bosea (ASV1)	83.1%	83.1%	83.1%	79.5%	79.5%	80.2%	78.3%	78.9%	89.3%		99.8%
Bosea (ASV2)	82.8%	82.8%	82.8%	79.3%	79.3%	80.0%	78.1%	78.7%	89.1%	99.8%	

Source: author (2022)

B2. SUPPLEMENTARY INFORMATION - ANAEROBIC BIOTRANSFORMATIONS OF CONTAMINANTS

Anaerobic medium:

1 L of anaerobic mineral medium composition (detailed procedure available in Edwards & Grbic-Galic, 1994):

- 10 mL of MM1 - Phosphate buffer: 27.2 g of KH_2PO_4 + 34.8 g K_2HPO_4 in 1 L of dH_2O
- 10 mL of MM2 - Salt solution: 7.0 g of $CaCl_2.6H_2O$ + 2.0 g of $FeCl_2.4H_2O$ in 1 L of dH_2O – special solution free of NH_4^+
- 2 mL of MM3 - Trace Minerals: 0.3 g of H_3BO_3 + 0.1 g of ZnCl + 0.1 g of $Na_2MoO_4.2H_2O$ + 0.75 g of $NiCl_2.6H_2O$ + 1.0 g of $MnCl_2.4H_2O$ + 0.1 g of $CuCl_2.2H_2O$ + 1.5 g of $CoCl_2.6H_2O$ + 0.02 g of Na_2SeO_3 + 0.1 g of $Al_2(SO_4)_3.18H_2O$ in 1 L of dH_2O
- 2 mL of MM4 - Magnesium solution: 50.8 g/L of $MgCl_2.6H_2O$ 50.8 g/L
- 1 mL of MM5 - Redox indicator: 1 g/L of resazurin
- 10 mL of MM6 - Saturated bicarbonate: 20 g $NaHCO_3$ in 100 mL dH2O
- 10 mL of MM7 - Vitamins: 0.02 g of Biotin + 0.02 g of Folic acid + 0.1 g of Pyridoxine HCl + 0.05 g of Riboflavin + 0.05 g of Thiamine + 0.05 g of Nicotinic acid + 0.05 g of Pantothenic acid + 0.05 g of PABA + 0.05 g of Cyanocobalamin (vitamin B12) + 0.05 g of Thioctic (lipoic) acid + 1.0 g of Coenzyme M in 100 mL dH2O
- 10 mL of MM8 - Amorphous Ferrous Sulfide: 13.9 g/500 mL $FeSO_4.7H_2O$ + 12.0 g/500 mL $Na_2S.9H_2O$

Table 10B2 - Electron added versus electron needed in microcosms

Ethanol (12 e^- per mol)	**e^- added (µM)**	**e^- needed (µM)**	**Ratio (added/needed)**
10.0 mg DCNB/L and 3 µL added	6165.4	520.8	11.8
13.3 mg DCNB/L and 6 µL added	8220.5	692.7	11.9
20.0 mg DCNB/L and 6 µL added	8220.5	1041.6	7.9
Lactate (12 e^- per mol)	**e^- added (µM)**	**e^- needed (µM)**	**Ratio (added/needed)**
10.0 mg DCNB/L and 33 µL added	2772.0	520.8	5.3
13.3 mg DCNB/L and 66 µL added	3696.0	692.7	5.3
20.0 mg DCNB/L and 66 µL added	3696.0	1041.6	3.5

Considering:

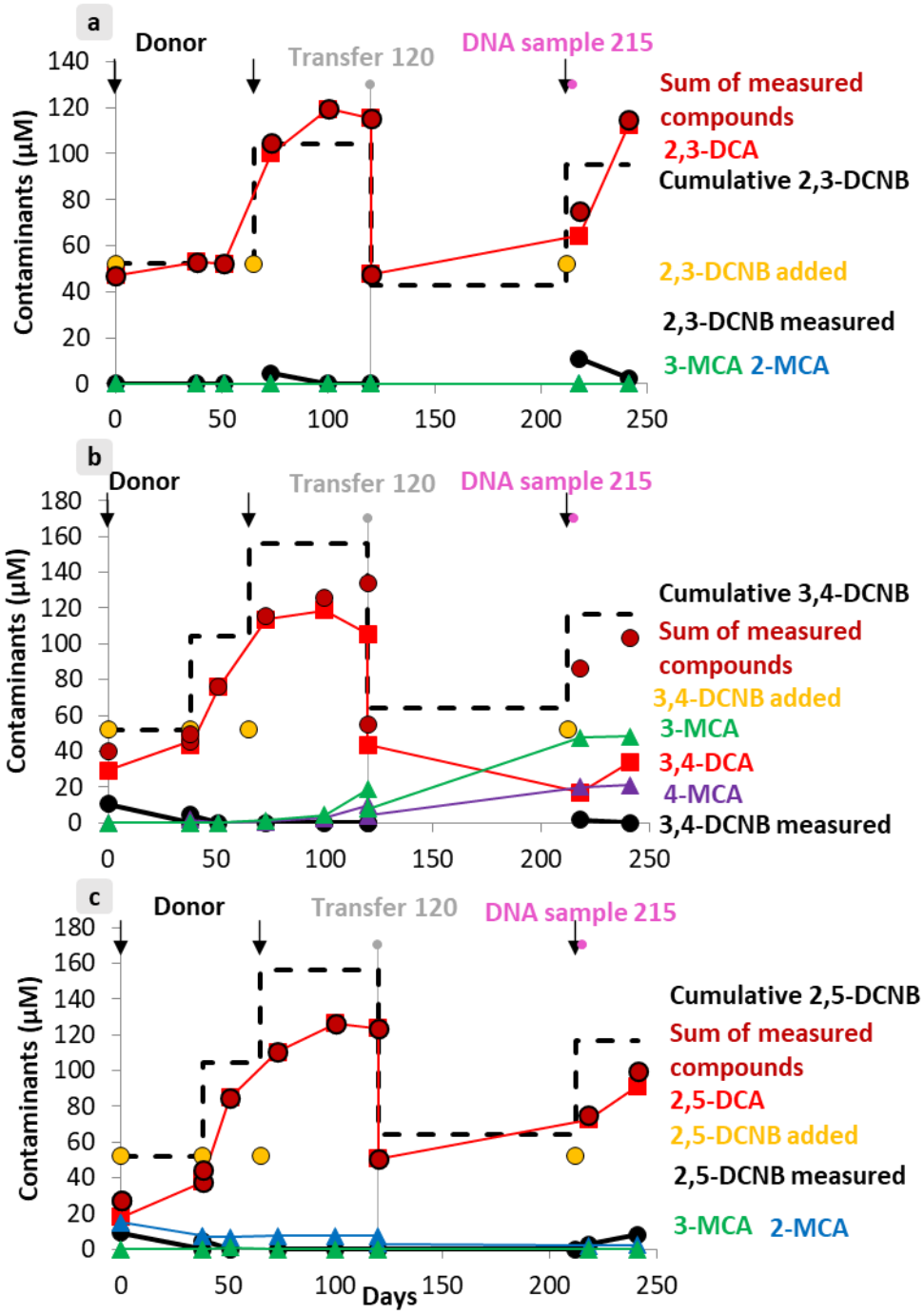
Lactate complete oxidation: $C_3H_6O_3 + 3O_2 \rightarrow 3CO_2 + 3H_2O$ (12 e^-)

Ethanol complete oxidation: $C_3H_6O + 3O_2 \rightarrow 2CO_2 + 3H_2O$ (12 e^-)

DCNB → Aniline: $C_6H_3Cl_2NO_2 + 5H_2 \rightarrow C_6H_5NH_2 + 2HCl + 2H_2O$ (10 e^-)

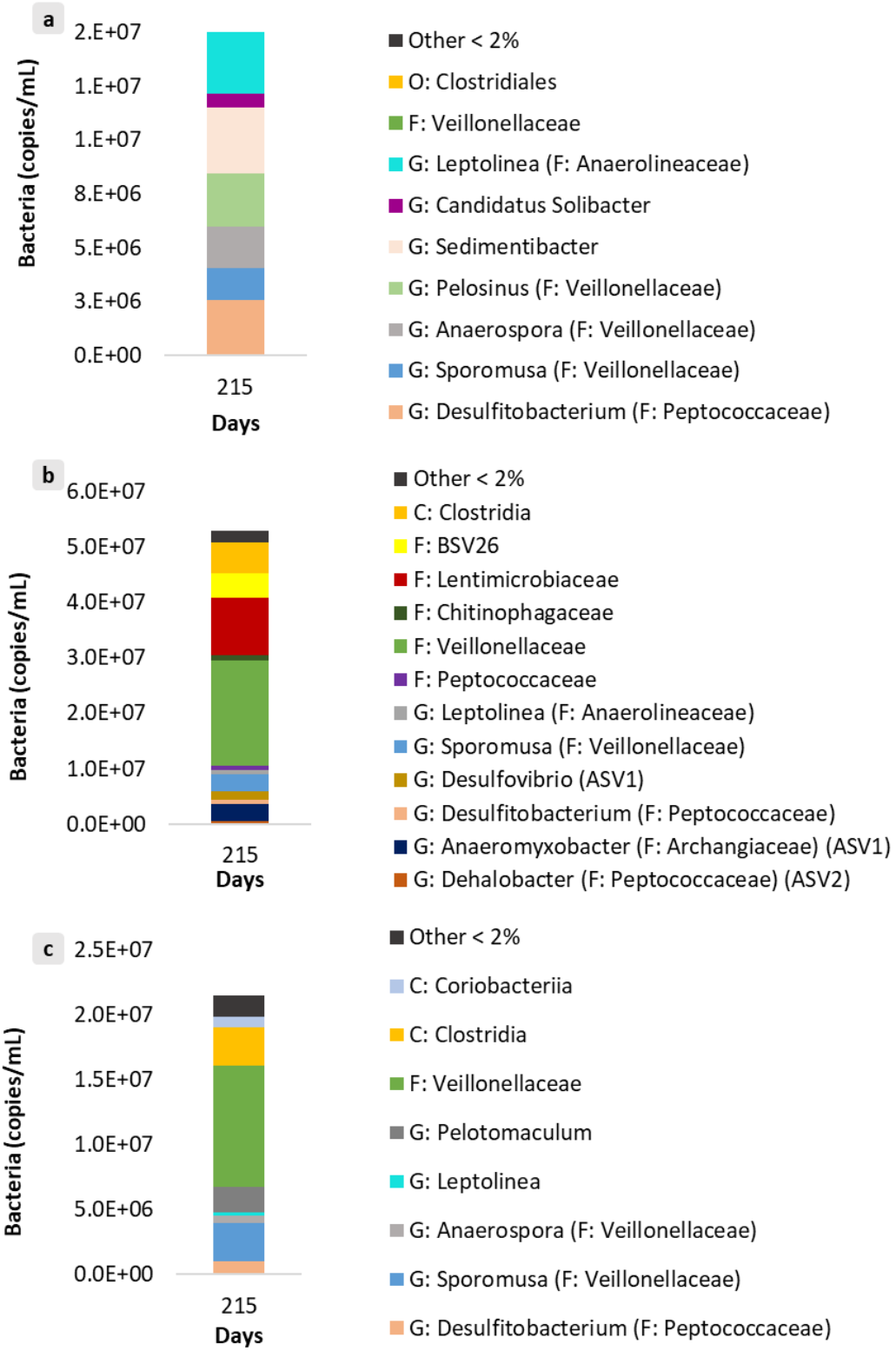
Source: author (2022)

Figure 61B2 - Concentration of contaminants in MC23DCNB-T1 (a), MC34DCNB-T1 (b), and MC25DCNB-T1 (c)



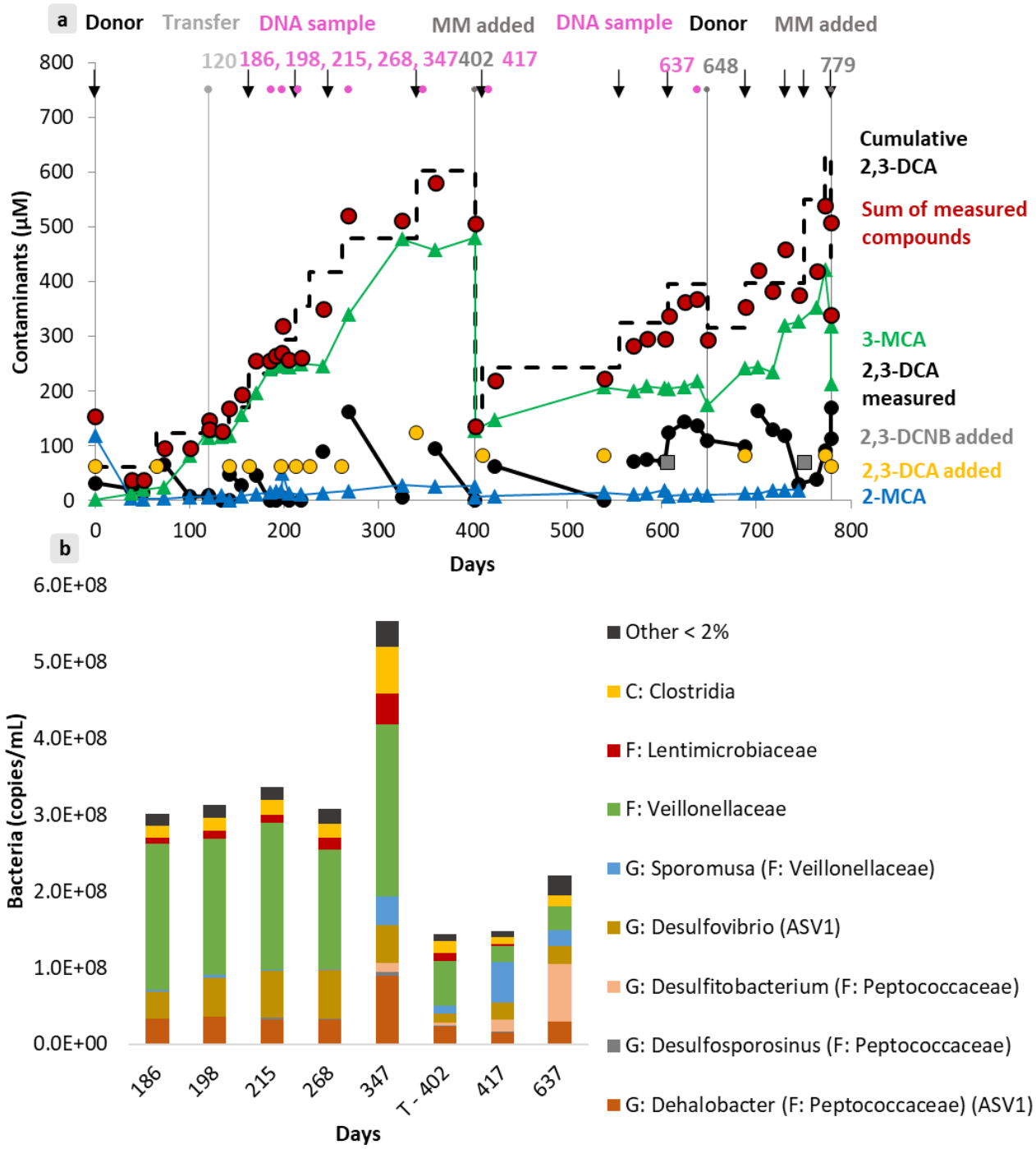
Source: author (2022)

Figure 62B2 - Bacterial community in MC23DCNB-T1 (a), MC34DCNB-T1 (b), and MC25DCNB-T1 (c)



Source: author (2022)

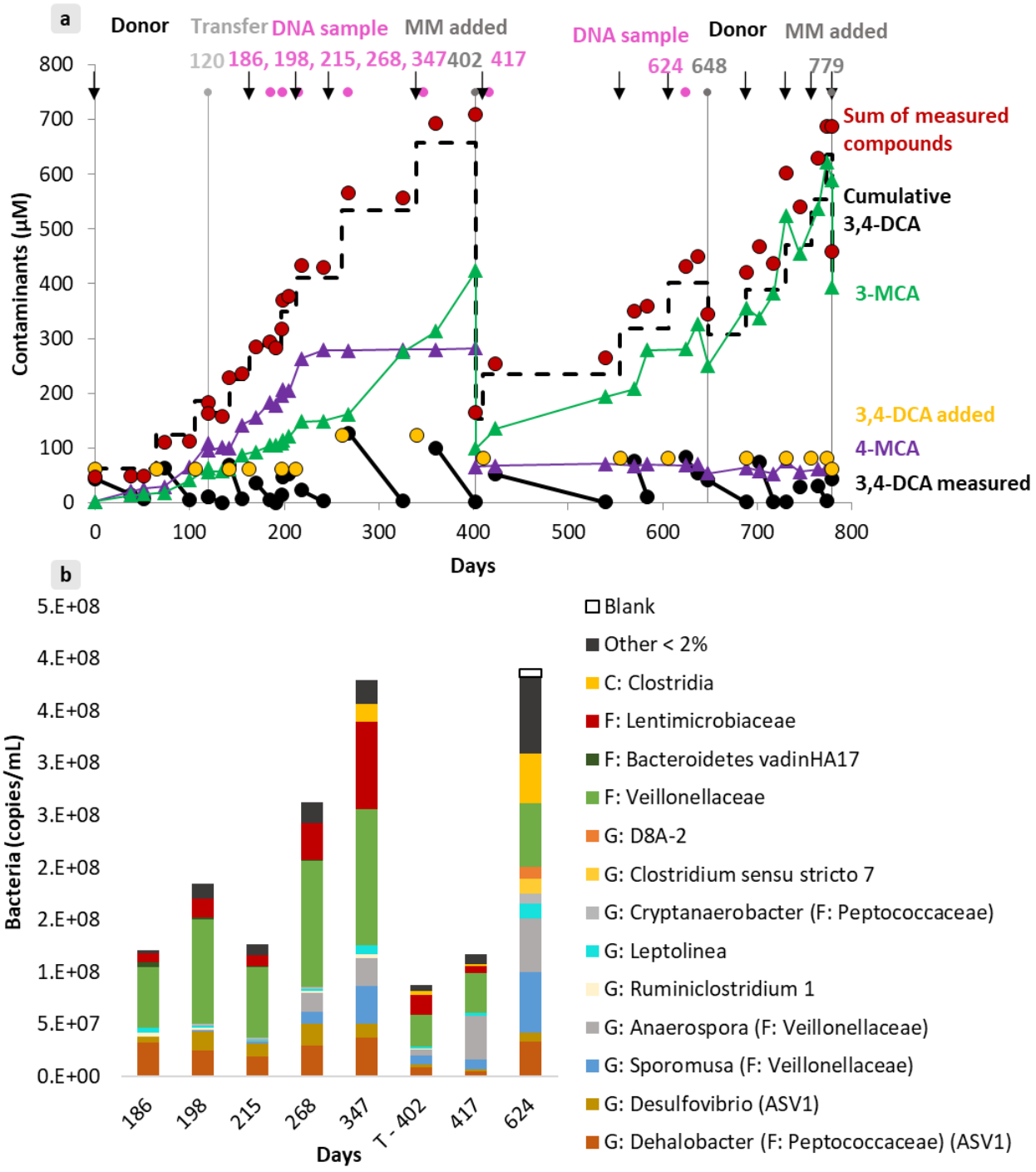
Figure 63B2 - Concentrations of contaminants (a) and bacterial community (b) of MC23DCA-T1 during the experimental period



T-402 is a theoretical data based on the dilution of the microcosm when mineral medium was added (abundance of day 347 was multiplied by the dilution factor of 0.3).

Source: author (2022)

Figure 64B2 - Concentrations of contaminants (a) and bacterial community (b) of MC34DCA-T1 during the experimental period



T-402 is a theoretical data based on the dilution of the microcosm when mineral medium was added (abundance of day 347 was multiplied by the dilution factor of 0.2).

Source: author (2022)

Table 11B2 - Archaea versus bacteria relative abundance in microcosms

Microcosms	**Transfer (T) - DNA sampling day**													
MC23DCNB	T1-215	T2-95	T2-148	T2-297	T2-517			T3A-100	T3A-170	T3A-390		T3B-100	T3B-170	T3B-390
Archaea	13%	1%	2%	2%	2%			1%	1%	1%		1%	1%	1%
Bacteria	87%	99%	98%	98%	98%			99%	99%	99%		99%	99%	99%
MC34DCNB	T1-215	T2-95	T2-148	T2-297	T2-517	T2-589	T3A-21	T3A-100	T3A-170	T3A-390	T3B-21	T3B-100	T3B-170	T3B-390
Archaea	96%	95%	94%	88%	56%	57%	53%	93%	88%	74%	69%	70%	70%	50%
Bacteria	4%	5%	6%	12%	44%	43%	47%	7%	12%	26%	31%	30%	30%	50%
MC25DCNB	T1-215	T2-95	T2-148	T2-297	T2-517		T3A-21	T3A-100	T3A-170	T3A-390	T3B-21	T3B-100	T3B-170	T3B-390
Archaea	52%	53%	56%	28%	13%		34%	34%	7%	2%	45%	24%	31%	18%
Bacteria	48%	47%	44%	72%	87%		66%	66%	93%	98%	55%	76%	69%	82%
MC23DCA	T1-186	T1-198	T1-215	T1-268	T1-347	T1-417	T1-637	T2-81	T2-134	T2-213	T2-283	T2-517		
Archaea	65%	67%	69%	71%	84%	75%	69%	50%	56%	74%	63%	73%		
Bacteria	35%	33%	31%	29%	16%	25%	31%	50%	44%	26%	37%	27%		
MC34DCA	T1-186	T1-198	T1-215	T1-268	T1-347	T1-417	T1-624	T2-95	T2-148	T2-227	T2-297	T2-517		
Archaea	83%	80%	78%	85%	88%	77%	84%	49%	54%	71%	56%	63%		
Bacteria	17%	20%	22%	15%	12%	23%	16%	51%	46%	29%	44%	37%		

Source: author (2022)

Table 12B2 - Archaea genera found in microcosms

Microcosms	**Transfer (T)-DNA sampling day**													
MC23DCNB	T1-215	T2-95	T2-148	T2-297	T2-517			T3A-100	T3A-170	T3A-390		T3B-100	T3B-170	T3B-390
Methanobacterium	14.6%	22.9%	12.6%	23.1%	24.0%				17.3%	71.2%		21.1%		45.6%
Methanocella		2.2%												
Methanomassiliicoccus	82.1%	71.6%	84.9%	76.9%	75.7%			100.0%	42.3%	6.0%		78.9%	70.8%	54.4%
Methanoregula		3.3%	1.1%	0.3%					19.2%	22.8%				
Methanosaeta									21.2%					
Methanosarcina	1.6%												29.2%	
Blank or <1%	1.7%		1.4%											
MC34DCNB	T1-215	T2-95	T2-148	T2-297	T2-517	T2-589	T3A-21	T3A-100	T3A-170	T3A-390	T3B-21	T3B-100	T3B-170	T3B-390
Methanocella	13.9%	9.4%	7.4%	9.0%	6.8%	4.0%	10.9%	1.1%	1.3%	0.9%	12.9%	13.1%	14.2%	19.7%
Methanomassiliicoccus					0.6%	0.4%				0.7%		5.9%	7.5%	8.1%
Methanoregula	85.1%	90.2%	92.0%	89.9%	92.6%	95.6%	88.2%	98.3%	97.9%	98.4%	86.3%	80.7%	78.0%	72.0%
Blank or <1%	0.9%		0.6%	0.7%			1.0%	0.6%	0.7%		0.8%			0.2%
MC25DCNB	T1-215	T2-95	T2-148	T2-297	T2-517		T3A-21	T3A-100	T3A-170	T3A-390	T3B-21	T3B-100	T3B-170	T3B-390
Methanocella	73.2%	25.1%	49.0%	78.8%	97.7%		43.3%	44.5%	58.9%	79.7%	30.5%	43.2%	59.5%	58.8%
Methanoregula	26.7%	74.8%	51.0%	21.2%	2.3%		56.7%	55.4%	41.1%	20.3%	63.1%	56.7%	40.3%	41.1%
Blank or <1%													6.0%	0.1%
MC23DCA	T1-186	T1-198	T1-215	T1-268	T1-347	T1-417	T1-637	T2-81	T2-134	T2-213	T2-283	T2-517		
Methanocella	28.2%	19.7%	29.3%	17.9%	9.9%	11.6%	19.1%	34.9%	23.4%	15.5%	15.5%	17.2%		
Methanomassiliicoccus								5.2%	3.7%	1.8%	1.1%			
Methanoregula	50.5%	60.2%	52.4%	63.3%	79.9%	77.7%	75.3%	51.8%	67.9%	80.8%	82.1%	82.2%		
Methanosaeta	10.5%	12.0%	7.9%	10.3%	6.0%	6.4%	3.2%	4.7%	2.8%	1.1%				
Blank or <1%	11.1%	7.6%	10.8%	9.1%	4.5%	4.6%	2.4%	3.0%	1.8%	1.0%	1.8%	0.6%		
MC34DCA	T1-186	T1-198	T1-215	T1-268	T1-347	T1-417	T1-624	T2-95	T2-148	T2-227	T2-297	T2-517		
Methanocella	72.5%	74.8%	74.5%	28.9%	22.9%	31.5%	29.2%	76.7%	65.0%	54.2%	43.8%	20.1%		
Methanomassiliicoccus	2.1%							5.9%	6.2%	5.2%	2.4%			
Methanoregula	13.3%	13.6%	17.5%	67.4%	70.4%	64.5%	69.0%	15.8%	18.2%	38.4%	42.5%	77.5%		
Methanosaeta	1.1%													
Methanosarcina	7.4%	9.2%	5.5%	2.1%	5.1%	2.4%	1.1%		9.8%	1.8%	11.1%	1.7%		
Blank or <1%	3.6%	2.8%	2.3%	1.3%	1.3%	1.6%	0.7%	1.8%	1.0%	0.2%	0.1%	0.7%		

Source: author (2022)

Table 13B2 - Methane measured in microcosms

	CH_4 measured (µM) in microcosms (Transfer T-day of sampling)													
Microcosms	T1-638	T2-518	T3A-78	T3A-113	T3A-155	T3A-176	T3A-292	T3A-391	T3B-78	T3B-113	T3B-155	T3B-176	T3B-292	T3B-391
MC23DCNB	-	0.0	3.4	2.7	3.4	1.5	1.0	1.4	3.6	2.4	3.7	1.0	0.9	0.0
MC34DCNB	-	52.6	129.9	60.5	155.3	104.6	19.2	1.7	8.3	2.9	5.5	1.5	0.4	0.0
MC25DCNB	-	2.1	4.4	0.0	1.9	1.1	0.4	1.7	3.9	0.0	0.3	0.0	0.5	0.0
MC23DCA	86.3	203.3	-	-	-	-	-	-	-	-	-	-	-	-
MC34DCA	228.2	137.0	-	-	-	-	-	-	-	-	-	-	-	-

- = not sampled

Source: author (2022)

ASV found in the communities:

Dehalobacter (genus level) (ASV1):

GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGAAGAACCTT
ACCAAGGCTTGACATCTACAGAATCCTTAAGAGATTAGGGAGTGCCTTTCGGGGAAC
TGTAAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAG
TCCCGCAACGAGCGCAACCCCTATATTTAGTTGCTAACAGGTAAAGCTGAGAACTCT
AGATAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATG
CCCCTTATGTCTTGGGCTACACACGTGCTACAATGGACGGTACAGACGGAAGCGAAG
CCGCGAGGTGAAGCAAATCCGAGAAAGCCGTTCTCAGTTCGGATTGCAGGCTGCAA
CTCGCCTGCATGAAGTCGGAATCGCTAGTAATCGCAGGTCAGCACACTGCGGTGAAT
ACGTTCCCGGGCCTT

Dehalobacter (genus level) (ASV2):

GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGAAGAACCTT
ACCAAGGCTTGACATCCAACTAATCCCGTAGAGATATGGGAGTGCCCTTCGGGGAAA
GTTGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAG
TCCCGCAACGAGCGCAACCCCTATATTTAGTTGCTAACAGGTAAAGCTGAGAACTCT
AGATAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATG
CCCCTTATGTCTTGGGCTACACACGTGCTACAATGGACGGTACAGACGGAAGCGAAG
CCGCGAGGTGAAGCAAATCCGAGAAAGCCGTTCTCAGTTCGGATTGCAGGCTGCAA
CTCGCCTGCATGAAGTCGGAATCGCTAGTAATCGCAGGTCAGCACACTGCGGTGAAT
ACGTTCCCGGGCCTT

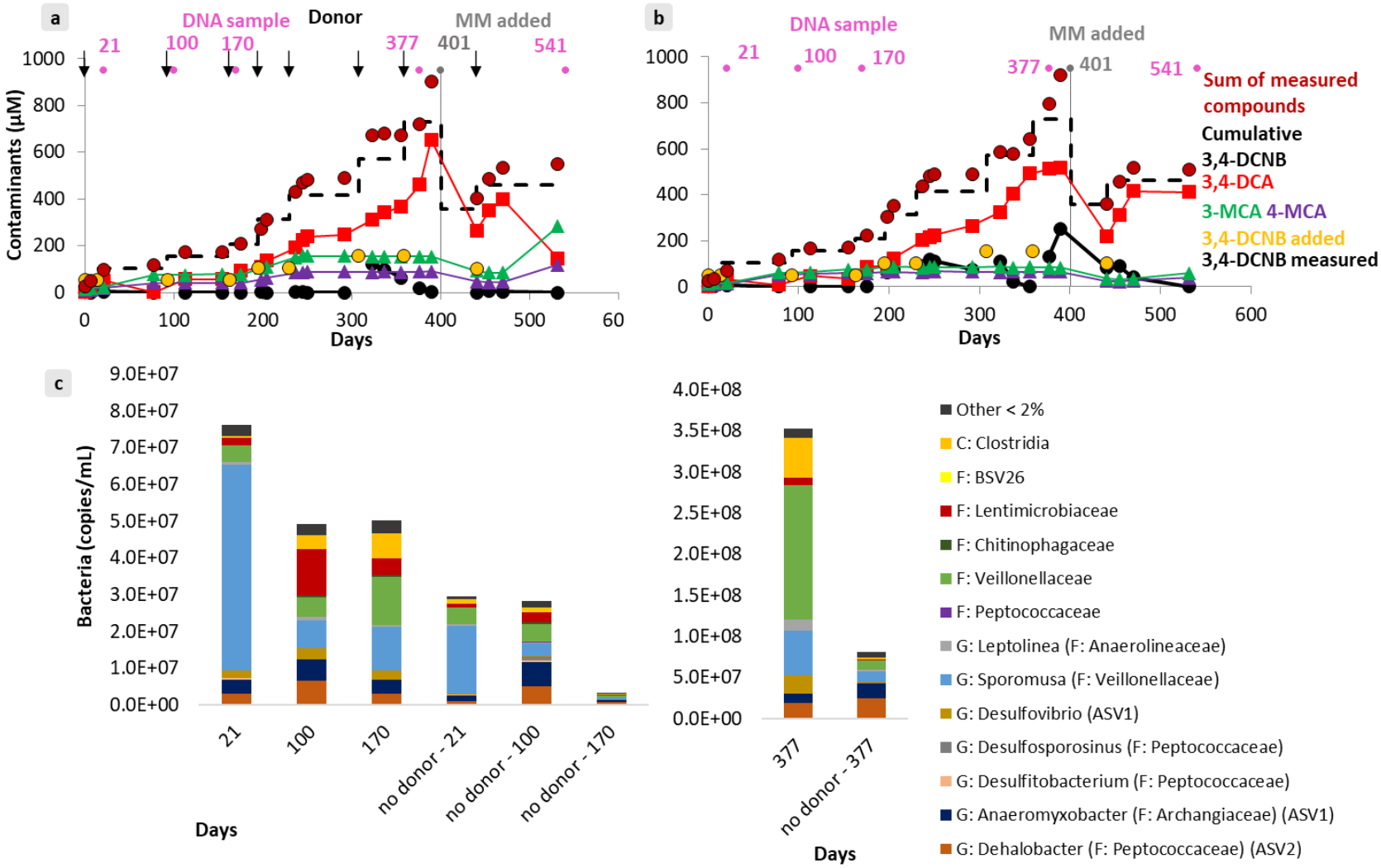
Anaeromyxobacter (genus level) (ASV1):

GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGCAGAACCTT
ACCTGGTCTTGACATCCTCGGAATCCTTCAGAGATGAGGGAGTGCCCGCAAGGGAAC
CGAGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAG
TCCCGCAACGAGCGCAACCCCTGCCGTTAGTTGCCATCATTCAGTTGGGCACTCTAA
CGGGACTGCCGGCGTCAAGCCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGC
CTTTATGACCAGGGCTACACACGTGCTACAATGGCCGGTACAGAGGGTCGCCAAGTC
GCGAGACGGAGCTAATCCCAGAAAACCGGTCTCAGTTCGGATTGGAGTCTGCAACTC

GACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATAC
GTTCCCGGGCCTT

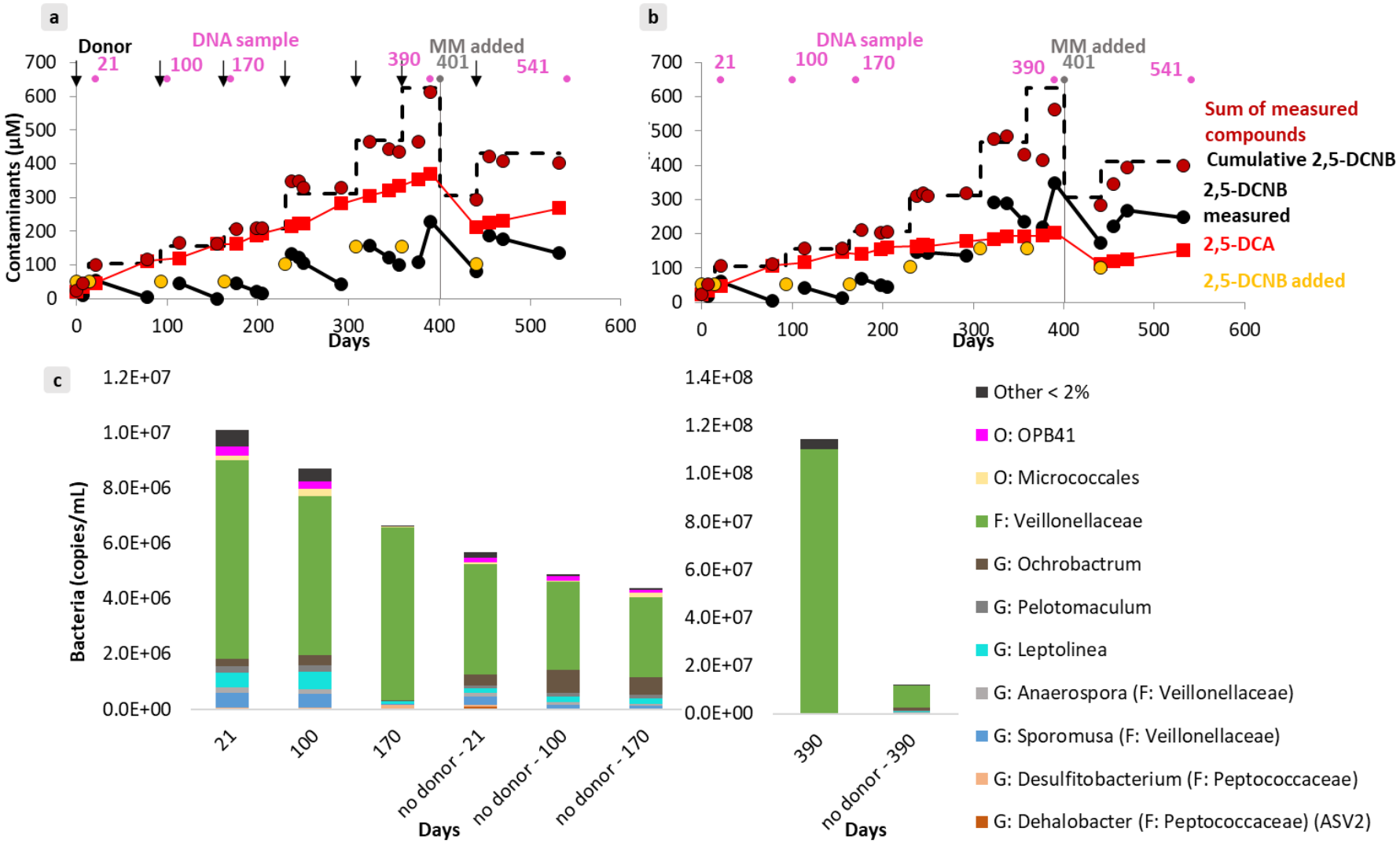
Desulfovibrio (genus level) (ASV1):
GGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTA
CCTGGGTTTGACATCCCTCGAATCCTCCGGAAATGGAGGAGTGCCCTTCGGGGAGCG
GGGTGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTGTCTTTAGTTGCCAGCGAGTAATGTCGGGCACTCTA
GAGAGACCGCCTCGGTCAACGGGGAGGAAGGTGGGGACGACGTCAAGTCATCATGG
CCCTTACGCCCAGGGCTACACACGTACTACAATGGTGGGTACAATGGGCTGCGAGAC
CGCAAGGTGGAGCCAATCCCAAAAAACCCATCCCAGTTCGGATCGGGGTCTGCAACT
CGACCCCGTGAAGTCGGAATCGCTAGTAATCGGAGATCAGCATGCTCCGGTGAATAC
GTTCCCGGGCCTT

Figure 65B2 - Concentrations of contaminants in MC34DCNB-T3A (with donor) (a) and MC34DCNB-T3B (without donor) (b) and their bacterial community (c) observed during the experimental period



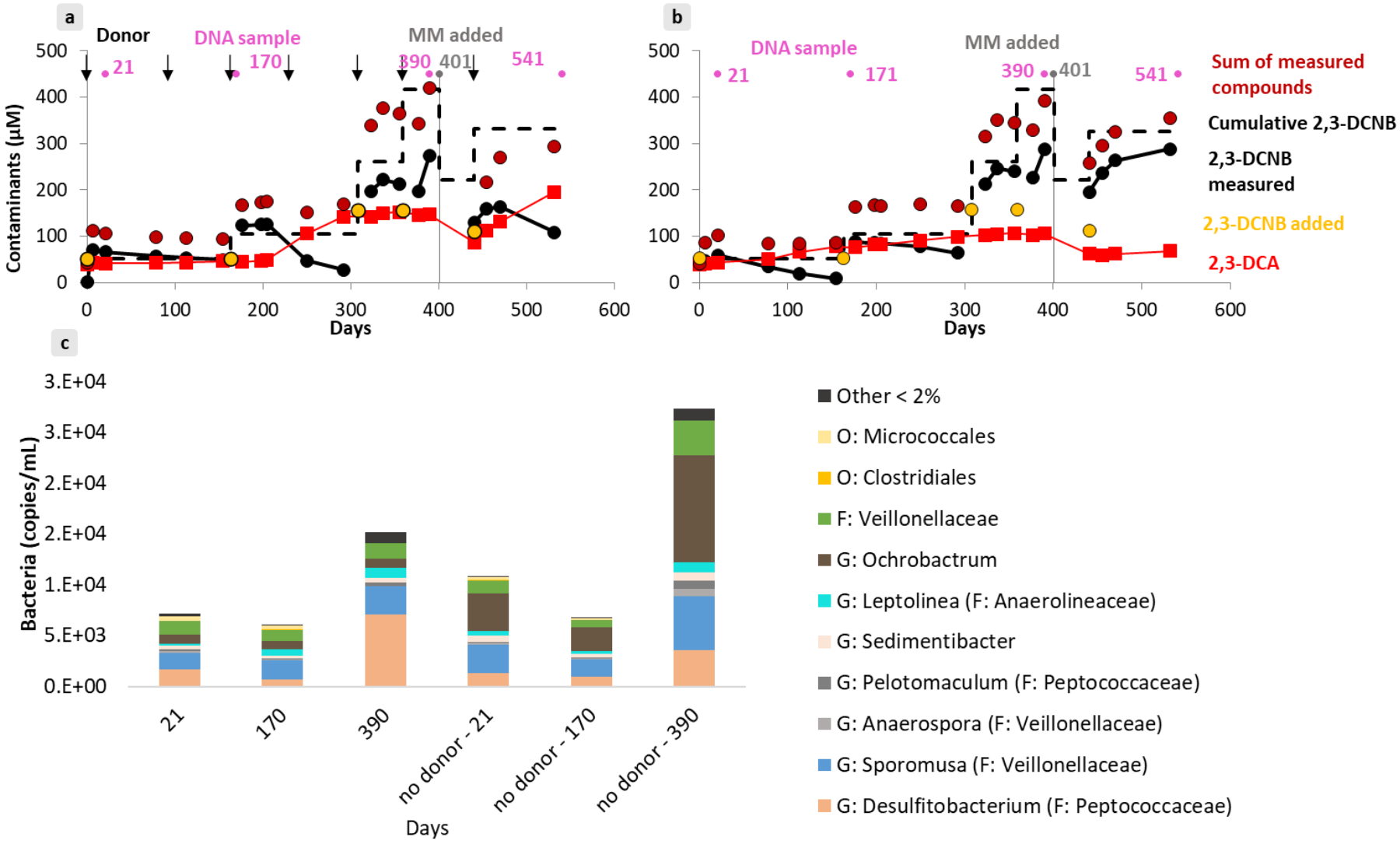
Source: author (2022)

Figure 66B2 - Concentrations of contaminants in MC25DCNB-T3A (with donor) (a) and MC25DCNB-T3B (without donor) (b) and their bacterial community (c) observed during the experimental period



Source: author (2022)

Figure 67B2 - Concentrations of contaminants in MC23DCNB-T3A (with donor) (a) and MC23DCNB-T3B (without donor) (b) and their bacterial community (c) observed during the experimental period



Source: author (2022)

Table 14B2 - Concentrations of chloride measured and expected to be released from biotransformation on days 7, 120, 518, and 644 in anaerobic microcosms

Microcosms	Days	Cl^- measured (µM)	Difference of measured Cl^- (µM)	MCA accumulated (equivalent to Cl^- released) (µM)	Difference of Cl^- released (µM)
MC23DCA-T2	7	1925.3		12.6	
	120	2064.7	139.4	139.0	126.3
	518	2450.5		374.6	
	644	2530.9	80.4	440.2	65.6
MC34DCA-T2	7	1804.6		19.6	
	120	1960.2	155.5	184.7	165.0
	518	2622.8		430.1	
	644	2888.8	265.9	726.1	296.0
MC34DCNB-T2	7	1869.30		16.8	
	120	2235.16	365.9	234.7	217.9
	518	2498.74		429.5	
	644	2699.69	200.9	552.9	123.4

The MCA accumulated is equivalent to the amount of chloride expected to be released.

Source: author (2022)

Table 15B2 - Calculations of *Dehalobacter* growth yields in terms of copies of cell per µmol of Cl^- released

***Dehalobacter* (ASV1 and ASV2) growth yields**					
Microcosms	Cl^- released from day 92 to 227 (µM)	Abundance of *Dehalobacter* (ASV1) on day 95	Abundance of *Dehalobacter* (ASV1) on day 227	Growth (copies/mL)	Growth yields (copies/µmol of Cl^- released)
MC23DCA-T2	220.56	3.84E+06	4.69E+07	4.31E+07	1.95E+08
MC34DCA-T2	136.27	1.30E+06	1.06E+07	9.26E+06	6.79E+07
Microcosms	Cl^- released from day 297 to 517 (µM)	Abundance of *Dehalobacter* (ASV1) on day 297	Abundance of *Dehalobacter* (ASV1) on day 517	Growth (copies/mL)	Growth yields (copies/µmol of Cl^- released)
MC23DCA-T2	249.54	7.96E+06	5.47E+07	4.67E+07	1.87E+08
MC34DCA-T2	355.72	4.03E+06	5.39E+07	4.99E+07	1.40E+08
Microcosm	Cl^- released from day 297 to 517 (µM)	Abundance of *Dehalobacter* (ASV2) on day 297	Abundance of *Dehalobacter* (ASV2) on day 517	Growth (copies/mL)	Growth yield (copies/µmol of Cl^- released)
MC34DCNB-T2	412.56	2.05E+06	7.80E+07	7.59E+07	1.84E+08

Dehalobacter growth yields were calculated for the periods between days 227 and 95 in MC23DCA-T2 and MC34DCA-T2 (ASV1) and between days 297 and 519 in MC23DCA-T2 and MC34DCA-T2 (ASV1) and in MC34DCNB-T2 (ASV2).

Source: author (2022)

Figure 68B2 - Matrix of the phylogenetic tree (anaerobic microcosms)

	Desulfovib...	Anaeromy...	Anaeromy...	Anaeromy...	Dehalobac...	Dehalobac...	Dehalobac...	Dehalobac...	Dehalobac...
Desulfovibrio (ASV1)		83.5%	82.7%	83.1%	81.6%	81.8%	81.8%	81.8%	81.8%
Anaeromyxobacter (A...	83.5%		99.1%	99.6%	85.7%	84.8%	84.8%	84.8%	84.8%
Anaeromyxobacter de...	82.7%	99.1%		99.6%	84.8%	78.5%	74.2%	74.2%	84.4%
Anaeromyxobacter de...	83.1%	99.6%	99.6%		85.3%	78.5%	74.3%	74.3%	84.8%
Dehalobacter (ASV1)	81.6%	85.7%	84.8%	85.3%		97.0%	97.0%	97.0%	97.0%
Dehalobacter restrictu...	81.8%	84.8%	78.5%	78.5%	97.0%		92.2%	92.2%	100%
Dehalobacter sp. DCA ...	81.8%	84.8%	74.2%	74.3%	97.0%	92.2%		100%	100%
Dehalobacter sp. CF (...	81.8%	84.8%	74.2%	74.3%	97.0%	92.2%	100%		100%
Dehalobacter (ASV2)	81.8%	84.8%	84.4%	84.8%	97.0%	100%	100%	100%	

Source: author (2022)

APPENDIX C - PRE-EXPERIMENTS PERFORMED AT THE SITE (2017-2019)

I am particularly proud of these pre-experiments because they were all a result of great teamwork performed by Lígia Carvalho and her awesome lab team, responsible for the analytical procedures: Jefferson Cruz, Evandro José, and Getúlio dos Reis. From out of the lab, these experiments were also done with the help of Paloma Carvalho, Ruy Silva, Marcílio Souza, and many others who collaborated. We also had the contribution of Professor Jim Spain, who spent one week with us, at the site, helping us to start some enrichment cultures.

Aiming to verify whether natural attenuation was likely to happen at the contaminated site, or whether bioestimulation or even bioaugmentation were strategies that would be needed as bioremediation strategies, the main objective of the tests performed in the industry was to evaluate the ability of native bacteria from soil and groundwater to aerobically degrade the contaminants of interest (COI): 2,3- and 3,4-dichloronitrobenzene isomers (DCNB), 2,3- and 3,4-dichloroaniline isomers (DCA), and 1,2-dichlorobenzene (DCB). Concentration of those contaminants and monochloroaniline isomers (2-, 3-, and 4-MCA) were measured through high-performance liquid chromatography (HPLC) in soil samples (extraction was performed using acetonitrile) and in water samples. The DNA samples collected from the microcosms/enrichments during the experimental period were sequenced by an external laboratory and processed by Line Lomheim (UofT).

The pre-experiments are divided into the following topics:

- Aerobic test with soil – microcosms containing 10 g of soil
- Aerobic teste with groundwater – microcosms containing 100 mL of groundwater
- Enrichment cultures
- Aerobic test with soil (bioaugmentation) – microcosms containing 10 g of soil
- Aerobic test with soil (bioaugmentation) – mesocosms containing 1 Kg of soil
- Anaerobic versus aerobic tests in water with active enrichments – microcosms containing 100 mL of water and enrichment
- Aerobic pH test with enrichment – microcosms containing 100 mL of water and enrichment
- Microbial community from 16S sequencing

AEROBIC TEST WITH SOIL – MICROCOSMS CONTAINING 10 G OF SOIL

The first test performed at the site was set by Laís Trento, Maria Cristina Silva Lemes, Sofia Pimentel and Suzana Kraus, in January 2018. The objective was to obtain the first insights on biodegradation by native microbes in contaminated soil under different conditions (sterile soil as a control, natural soil with no pH adjustment, neutralized soil with pH adjusted to 7.0, and soil inoculated with organic and clean soil). For that, 2 different mixtures of soil samples were used for 2 independent sets of microcosms; contamination in set 1 was in the range of 5 to 35 mg/Kg for each COI, while set 2 had contamination in the range of 50 to 700 mg/Kg. The microcosms consisted of 10 g of soil in a 40 mL glass vail (pictures of the test are shown in Figure 69), under the following four different conditions:

- STE - Sterile control (set 1 and 2): 10g of contaminated soil from the site autoclaved
- NAT- Natural soil (set 1 and 2): 10 g of contaminated soil from the site
- NEU - Neutralized soil pH = 7.0 (set 1 and 2): 10 g of contaminated soil from the site with buffer solution made of K_2HPO_4 (1 M)
- POS - Positive control (set 1 and 2): 9 g of contaminated soil from the site and 1 g of organic and clean soil, not from the site

Microcosms for the two sets were prepared in 10 replicates for each of the four conditions. On each sampling day, the full mass of soil from 2 vials from each condition was sacrificed to be analyzed by the HPLC method.

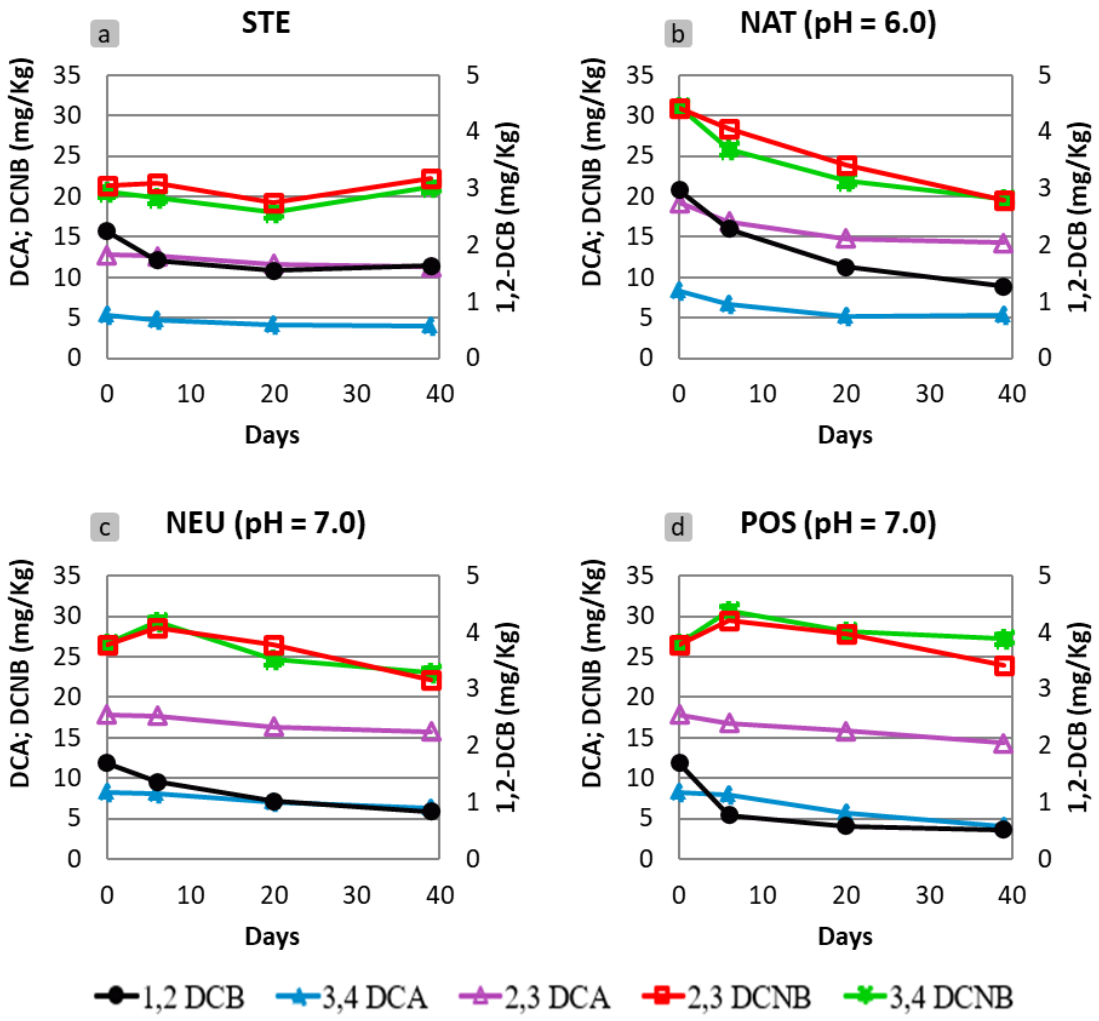
Figure 69C - Pictures of the first soil test in 40 mL with 10 g of soil

Source: author (2022)

Findings:

Figures 70 and 71 show the results of the test with set 1 and set 2 of soil. The monitoring period of one month was not enough to verify significant transformation of the contaminants in the soil. Possible reasons for the lag phase or inactivity were thought to be the low water content (12% of moisture content), high COI concentration, or even absence of active bacteria even in the positive control.

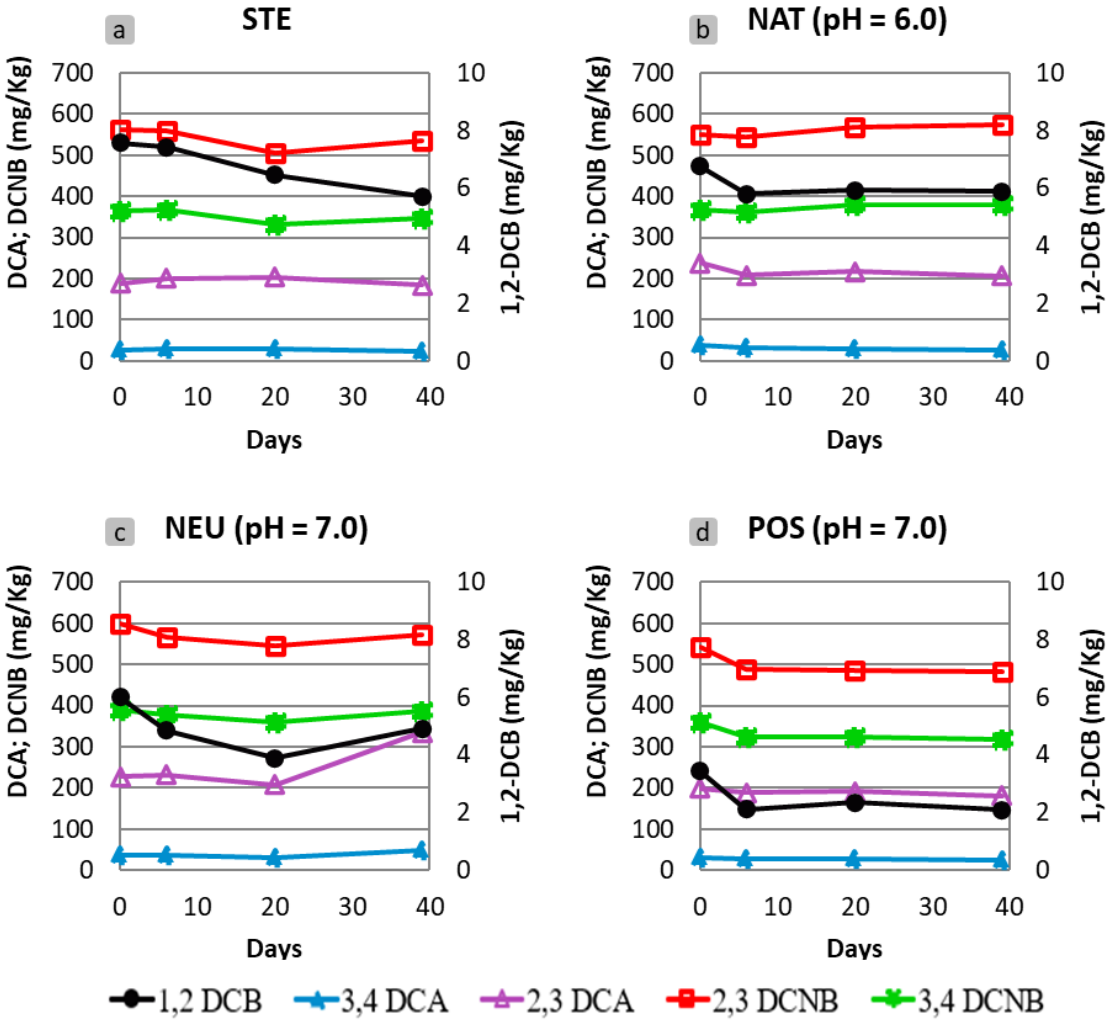
Figure 70C - Concentrations of contaminants in set 1 of microcosms with soil



STE - Sterile control (set 1 and 2): 10g of contaminated soil from the site autoclaved (a), NAT- Natural soil (set 1 and 2): 10 g of contaminated soil from the site (b), NEU - Neutralized soil pH = 7.0 (set 1 and 2): 10 g of contaminated soil from the site with buffer solution made of K_2HPO_4 (1 M) (c), and POS - Positive control (set 1 and 2): 9 g of contaminated soil from the site and 1 g of organic and clean soil, not from the site (d).

Source: author (2022)

Figure 71C - Concentrations of contaminants in set 2 of microcosms with soil



STE - Sterile control (set 1 and 2): 10g of contaminated soil from the site autoclaved (a), NAT- Natural soil (set 1 and 2): 10 g of contaminated soil from the site (b), NEU - Neutralized soil pH = 7.0 (set 1 and 2): 10 g of contaminated soil from the site with buffer solution made of K_2HPO_4 (1 M) (c), and POS - Positive control (set 1 and 2): 9 g of contaminated soil from the site and 1 g of organic and clean soil, not from the site (d).

Source: author (2022)

AEROBIC TESTE WITH GROUNDWATER – MICROCOSMS CONTAINING 100 ML OF GROUNDWATER

In addition to the initial tests with soil, we also set up microcosms with groundwater from the site, collected from five different wells (PM-30, PM-30, PM-11, PM-08, PM-06,

PM-13). The objective was to find native microbes able to biodegrade contaminants in groundwater. For that, five sets of microcosms were started in boston bottles of 250 mL with 100 mL of groundwater from each well (Figure 72). For each set of microcosms, the following conditions were tested in triplicate:

- STE - Sterilized groundwater (autoclaved)
- NAT - Natural pH with no adjustment
- NEU - Neutralized pH (7.0) with buffer solution made of K_2HPO_4 (1 M)

If the groundwater from any wells was not contaminated, we amended the microcosms with the target compounds in the range of 5 mg/L (34 μM) for DCB, 5 mg/L (31 μM) for DCA isomers, 10 mg/L (52 μM) for 2,3-DCNB, and 15 mg/L (78 μM) for 3,4-DCNB.

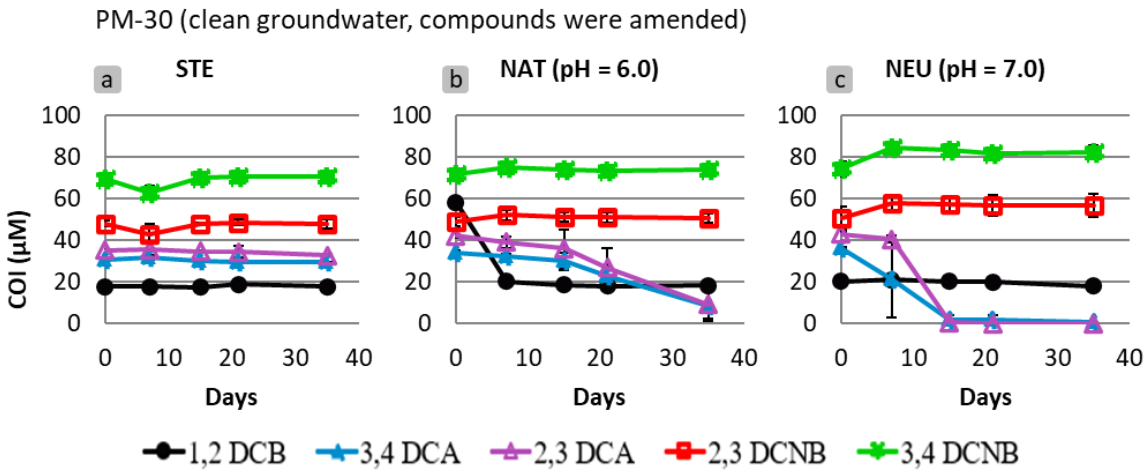
Figure 72C - Microcosms with groundwater

Source: author (2022)

Findings:

Each set of microcosms was monitored during around 30 days, except for the microcosms with groundwater from the well PM-08, which were analyzed again around day 50. Figures 73, 74, 75, 76, and 77 show the results of the monitoring period for microcosms set with groundwater from PM-30, PM-11, PM-08, PM-06, and PM-13, respectively.

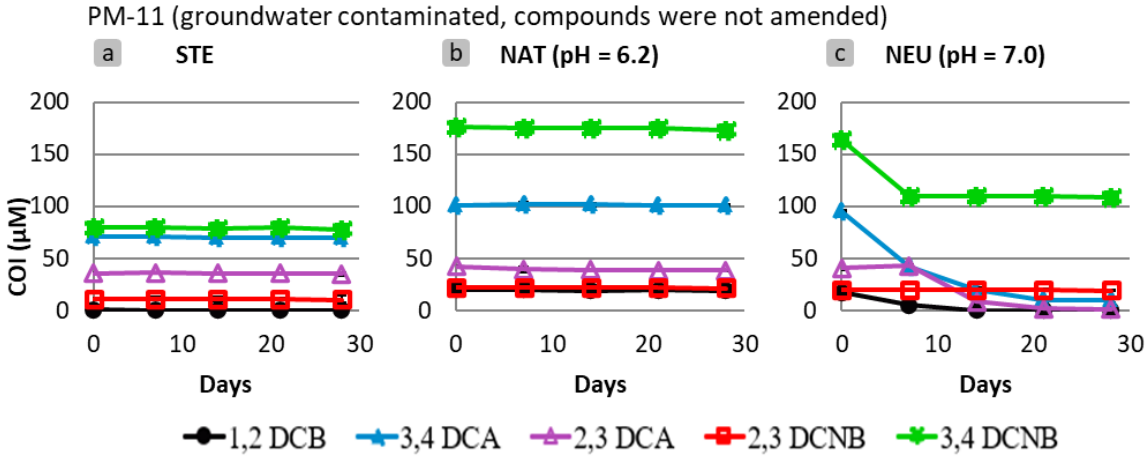
Figure 73C - Concentrations of contaminants in microcosms set with groundwater from PM-30 (compounds amended)



STE - Sterilized groundwater (autoclaved) (a), NAT - Natural pH with no adjustment (b), and NEU - Neutralized pH (7.0) with buffer solution made of K_2HPO_4 (1 M) (c).

Source: author (2022)

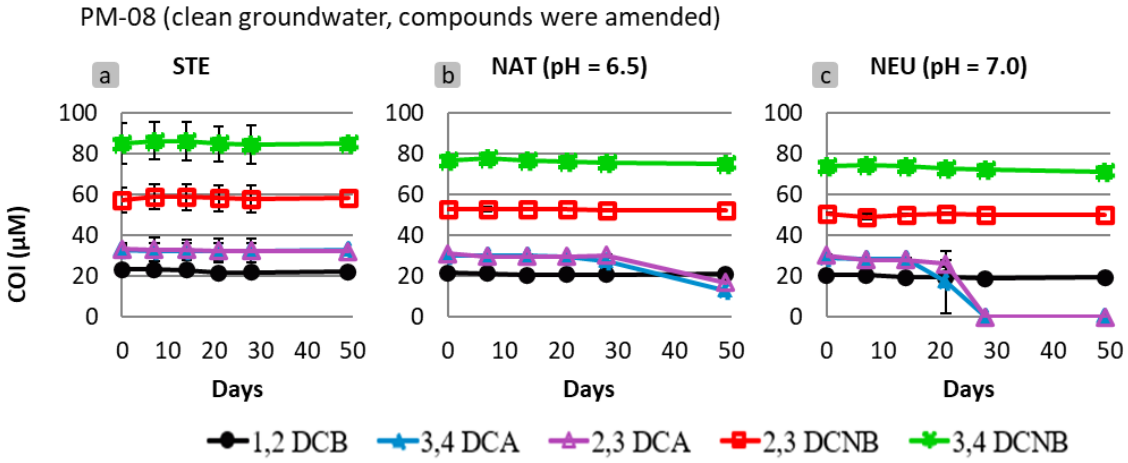
Figure 74C - Concentrations of contaminants in microcosms set with groundwater from PM-11 (already contaminated)



STE - Sterilized groundwater (autoclaved) (a), NAT - Natural pH with no adjustment (b), and NEU - Neutralized pH (7.0) with buffer solution made of K_2HPO_4 (1 M) (c).

Source: author (2022)

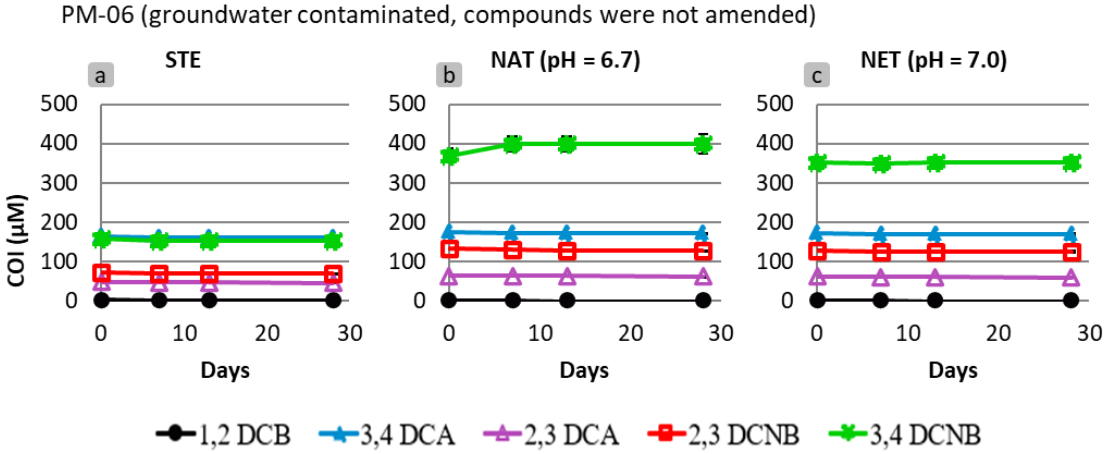
Figure 75C - Concentrations of contaminants in microcosms set with groundwater from PM-08 (compounds amended)



STE - Sterilized groundwater (autoclaved) (a), NAT - Natural pH with no adjustment (b), and NEU - Neutralized pH (7.0) with buffer solution made of K_2HPO_4 (1 M) (c).

Source: author (2022)

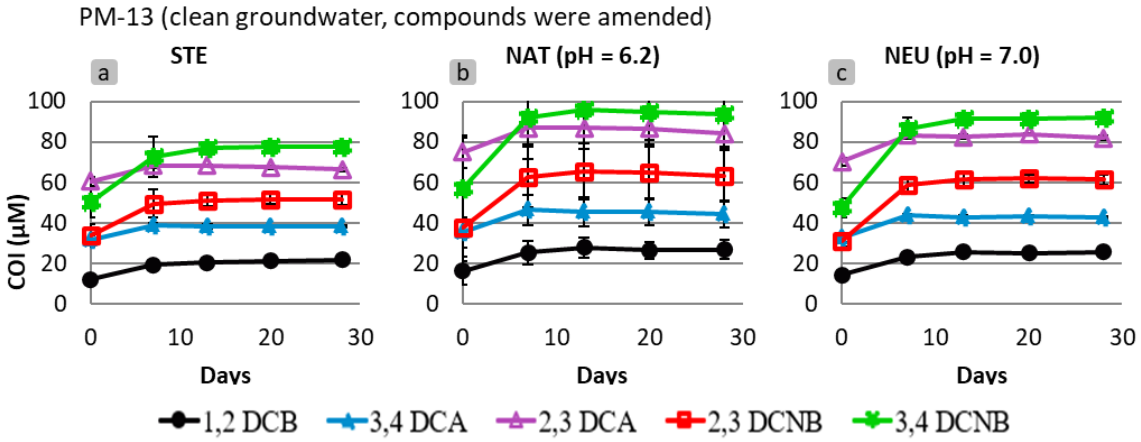
Figure 76C - Concentrations of contaminants in microcosms set with groundwater from PM-06 (already contaminated)



STE - Sterilized groundwater (autoclaved) (a), NAT - Natural pH with no adjustment (b), and NEU - Neutralized pH (7.0) with buffer solution made of K_2HPO_4 (1 M) (c).

Source: author (2022)

Figure 77C - Concentrations of contaminants in microcosms set with groundwater from PM-13 (compounds amended)



STE - Sterilized groundwater (autoclaved) (a), NAT - Natural pH with no adjustment (b), and NEU - Neutralized pH (7.0) with buffer solution made of K_2HPO_4 (1 M) (c).
Source: author (2022)

All the sterilized groundwater showed no changes in contaminants concentration, as expected, indicating that there were no abiotic losses happening in the microcosms. However, the initial concentration of some contaminants in the autoclaved groundwater for the sterile control (Figure 73a to 77a – STE condition) was lower, suggesting that the COI were lost through volatilization during the preparation of these microcosms.

In microcosms with groundwater from PM-30 (Figure 73), DCA isomers were not completely degraded in 35 days under natural pH (Figure 73b, NAT condition). With the pH adjusted to 7.0, in NEU (Figure 73), concentration of both DCA isomers decays to zero in 15 days in NEU, indicating that the pH adjustment improved the activity. In this groundwater sample, microbes were not able to degrade the DCNB isomers and the DCA even after 118 days (not shown).

The microbial community from the groundwater collected from PM-11 (Figure 74) was able to decrease the contamination of the DCB, the DCA isomers and part of the 3,4-DCNB with pH adjustment (Figure 74c – NEU condition).

Microbes from PM-08 (Figure 75) were able to remove some of both isomers of DCA from groundwater (Figure 75b), but when the pH was adjusted (Figure 75c), the removal occurred faster and complete.

Microcosms set with groundwater from PM-06 (Figure 76) and PM-13 (Figure 77) were not active for the contaminants and conditions tested. The 2,3-DCNB was not degraded in any of the microcosms of this test.

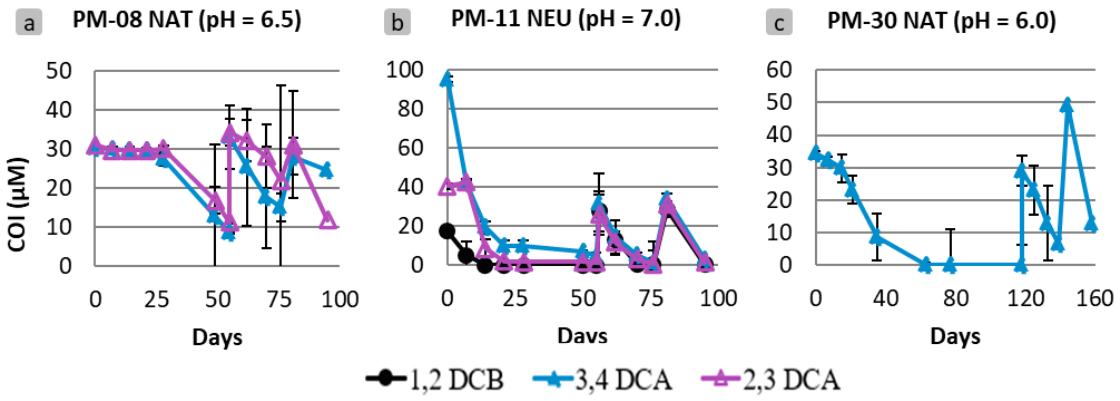
A summary of what was observed from the microcosms in presented in Table 16. The active microcosms which removed any of the compounds were then re-amended; after that, if activity was again observed in these microcosms (Table 16, highlighted in red boxes) DNA samples were collected and sequenced. Any culture was able to degrade all five contaminants together. The activity of the microcosms re-amended and sampled for DNA sequencing is presented in the Figure 78.

Table 16C - Summary of the microcosms' activity

WELL Natural pH Spiked?	**COI degraded?** **In which conditions?**				
	1,2-DCB	**3,4-DCA**	**2,3-DCA**	**3,4-DCNB**	**2,3-DCNB**
PM-08 6.5 yes	No	Yes NAT NEU	Yes NAT NEU	No	No
PM-11 6.2 no	Yes NEU	Yes NEU	Yes NEU	Yes NEU	No
PM-13 6.2 yes	No	No	No	No	No
PM-06 6.7 no	No	No	No	No	No
PM-30 6.0 yes	No	Yes NAT NEU	Yes NAT NEU	No	No

Source: author (2022)

Figure 78C - Concentrations of contaminants in active and re-amended microcosms, sampled for DNA sequencing



Microcosms PM-08 NAT (a), PM-11 NEU (b), and PM-30 NAT (c).
Source: author (2022)

ENRICHMENT CULTURES

As we did not see significant degradation of all five target contaminants in the first tests (with soil and groundwater), enrichment cultures from another source of microbes were cultivated aiming to obtain a positive control able to degrade the COI in the following tests. The enrichments were composed of the inoculum (source of microbes), phosphate buffer in boiled tap water (K_2HPO_4 - 1M), and contaminants with an initial feeding concentration in the range of 5 mg/L (34 µM), 5 mg/L (31 µM), 10 mg/L (52 µM), and 15 mg/L (78 µM) for DCB, DCA isomers, 2,3-DCNB, and 3,4-DCNB, respectively. Enrichments were set in 1 L glass bottles and aerated through manually mixing and by letting the bottles open inside a fumehood, at least once per week.

Among many sources of microbes tested, two are included here:

- E-CS: 100 mL of sludge from the wastewater treatment plant of the industrial complex in 900 mL of buffer solution
- E-DS: sediment collected from a contaminated equipment installed at the site in 1 L of buffer solution

Findings:

Figure 79 shows the result of the two mentioned enrichments (E-CS and E-DS). E-CS (Figure 79a) was first amended with 2,3-DCNB only (20 mg/L) until day 100, and the contaminant

was rapidly reduced into 2,3-DCA even with the aeration provided. For this reason, around day 30, we started aerating, at least 3 times a week, and the reduction of the nitro group got slower. On day 75 a magnetic stirring bar was added into the bottle, which was transferred to a magnetic plate, to improve aeration.

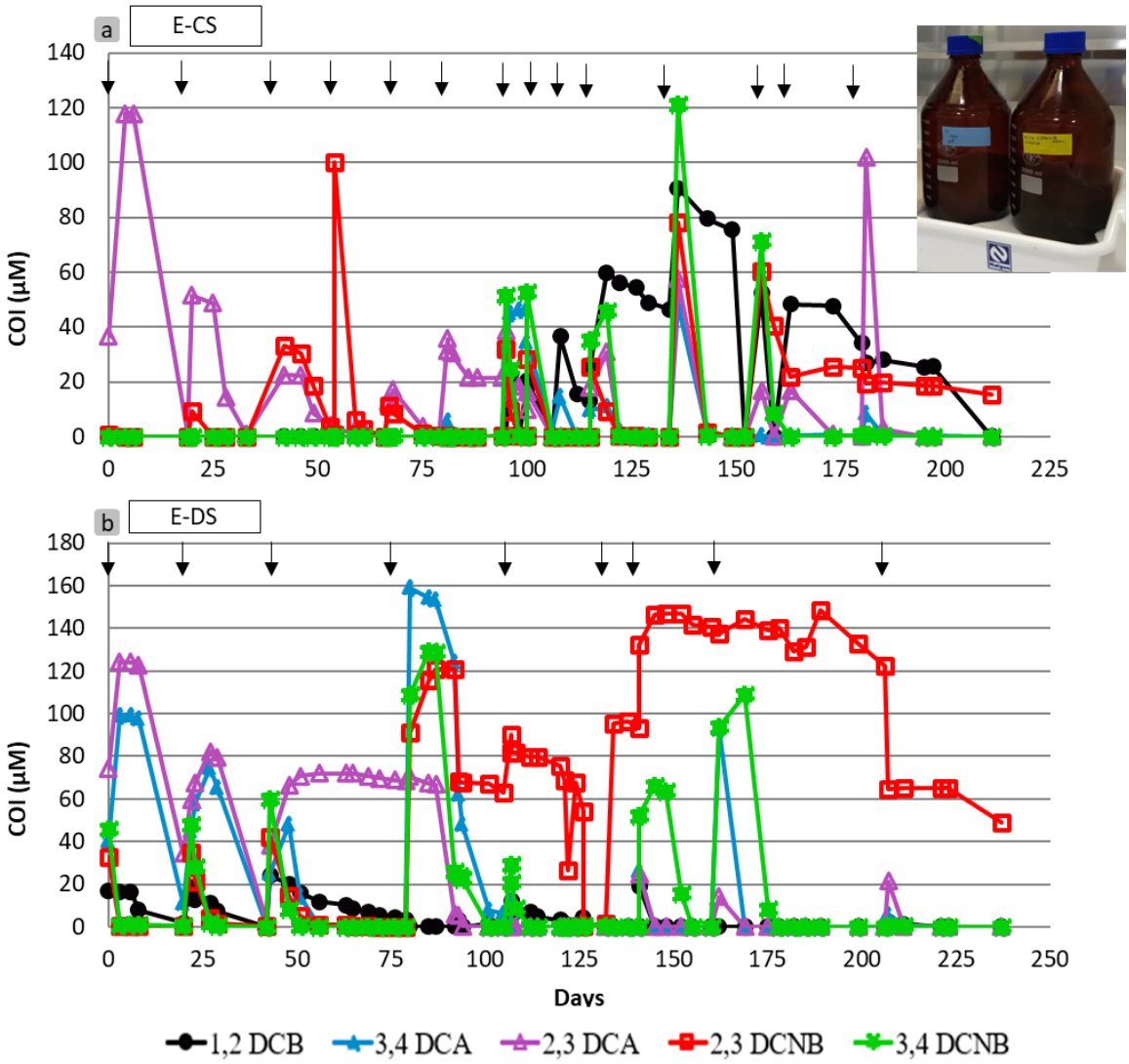
As of day 100, all target contaminants (1,2-DCB, 2,3- and 3,4-DCA and 2,3- and 3,4-DCNB) were added as feeding substrate in the range mentioned previously, and as of day 125, we doubled the contaminants' feeding concentration. The decrease of the DCB concentration was not as consistent as the other compounds'; even though, the concentration of all five contaminants was decreased to zero multiple times, indicating that the microbial community was able to biotransform the target compounds. As the monochloroaniline isomers were not detected when concentration of the DCA isomers decreased, it is possible that complete oxidation of the contaminants was taking place. The 2,3-DCNB, however, was not biotransformed when intense aeration was provided (as of day 125)

The second enrichment, named E-DS (Figure 79b), was initially aerated once a week. E-DS was amended with all five contaminants since the beginning and it was able to reduce both DCNB isomers, forming the respective DCA isomers, which were then potentially mineralized through oxidative process. Similarly to what was applied to E-CS, on day 75, E-DS was transferred to the magnetic plate and a stirring bar was dropped into the bottle in improve aeration. After that, 2,3-DCNB accumulated in the enrichment.

The differences in the behaviour observed in both enrichments when more intense aeration was provided indicates that the aerobic microbes able to biotransform the 2,3-DCNB were not present in the enrichment or not able to use the isomer. On the other hand, it suggests the presence of organisms able to reduce the nitro group from the contaminant under less aerated condition.

After achieving biotransformation of contaminants with the enrichment cultures, samples for DNA sequencing were collected from both enrichments on the last sampling day presented in Figure 79 and other tests using them as inocula were planned. The enrichments were used as inoculum for bioaugmentation tests with soil (10 g and 1 Kg) and water (100 mL) under aerobic versus anaerobic conditions and under different pH values. The tests are briefly discussed in the following topics.

Figure 79C - Concentrations of contaminants in E-CS (a) and E-DS (b)



E-CS: 100 mL of sludge from the wastewater treatment plant of the industrial complex in 900 mL of buffer solution, E-DS: sediment collected from a contaminated equipment installed at the site in 1 L of buffer solution. Days when COI were added (↓).

Source: author (2022)

AEROBIC TEST WITH SOIL (BIOAUGMENTATION) – MICROCOSMS CONTAINING 10 G OF SOIL

The E-CS was then used as inoculum in a soil test composed of 10 g of two different mixtures of soil samples (mix of soil 1 and mix of soil 2 – with higher contamination) added to 40 mL glass vial. For that, six different conditions were tests to verify the activity of the inoculum (E-

CS) in contaminated soil, based on inoculated and non-inoculated soil, different moisture contents, pH values, and contamination levels:

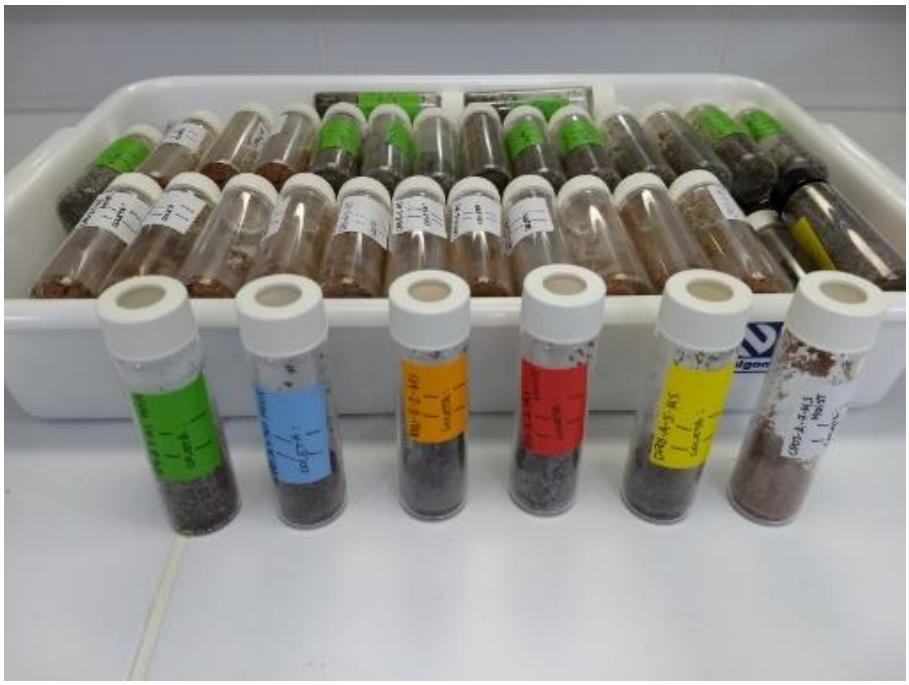
- STE: Autoclaved soil (18% of moisture content; mixture of soil 1)
- NEU: Soil + phosphate buffer (pH 7.0) (18% of moisture content; mixture of soil 1)
- NAT.E-CS: Soil + E-CS (pH 6.2) (18% of moisture content; mixture of soil 1)
- NEU.E-CS: Soil + phosphate buffer + E-CS (pH 7.0) (18% of moisture content; mixture of soil 1)
- DNEU.E-CS: Soil + phosphate buffer + E-CS (pH 7.0) (14% of moisture content; mixture of soil 1)
- CNEU.E-CS: Soil + phosphate buffer + E-CS + COI in higher concentration (pH 7.0) (15% of moisture content; mixture of soil 2)

The soil samples used for this test were mixed in a way we had two different mixtures: mixture of soil 1 had lower concentration of contaminants than mixture of soil 2. The buffer used in this test was the same from previous tests (K_2HPO_4 - 1M). Microcosms were set in 16 replicates for each of the 6 conditions tested. In addition, 2 extra vials for each condition were set for moisture content measurement.

The 2 mixtures of soil were first prepared and split in glass bottles for each condition: 180 g for each of the six conditions. Then, we proceeded to the conditions' preparation. The amount of 10 mL of E-CS was added to the conditions with enrichment (NAT.E-CS, NEU.E-CS, DNEU.E-CS, and CNEU.E-CS) and 10 mL of buffer was added to adjust the pH to 7 (NEU, NEU.E-CS, DNEU.E-CS, and CNEU.E-CS). In those microcosms without buffer or enrichment, we added the corresponding amount of sterilized water, to ensure the same moisture content for all conditions; STE microcosms received 20 mL of water, while NAT.E-CS and NEU.E-CS received 10 mL each. The dry condition (DNEU.E-CS) was set by letting the 180 g of soil open in the fumehood for 5 hours.

On each sampling day, the full mass of soil from 2 vials from each condition was sacrificed to be analyzed by the HPLC method. Figure 80 shows the microcosms set for this test.

Figure 80C - Soil test in 40 mL with 10 g of soil and E-CS



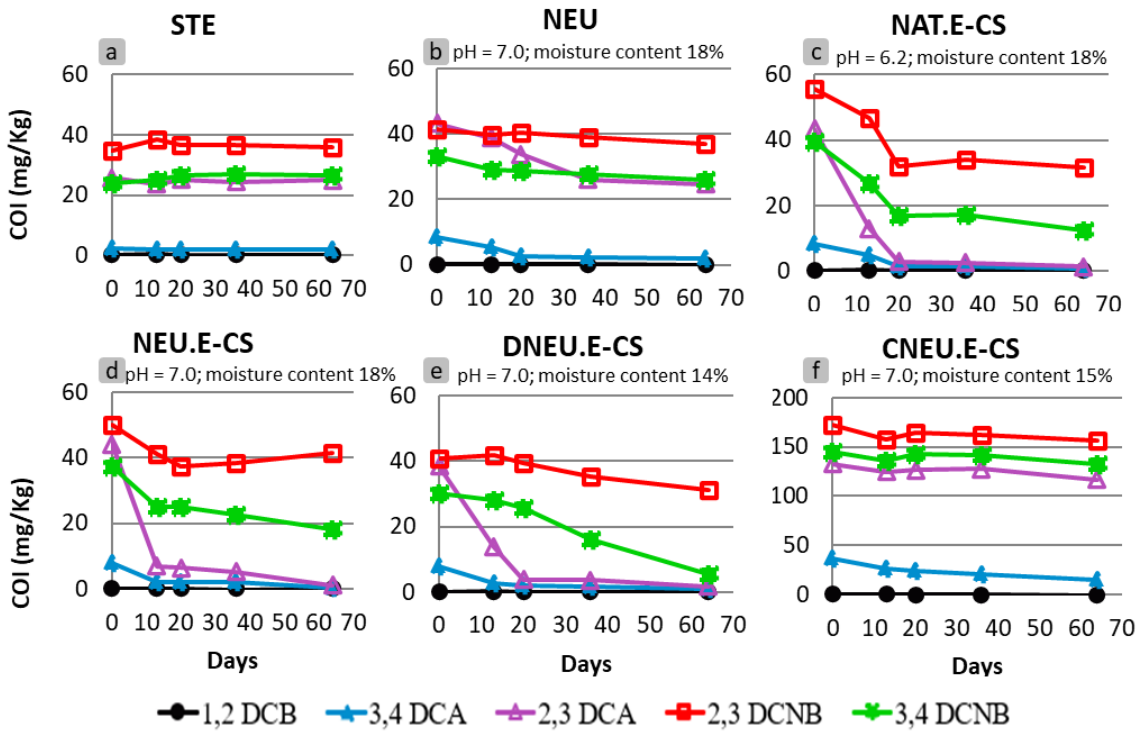
Source: author (2022)

Findings:

Figure 81 shows the results obtained from each of the 6 conditions tested. As expected, the autoclaved soil (STE) did not show activity related to transformation of COI (Figure 81a). In NEU, with soil and buffer (best conditions found from previous tests), but not inoculated with the E-CS, only the concentration of 2,3-DCA was slightly decreased, indicating that the microbial community present in mix of soil 1 was not able to biotransform the COI (Figure 81b).

The results obtained from NAT.E-CS, composed of the same mix of soil and the enrichment E-CS, showed promising activity of the inoculum as the concentration of the DCNB and DCA isomers was decreased significantly, the latter being decreased to zero in the first 20 days (Figure 81c). As in NAT.E-CS, similar results were observed in NEU.E-CS and DNEU.E-CS, meaning that the addition of the buffer (NEU.E-CS) and the different moisture content tested in DNEU.E-CS (14%) did not influence the activity of the enrichment in the soil mixture (Figures 81d and 81e). Finally, when contaminants were in higher concentration (CNEU.E-CS), the activity of the E-CS was not enough to provide biotransformation, thus, in CNEU.E-CS, the bioaugmentation did not work (Figure 81f). Among all compounds, the 2,3-DCNB was again the most persistent, which was stable in all conditions.

Figure 81C - Contaminants concentrations in microcosms set with soil (bioaugmentation test)



STE – Autoclaved soil (18% of moisture content; mixture of soil 1) (a), NEU: Soil + phosphate buffer (pH 7.0) (18% of moisture content; mixture of soil 1) (b), NAT.E-CS: Soil + E-CS (pH 6.2) (18% of moisture content; mixture of soil 1) (c), NEU.E-CS: Soil + phosphate buffer + E-CS (pH 7.0) (18% of moisture content; mixture of soil 1) (d), DNEU.E-CS: Soil + phosphate buffer + E-CS (pH 7.0) (14% of moisture content; mixture of soil 1) (e), and CNEU.E-CS: Soil + phosphate buffer + E-CS + COI in higher concentration (pH 7.0) (15% of moisture content; mixture of soil 2) (f).

Source: author (2022)

AEROBIC TEST WITH SOIL (BIOAUGMENTATION) – MESOCOSMS CONTAINING 1 KG OF SOIL

After verifying the activity of the E-CS in 10 g of soil for some of the COI, we then aimed to scale up the test to 1 Kg of soil. The objective of this test was to check the activity of both enrichments (E-CS, from wastewater treatment plant, and E-DS, from sediment collected at the industrial site) in a bigger pile of soil collected from the site. Checking activity of E-DS was important since this culture was enriched from sediment collected at the site, thus making the bioaugmentation strategy more feasible, considering the steps the industry need to face to get the approval from the environmental protection agencies.

Three mesocosms were set in 4 L glass bottles with autoclaved and contaminated soil (Figure 82):

- STE – Sterile contaminated soil (1 Kg) + 50 mL of sterile water
- E-DS - Sterile contaminated soil (1 Kg) + 50 mL of E-DS
- E-CS - Sterile contaminated soil (1 Kg) + 50 mL of E-CS

The initial pH of the three mesocosms was 6.5, the moisture content was 13%. All the bottles were manually mixed and aerated by letting them open inside the fumehood for 1 hour every day. Differently from the previous tests with soil, for this test, 10 g of soil were sampled from the 1 Kg of each bottle for the HPLC analysis.

Figure 82C - Mesocosms with 1 Kg of soil

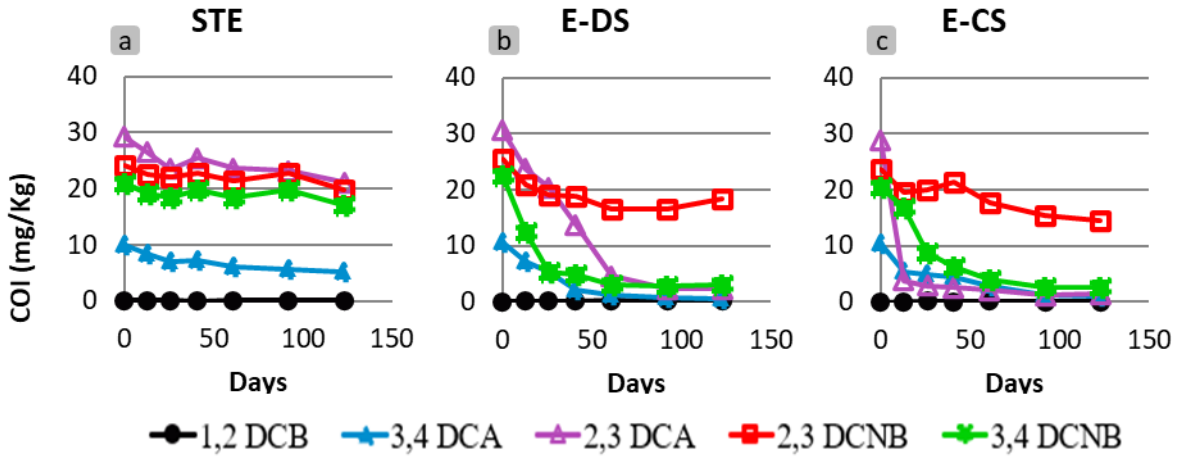
Source: author (2022)

Findings:

The mesocosms were monitored for almost 150 days (Figure 83). The autoclaved soil (STE) did not show any changes in contaminants concentrations (Figure 83a). The mesocosms bioaugmented with E-DS and E-CS showed similar behaviour. Both isomers of DCA and the 3,4-

DCNB were biotransformed with no subproduct being detected. However, the 2,3-DCNB was persistent for the 2 microbial communities (Figure 83b and 83c).

Figure 83C - Concentration of contaminants in of mesocosms set with soil (bioaugmentation test)



STE – Sterile contaminated soil (1 Kg) + 50 mL of sterile water (a), E-DS - Sterile contaminated soil (1 Kg) + 50 mL of E-DS (b), and E-CS - Sterile contaminated soil (1 Kg) + 50 mL of E-CS (c).
Source: author (2022)

ANAEROBIC VERSUS AEROBIC TESTS IN WATER WITH ACTIVE ENRICHMENTS – MICROCOSMS CONTAINING 100 ML OF WATER AND ENRICHMENT

Given the differences in the behaviour observed when the enrichment cultures were submitted to manual versus intense aeration (once per week versus kept in a magnetic plate with a magnetic stirring bar), an anaerobic versus aerobic test in clean water was performed. The objective of this test was then to evaluate the activity of the E-CS related to biotransformation of contaminants when it is submitted to aerobic and anaerobic conditions.

The conditions tested follow:

- ANAE - STE-NE: Mineral medium (180 mL), ethanol as the electron donor (20 μL), COI, clean tap water (20 mL)
- ANAE – ENR: Clean tap water (180 mL), E-CS (20 mL), COI
- ANAE - ENR-N: Mineral medium (180 mL), E-CS (20 mL), COI
- ANAE - ENR-NE: Mineral medium (180 mL), ethanol as the electron donor (20 μL), E-CS (20 mL), COI
- ANAE - ENR-E: Clean tap water (180 mL), ethanol as the electron donor (20 μL), E-CS (20 mL), COI
- AE - STE Clean tap water (200 mL), COI
- AE - ENR Clean tap water (180 mL), E-CS (20 mL), COI

The anaerobic control and microcosms were set in triplicate, in boston bottles of 250 mL containing 200 mL of liquid composed of clean tap water boiled and/or mineral medium solution (Edwards & Grbic-Galic, 1994) and/or enrichment cultures. The aerobic control and microcosm were set in one only bottle The anaerobic bottles (ANAE) were kept inside the glovebox (atmosphere composed of N_2:CO_2:H_2 in the proportion of 80%:10%:10%); while the aerobic ones (AE) were kept in a magnetic plate with a stirring magnetic bar to ensure agitation (Figure 84). Contaminants were added in the range of 3 mg/L (23 μM), 3 mg/L (21 μM), 7 mg/L (35 μM), and 10 mg/L (52 μM) for DCB, DCA isomers, 2,3-DCNB, and 3,4-DCNB, respectively.

Figure 84C - Anaerobic and aerobic microcosms and control bottles

Source: author (2022)

Findings:

The concentration of the contaminants added into the microcosms was monitored during almost 2 months by measuring the COI, ethanol and the acetate (Figures 85, 86, 87, 88, and 89). The anaerobic sterile control with nutrients and electron donor (ANAE-STE-NE) did not have any activity during the monitoring period; in this microcosm, concentration of the COI and ethanol was stable, and acetate was not formed (Figure 85).

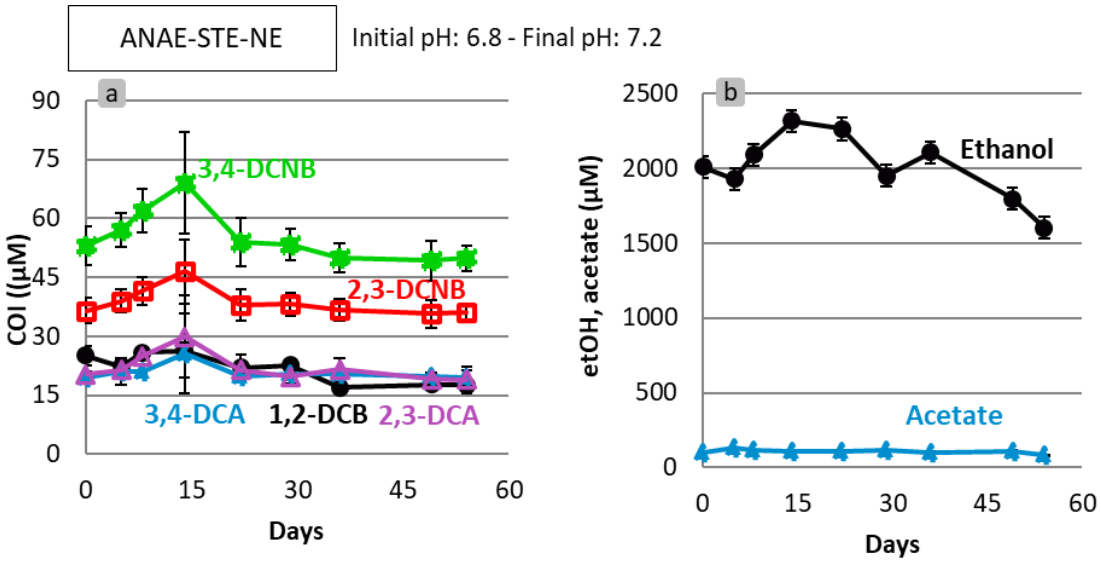
From the other four anaerobic microcosms (ANAE-ENR-NE ANAE-ENR-E, ANAE-ENR-N, and ANAE-ENR), it could be verified that the E-CS was able to reduce both dichloronitrobenzene isomers (2,3- and 3,4-DCA) into their respective DCA isomer. Enrichment was not able to reduce DCA isomers anaerobically, which accumulated in all the anaerobic bottles. The DCB was not transformed in any of those anaerobic conditions. Faster DCNB reduction under anaerobic conditions were found in NE (where enrichment, electron and mineral medium were provided) (Figures 86a, 87a, and 88a-b) The ethanol added as electron donor in ANAE-ENR-NE and ANAE-ENR-E was converted into acetate by the microbial community (Figures 86b and 87b).

The aerobic sterile control (AE-STE), there was no change in the contaminant's concentration. In the aerobic microcosms (AE-ENR), the DCA isomers' and 3,4-DCNB concentration were decreased to zero in less than 10 days, 2,3 DCNB concentration decayed, but

not completely until the end of the experiment. Under aerobic condition, DCB was decreased to zero in less than 5 days, but it was lost in the sterile control in less than 15 days (Figure 89b).

Results indicate that the enrichment E-CS has different mechanisms to deal with contaminants in the presence and in the absence of free oxygen, as it was hypothesized from previous tests. The 3,4-DCNB is biotransformed under both conditions but forming DCA without oxygen in the medium. E-CS was proven to be able to reduce the nitro group from the DCNB isomers under anaerobic conditions, but it was not able to deal with the 2,3-DCNB when oxygen was available. On the other hand, the DCA isomers and the DCB accumulated when oxygen was not in the medium. The active bottles from this experiment were filtered for DNA 16S sequencing.

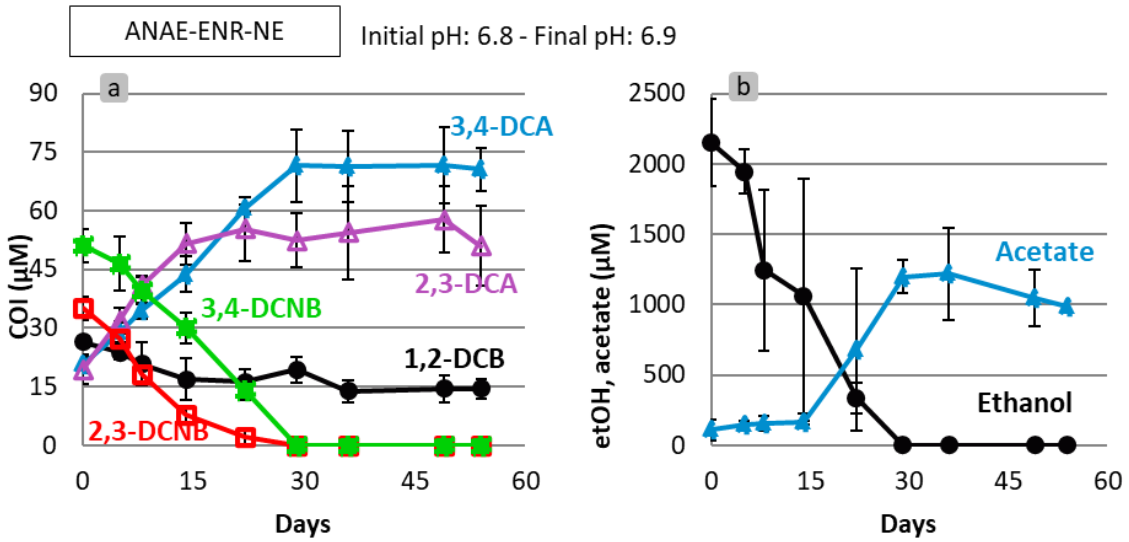
Figure 85C - Concentrations of the contaminants (a), ethanol and acetate (b) in ANAE-STE-NE



ANAE-STE-NE contains: Mineral medium (180 mL), ethanol as the electron donor (20 µL), COI, clean tap water (20 mL).

Source: author (2022)

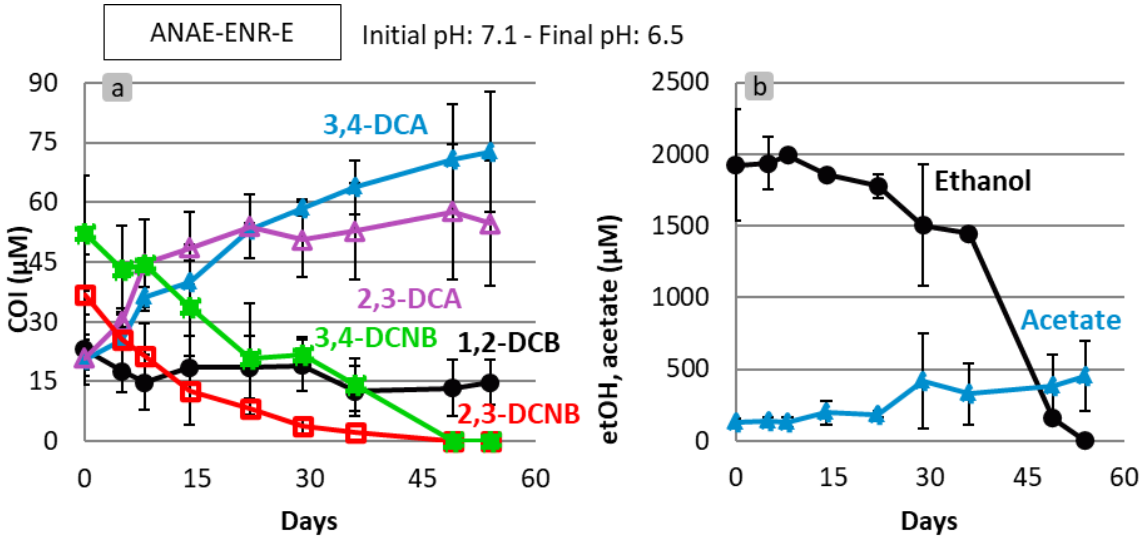
Figure 86C – Concentrations of the contaminants (a), ethanol and acetate (b) in ANAE-ENR-NE



ANAE-ENR-NE contains: Mineral medium (180 mL), ethanol as the electron donor (20 µL), E-CS (20 mL), COI.

Source: author (2022)

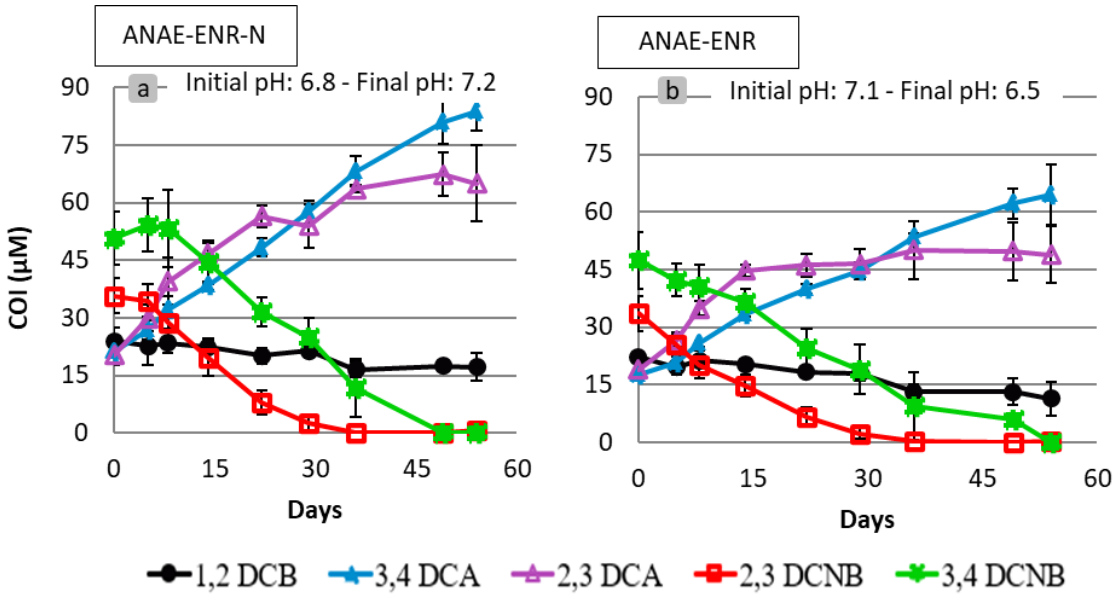
Figure 87C – Concentrations of the contaminants (a), ethanol and acetate (b) in ANAE-ENR-E



ANAE-ENR-E contains: Clean tap water (180 mL), ethanol as the electron donor (20 µL), E-CS (20 mL), COI.

Source: author (2022)

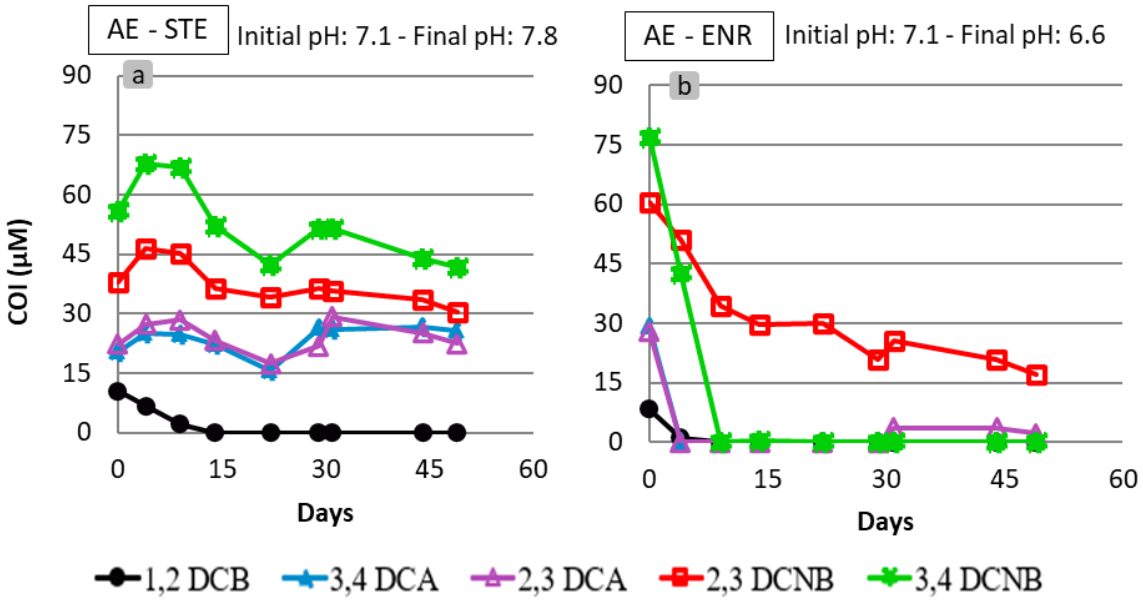
Figure 88C – Concentrations of the contaminants in ANAE-ENR-N (a) and ANAE-ENR (b)



ANAE-ENR-N contains: Mineral 194édium (180 mL), E-CS (20 mL), COI; ANAE-ENR contains: Clean tap water (180 mL), E-CS (20 mL), COI.

Source: author (2022)

Figure 89C – Concentrations of the contaminants in AE-STE (a) and AE-ENR (b)



AE-STE contains: Clean tap water (200 mL), COI; AE-ENR contains: Clean tap water (180 mL), E-CS (20 mL), COI.

Source: author (2022)

AEROBIC PH TEST WITH ENRICHMENT – MICROCOSMS CONTAINING 100 ML OF WATER AND ENRICHMENT

The last experiment performed at the industrial site was an activity test of the E-CS under different pH values. The objective of this test was to evaluate the possibility of using this inoculum for bioaugmentation in areas where the pH is lower than 7.0.

Aiming that, a set of 5 microcosms were inoculated with 20 mL of E-CS in 80 mL of boiled and clean tap water further amended with the five COI. The bottles were kept in a magnetic plate for agitation (Figure 90).

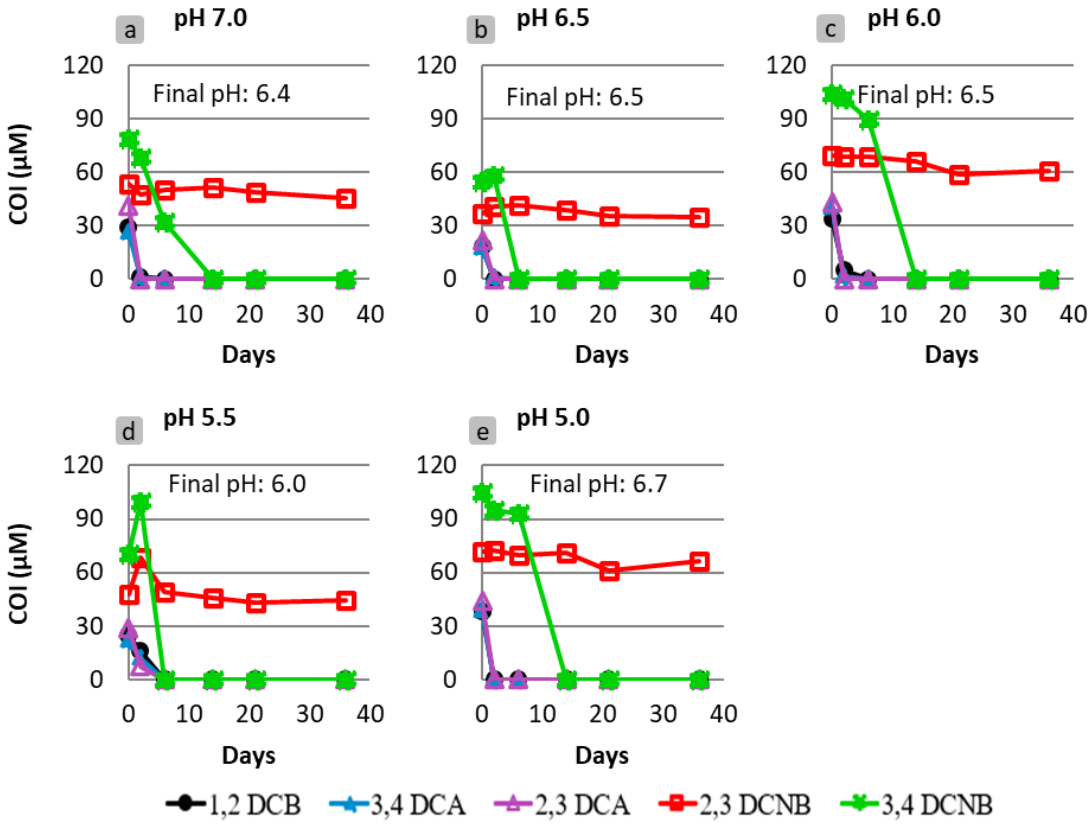
Figure 90C - Microcosms set for activity test of E-CS under different initial values of pH

Source: author (2022)

Findings:

Excepting the 2,3-DCNB, the enrichment E-CS used as inoculum for this experiment was able to biodegrade the contaminants of interest under the different initial pH values tested (Figure 91). Thus, the culture could be used as source of active bacteria able to degrade the 1,2-DCB, the DCA isomers and the 3,4-DCNB in areas at the site where the pH is in the range of 7.0 to 5.0.

Figure 91C - Concentrations of contaminants in E-CS under initial pH values adjusted to 7.0 (a), 6.5 (b), 6.0 (c), 5.5 (d), and 5.0 (e)



Microcosms were inoculated with 20 mL of E-CS in 80 mL of boiled and clean tap water further amended with the five COI.

Source: author (2022)

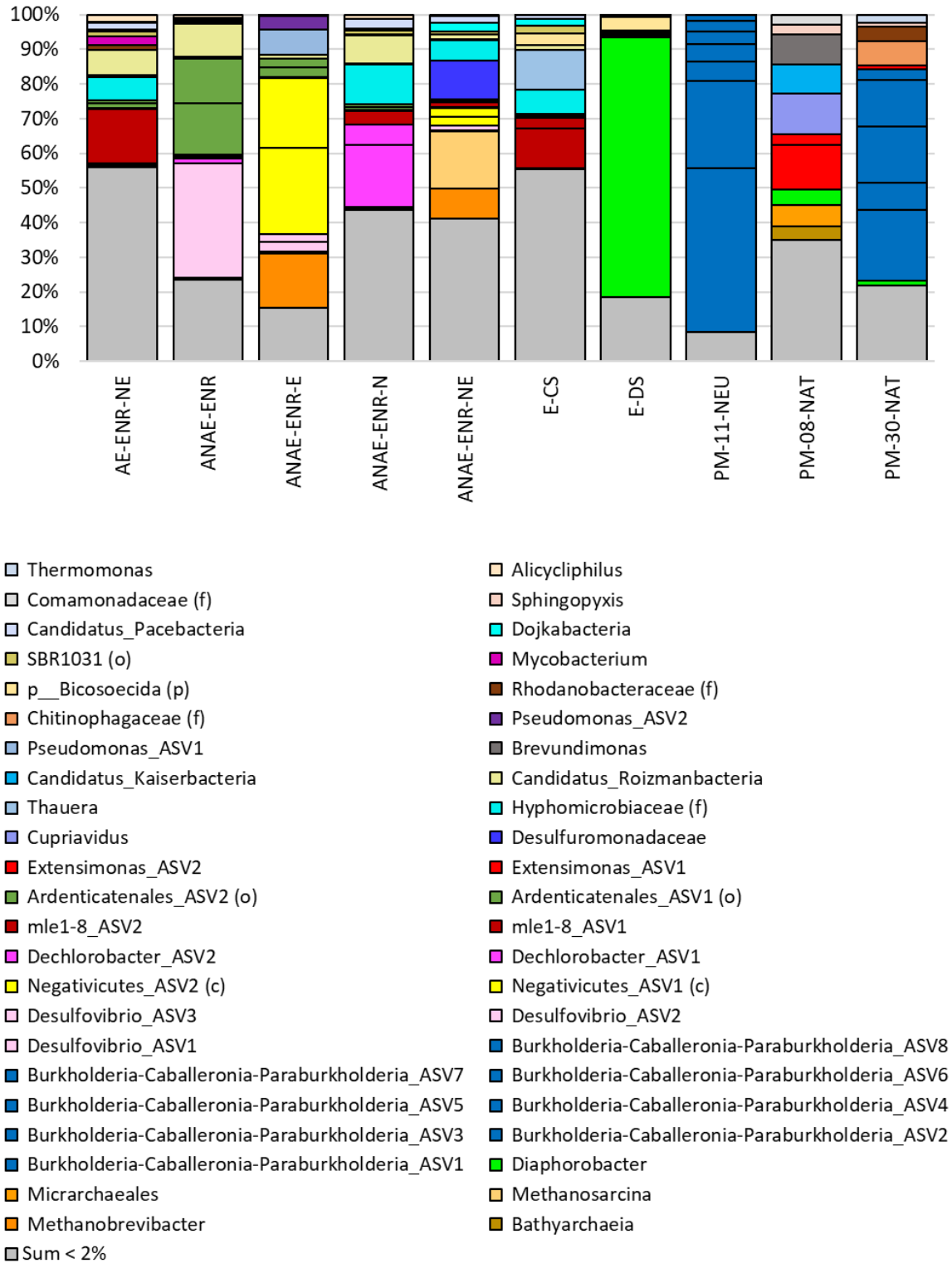
MICROBIAL COMMUNITY FROM 16S SEQUENCING

The samples for DNA sequencings were collected from microcosms/enrichment on their last day of the monitoring period. The following 10 samples were sequenced:

- Microcosms from aerobic test with groundwater: PM-30-NAT; PM-08-NAT; PM-11-NEU (3 samples)
- Enrichments: E-CS and E-DS (2 samples)
- Microcosms from anaerobic versus aerobic test: ENR; ENR-N; ENR-NE; ENR-E; AE-ENR (5 samples)

The DNA data processing and the Figure 92 were made by Line Lomheim (UofT).

Figure 92C - Microbial community sequenced from DNA samples



Source: elaborated by Line Lomheim (UofT)

Findings:

The microbial community observed in the aerobic tests with groundwater in PM-11-NEU and PM-30-NAT is mainly composed of *Burkholderia-Caballeronia-Paraburkholderia*. This group was highly enriched in aerobic microcosms discussed in section 4.1 and likewise in here, it was likely to be responsible for the degradation of DCA and MCA.

A more diverse community was observed in the result from PM-08-NAT. However, the genus *Diaphorobacter*, present in the community, was previously isolated from a microbial source from the same contaminated site by Palatucci *et al.*, (2019). The authors reported the genus as degrader of dichloronitrobenzene (2,3-, and 3,4- isomers). The same genus was predominant in the enrichment E-DS; the culture enriched from the sediment collected at the site, which was able to degrade 1,2-DCA, DCA isomers, and 3,4-DCNB.

The microbial community sequenced from the enrichment E-CS (inoculated with sludge from the wastewater treatment plant of the industrial complex) and from the microcosms inoculated with it (ANAE-ENR-NE, ANAE-ENR-N, ANAE-ENR-E, ANAE-ENR, and AE-ENR-NE) presented higher diversity. The AE-ENR-NE (aerobic microcosms) had the most similar community to the E-CS; and both had more than 50% of the community composed of organisms grouped as lower than 2% in relative abundance. When the E-CS was submitted to anaerobic conditions, some of these organisms were enriched, including some archaea and some bacteria from the genus *Desulfovibrio*, *Dechlorobacter*, and *Negativicute*s. The significant difference in the microbial composition found in the aerobic and anaerobic microcosms inoculated with the same enrichment (E-CS) explains the difference in the biodegradation behaviour observed in the tests when intense aeration was provided to the cultures.