Exploring Antibiotic Biotransformation In Denitrifying Wastewater Treatment Processes

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Silvana Inés Quiton Tapia, MSc.

Aus *La Paz, Bolivia*

Berichter*:* *Universitätsprofessor Dr. Andreas Schäffer*

Universitätsprofessor Dr. Francisco Omil Prieto

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WIDMUNG

Pienso constantemente en mi abuela Clotilde mientras escribo mi tesis. Me imagino tenerla a lado mío cuando defienda mi tesis con éxito. Es como si fuera mi manera de honrarla. Pero también siento que es algo más profundo. No es solo por ella, sino por la niña que fuí cuando estaba con ella. Terminar esta tesis y llegar hasta el final es mi manera de decirle a esa niña que llegaría a cumplir retos grandes, que su esfuerzo no fue en vano y que, aunque el tiempo haya pasado, ella y su abuela celebran los logros cumplidos y se llenan de orgullo.

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Summary

Chapter 1: Introduction and main objectives

Antibiotic usage has fundamentally transformed modern healthcare by effectively treating human and animal infections, increasing agricultural productivity, and supporting global aquaculture. Yet, the rising global demand for antibiotics, often coupled with unregulated or excessive application, has led to accumulating concentrations in the environment and the proliferation of antibiotic resistance genes in aquatic ecosystems. A significant fraction of these compounds, excreted as parent compounds or as partially active transformation products, persists in wastewater. Furthermore, conventional wastewater treatment plants (WWTPs), primarily designed for nutrient and organic load removal, only partially eliminate many of these organic micropollutants (OMPs). The overuse and increasing antibiotic consumption and uncomplete treatment result in a widespread antibiotic presence across all water cycle (surface water, treated effluents, groundwater).

Antibiotic concentrations in aquatic environments exhibit considerable variability, primarily due to differences in consumption patterns, access to antibiotics, the biodegradability of each molecule, and the removal efficiencies of full-scale WWTPs. While WWTPs function as a frontline defense against antibiotic dispersion to the environment, they are recognized as the main source of antibiotics into the water cycle. The removal efficiency of each OMPs, including antibiotic in WWTPs, depends on the intrinsic molecular characteristics of the molecule, their interactions with the surrounding environment, and the specific type of treatment process employed. Therefore, enhancing water and sanitation infrastructures is crucial for reducing antibiotic pollution, given the pivotal role that WWTPs play in mitigating the release of these compounds into the environment.

Biological nutrient treatment, primarily through activated sludge processes, is a fundamental component of WWTPs and drives most biotransformation mechanisms. Biotransformation occurs either metabolically or cometabolically, with cometabolic processes being the predominant mechanism in full-scale WWTPs. A common approach to enhance cometabolic biotransformation of OMPs involves increasing the concentration of a growth substrate, as demonstrated by several studies on heterotrophic and nitrifying activated sludge systems. While extensive research has explored antibiotic biodegradation under aerobic conditions, the pathways, rates, and key factors influencing micropollutant removal under anoxic conditions, especially through denitrification, are less well understood. Denitrification, a crucial biological nutrient removal process, provides an alternative electron acceptor pathway that may facilitate cometabolic antibiotic degradation.

However, the complex interactions between operational parameters, microbial community dynamics, enzymatic activities, and the fate of micropollutants under varying redox states remain unclear.

Addressing these knowledge gaps is essential for optimizing treatment processes, ensuring compliance with increasingly stringent regulations, and preventing the global spread of antibiotic resistance. Additionally, understanding the formation of transformation products during denitrifying conditions is vital, as these intermediates can retain antimicrobial properties or exhibit new toxic effects. Comprehensive insights into these processes will enable more effective wastewater treatment strategies and mitigate the environmental impact of antibiotic pollution.

This doctoral research aims to address these challenges by investigating antibiotic biotransformation under denitrifying conditions. The work focuses on three main objectives: (1) assessing cometabolic biotransformation by increasing denitrifying activity through increasing nitrogen loading rates (NLRs), (2) elucidating how microbial community shifts and enzymatic responses influence antibiotic biotransformation, and (3) understanding the fate and transformation pathways of antibiotics and their metabolites during heterotrophic denitrification systems. By examining these aspects, this thesis seeks to offer new insights into the microbial and operational factors that govern antibiotic removal, ultimately contributing to more effective wastewater treatment strategies and reduced environmental impacts.

Chapter 3: Microbial Community Shifts and Cometabolic Limitations in Heterotrophic Denitrification

This chapter investigates whether increasing nitrogen loading rates (NLRs) in a heterotrophic denitrification system enhances antibiotic removal through cometabolism and examines how long-term shifts in microbial community structure affect removal efficiencies. Although heterotrophic denitrification is widely applied in wastewater treatment, its biotransformation mechanisms remain only partially understood. For this purpose, two continuous-flow denitrifying reactors, with and without antibiotics feeding were operated at long-term (262 days) in an analogous way. Results showed that a progressive increase in the NLR from 25 ± 6 mg $N–NO_3^-$ L^{-1} d^{-1} to 277 ± 38 mg $N–NO_3^-$ L^{-1} d^{-1}, did not result an increase in OMP removal following a cometabolic tendency despite the increase in denitrifying activity. Rather, the high NLR favored fast-growing denitrifiers, which reduced the prevalence of specialized taxa crucial for antibiotic biotransformation. Functional and taxonomic richness thus proved most influential for effective OMP removal, with denitrification activity negatively correlating with antibiotic biotransformation. These observations reinforce that microbial community

composition, more than merely high denitrifying activities, governs the extent of antibiotic removal.

DNA metabarcoding analyses revealed that even low antibiotic concentrations (5 µg L^{-1}) significantly reshaped the microbial community, highlighting the critical role of community composition and diversity in OMP removal. Furthermore, taxa from the order *Rhizobiales* emerged as key contributors to antibiotic biotransformation under these conditions. However, when NLRs were increased, the relative abundance of Rhizobiales declined, and these populations were outcompeted by other taxa better adapted to high-load environments, leading to diminished antibiotic removal efficiencies. Overall, this work underscores the functional trade-offs between maintaining high NLRs for enhanced denitrification and preserving metabolic functions like antibiotic biotransformation. Understanding and balancing these trade-offs is essential to optimizing reactor performance under varying operational conditions.

Chapter 4: How efficiently does a metabolically enhanced system with denitrifying anaerobic methane oxidizing microorganisms remove antibiotics?

In this chapter, we investigate the novel nitrite-dependent anaerobic methane oxidation (N-Damo) process at high biomass activities for its potential to simultaneously remove nitrite, methane, and trace antibiotics commonly encountered in sewage. Two membrane bioreactors (MBRs) were operated at three elevated nitrite loading rates (NLRs),76 ± 9.9, 161.5 ± 11.4, and 215.2 ± 24.2 mg $N–NO_2$ L^{-1} d^{-1}, over prolonged periods, achieving specific denitrifying activities of up to 540 mg $N–NO_2$ g^{-1} VSS d^{-1}. Remarkably, these activities rival conventional heterotrophic systems in terms of nitrite removal rates. A key differentiator from previous studies was the addition of cerium as a trace element in the feeding medium, allowing stable reactor operation under these high loads.

Microbial community analyses showed that N-Damo bacteria (*Candidatus Methylomirabilis*) initially dominated both MBRs (67–87% relative abundance during different operational periods). However, as overall biomass activity and concentration rose, the dominance of these functional N-Damo populations declined. Concurrently, other minor families such as Hypomicrobiaceae and Xanthobacteraceae proliferated, indicating a shift in community structure.

Under these conditions, several antibiotics, including sulfamethoxazole, trimethoprim, cefalexin, and azithromycin, displayed high biotransformation rates. By contrast, roxithromycin and clarithromycin showed only partial removal (20–35%), and ciprofloxacin remained largely unaffected. Intriguingly, despite the elevated N-Damo activity and the novel operational strategy, no clear improvement

in antibiotic removal was observed over the course of the experiment. As noted in the heterotrophic denitrification systems examined in Chapter 3, these results underscore that microbial community composition exerts a more decisive influence on antibiotic removal than elevated denitrifying activity alone. Ultimately, managing and steering the microbial community structure emerges as critical for enhancing antibiotic biotransformation in N-Damo reactors.

Chapter 5: Enzymatic Underpinnings of N-Damo Systems Revealed by Metaproteomics

Building on the microbial shifts observed in Chapter 4, this chapter explores the enzymatic foundation of the nitrite-dependent anaerobic methane oxidation (N-Damo) process, a promising pathway for achieving carbon-neutral wastewater treatment in line with sustainable development goals. Utilizing metaproteomic analyses, we elucidated the complete enzymatic pathways of N-Damo, with a particular emphasis on metalloenzymes. The analyses confirmed that *Candidatus Methylomirabilis Lanthanidiphila* was the predominant N-Damo species, representing 67–87% of the relative protein abundance. This dominance was driven by the induction of its lanthanide-binding methanol dehydrogenase in the presence of supplemented cerium, highlighting the essential role of lanthanides in supporting high nitrogen removal rates.

Further metaproteomic data revealed the active involvement of accompanying taxa in denitrification, methylotrophy, and methanotrophy, demonstrating the synergistic interactions within the microbial community. The most abundant functional metalloenzymes identified required copper, iron, and cerium as cofactors, which correlated with the metal consumption patterns observed in the bioreactor. Despite the enhanced enzymatic activity supporting these core metabolic functions, there was no corresponding improvement in antibiotic removal efficiencies. This finding underscores that while optimizing micronutrient availability and enzymatic pathways can sustain robust N-Damo activity, effective antibiotic biotransformation likely requires targeting specific enzymes and metabolic routes directly involved in pollutant degradation.

Overall, this study highlights the utility of metaproteomics in evaluating enzymatic activities within engineered systems, providing critical insights for optimizing microbial management. By understanding the intricate balance between maintaining high N-Damo activity and preserving the metabolic functions necessary for antibiotic biotransformation, we can better design and operate wastewater treatment processes to achieve both efficient nutrient removal and pollutant degradation.

Chapter 6: Physico-Chemical Drivers of Antibiotic Fate and Transformation Pathways

The final experimental chapter explores the fate and transformation of antibiotics sulfamethoxazole (SMX) and triclocarban (TCC) under heterotrophic denitrification using radiolabeled techniques with two labelled molecules ^{14C}-SMX and ^{14C}-TCC. The aim of the study was to elucidate how the chemical structure and physicochemical properties of these micropollutants significantly influence their behavior in denitrifying environments.

TCC, characterized by its hydrophobic and neutral nature, exhibited persistent behavior under denitrifying conditions. Over a 14-day period, more than 59% of TCC sorbed onto sludge, remaining primarily in extractable forms with negligible mineralization. This high sorption likely limited TCC's bioavailability, thereby preventing extensive biotransformation. Additionally, extractable fractions of TCC declined over time, suggesting remobilization driven by chemical gradients and shear forces during mixing.

In contrast, SMX, a highly polar compound existing mostly as an anion, underwent rapid biotransformation with approximately 80% of the parent compound transformed within one day. However, mineralization of SMX remained low as it was converted into more polar and persistent metabolites that accumulated in the aqueous phase. These transformation products, potentially exhibiting antibiotic activity, highlight the insufficiency of focusing solely on parent compound removal. Moreover, extractable fractions for both SMX and TCC decreased over time, indicating remobilization of residues, particularly pronounced for TCC due to its high presence in the extractable fraction. Non-extractable residues (NER) also showed remobilization, especially for SMX, underscoring the dynamic behavior of these compounds.

The findings emphasize the necessity of integrated approaches that consider both biotic and abiotic factors to effectively minimize the persistence of harmful micropollutants. Evaluating the fate of antibiotics must encompass not only the parent compounds but also their transformation products and non-extractable residues. These persistent and potentially bioactive metabolites, along with remobilized fractions, pose ongoing ecological and health concerns, necessitating comprehensive fate studies that characterize biotransformation pathways and assess the antibiotic activity of transformation products.

Chapter 7: General Discussion

This chapter synthesizes the insights and findings presented throughout the thesis, integrating results from each preceding chapter. The collective findings challenge the prevailing assumption that enhancing denitrification inherently leads to

improved antibiotic removal. Instead, the study highlights that microbial community structure, diversity, and the presence of specific antibiotic-transforming taxa and enzymes are critical determinants of antibiotic biotransformation. Both heterotrophic and autotrophic (N-Damo) systems were capable of achieving high nitrogen removal rates; however, neither intensified denitrification nor enriched enzymatic capabilities alone ensured enhanced antibiotic removal. Additionally, the complexity of antibiotic fate—driven by compound-specific properties and the formation of recalcitrant metabolites—further complicates the development of effective treatment strategies. These results underscore the necessity of considering microbial ecology and biochemical pathways when designing and optimizing wastewater treatment processes for antibiotic removal.

Chapter 8: Conclusion and Future Perspectives

This concluding chapter outlines the main outcomes of the research and proposes directions for future studies. In summary, this thesis advances our understanding of the intricate relationships between operational parameters, microbial community dynamics, enzymatic activity, and micropollutant properties in denitrifying wastewater treatment systems. The findings emphasize that effective antibiotic removal cannot be achieved by simply enhancing denitrification activity. Instead, it requires a holistic approach that maintains microbial diversity, targets specific biotransformation pathways, and considers the physicochemical properties of pollutants. These insights will guide future innovations in wastewater treatment design and operation, ultimately supporting safer effluent discharge, protecting aquatic environments, and contributing to global efforts to mitigate antibiotic resistance.

Such refinements are increasingly vital as wastewater treatment plants face stricter regulations, the need for more energy-efficient operations, and the imperative to control the spread of antibiotic resistance. By focusing on these areas, denitrification processes can become more effective and sustainable, addressing the complex challenges of modern wastewater management. Ultimately, this research contributes to the advancement of wastewater treatment technologies, promoting environmental protection and public health through improved antibiotic removal and resistance mitigation.

Significance and Outlook

This doctoral thesis provides critical insights into the complex interplay between operational parameters, microbial ecology, enzymatic activity, and micropollutant properties in governing antibiotic fate during denitrification. By integrating molecular, metabolic, and chemical analyses, the research advances our understanding of the conditions necessary to harness denitrifying processes for

improved antibiotic removal. Key findings reveal that while increasing nitrogen loading rates (NLRs) can enhance denitrifying activity, this does not necessarily lead to continuous improvements in the removal of organic micropollutants (OMPs). High NLRs were shown to induce significant shifts in microbial community structure, favoring fast-growing denitrifiers over specialized taxa such as those within the order Rhizobiales, which are crucial for antibiotic biotransformation.

Moreover, the study highlights that the functional and taxonomic richness of the microbial community is a critical determinant for effective antibiotic biotransformation. Denitrification activity was found to negatively correlate with antibiotic removal efficiency, emphasizing the importance of maintaining a diverse and specialized microbial community rather than solely focusing on enhancing denitrifying activity. DNA metabarcoding analyses demonstrated that antibiotic exposure, even at low concentrations, significantly alters microbial community composition, particularly over long-term operations, further impacting biotransformation outcomes.

Enzymatic investigations within the N-Damo process underscored that optimizing micronutrient availability and enhancing enzymatic pathways can sustain high denitrification rates. However, achieving improved antibiotic biotransformation requires targeting specific enzymes and metabolic routes directly involved in pollutant degradation. This indicates that a nuanced approach to microbial and enzymatic management is necessary to balance denitrification efficiency with antibiotic removal capabilities.

These findings underscore the functional trade-offs between maintaining high NLRs for enhanced denitrification and preserving the metabolic functions essential for antibiotic biotransformation. Understanding and balancing these trade-offs is essential for optimizing reactor performance under varying operational conditions. The research suggests that future innovations in wastewater treatment design should focus on identifying and stimulating specific microbial strains and enzymes involved in antibiotic degradation, diversifying carbon sources, and exploring targeted co-metabolic substrates and tailored micronutrient supplementation. Additionally, the development of engineered microbial consortia presents a promising avenue for enhancing micropollutant removal within denitrifying systems.

Ultimately, this thesis contributes to the advancement of wastewater treatment technologies by providing a comprehensive framework for optimizing microbial community structure and enzymatic activity to achieve both efficient nutrient removal and effective antibiotic degradation. These advancements support safer effluent discharge, protect aquatic environments, and contribute to global efforts to mitigate the spread of antibiotic resistance, aligning with sustainable development goals and addressing the pressing challenges of modern wastewater management.

Resumen

Capítulo 1: Introducción y Objetivos Principales

El uso de antibióticos ha transformado fundamentalmente la calidad de vida y atención sanitaria al tratar eficazmente infecciones humanas y animales, aumentar la productividad agrícola y apoyar la acuicultura global. Sin embargo, la creciente demanda mundial de antibióticos, a menudo acompañada de aplicaciones no reguladas o excesivas, ha llevado a la acumulación de concentraciones en el medio ambiente y a la proliferación de genes de resistencia a antibióticos en los ecosistemas acuáticos. Una fracción significativa de estos compuestos, excretados como compuestos parentales o como productos de transformación parcialmente activos, persiste en las aguas residuales. Además, las plantas de tratamiento de aguas residuales convencionales (PTAR), diseñadas principalmente para la eliminación de cargas de nutrientes y orgánicas, solo eliminan parcialmente muchos de estos microcontaminantes orgánicos (MPO). El uso excesivo y el creciente consumo de antibióticos, junto con un tratamiento incompleto, resultan en una presencia generalizada de antibióticos a lo largo de todo el ciclo del agua (aguas superficiales, efluentes tratados, aguas subterráneas).

Las concentraciones de antibióticos en ambientes acuáticos exhiben una considerable variabilidad, principalmente debido a diferencias en los patrones de consumo, el acceso a antibióticos, la biodegradabilidad de cada molécula y las eficiencias de eliminación de las PTAR a gran escala. Aunque las PTAR funcionan como una defensa primaria contra la dispersión de antibióticos al medio ambiente, se reconocen como la principal fuente de antibióticos en el ciclo del agua. La eficiencia de eliminación de cada MPO, incluidos los antibióticos en las PTAR, depende de las características moleculares intrínsecas de la molécula, sus interacciones con el entorno circundante y el tipo específico de proceso de tratamiento empleado. Por lo tanto, mejorar las infraestructuras de agua y saneamiento es crucial para reducir la contaminación por antibióticos, dado el papel fundamental que las PTAR desempeñan en mitigar la liberación de estos compuestos al medio ambiente.

El tratamiento biológico de nutrientes, principalmente a través de procesos de lodo activado, es un componente fundamental de las PTAR y propulsa la mayoría de los mecanismos de biotransformación. La biotransformación ocurre ya sea metabólicamente o cometabólicamente, siendo los procesos cometabólicos el mecanismo predominante en las PTAR a gran escala. Un enfoque común para mejorar la biotransformación cometabólica de MPOs implica aumentar la concentración de un sustrato de crecimiento, como lo han demostrado varios

estudios en sistemas de lodo activado heterotrófico y nitrificante. Aunque se ha investigado extensamente la biodegradación de antibióticos en condiciones aeróbicas, las vías, tasas y factores clave que influyen en la eliminación de microcontaminantes en condiciones anóxicas, especialmente a través de la desnitrificación, están menos comprendidos. La desnitrificación, un proceso crucial de eliminación biológica de nutrientes, proporciona una vía alternativa de aceptador de electrones que puede facilitar la degradación cometabólica de antibióticos. Sin embargo, las complejas interacciones entre parámetros operativos, dinámicas de la comunidad microbiana, actividades enzimáticas y el destino de los microcontaminantes bajo diferentes estados redox aún no están claras.

Abordar estas lagunas de conocimiento es esencial para optimizar los procesos de tratamiento, asegurar el cumplimiento de regulaciones cada vez más estrictas y prevenir la propagación global de la resistencia a antibióticos. Además, comprender la formación de productos de transformación durante condiciones desnitrificantes es vital, ya que estos intermedios pueden retener propiedades antimicrobianas o exhibir nuevos efectos tóxicos. Una comprensión integral de estos procesos permitirá estrategias de tratamiento de aguas residuales más efectivas y mitigará el impacto ambiental de la contaminación por antibióticos.

Esta investigación doctoral tiene como objetivo abordar estos desafíos investigando la biotransformación de antibióticos en condiciones desnitrificantes. El trabajo se centra en tres objetivos principales: (1) evaluar la biotransformación cometabólica aumentando la actividad desnitrificante mediante el incremento de las tasas de carga de nitrógeno (NLRs), (2) elucidar cómo los cambios en la comunidad microbiana y las respuestas enzimáticas influyen en la biotransformación de antibióticos, y (3) comprender el destino y las vías de transformación de los antibióticos y sus metabolitos durante sistemas de desnitrificación heterotrófica. Al examinar estos aspectos, esta tesis busca ofrecer nuevas perspectivas sobre los factores microbianos y operativos que rigen la eliminación de antibióticos, contribuyendo en última instancia a estrategias de tratamiento de aguas residuales más efectivas y a la reducción de impactos ambientales.

Capítulo 3: Cambios en la Comunidad Microbiana y Limitaciones Cometabólicas en la Desnitrificación Heterotrófica

Este capítulo investiga si el aumento de las tasas de carga de nitrógeno (NLRs) en un sistema de desnitrificación heterotrófica mejora la eliminación de antibióticos a través del cometabolismo y examina cómo los cambios a largo plazo en la estructura de la comunidad microbiana afectan las eficiencias de eliminación. Aunque la desnitrificación heterotrófica se aplica ampliamente en el tratamiento de aguas residuales, sus mecanismos de biotransformación solo se comprenden parcialmente. Para este propósito, se operaron dos reactores desnitrificantes de flujo continuo, con y sin alimentación de antibióticos, de manera análoga durante un

largo período (262 días). Los resultados mostraron que un aumento progresivo en la NLR de 25 ± 6 mg $N–NO_3^-$ L^{-1} d^{-1} a 277 ± 38 mg $N–NO_3^-$ L^{-1} d^{-1} no resultó en un aumento en la eliminación de MPO siguiendo una tendencia cometabólica a pesar del aumento en la actividad desnitrificante. Por el contrario, la alta NLR favoreció a desnitrificantes de rápido crecimiento, lo que redujo la prevalencia de táxones especializados cruciales para la biotransformación de antibióticos. La riqueza funcional y taxonómica resultó ser la más influyente para la eliminación efectiva de MPO, con la actividad desnitrificante correlacionándose negativamente con la biotransformación de antibióticos. Estas observaciones refuerzan que la composición de la comunidad microbiana, más que simplemente altas actividades desnitrificantes, gobierna el alcance de la eliminación de antibióticos.

Los análisis de metabarcoding de ADN revelaron que incluso concentraciones bajas de antibióticos (5 μg L^{-1}) remodelaron significativamente la comunidad microbiana, destacando el papel crítico de la composición y diversidad comunitaria en la eliminación de MPO. Además, táxones del orden Rhizobiales emergieron como contribuyentes clave para la biotransformación de antibióticos en estas condiciones. Sin embargo, cuando se aumentaron las NLRs, la abundancia relativa de Rhizobiales disminuyó, y estas poblaciones fueron superadas por otros táxones mejor adaptados a ambientes de alta carga, lo que llevó a una disminución en las eficiencias de eliminación de antibióticos. En general, este trabajo subraya los compromisos funcionales entre mantener altas NLRs para una desnitrificación mejorada y preservar funciones metabólicas como la biotransformación de antibióticos. Comprender y equilibrar estos compromisos es esencial para optimizar el rendimiento del reactor bajo diversas condiciones operativas.

Capítulo 4: ¿Qué tan eficientemente un sistema metabólicamente mejorado con microorganismos oxidadores de metano anaeróbicos desnitrificantes elimina antibióticos?

En este capítulo, investigamos el novedoso proceso de oxidación anaeróbica de metano dependiente de nitrito (N-Damo) a altas actividades de biomasa por su potencial para eliminar simultáneamente nitrito, metano y trazas de antibióticos comúnmente encontrados en aguas residuales. Se operaron dos biorreactores de membrana (MBRs) a tres tasas elevadas de carga de nitrito (NLRs): 76 ± 9.9, 161.5 ± 11.4 y 215.2 ± 24.2 mg $N–NO_2$ L^{-1} d^{-1}, durante períodos prolongados, logrando actividades desnitrificantes específicas de hasta 540 mg $N–NO_2$ g^{-1} VSS d^{-1}. Notablemente, estas actividades rivalizan con los sistemas heterotróficos convencionales en términos de tasas de eliminación de nitrito. Un diferenciador clave respecto a estudios anteriores fue la adición de cerio como elemento traza en el medio de alimentación, lo que permitió una operación estable del reactor bajo estas altas cargas.

Los análisis de la comunidad microbiana mostraron que las bacterias N-Damo (*Candidatus Methylomirabilis*) dominaban inicialmente ambos MBRs (67–87% de abundancia relativa durante diferentes períodos operativos). Sin embargo, a medida que aumentaba la actividad y concentración total de biomasa, la dominancia de estas poblaciones funcionales de N-Damo disminuyó. Concurrentemente, proliferaron otras familias menores como *Hypomicrobiaceae y Xanthobacteraceae,* indicando un cambio en la estructura comunitaria.

Bajo estas condiciones, varios antibióticos, incluidos sulfametoxazol, trimetoprim, cefalexina y azitromicina, mostraron altas tasas de biotransformación. En contraste, roxitromicina y claritromicina solo mostraron una eliminación parcial (20–35%), y ciprofloxacino permaneció mayormente sin afectar. Intrigantemente, a pesar de la elevada actividad N-Damo y la nueva estrategia operativa, no se observó una mejora clara en la eliminación de antibióticos a lo largo del experimento. Como se observó en los sistemas de desnitrificación heterotrófica examinados en el Capítulo 3, estos resultados subrayan que la composición de la comunidad microbiana ejerce una influencia más decisiva en la eliminación de antibióticos que la elevada actividad desnitrificante por sí sola. En última instancia, la gestión y dirección de la estructura de la comunidad microbiana emerge como crítica para mejorar la biotransformación de antibióticos en reactores N-Damo.

Capítulo 5: Fundamentos Enzimáticos de los Sistemas N-Damo Revelados por Metaproteómica

Construyendo sobre los cambios microbianos observados en el Capítulo 4, este capítulo explora la base enzimática del proceso de oxidación anaeróbica de metano dependiente de nitrito (N-Damo), una vía prometedora para lograr un tratamiento de aguas residuales neutro en carbono en línea con los objetivos de desarrollo sostenible. Utilizando análisis metaproteómicos, elucidamos las rutas enzimáticas completas de N-Damo, con un énfasis particular en las metaloenzimas. Los análisis confirmaron que *Candidatus Methylomirabilis Lanthanidiphila* era la especie N-Damo predominante, representando el 67–87% de la abundancia relativa de proteínas. Esta dominancia fue impulsada por la inducción de su metanol deshidrogenasa ligadora de lantánidos en presencia de cerio suplementado, destacando el papel esencial de los lantánidos en el apoyo a altas tasas de eliminación de nitrógeno.

Datos metaproteómicos adicionales revelaron la participación activa de táxones acompañantes en la desnitrificación, metilotrofia y metanotrofia, demostrando las interacciones sinérgicas dentro de la comunidad microbiana. Las metaloenzimas funcionales más abundantes identificadas requerían cobre, hierro y cerio como cofactores, lo que se correlacionó con los patrones de consumo de metales observados en el biorreactor. A pesar de la actividad enzimática mejorada que apoyaba estas funciones metabólicas centrales, no hubo una mejora

correspondiente en las eficiencias de eliminación de antibióticos. Este hallazgo subraya que, aunque la optimización de la disponibilidad de micronutrientes y las rutas enzimáticas puede sostener una robusta actividad N-Damo, la biotransformación efectiva de antibióticos probablemente requiere el enfoque enzimático y de rutas metabólicas específicas directamente involucradas en la degradación de contaminantes.

En general, este estudio destaca la utilidad de la metaproteómica en la evaluación de las actividades enzimáticas dentro de sistemas de tratamiento ingenieriles, proporcionando conocimientos críticos para optimizar la gestión microbiana. Al comprender el equilibrio intrincado entre mantener una alta actividad N-Damo y preservar las funciones metabólicas necesarias para la biotransformación de antibióticos, podemos diseñar y operar mejor los procesos de tratamiento de aguas residuales para lograr tanto una eliminación eficiente de nutrientes como una degradación efectiva de contaminantes.

Capítulo 6: Controladores Físico-Químicos del Destino de los Antibióticos y las Vías de Transformación

Este capítulo explora el destino y la transformación de los antibióticos sulfametoxazol (SMX) y triclocarban (TCC) bajo procesos de desnitrificación heterotrófica utilizando técnicas radiomarcadas con dos moléculas etiquetadas, ^{14}C-SMX y ^{14}C-TCC. El objetivo del estudio fue dilucidar cómo la estructura química y las propiedades fisicoquímicas de estos microcontaminantes influyen significativamente en su comportamiento en entornos desnitrificantes.

El TCC, caracterizado por su naturaleza hidrofóbica y neutra, exhibió un comportamiento persistente bajo condiciones de desnitrificación. Durante un período de 14 días, más del 59% del TCC se adsorbió al lodo, permaneciendo principalmente en formas extraíbles con una mineralización negligente. Esta alta adsorción probablemente limitó la biodisponibilidad del TCC, impidiendo así una biotransformación extensa. Además, las fracciones extraíbles de TCC disminuyeron con el tiempo, lo que sugiere una remobilización impulsada por gradientes químicos y fuerzas de cizallamiento durante la mezcla.

En contraste, el SMX, un compuesto altamente polar que existe principalmente como un anión, sometió a una rápida biotransformación con aproximadamente el 80% del compuesto parental transformado en un día. Sin embargo, la mineralización del SMX se mantuvo baja, ya que se convirtió en metabolitos más polares y persistentes que se acumularon en la fase acuosa. Estos productos de transformación, que potencialmente exhiben actividad antibiótica, destacan la insuficiencia de centrarse únicamente en la eliminación del compuesto parental. Además, las fracciones extraíbles tanto de SMX como de TCC disminuyeron con el tiempo, indicando la

remobilización de residuos, particularmente pronunciada para TCC debido a su alta presencia en la fracción extraíble. Los residuos no extraíbles (NER) también mostraron remobilización, especialmente para SMX, subrayando el comportamiento dinámico de estos compuestos.

Los hallazgos enfatizan la necesidad de enfoques integrados que consideren tanto factores bióticos como abióticos para minimizar efectivamente la persistencia de microcontaminantes nocivos. Evaluar el destino de los antibióticos debe abarcar no solo los compuestos parentales, sino también sus productos de transformación y residuos no extraíbles. Estos metabolitos persistentes y potencialmente bioactivos, junto con las fracciones remobilizadas, plantean preocupaciones ecológicas y de salud continuas, lo que requiere estudios de destino comprensivos que caractericen las vías de biotransformación y evalúen la actividad antibiótica de los productos de transformación.

Capítulo 7: Discusión General

Este capítulo sintetiza los conocimientos y hallazgos presentados a lo largo de la tesis, integrando resultados de cada capítulo precedente. Los hallazgos colectivos desafían la suposición prevalente de que mejorar la desnitrificación conduce inherentemente a una mejor eliminación de antibióticos. En cambio, el estudio destaca que la estructura de la comunidad microbiana, la diversidad y la presencia de táxones y enzimas específicas que transforman antibióticos son determinantes críticos de la biotransformación de antibióticos. Tanto los sistemas heterotróficos como los autotróficos (N-Damo) fueron capaces de lograr altas tasas de eliminación de nitrógeno; sin embargo, ni la desnitrificación intensificada ni las capacidades enzimáticas enriquecidas por sí solas aseguraron una eliminación mejorada de antibióticos. Además, la complejidad del destino de los antibióticos —impulsada por propiedades específicas de los compuestos y la formación de metabolitos recalcitrantes— complica aún más el desarrollo de estrategias de tratamiento efectivas. Estos resultados subrayan la necesidad de considerar la ecología microbiana y las vías bioquímicas al diseñar y optimizar los procesos de tratamiento de aguas residuales para la eliminación de antibióticos.

Capítulo 8: Conclusión y Perspectivas Futuras

Este capítulo final describe los principales resultados de la investigación y propone direcciones para estudios futuros. En resumen, esta tesis avanza en nuestra comprensión de las relaciones intrincadas entre parámetros operativos, dinámicas de la comunidad microbiana, actividad enzimática y propiedades de microcontaminantes en sistemas de tratamiento de aguas residuales desnitrificantes. Los hallazgos enfatizan que la eliminación efectiva de antibióticos no puede lograrse simplemente mejorando la actividad de desnitrificación. En

cambio, requiere un enfoque holístico que mantenga la diversidad microbiana, apunte a rutas específicas de biotransformación y considere las propiedades fisicoquímicas de los contaminantes. Estos conocimientos guiarán futuras innovaciones en el diseño y operación de tratamientos de aguas residuales, apoyando en última instancia una descarga de efluentes más segura, protegiendo los ambientes acuáticos y contribuyendo a esfuerzos globales para mitigar la resistencia a antibióticos.

Tales refinamientos son cada vez más vitales a medida que las PTAR enfrentan regulaciones más estrictas, la necesidad de operaciones más eficientes en términos de energía y el imperativo de controlar la propagación de la resistencia a antibióticos. Al centrarse en estas áreas, los procesos de desnitrificación pueden volverse más efectivos y sostenibles, abordando los complejos desafíos de la gestión moderna de aguas residuales. En última instancia, esta investigación contribuye al avance de las tecnologías de tratamiento de aguas residuales, promoviendo la protección ambiental y la salud pública a través de una mejora en la eliminación de antibióticos y la mitigación de la resistencia.

Significado y Perspectiva

Esta tesis doctoral proporciona conocimientos críticos sobre la compleja interacción entre parámetros operativos, ecología microbiana, actividad enzimática y propiedades de microcontaminantes en la determinación del destino de los antibióticos durante la desnitrificación. Al integrar análisis moleculares, metabólicos y químicos, la investigación avanza nuestra comprensión de las condiciones necesarias para aprovechar los procesos desnitrificantes para una mejor eliminación de antibióticos. Los hallazgos clave revelan que, aunque aumentar las tasas de carga de nitrógeno (NLRs) puede mejorar la actividad desnitrificante, esto no necesariamente conduce a mejoras continuas en la eliminación de microcontaminantes orgánicos (OMPs). Se demostró que las altas NLRs inducen cambios significativos en la estructura de la comunidad microbiana, favoreciendo a desnitrificantes de rápido crecimiento sobre táxones especializados como los del orden Rhizobiales, que son cruciales para la biotransformación de antibióticos.

Además, el estudio destaca que la riqueza funcional y taxonómica de la comunidad microbiana es un determinante crítico para la biotransformación efectiva de antibióticos. Se encontró que la actividad de desnitrificación se correlaciona negativamente con la eficiencia de eliminación de antibióticos, enfatizando la importancia de mantener una comunidad microbiana diversa y especializada en lugar de centrarse exclusivamente en mejorar la actividad desnitrificante. Los análisis de metabarcoding de ADN demostraron que la exposición a antibióticos, incluso en concentraciones bajas, altera significativamente la composición de la

comunidad microbiana, particularmente en operaciones a largo plazo, impactando además los resultados de biotransformación.

Las investigaciones enzimáticas dentro del proceso N-Damo subrayaron que optimizar la disponibilidad de micronutrientes y mejorar las vías enzimáticas puede sostener altas tasas de desnitrificación. Sin embargo, lograr una biotransformación mejorada de antibióticos requiere apuntar a enzimas específicas y rutas metabólicas directamente involucradas en la degradación de contaminantes. Esto indica que se necesita un enfoque matizado para la gestión microbiana y enzimática para equilibrar la eficiencia de desnitrificación con las capacidades de eliminación de antibióticos.

Estos hallazgos subrayan los compromisos funcionales entre mantener altas NLRs para una mejor desnitrificación y preservar las funciones metabólicas esenciales para la biotransformación de antibióticos. Comprender y equilibrar estos compromisos es esencial para optimizar el rendimiento del reactor bajo diversas condiciones operativas. La investigación sugiere que futuras innovaciones en el diseño del tratamiento de aguas residuales deberían centrarse en identificar y estimular cepas microbianas y enzimas específicas involucradas en la degradación de antibióticos, diversificando las fuentes de carbono y explorando sustratos co-metabólicos dirigidos y suplementación personalizada de micronutrientes. Además, el desarrollo de consorcios microbianos diseñados presenta una vía prometedora para mejorar la eliminación de microcontaminantes dentro de sistemas desnitrificantes.

En última instancia, esta tesis contribuye al avance de las tecnologías de tratamiento de aguas residuales al proporcionar un marco comprensivo para optimizar la estructura de la comunidad microbiana y la actividad enzimática para lograr tanto una eliminación eficiente de nutrientes como una degradación efectiva de antibióticos. Estos avances apoyan una descarga de efluentes más segura, protegen los ambientes acuáticos y contribuyen a los esfuerzos globales para mitigar la propagación de la resistencia a antibióticos, alineándose con los objetivos de desarrollo sostenible y abordando los desafíos urgentes de la gestión moderna de aguas residuales.

Resumo

Capítulo 1: Introdución e Obxectivos Principais

O uso de antibióticos transformou fundamentalmente a atención sanitaria moderna ao tratar eficazmente infeccións humanas e animais, incrementar a produtividade agrícola e apoiar a acuacultura global. Con todo, a crecente demanda global de antibióticos, a miúdo xuntada coa aplicación non regulada ou excesiva, levou á acumulación de concentracións no medio ambiente e á proliferación de xenes de resistencia a antibióticos en ecosistemas acuáticos. Unha fracción significativa destes compostos, excretados como compostos parentais ou como produtos de transformación parcialmente activos, persiste nas augas residuais. Ademais, as plantas convencionais de tratamento de augas residuais (PTAR), deseñadas principalmente para a eliminación de cargas de nutrientes e orgánicas, só eliminan parcialmente moitos destes microcontaminantes orgánicos (MPOs). O uso excesivo e o aumento do consumo de antibióticos, xunto cun tratamento incompleto, resultan nunha ampla presenza de antibióticos a través de todo o ciclo da auga (auga superficial, efluentes tratados, auga subterránea).

As concentracións de antibióticos en ambientes acuáticos exhiben unha considerable variabilidade, principalmente debido ás diferenzas nos patróns de consumo, o acceso aos antibióticos, a biodegradabilidade de cada molécula e as eficiencias de eliminación das PTAR a escala completa. Aínda que as PTAR funcionan como unha defensa fronteira contra a dispersión de antibióticos ao medio ambiente, recoñécense como a principal fonte de antibióticos no ciclo da auga. A eficiencia de eliminación de cada MPO, incluíndo antibióticos nas PTAR, depende das características moleculares intrínsecas da molécula, das súas interaccións co entorno circundante e do tipo específico de proceso de tratamento empregado. Polo tanto, mellorar as infraestruturas de auga e saneamento é crucial para reducir a contaminación por antibióticos, dado o papel fundamental que as PTAR desempeñan na mitigación da liberación destes compostos ao medio ambiente.

O tratamento biolóxico de nutrientes, principalmente a través de procesos de lodos activados, é un componente fundamental das PTAR e impulsa a maioría dos mecanismos de biotransformación. A biotransformación ocorre xa sexa metabólicamente ou cometabólicamente, sendo os procesos cometabólicos o mecanismo predominante nas PTAR a escala completa. Unha aproximación común para mellorar a biotransformación cometabólica de MPOs implica incrementar a concentración dun substrato de crecemento, como demostraron varios estudos en sistemas de lodos activados heterotróficos e nitrificantes. Aínda que se investigou amplamente a biodegradación de antibióticos en condicións aeróbicas, as vías, as taxas e os factores clave que inflúen na eliminación de microcontaminantes en condicións anóxicas, especialmente a través da desnitrificación, están menos ben

comprendidos. A desnitrificación, un proceso crucial de eliminación biolóxica de nutrientes, proporciona unha vía alternativa de aceptores de electrón que pode facilitar a degradación cometabólica de antibióticos. Non obstante, as interaccións complexas entre parámetros operativos, dinámicas da comunidade microbiana, actividades enzimáticas e o destino dos microcontaminantes en diferentes estados redox aínda non están claras.

Abordar estas lacunas de coñecemento é esencial para optimizar os procesos de tratamento, asegurar o cumprimento de regulacións cada vez máis estritas e previr a difusión global da resistencia a antibióticos. Ademais, comprender a formación de produtos de transformación durante condicións desnitrificantes é vital, xa que estes intermedios poden manter propiedades antimicrobianas ou exhibir novos efectos tóxicos. Unha comprensión completa destes procesos permitirá estratexias de tratamento de augas residuales máis efectivas e mitigará o impacto ambiental da contaminación por antibióticos.

Esta investigación doutoral ten como obxectivo abordar estes desafíos investigando a biotransformación de antibióticos en condicións desnitrificantes. O traballo céntrase en tres obxectivos principais: (1) avaliar a biotransformación cometabólica incrementando a actividade desnitrificante mediante o aumento das taxas de carga de nitróxeno (NLRs), (2) elucidando como os cambios na comunidade microbiana e as respostas enzimáticas inflúen na biotransformación de antibióticos, e (3) comprender o destino e as vías de transformación dos antibióticos e os seus metabólitos durante sistemas de desnitrificación heterotrófica. Ao examinar estes aspectos, esta tese busca ofrecer novas perspectivas sobre os factores microbianos e operativos que gobernan a eliminación de antibióticos, contribuíndo finalmente a estratexias de tratamento de augas residuales máis efectivas e a reducir os impactos ambientais.

Capítulo 3: Cambios na Comunidade Microbiana e Limitacións Cometabólicas na Desnitrificación Heterotrófica

Este capítulo investiga se o aumento das taxas de carga de nitróxeno (NLRs) nun sistema de desnitrificación heterotrófica mellora a eliminación de antibióticos a través da cometabolía e examina como os cambios a longo prazo na estrutura da comunidade microbiana afectan as eficiencias de eliminación. Aínda que a desnitrificación heterotrófica está amplamente aplicada no tratamento de augas residuales, os seus mecanismos de biotransformación só están parcialmente comprendidos. Para este propósito, operáronse dous reactores desnitrificantes de fluxo continuo, con e sen a adición de antibióticos, durante longos períodos (262 días) de xeito análogo. Os resultados amosaron que un incremento progresivo na NLR desde 25 ± 6 mg $N–NO_3^-$ L^{-1} d^{-1} ata 277 ± 38 mg $N–NO_3^-$ L^{-1} d^{-1} non resultou nun aumento na eliminación de MPOs seguindo unha tendencia cometabólica a pesar do aumento na actividade desnitrificante. Polo contrario, a alta NLR favoreceu

aos desnitrificantes de crecemento rápido, reducindo a prevalencia de taxa especializadas cruciais para a biotransformación de antibióticos. A riqueza funcional e taxonómica demostrou ser a máis influente para a eliminación efectiva de MPOs, coa actividade de desnitrificación correlacionándose negativamente coa biotransformación de antibióticos. Estas observacións reforzan que a composición da comunidade microbiana, máis aló de simplemente altas actividades desnitrificantes, governa a extensión da eliminación de antibióticos.

As análises de metabarcoding de ADN revelaron que incluso concentracións baixas de antibióticos (5 μg L^{-1}) remodelaron significativamente a comunidade microbiana, destacando o papel crítico da composición e diversidade comunitaria na eliminación de MPOs. Ademais, taxas da orde *Rhizobiales* emerxeron como contribuintes clave á biotransformación de antibióticos nestas condicións. Non obstante, cando se incrementaron as NLRs, a abundancia relativa de Rhizobiales diminuíu, e estas poboacións foron superadas por outras taxas mellor adaptadas a ambientes de alta carga, levando a diminucións nas eficiencias de eliminación de antibióticos. En xeral, este traballo subliña os compromisos funcionais entre manter altas NLRs para mellorar a desnitrificación e preservar funcións metabólicas como a biotransformación de antibióticos. Comprender e equilibrar estes compromisos é esencial para optimizar o rendemento do reactor en condicións operativas variables.

Capítulo 4: ¿Qué tan eficazmente un sistema metabólicamente mellorado con microorganismos oxidantes de metano anaeróbicos desnitrificantes elimina antibióticos?

Neste capítulo, investigamos o novo proceso de oxidación anaeróbica de metano dependente de nitrito (N-Damo) con altas actividades de biomasa polo seu potencial para eliminar simultaneamente nitrito, metano e antibióticos traza comúns en asfalto. Dous bioreactores de membrana (MBRs) operáronse a tres taxas elevadas de carga de nitrito (NLRs)—76 ± 9.9, 161.5 ± 11.4 e 215.2 ± 24.2 mg $N–NO_2$ L^{-1} d^{-1}—durante períodos prolongados, achegándose a actividades específicas de desnitrificación de ata 540 mg $N–NO_2$ g^{-1} VSS d^{-1}. Notablemente, estas actividades rivalizan cos sistemas heterotróficos convencionais en termos de taxas de eliminación de nitrito. Un diferenciador clave respecto a estudos previos foi a adición de cério como elemento traza no medio de alimentación, permitindo unha operación estable do reactor baixo estas altas cargas.

As análises da comunidade microbiana amosaron que as bacterias N-Damo (*Candidatus Methylomirabilis*) dominaban inicialmente ambos os MBRs (67–87% de abundancia relativa durante diferentes períodos operativos). Con todo, a medida que a actividade e concentración global da biomasa aumentaban, a dominancia destas poboacións funcionais N-Damo diminuía. Concurrentemente, outras familias

menores como Hypomicrobiaceae e Xanthobacteraceae proliferaron, indicando un cambio na estrutura comunitaria.

Nestes condicións, varios antibióticos—incluíndo sulfametoxazol, trimetoprim, cefalexina e azitromicina—exhibiron altas taxas de biotransformación. En contraste, roxitromicina e claritromicina amosaron só unha eliminación parcial (20–35%) e a ciprofloxacina permaneceu amplamente afectada. Intrigantemente, a pesar da elevada actividade N-Damo e da nova estratexia operativa, non se observou unha clara mellora na eliminación de antibióticos durante o curso do experimento. Como se observou nos sistemas de desnitrificación heterotrófica examinados no Capítulo 3, estes resultados subliñan que a composición da comunidade microbiana exerce unha influencia máis decisiva na eliminación de antibióticos que a elevada actividade desnitrificante só. En última instancia, a xestión e dirección da estrutura da comunidade microbiana xérase crítica para mellorar a biotransformación de antibióticos nos reactores N-Damo.

Capítulo 5: Fundamentos Enzimáticos dos Sistemas N-Damo Revelados por Metaproteómica

Baseándose nos cambios microbianos observados no Capítulo 4, este capítulo explora a base enzimática do proceso de oxidación anaeróbica de metano dependente de nitrito (N-Damo), unha vía prometedora para lograr un tratamento de augas residuales neutro en carbono en liña cos obxectivos de desenvolvemento sostible. Utilizando análises metaproteómicas, elucidáronse as vías enzimáticas completas de N-Damo, cun énfasis particular nas metaloenzimas. As análises confirmaron que *Candidatus Methylomirabilis Lanthanidiphila* era a especie N-Damo predominante, representando o 67–87% da abundancia relativa de proteínas. Esta dominancia foi impulsada pola indución da súa dehidrogenasa de metanol ligadora de lantánidos na presenza de cério suplementado, destacando o papel esencial dos lantánidos no sostén das altas taxas de eliminación de nitróxeno.

Ademais, os datos metaproteómicos revelaron a participación activa das taxas acompañantes na desnitrificación, metilotrofía e metanotrofía, demostrando as interaccións sinérxicas dentro da comunidade microbiana. As metaloenzimas funcionais máis abundantes identificadas requirían cobre, ferro e cério como cofactors, o que correlacionaba cos patróns de consumo de metais observados no bioreactor. A pesar da actividade enzimática mellorada que sostivo estas funcións metabólicas centrais, non houbo mellora correspondente nas eficiencias de eliminación de antibióticos. Este achado subliña que, aínda que optimizar a dispoñibilidade de micronutrientes e as vías enzimáticas pode sostecer unha robusta actividade N-Damo, a biotransformación efectiva de antibióticos probablemente requira dirixirse a enzimás específicas e rutas metabólicas directamente involucradas na degradación de contaminantes.

En xeral, este estudo destaca a utilidade da metaproteómica na avaliación das actividades enzimáticas dentro de sistemas xerados, proporcionando perspectivas críticas para optimizar a xestión microbiana. Ao comprender o equilibrio intricado entre manter unha alta actividade N-Damo e preservar as funcións metabólicas necesarias para a biotransformación de antibióticos, podemos deseñar e operar mellor os procesos de tratamento de augas residuales para lograr tanto unha eficiente eliminación de nutrientes como a degradación de contaminantes.

Capítulo 6: Controladores Físico-Químicos do Destino dos Antibióticos e das Vías de Transformación

Neste último capítulo experimental explora o destino e a transformación dos antibióticos sulfametoxazol (SMX) e triclocarban (TCC) baixo procesos de desnitrificación heterotrófica empregando técnicas radiolabeladas con dúas moléculas etiquetadas, ^{14}C-SMX e ^{14}C-TCC. O obxectivo do estudo foi elucidar como a estrutura química e as propiedades físico-químicas destes microcontaminantes inflúen significativamente no seu comportamento en ambientes desnitrificantes.

O TCC, caracterizado pola súa natureza hidrofóbica e neutra, exhibiu un comportamento persistente en condicións de desnitrificación. Durante un período de 14 días, máis do 59% do TCC adsorbeuse ao fango, permanecendo principalmente en formas extraíbles cunha mineralización negligente. Esta alta adsorción probablemente limitou a bio dispoñibilidade do TCC, prevenindo así unha biotransformación extensa. Ademais, as fraccións extraíbles de TCC diminuíron co tempo, o que suxire unha remobilización impulsada por gradientes químicos e forzas de corte durante a mestura.

En contraste, o SMX, un composto altamente polar que existe principalmente como un anión, someteuse a unha rápida biotransformación cunha aproximación do 80% do composto parent transformado en un día. Non obstante, a mineralización do SMX mantívose baixa ao converterse en metabólitos máis polares e persistentes que acumularon na fase acuosa. Estes produtos de transformación, que potencialmente exhiben actividade antibiótica, destacan a insuficiencia de centrar-se exclusivamente na eliminación do composto parent. Ademais, as fraccións extraíbles tanto de SMX como de TCC diminuíron co tempo, indicando remobilización de residuos, particularmente pronunciada para TCC debido á súa alta presenza na fracción extraíble. Os residuos non extraíbles (NER) tamén mostraron remobilización, especialmente para SMX, subliñando o comportamento dinámico destes compostos.

Os achados enfatizan a necesidade de enfoques integrados que consideren tanto factores bióticos como abióticos para minimizar efectivamente a persistencia de microcontaminantes nocivos. Avaliar o destino dos antibióticos debe abarcar non só os compostos parent senón tamén os seus produtos de transformación e os residuos

non extraíbles. Estes metabólitos persistentes e potencialmente bioactivos, xunto cos fraccionados remobilizados, representan preocupacións ecolóxicas e de saúde continuas, requirindo estudos de destino comprensivos que caractericen as rutas de biotransformación e avalíen a actividade antibiótica dos produtos de transformación.

Capítulo 7: Discusión Xeral

Este capítulo sintetiza as perspectivas e os achados presentados ao longo da tese, integrando os resultados de cada capítulo precedente. Os achados colectivos desafían a suposición predominante de que mellorar a desnitrificación leva inerentemente a unha mellora na eliminación de antibióticos. Ao contrario, o estudo destaca que a estrutura da comunidade microbiana, a diversidade e a presenza de taxas e enzimás específicas transformadoras de antibióticos son determinantes críticos da biotransformación de antibióticos. Tanto os sistemas heterotróficos como os autotróficos (N-Damo) foron capaces de lograr altas taxas de eliminación de nitróxeno; non obstante, nin a desnitrificación intensificada nin as capacidades enzimáticas enriquecidas por si só aseguraron unha eliminación mellorada de antibióticos. Ademais, a complexidade do destino dos antibióticos—impulsada por propiedades específicas dos compostos e a formación de metabólitos recalcitrantes—complica ademais o desenvolvemento de estratexias de tratamento efectivas. Estes resultados subliñan a necesidade de considerar a ecología microbiana e as vías bioquímicas ao deseñar e optimizar os procesos de tratamento de augas residuales para a eliminación de antibióticos.

Capítulo 8: Conclusión e Perspectivas Futuras

Este capítulo final describe os principais resultados da investigación e propón direccións para estudos futuros. En resumo, esta tese avanza a nosa comprensión das relacións intrincadas entre parámetros operativos, dinámicas da comunidade microbiana, actividade enzimática e propiedades dos microcontaminantes en sistemas de tratamento de augas residuales desnitrificantes. Os achados enfatizan que a eliminación efectiva de antibióticos non pode lograrse simplemente mellorando a actividade de desnitrificación. Ao contrario, require un enfoque holístico que mantén a diversidade microbiana, diríxase a vías de biotransformación específicas e considere as propiedades fisicoquímicas dos contaminantes. Estas perspectivas guiarán as innovacións futuras no deseño e operación do tratamento de augas residuales, apoiando finalmente unha descarga de efluentes máis segura, protexendo os ambientes acuáticos e contribuíndo aos esforzos globais para mitigar a resistencia a antibióticos.

Tales refinamentos son cada vez máis vitais a medida que as PTAR afrontan regulacións máis estritas, a necesidade de operacións máis eficientes enerxéticamente e a imperativa de controlar a difusión da resistencia a antibióticos.

Ao centrarse nestas áreas, os procesos de desnitrificación poden volverse máis efectivos e sostibles, abordando os desafíos complexos da xestión moderna de augas residuales. En última instancia, esta investigación contribúe ao avance das tecnoloxías de tratamento de augas residuales, promovendo a protección ambiental e a saúde pública mediante unha mellorada eliminación de antibióticos e a mitigación da resistencia.

Significado e Perspectiva

Esta tese doutoral proporciona perspectivas críticas sobre a interacción complexa entre parámetros operativos, ecología microbiana, actividade enzimática e propiedades dos microcontaminantes no destino dos antibióticos durante a desnitrificación. Ao integrar análises moleculares, metabólicas e químicas, a investigación avanza a nosa comprensión das condicións necesarias para aproveitar os procesos desnitrificantes para mellorar a eliminación de antibióticos. Os achados clave revelan que, aínda que incrementar as taxas de carga de nitróxeno (NLRs) pode aumentar a actividade desnitrificante, isto non leva necesariamente a melloras continuas na eliminación de microcontaminantes orgánicos (MPOs). As altas NLRs demostraron inducir cambios significativos na estrutura da comunidade microbiana, favorecendo aos desnitrificantes de crecemento rápido sobre taxas especializadas como aquelas dentro da orde Rhizobiales, que son cruciais para a biotransformación de antibióticos.

Ademais, o estudo destaca que a riqueza funcional e taxonómica da comunidade microbiana é un determinante crítico para a biotransformación efectiva de antibióticos. A actividade de desnitrificación corrobora unha correlación negativa coa eficiencia de eliminación de antibióticos, enfatizando a importancia de manter unha comunidade microbiana diversa e especializada en lugar de centrarse exclusivamente en aumentar a actividade desnitrificante. As análises de metabarcoding de ADN demostraron que a exposición a antibióticos, incluso a concentracións baixas, altera significativamente a composición da comunidade microbiana, particularmente durante operacións a longo prazo, impactando ademais os resultados da biotransformación.

As investigacións enzimáticas dentro do proceso N-Damo subliñaron que optimizar a dispoñibilidade de micronutrientes e mellorar as vías enzimáticas pode sostener altas taxas de desnitrificación. Non obstante, lograr unha biotransformación mellorada de antibióticos require dirixirse a enzimás específicas e rutas metabólicas directamente involucradas na degradación de contaminantes. Isto indica que un enfoque matizado para a xestión microbiana e enzimática é necesario para equilibrar a eficiencia de desnitrificación coas capacidades de eliminación de antibióticos.

Estes achados subliñan os compromisos funcionais entre manter altas NLRs para unha mellora da desnitrificación e preservar as funcións metabólicas esenciais para a biotransformación de antibióticos. Comprender e equilibrar estes compromisos é esencial para optimizar o rendemento do reactor en condicións operativas variables. A investigación suxire que as innovacións futuras no deseño do tratamento de augas residuales deberían centrarse en identificar e estimular taxas microbianas específicas e enzimás involucradas na degradación de antibióticos, diversificar fontes de carbono e explorar substratos co-metabólicos dirixidos e suplementación personalizada de micronutrientes. Ademais, o desenvolvemento de consorcios microbianos deseñados presenta unha vía prometedora para mellorar a eliminación de microcontaminantes dentro de sistemas desnitrificantes.

En última instancia, esta tese contribúe ao avance das tecnoloxías de tratamento de augas residuales proporcionando un marco comprensivo para optimizar a estrutura da comunidade microbiana e a actividade enzimática para lograr tanto unha eficiente eliminación de nutrientes como unha efectiva degradación de antibióticos. Estes avances apoian unha descarga de efluentes máis segura, protexen os ambientes acuáticos e contribúen aos esforzos globais para mitigar a difusión da resistencia a antibióticos, aliándose cos obxectivos de desenvolvemento sostible e abordando os desafíos apremiantes da xestión moderna de augas residuales.

Übersicht

Kapitel 1: Einführung und Hauptziele

Der Einsatz von Antibiotika hat die moderne Gesundheitsversorgung grundlegend transformiert, indem er menschliche und tierische Infektionen wirksam behandelt, die landwirtschaftliche Produktivität steigert und die globale Aquakultur unterstützt. Dennoch hat die steigende weltweite Nachfrage nach Antibiotika, oft verbunden mit unregulierten oder übermäßigen Anwendungen, zu einer Ansammlung von Konzentrationen in der Umwelt und zur Vermehrung von Antibiotikaresistenzgenen in aquatischen Ökosystemen geführt. Ein erheblicher Anteil dieser Verbindungen, ausgeschieden als Ausgangsverbindungen oder als teilweise aktive Transformationsprodukte, bleibt in Abwässern bestehen. Darüber hinaus beseitigen konventionelle Kläranlagen (WWTPs), die hauptsächlich für die Entfernung von Nährstoffen und organischer Last ausgelegt sind, viele dieser organischen Mikropollutanten (OMPs) nur teilweise. Der übermäßige und zunehmende Antibiotikaverbrauch sowie eine unvollständige Behandlung führen zu einer weit verbreiteten Anwesenheit von Antibiotika im gesamten Wasserkreislauf (Oberflächenwasser, behandelte Efluente, Grundwasser).

Die Antibiotikkonzentrationen in aquatischen Umgebungen zeigen eine beträchtliche Variabilität, hauptsächlich bedingt durch Unterschiede in den Konsummustern, dem Zugang zu Antibiotika, der Abbaubarkeit jeder einzelnen Molekül und den Entfernungseffizienzen vollskaliger WWTPs. Obwohl WWTPs als erste Verteidigungslinie gegen die Verbreitung von Antibiotika in die Umwelt fungieren, werden sie als Hauptquelle von Antibiotika im Wasserkreislauf anerkannt. Die Entfernungseffizienz jedes OMPs, einschließlich Antibiotika in WWTPs, hängt von den intrinsischen molekularen Eigenschaften der Moleküle, ihren Wechselwirkungen mit der umgebenden Umwelt und dem spezifischen eingesetzten Behandlungstyp ab. Daher ist die Verbesserung der Wasser- und Sanitärinfrastrukturen entscheidend zur Reduzierung der Antibiotikaverunreinigung, angesichts der zentralen Rolle, die WWTPs bei der Minderung der Freisetzung dieser Verbindungen in die Umwelt spielen.

Die biologische Nährstoffbehandlung, hauptsächlich durch Prozesse mit aktiviertem Schlamm, ist ein grundlegender Bestandteil von WWTPs und treibt die meisten Biotransformationsmechanismen an. Die Biotransformation erfolgt entweder metabolisch oder cometabolisch, wobei cometabolische Prozesse der vorherrschende Mechanismus in vollskaligen WWTPs sind. Ein gängiger Ansatz zur Verbesserung der cometabolischen Biotransformation von OMPs besteht darin, die Konzentration eines Wachstumssubstrats zu erhöhen, wie durch mehrere Studien an heterotrophen und nitrifizierenden aktivierten Schlamm-Systemen gezeigt wurde. Während umfangreiche Forschungen die Antibiotikabioabbaus unter

aeroben Bedingungen untersucht haben, sind die Wege, Raten und Schlüsselfaktoren, die die Entfernung von Mikropollutanten unter anoxischen Bedingungen, insbesondere durch Denitrifikation, beeinflussen, weniger gut verstanden. Die Denitrifikation, ein entscheidender biologischer Nährstoffentfernungsprozess, bietet einen alternativen Elektronenakzeptorweg, der die cometabolische Antibiotikadegradation erleichtern könnte. Allerdings bleiben die komplexen Wechselwirkungen zwischen Betriebsparametern, Dynamiken der mikrobiellen Gemeinschaft, enzymatischen Aktivitäten und dem Schicksal von Mikropollutanten unter variierenden Redoxzuständen unklar.

Das Schließen dieser Wissenslücken ist essenziell zur Optimierung der Behandlungsprozesse, zur Sicherstellung der Einhaltung zunehmend strengerer Vorschriften und zur Verhinderung der globalen Ausbreitung von Antibiotikaresistenzen. Zudem ist das Verständnis der Bildung von Transformationsprodukten unter denitrifizierenden Bedingungen von entscheidender Bedeutung, da diese Zwischenprodukte antimikrobielle Eigenschaften behalten oder neue toxische Effekte aufweisen können. Umfassende Einblicke in diese Prozesse ermöglichen effektivere Strategien zur Abwasserbehandlung und mildern die Umweltauswirkungen der Antibiotikaverunreinigung.

Diese Doktorarbeit zielt darauf ab, diese Herausforderungen zu adressieren, indem sie die Biotransformation von Antibiotika unter denitrifizierenden Bedingungen untersucht. Die Arbeit konzentriert sich auf drei Hauptziele: (1) die Bewertung der cometabolischen Biotransformation durch Erhöhung der denitrifizierenden Aktivität mittels Steigerung der Stickstoffbelastungsraten (NLRs), (2) die Aufklärung, wie Verschiebungen in der mikrobiellen Gemeinschaft und enzymatische Reaktionen die Biotransformation von Antibiotika beeinflussen, und (3) das Verständnis des Schicksals und der Transformationswege von Antibiotika und ihren Metaboliten während heterotropher Denitrifizierungssysteme. Durch die Untersuchung dieser Aspekte soll diese These neue Einblicke in die mikrobiellen und operativen Faktoren bieten, die die Entfernung von Antibiotika steuern, und letztlich zu effektiveren Strategien zur Abwasserbehandlung und zu reduzierten Umweltauswirkungen beitragen.

Kapitel 3: Veränderungen in der mikrobiellen Gemeinschaft und cometabolische Einschränkungen bei der heterotrophen Denitrifikation

Dieses Kapitel untersucht, ob die Erhöhung der Stickstoffbelastungsraten (NLRs) in einem heterotrophen Denitrifikationssystem die Entfernung von Antibiotika durch Cometabolismus verbessert und wie langfristige Veränderungen in der Struktur der mikrobiellen Gemeinschaft die Entfernungseffizienzen beeinflussen. Obwohl die heterotrophe Denitrifikation weit verbreitet in der Abwasserbehandlung angewendet wird, sind ihre Biotransformationsmechanismen nur teilweise

verstanden. Zu diesem Zweck wurden zwei kontinuierlich durchströmte denitrifizierende Reaktoren, mit und ohne Antibiotikazufuhr, über einen langen Zeitraum (262 Tage) auf ähnliche Weise betrieben. Die Ergebnisse zeigten, dass eine progressive Erhöhung der NLR von 25 ± 6 mg $N–NO_3^-$ L^{-1} d^{-1} auf 277 ± 38 mg $N–NO_3^-$ L^{-1} d^{-1} trotz der Steigerung der denitrifizierenden Aktivität nicht zu einer erhöhten Entfernung von OMPs im Einklang mit einer cometabolischen Tendenz führte. Stattdessen begünstigte die hohe NLR schnell wachsende Denitrifikanten, was die Prävalenz spezialisierter Taxa, die für die Biotransformation von Antibiotika entscheidend sind, verringerte. Funktionale und taxonomische Vielfalt erwies sich somit als am einflussreichsten für die effektive Entfernung von OMPs, wobei die Denitrifikationsaktivität negativ mit der Biotransformation von Antibiotika korrelierte. Diese Beobachtungen verstärken die Erkenntnis, dass die Zusammensetzung der mikrobiellen Gemeinschaft, mehr als nur hohe denitrifizierende Aktivitäten, das Ausmaß der Antibiotikaentfernung bestimmt.

DNA-Metabarcoding-Analysen zeigten, dass selbst niedrige Antibiotikkonzentrationen (5 µg L^{-1}) die mikrobielle Gemeinschaft signifikant umgestalteten, was die kritische Rolle der Gemeinschaftszusammensetzung und -diversität bei der Entfernung von OMPs hervorhebt. Darüber hinaus traten Taxa aus der Ordnung *Rhizobiales* als Schlüsselbeiträge zur Biotransformation von Antibiotika unter diesen Bedingungen hervor. Jedoch sank die relative Häufigkeit von Rhizobiales, als die NLRs erhöht wurden, und diese Populationen wurden von anderen Taxa, die besser an hochbelastete Umgebungen angepasst sind, verdrängt, was zu einer Verringerung der Effizienz der Antibiotikaentfernung führte. Insgesamt unterstreicht diese Arbeit die funktionalen Kompromisse zwischen der Aufrechterhaltung hoher NLRs zur Verbesserung der Denitrifikation und der Erhaltung metabolischer Funktionen wie der Biotransformation von Antibiotika. Das Verständnis und die Balance dieser Kompromisse sind wesentlich, um die Reaktorausführung unter verschiedenen Betriebsbedingungen zu optimieren.

Kapitel 4: Wie effizient entfernt ein metabolisch verbessertes System mit denitrifizierenden anaeroben methanoxidierenden Mikroorganismen Antibiotika?

In diesem Kapitel untersuchen wir den neuartigen nitritabhängigen anaeroben Methanoxidationsprozess (N-Damo) bei hohen Biomasseaktivitäten hinsichtlich seines Potenzials, gleichzeitig Nitrit, Methan und Spuren von in Abwässern häufig vorkommenden Antibiotika zu entfernen. Zwei Membranbioreaktoren (MBRs) wurden bei drei erhöhten Nitritbelastungsraten (NLRs) betrieben: 76 ± 9,9, 161,5 ± 11,4 und 215,2 ± 24,2 mg $N–NO_2$ L^{-1} d^{-1}, über längere Zeiträume, wobei spezifische denitrifizierende Aktivitäten von bis zu 540 mg $N–NO_2$ g^{-1} VSS d^{-1} erreicht wurden. Bemerkenswerterweise rivalisieren diese Aktivitäten mit konventionellen heterotrophen Systemen in Bezug auf Nitritentfernungsraten. Ein wesentlicher

Unterschied zu früheren Studien war die Zugabe von Cerium als Spurenelement in das Fütterungsmedium, was eine stabile Reaktorbetrieb unter diesen hohen Lasten ermöglichte.

Die Analysen der mikrobiellen Gemeinschaft zeigten, dass N-Damo-Bakterien (*Candidatus Methylomirabilis)* anfänglich beide MBRs dominierten (67–87% relative Häufigkeit während verschiedener Betriebsperioden). Jedoch, mit zunehmender Gesamtaktivität und Konzentration der Biomasse, nahm die Dominanz dieser funktionalen N-Damo-Populationen ab. Gleichzeitig proliferierten andere kleinere Familien wie Hypomicrobiaceae und Xanthobacteraceae, was auf eine Verschiebung in der Gemeinschaftsstruktur hinweist.

Unter diesen Bedingungen zeigten mehrere Antibiotika, einschließlich Sulfamethoxazol, Trimetoprim, Cefalexin und Azithromycin, hohe Biotransformationsraten. Im Gegensatz dazu zeigten Roxithromycin und Clarithromycin nur eine partielle Entfernung (20–35%), und Ciprofloxacin blieb weitgehend unbeeinflusst. Interessanterweise wurde trotz der erhöhten N-Damo-Aktivität und der neuartigen Betriebsstrategie keine klare Verbesserung der Antibiotikaentfernung im Verlauf des Experiments beobachtet. Wie in den in Kapitel 3 untersuchten heterotrophen Denitrifikationssystemen festgestellt, unterstreichen diese Ergebnisse, dass die Zusammensetzung der mikrobiellen Gemeinschaft einen entscheidenderen Einfluss auf die Antibiotikaentfernung ausübt als die allein erhöhte denitrifizierende Aktivität. Letztlich erweist sich die Verwaltung und Steuerung der Struktur der mikrobiellen Gemeinschaft als kritisch für die Verbesserung der Antibiotika-Biotransformation in N-Damo-Reaktoren.

Kapitel 5: Enzymatische Grundlagen der N-Damo-Systeme, enthüllt durch Metaproteomik

Aufbauend auf den in Kapitel 4 beobachteten mikrobiellen Verschiebungen untersucht dieses Kapitel die enzymatische Grundlage des nitritabhängigen anaeroben Methanoxidationsprozesses (N-Damo), einen vielversprechenden Weg zur Erreichung einer kohlenstoffneutralen Abwasserbehandlung im Einklang mit den Zielen nachhaltiger Entwicklung. Durch die Nutzung von Metaproteomik-Analysen haben wir die vollständigen enzymatischen Wege von N-Damo erläutert, wobei ein besonderer Schwerpunkt auf Metalloenzyme gelegt wurde. Die Analysen bestätigten, dass *Candidatus Methylomirabilis Lanthanidiphila* die vorherrschende N-Damo-Spezies war und 67–87% der relativen Proteinmenge repräsentierte. Diese Dominanz wurde durch die Induktion seiner Lanthanid-bindenden Methanol-Dehydrogenase in Anwesenheit von ergänztem Cerium angetrieben, was die wesentliche Rolle der Lanthanide bei der Unterstützung hoher Stickstoffentfernungsraten hervorhebt.

Weitere metaproteomische Daten zeigten die aktive Beteiligung begleitender Taxa an der Denitrifikation, Methylotrophie und Methanotrophie, was die synergetischen Interaktionen innerhalb der mikrobiellen Gemeinschaft demonstriert. Die identifizierten funktionell reichhaltigsten Metalloenzyme erforderten Kupfer, Eisen und Cerium als Cofaktoren, was mit den beobachteten Metallverbrauchsmustern im Bioreaktor korrelierte. Trotz der verbesserten enzymatischen Aktivität, die diese Kernstoffwechselprozesse unterstützte, gab es keine entsprechende Verbesserung der Antibiotikaentfernungseffizienz. Dieses Ergebnis unterstreicht, dass zwar die Optimierung der Mikronährstoffverfügbarkeit und der enzymatischen Wege eine robuste N-Damo-Aktivität aufrechterhalten kann, eine effektive Antibiotika-Biotransformation jedoch wahrscheinlich die gezielte Ansprache spezifischer Enzyme und metabolischer Routen erfordert, die direkt am Schadstoffabbau beteiligt sind.

Insgesamt hebt diese Studie die Nützlichkeit der Metaproteomik bei der Bewertung enzymatischer Aktivitäten innerhalb ingenieurstechnischer Systeme hervor und liefert kritische Einblicke zur Optimierung des mikrobiellen Managements. Durch das Verständnis des komplexen Gleichgewichts zwischen der Aufrechterhaltung hoher N-Damo-Aktivitäten und der Erhaltung der notwendigen Stoffwechselfunktionen für die Antibiotika-Biotransformation können wir Abwasserbehandlungsprozesse besser entwerfen und betreiben, um sowohl eine effiziente Nährstoffentfernung als auch eine Schadstoffabbau zu erreichen.

Kapitel 6: Physiko-Chemische Treiber des Antibiotika-Schicksals und der Transformationswege

Das finale experimentelle Kapitel untersucht das Schicksal und die Transformation der Antibiotika Sulfamethoxazol (SMX) und Triclocarban (TCC) unter heterotropher Denitrifikation mithilfe radiolabelierter Techniken mit zwei markierten Molekülen, ^{14}C-SMX und ^{14}C-TCC. Ziel der Studie war es, zu ergründen, wie die chemische Struktur und die physikochemischen Eigenschaften dieser Mikropollutanten ihr Verhalten in denitrifizierenden Umgebungen signifikant beeinflussen.

TCC, charakterisiert durch seine hydrophobe und neutrale Natur, zeigte ein persistentes Verhalten unter denitrifizierenden Bedingungen. Über einen Zeitraum von 14 Tagen wurden mehr als 59% des TCC am Schlamm adsorbiert und blieben hauptsächlich in extrahierbaren Formen mit vernachlässigbarer Mineralisierung. Diese hohe Adsorption begrenzte wahrscheinlich die Bioverfügbarkeit von TCC, wodurch eine umfassende Biotransformation verhindert wurde. Darüber hinaus nahmen die extrahierbaren Fraktionen von TCC im Laufe der Zeit ab, was auf eine Remobilisierung durch chemische Gradienten und Scherkräfte während des Mischens hinweist.

Im Gegensatz dazu durchlief SMX, ein hochpolares Molekül, das überwiegend als Anion vorliegt, eine schnelle Biotransformation, wobei etwa 80% der Ausgangsverbindung innerhalb eines Tages transformiert wurden. Die Mineralisierung von SMX blieb jedoch niedrig, da es in polarere und persistente Metaboliten umgewandelt wurde, die sich in der wässrigen Phase anhäuften. Diese Transformationsprodukte, die potenziell antibiotische Aktivität aufweisen, unterstreichen die Unzulänglichkeit, sich ausschließlich auf die Entfernung der Ausgangsverbindung zu konzentrieren. Darüber hinaus nahmen die extrahierbaren Fraktionen sowohl von SMX als auch von TCC im Laufe der Zeit ab, was auf eine Remobilisierung von Rückständen hinweist, die bei TCC aufgrund seiner hohen Anwesenheit in der extrahierbaren Fraktion besonders ausgeprägt ist. Nicht extrahierbare Rückstände (NER) zeigten ebenfalls eine Remobilisierung, insbesondere bei SMX, was das dynamische Verhalten dieser Verbindungen unterstreicht.

Die Ergebnisse betonen die Notwendigkeit integrierter Ansätze, die sowohl biotische als auch abiotische Faktoren berücksichtigen, um die Persistenz schädlicher Mikropollutanten effektiv zu minimieren. Die Bewertung des Schicksals von Antibiotika muss nicht nur die Ausgangsverbindungen, sondern auch ihre Transformationsprodukte und nicht extrahierbaren Rückstände umfassen. Diese persistierenden und potenziell bioaktiven Metaboliten sowie die remobilisierten Fraktionen stellen fortdauernde ökologische und gesundheitliche Bedenken dar, was umfassende Schicksalsstudien erforderlich macht, die Biotransformationswege charakterisieren und die antibiotische Aktivität der Transformationsprodukte bewerten.

Kapitel 7: Allgemeine Diskussion

Dieses Kapitel fasst die im gesamten Umfang der Arbeit präsentierten Einsichten und Ergebnisse zusammen, indem es die Ergebnisse aus jedem vorangegangenen Kapitel integriert. Die kollektiven Ergebnisse stellen die vorherrschende Annahme in Frage, dass die Verbesserung der Denitrifikation inhärent zu einer verbesserten Entfernung von Antibiotika führt. Stattdessen hebt die Studie hervor, dass die Struktur der mikrobiellen Gemeinschaft, die Diversität und die Präsenz spezifischer antibiotika-transformierender Taxa und Enzyme kritische Determinanten der Antibiotika-Biotransformation sind. Sowohl heterotrophe als auch autotrophe (N-Damo) Systeme waren in der Lage, hohe Stickstoffentfernungsraten zu erreichen; jedoch stellten weder die intensivierte Denitrifikation noch die angereicherten enzymatischen Fähigkeiten allein eine verbesserte Antibiotikaentfernung sicher. Darüber hinaus verkompliziert die Komplexität des Antibiotika-Schicksals — angetrieben durch compoundspezifische Eigenschaften und die Bildung rezistente Metaboliten — weiter die Entwicklung effektiver Behandlungsstrategien. Diese Ergebnisse unterstreichen die Notwendigkeit, mikrobielle Ökologie und

biochemische Wege bei der Planung und Optimierung von Abwasserbehandlungsprozessen zur Entfernung von Antibiotika zu berücksichtigen.

Kapitel 8: Schlussfolgerung und Zukunftsperspektiven

Dieses abschließende Kapitel skizziert die Hauptresultate der Forschung und schlägt Richtungen für zukünftige Studien vor. Zusammenfassend trägt diese These zu unserem Verständnis der komplexen Beziehungen zwischen Betriebsparametern, Dynamiken der mikrobiellen Gemeinschaft, enzymatischer Aktivität und Eigenschaften von Mikropollutanten in denitrifizierenden Abwasserbehandlungssystemen bei. Die Ergebnisse betonen, dass eine effektive Entfernung von Antibiotika nicht durch einfaches Verbessern der Denitrifikationsaktivität erreicht werden kann. Stattdessen erfordert es einen ganzheitlichen Ansatz, der die mikrobielle Diversität aufrechterhält, spezifische Biotransformationswege anvisiert und die physikochemischen Eigenschaften von Schadstoffen berücksichtigt. Diese Erkenntnisse werden zukünftige Innovationen im Design und Betrieb von Abwasserbehandlungsanlagen leiten und letztlich sicherere Efluentfreisetzungen unterstützen, aquatische Umgebungen schützen und zu globalen Bemühungen zur Minderung der Antibiotikaresistenz beitragen.

Solche Verfeinerungen sind zunehmend entscheidend, da Abwasserbehandlungsanlagen strengeren Vorschriften, dem Bedarf an energieeffizienteren Betriebsabläufen und dem Imperativ zur Kontrolle der Verbreitung von Antibiotikaresistenzen gegenüberstehen. Durch die Fokussierung auf diese Bereiche können Denitrifikationsprozesse effektiver und nachhaltiger werden und die komplexen Herausforderungen der modernen Abwasserbewirtschaftung bewältigen. Letztlich trägt diese Forschung zur Weiterentwicklung der Technologien der Abwasserbehandlung bei, indem sie den Umweltschutz und die öffentliche Gesundheit durch verbesserte Entfernung von Antibiotika und die Minderung von Resistenzen fördert.

Bedeutung und Ausblick

Diese Doktorarbeit bietet kritische Einsichten in das komplexe Zusammenspiel zwischen Betriebsparametern, mikrobieller Ökologie, enzymatischer Aktivität und Eigenschaften von Mikropollutanten bei der Bestimmung des Antibiotika-Schicksals während der Denitrifikation. Durch die Integration molekularer, metabolischer und chemischer Analysen erweitert die Forschung unser Verständnis der notwendigen Bedingungen zur Nutzung denitrifizierender Prozesse für eine verbesserte Entfernung von Antibiotika. Wichtige Erkenntnisse zeigen, dass obwohl die Erhöhung der Stickstoffbelastungsraten (NLRs) die denitrifizierende Aktivität steigern kann, dies nicht notwendigerweise zu kontinuierlichen Verbesserungen bei der Entfernung organischer Mikropollutanten (OMPs) führt. Hohe NLRs führten zu

signifikanten Verschiebungen in der Struktur der mikrobiellen Gemeinschaft, wobei schnell wachsende Denitrifikanten gegenüber spezialisierten Taxa wie denen der Ordnung Rhizobiales bevorzugt wurden, die für die Antibiotika-Biotransformation entscheidend sind.

Darüber hinaus hebt die Studie hervor, dass die funktionale und taxonomische Vielfalt der mikrobiellen Gemeinschaft ein kritischer Faktor für die effektive Biotransformation von Antibiotika ist. Es wurde festgestellt, dass die Denitrifikationsaktivität negativ mit der Effizienz der Antibiotikaentfernung korreliert, was die Bedeutung der Aufrechterhaltung einer vielfältigen und spezialisierten mikrobiellen Gemeinschaft betont, anstatt sich ausschließlich auf die Steigerung der denitrifizierenden Aktivität zu konzentrieren. DNA-Metabarcoding-Analysen zeigten, dass die Antibiotikaexposition, selbst bei niedrigen Konzentrationen, die Zusammensetzung der mikrobiellen Gemeinschaft signifikant verändert, insbesondere bei Langzeitbetrieben, was die Biotransformationsergebnisse weiter beeinflusst.

Enzymatische Untersuchungen im Rahmen des N-Damo-Prozesses unterstrichen, dass die Optimierung der Verfügbarkeit von Mikronährstoffen und die Verbesserung enzymatischer Wege hohe Denitrifikationsraten aufrechterhalten kann. Jedoch erfordert die Erreichung einer verbesserten Antibiotika-Biotransformation das gezielte Ansprechen spezifischer Enzyme und metabolischer Routen, die direkt am Schadstoffabbau beteiligt sind. Dies deutet darauf hin, dass ein nuancierter Ansatz im mikrobiellen und enzymatischen Management notwendig ist, um die Denitrifikations-Effizienz mit den Fähigkeiten zur Antibiotikaentfernung auszubalancieren.

Diese Erkenntnisse unterstreichen die funktionalen Kompromisse zwischen der Aufrechterhaltung hoher NLRs zur Verbesserung der Denitrifikation und der Erhaltung der essentiellen metabolischen Funktionen für die Antibiotika-Biotransformation. Das Verständnis und die Balance dieser Kompromisse sind essenziell, um die Reaktorausführung unter unterschiedlichen Betriebsbedingungen zu optimieren. Die Forschung legt nahe, dass zukünftige Innovationen im Design der Abwasserbehandlung sich darauf konzentrieren sollten, spezifische mikrobielle Stämme und Enzyme zu identifizieren und zu stimulieren, die an der Antibiotikaabbau beteiligt sind, Kohlenstoffquellen zu diversifizieren und gezielte co-metabolische Substrate sowie maßgeschneiderte Mikronährstoffergänzungen zu erforschen. Darüber hinaus stellt die Entwicklung von konstruierten mikrobiellen Konsortien eine vielversprechende Möglichkeit dar, die Entfernung von Mikropollutanten in denitrifizierenden Systemen zu verbessern.

Letztlich trägt diese These zur Weiterentwicklung der Technologien der Abwasserbehandlung bei, indem sie einen umfassenden Rahmen für die Optimierung der Struktur der mikrobiellen Gemeinschaft und der enzymatischen

Aktivität bereitstellt, um sowohl eine effiziente Nährstoffentfernung als auch eine effektive Antibiotika-Degradation zu erreichen. Diese Fortschritte unterstützen eine sicherere Efluentfreisetzung, schützen aquatische Umgebungen und tragen zu den globalen Bemühungen bei, die Ausbreitung von Antibiotikaresistenzen zu mildern, im Einklang mit den Zielen der nachhaltigen Entwicklung und der Bewältigung der drängenden Herausforderungen der modernen Abwasserbewirtschaftung.

CHAPTER 1

Introduction and Objectives

1.1. OVERVIEW OF ANTIBIOTIC USE AND IMPACT

The use of antibiotics has revolutionized modern medicine by controlling infections in humans, animals, livestock and aquaculture systems all over the world. Beyond their medical applications, antibiotics are also used for commercial purposes as growth promoter in animal husbandry. However, this practice has been prohibited under current EU legislation (Carvalho and Santos, 2016; Oberoi et al., 2019; Tran et al., 2016). Among the various classes of antibiotics, β-lactams, macrolides, fluoroquinolones and tetracyclines are the most commonly prescribed antibiotics, in that order (Klein et al., 2024). Without interventions to reduce current consumption rates, global antibiotic use is projected to increase by 52% reaching 75.1 billion defined daily doses (DDDs) by 2030 (Klein et al., 2024). Moreover, the amount and type of antibiotics consumed varies geographically (**Figure 1.1** and **Figure 1.2**) (Browne et al., 2021; Klein et al., 2024). For instance, South Asia has a large consumption in fluoroquinolones and cephalosporins compared to mainland Europe (Browne et al., 2021). In South American countries such as Peru, macrolides are the most frequently consumed antibiotic (Klein et al., 2024). However, in many low to medium income countries, there is a lack of available data and standardized data collection methods across different geographical regions presents an ongoing challenge (Naghavi et al., 2024).

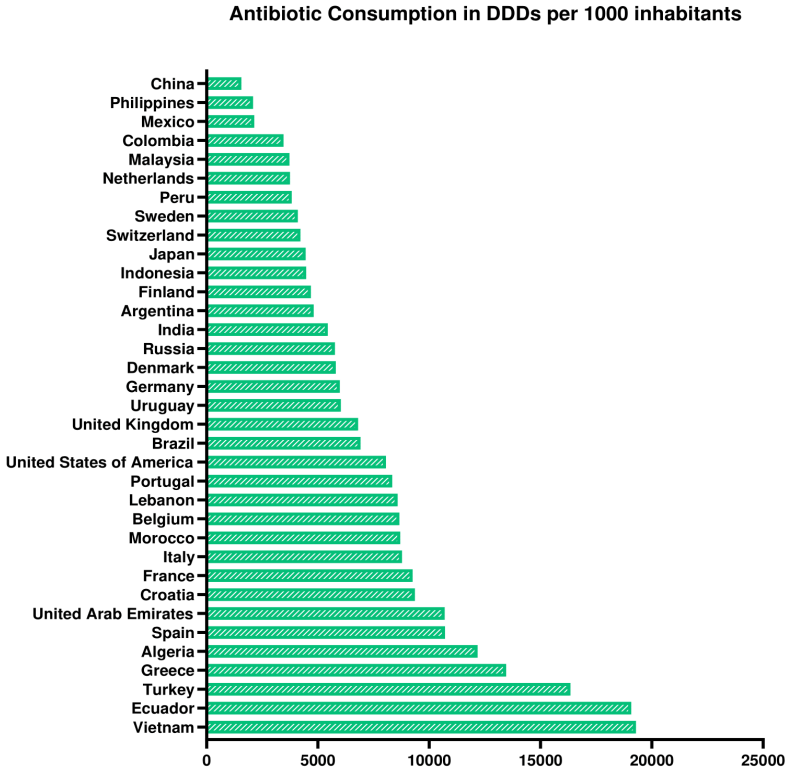


Figure 1.1. Global antibiotic consumption measured in defined daily doses (DDDs) by country for 2023. Data derived from pharmaceutical sales data licensed from

IQVIA MIDAS, as reported in Klein et al., (2024), and normalized by 2024 population estimates from (Our World in Data, 2024).

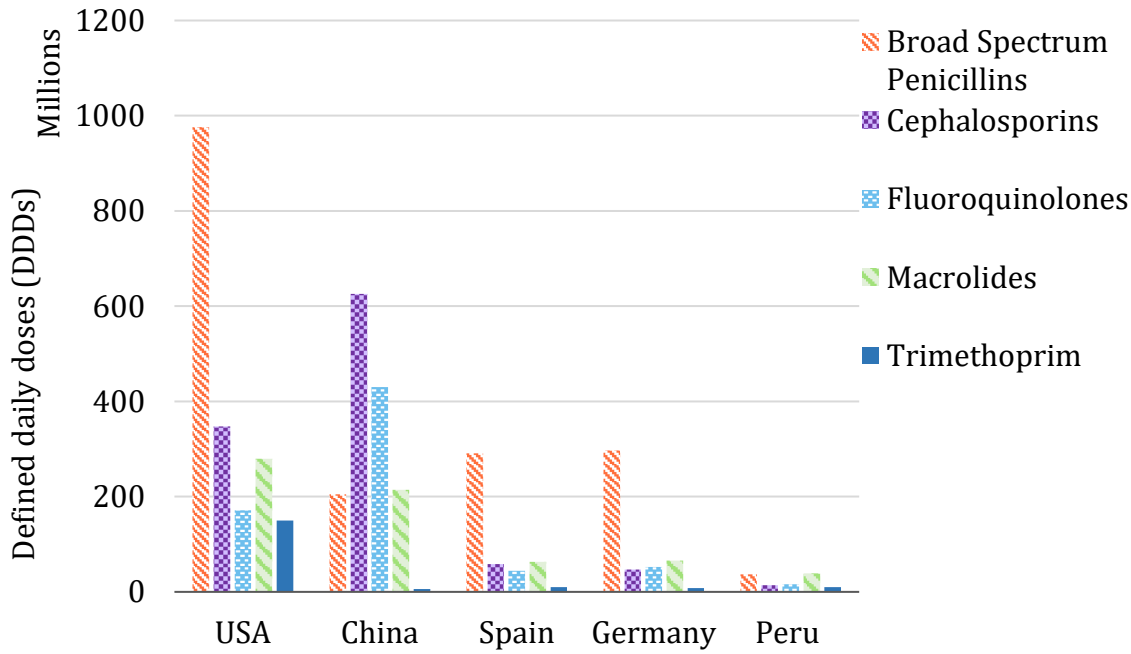


Figure 1.2. Antibiotic consumption by antibiotic class. Amounts in DDDs for the top five antibiotic classes by consumption amount. Data derived from pharmaceutical sales data licensed from IQVIA MIDAS, as reported in Klein et al., (2024)

The increasing global consumption of antibiotics has significant environmental and public health implications. Antibiotics cannot be fully mineralized; it is estimated that 50 to over 70% of antibiotic dosage is excreted via urine and feces as a mixture of parent compound and transformation metabolites, ending up in wastewater streams and, consequently, aquatic environments (Kovalakova et al., 2020; Kümmerer, 2009; Tran et al., 2016). The overuse of antibiotics and their incomplete removal during wastewater treatment have significantly contributed to the escalation of antibiotic resistance, now recognized as a leading cause of mortality worldwide. According to **Figure 1.3**, approximately 1.14 million deaths are attributable to antibiotic-resistant infections. This figure represents individuals who would not have succumbed to infections if effective treatments had been available, highlighting the dire consequences of resistance. Additionally, associated deaths from complications and secondary infections further emphasize the critical public health threat posed by antibiotic resistance. These statistics underscore the urgent need for improved wastewater treatment strategies that effectively remove antibiotics and mitigate the spread of resistance genes in the environment. (IHME Institute for Health Metrics and Evaluation, 2024).

Notably, low-income countries are projected to have the highest rate of mortality associated with bacterial resistance despite lower consumption rates (Klein et al., 2024; Naghavi et al., 2024). Low-income countries are particularly vulnerable due to deficiencies in sanitation infrastructure as well as the lack of regulatory frameworks on antimicrobial consumption (*WHO Report on Surveillance of Antibiotic Consumption*, 2016).

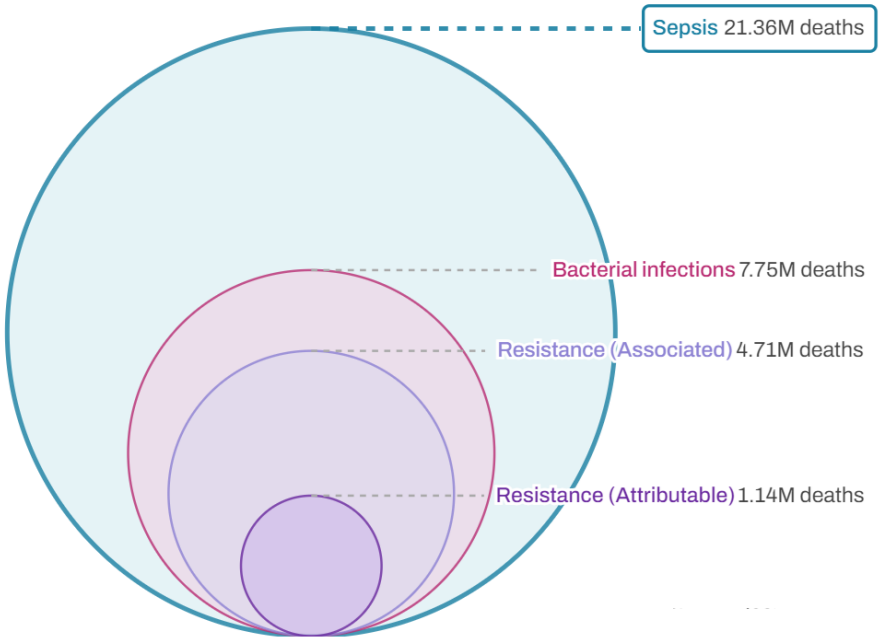


Figure 1.3. Composition of global infection-related deaths to antimicrobial resistance. Source: IHME Institute for Health Metrics and Evaluation, (2024).

1.2. OCCURRENCE OF ANTIBIOTICS IN AQUATIC ENVIRONMENTS

Similar to consumption patterns, the occurrence and type of antibiotics in aquatic environments also varies worldwide (Tran et al., 2018, 2016). In Europe and North America, the detection frequency and concentration of macrolides such erythromycin was on average 142 ng L^{-1} in freshwaters, although highest concentration of erythromycin (3847 ng L^{-1}) was observed in the surface water of the Madrid region(Li et al., 2022; Valcárcel et al., 2011). Concentrations of most organic micropollutants (OMPs), including antibiotics in raw influent of WWTPs in Asian regions tend to be higher to those in European and North American countries(Tran et al., 2018). Notably, β-lactams have been detected in multiple European countries (France, Germany, Greece, Italy, Romania, Spain and UK) across different aqueous environmental matrices from river water, groundwater and effluents of WWTPs (Carvalho and Santos, 2016).

While antibiotic consumption significantly contributes to the presence of antibiotics in the environment, their occurrence in water primarily depends on the removal efficiency of wastewater treatment plants (WWTPs) (Jiang et al., 2024). WWTPs are recognized as the main source of antibiotics in aquatic environments (Felis et al., 2020). The removal efficiency achieved for each antibiotic in WWTPs is influenced by intrinsic molecular characteristics, their interactions with the environment, and the type of treatment process employed (Jiang et al., 2024; Langbehn et al., 2021). For example, β-lactams, including cephalexin, are easily degraded during conventional activated sludge in WWTPs (Mutuku et al., 2022). Their higher removal rates help offset the significant amounts introduced into the environment by their high consumption, reducing their prevalence in treated water. In contrast, sulfonamides, despite lower consumption levels compared to β-lactams, are less efficiently removed during activated sludge, making them more frequently detected in aquatic environments. Sulfamethoxazole, in particular, is a notable sulfonamide antibiotic frequently detected in various water matrices (Oberoi et al., 2019; Tran et al., 2018). Additionally, other types of antibiotics such as fluoroquinolones and tetracyclines, show the highest adsorption coefficients (K_d) between different classes of antibiotics. Therefore, their environmental interactions are dominated by hydrophobic force and their removal is mainly by sorption by any solid matrix (EPS, sludge, sediments and soils) (Harrower et al., 2021; Liu et al., 2019). Overall, the heterogeneity of antibiotics makes them challenging to uniformly treat and remove in WWTPs, requiring diverse and tailored treatment strategies for efficient removal.

Antibiotic concentrations in the aquatic environment exhibit significant variability (Jiang et al., 2024; Li et al., 2022; Luo et al., 2014a). Similarly, the removal efficiencies during full-scale biological treatment WWTPs vary widely, ranging from 12.5 up to 100% (Jiang et al., 2024; Luo et al., 2014a; Oberoi et al., 2019; Verlicchi et al., 2012). Large concentration ranges can be observed even within a single compound due to varying sources, environmental conditions, and the diverse treatment processes employed by wastewater treatment plants. This variability is illustrated in **Table 1.1**, which represents the detection of selected antibiotics among raw wastewater, treated wastewater and receiving surface water. To enhance the overall performance of WWTPs in removing micropollutants, including antibiotics, a combination of different treatment processes can be employed, incorporating quaternary treatments as polishing steps (Pistocchi et al., 2022; Rattier et al., 2014). While incorporating advanced treatment processes significantly improves micropollutant removal, it also entails high costs related to investment, operation, and maintenance, which must be considered (Luo et al., 2014a; Pistocchi et al., 2022). Therefore, improving water and sanitation infrastructures is, therefore, pivotal to reduce antibiotic pollution in the environment, as WWTPs function both

as a frontline defense against antibiotic dispersion to the environment and as a diffusion point (Bürgmann et al., 2018).

The efficiency of advanced treatment process requires the proper implementation of nutrient removal steps earlier in treatment sequence to be effective (Pistocchi et al., 2022). In a comparison study between urban WWTPs effluents in India and the USA using the same methodology and analytical techniques, showed that most pharmaceuticals were on average 50% lower in the USA and that removal efficiencies were 10-30% higher compared to the Indian WWTPs, most likely due to the advanced biological treatment steps in North American WWTPs (Mohapatra et al., 2016). Despite major advancements, engineered biological wastewater treatment systems are only moderately effective (48–77%) in the removal of antibiotics. Moreover, the integration of multiple bioprocesses can synergistically enhance antibiotic degradation while reducing associated costs (Langbehn et al., 2021). In the following section, we focus on biological treatment as it is a key stage to minimize the antibiotic load released into the environment while keeping the cost of investment and operation low.

Table 1.1. Antibiotic concentrations in three different water matrices, influent and effluent of WWTPs and receiving rivers.

Compounds used in this thesis		CLA	ROX	AZI	SMX	CFX	TMP	CIP	TCC	Ref
Raw Wastewater [ng L^{-1}]	Range	-	-	-	<3—980	-	60—680	-	-	2
		1201—1854	-	1537—2951	893—1389	-	197.6—251.2	2241—6453	423.9—933.9	3
		nd—122	-	1083	52—2000	-	-	0.82—6453	-	5
		<LOQ—10491	nd—38.7	nd—22730	<LOD—5490	-	nd—6796	nd—4391	14—4644	6
		64—248	-	-	0.65—4255	nd—13818	409—1620	-	64—335	11
		-	2.3—9	-	3—30.5	-	29—221	66—382	-	12
		115—551	-	nd—214.5	330—417	-	87.8—179.5	639.1—1307	-	7
	Median	1497	-	-	802	-	235.5	-	700.9	3
		136	-	-	2007	5912	992	-	128	11
Treated Wastewater [ng L^{-1}]	Range	-	-	-	<3—1150	-	<10—3050	-	-	2
		387.3—637.1	-	367.3—980	301.5—463.4	-	124.9—178.6	321.3—524.1	143.1—214.5	3
		8—460	-	0—380	0,6—1147	-	-	0.4—920	-	5
		nd—2517	nd—42.3	nd—20178	nd—2070	-	nd—3052	nd—5692	<LOQ—617	6
		92.3—129	-	75.5—135	56.2—73	-	97.2—125	108—174.8	-	7
		18—91	-	-	4145	nd—5624	3—894	919	nd—67	11
		-	0.7—41	-	2.5—89	-	2.4—25	12—231	-	12
	Median	531.7	-	469.5	311.3	-	151.6	495.5	199	3
		66	-	-	342	1750	149	257	15	11
		-	-	-	164.1	-	178	82.1	-	1
Receiving surface water [ng L^{-1}]	Range	-	-	-	-	28.4—55.63	-	-	-	4
		75—91	-	240	0.3-360	-	-	14.9—21.3	-	5
		-	37.5—66.5	-	-	-	-	-	-	9
		235.5 ± 66.1	-	6.4±3.4	1013.2 ± 294.2	-	898.7 ± 303	-	-	10
	Median	-	402.3	-	78.9	-	61	238.3	-	8
		-	50.2	-	-	-	-	-	-	9

nd: not detected, LOQ: limit of quantification. Clarithromycin (CLA), Roxithromycin (ROX), Azithromycin (AZI), Sulfamethoxazole (SMX), Cephalexin (CFX), Trimethoprim (TMP), Ciprofloxacin (CIP), Triclocarban (TCC)

Ref Table 1

[1](Loos et al., 2013),[2](Luo et al., 2014a), [3] (Tran et al., 2016), [4](Ribeiro et al., 2018), [5] (Felis et al., 2020),[6](Jiang et al., 2024), [7] (Rodriguez-Mozaz et al., 2015), [8] (Jiang et al., 2014), [9] (Liu et al., 2015), [10] (Archer et al., 2017), [11](Mohapatra et al., 2016), [12](Tewari et al., 2013).

1.3. ANTIBIOTIC REMOVAL MECHANISMS DURING BIOLOGICAL WASTEWATER TREATMENT

Municipal WWTPs were originally designed to remove carbon, nitrogen, and phosphorus compounds, as well as microbiological contaminants, rather than OMPs (Krzeminski et al., 2019; Verlicchi et al., 2012). However, the growing concern regarding the occurrence and environmental impacts of OMPs, particularly antibiotics, has prompted WWTPs to integrate OMP removal as an additional design criterion. This shift has also forced the re-evaluation and retrofitting of existing processes to enhance OMP elimination (Krzeminski et al., 2019). Furthermore, the introduction of new regulations aimed at safeguarding water quality underscores the increasing need to monitor and effectively remove OMPs during wastewater treatment, as these compounds are often only partially eliminated. There are a number of ways in which WWTPs can contribute to the removal of antibiotics, including, abiotic and biotic processes. However, biological process are typically the most applied approaches in WWTPs due to their significant impact on the removal of antibiotics (Nguyen et al., 2021). By optimizing biological treatment processes, WWTPs can achieve greater sustainability and efficacy, thereby ensuring improved water quality and contributing to the protection of ecological health.

1.3.1. Fate and removal mechanisms of antibiotic in biological wastewater processes

During biological treatment, sorption and biotransformation are the primary mechanisms for the removal of antibiotics, whereas hydrolysis and volatilization are generally insignificant for most antibiotics (Oberoi et al., 2019). However, OMP removal seem to be highly dependent on both the compound characteristics and type of treatment process employed (Grandclément et al., 2017). The physico- chemical characteristics of each antibiotic such as hydrophobicity, sorption coefficient (K_d), and charge, together with the physico-chemical properties of the sludge and operational conditions, including pH, significantly influence the roles of sorption and biotransformation during treatment (Harrower et al., 2021; Nguyen et al., 2021; Oberoi et al., 2019). For example, the higher the K_d of a compound, the more propense it is for sorption, which can make it less available for biotransformation (Harrower et al., 2021; Ortega-Calvo et al., 2020). Hydrophilic yet ionizable compounds may exhibit variable sorption behavior depending on whether the environmental pH favors their neutral or charged forms. The presence of certain functional groups can also influence in the biodegradability of a compound. For instance, hydroxyl groups (R–OH) typically improve biodegradability, whereas halogens (Cl, F) are usually associated to higher recalcitrance or slower biodegradation (Rios-Miguel et al., 2023).

Biotransformation of OMPs, extends beyond their molecular structure and biodegradability. It critically depends on the presence of microorganisms with the necessary metabolic capabilities to degrade these compounds and on the bioavailability of the contaminant for microbial uptake (Ortega-Calvo et al., 2020). Effective biotransformation also requires a sufficient abundance of OMP degrading microorganisms, a condition generally supported by the diverse and rich microbiomes present in biological wastewater systems (Helbling et al., 2012a). The persistence of chemicals is commonly assessed through their biodegradation potential, quantified as the time required to remove their concentration by half, often referred to as half-life (DT_{50}). The accumulation of persistent antibiotics can exert toxic effects on the microbiome, leading to decreased microbial activity, diversity and richness, which may compromise the resilience and functionality of wastewater treatment ecosystems (Huemer et al., 2020).

Differences between removal efficiencies for a given molecule are often attributed to changes in operating parameters within biological systems. Key parameters influencing these processes include Solid Retention Time (SRT), Hydraulic Retention Time (HRT), temperature, pH, and redox condition (Alvarino et al., 2018; Grandclément et al., 2017; Le-Minh et al., 2010; Polesel et al., 2016). For example, longer SRTs support the growth of slow-growing microorganisms, such as nitrifiers, which contribute to greater enzymatic diversity and extended biomass retention, thereby enhancing biotransformation potential (Polesel et al., 2016). Similarly, extended HRT provides sufficient reaction time for either biotransformation or sorption to the sludge, allowing the system to reach maximum efficiency at equilibrium (Le-Minh et al., 2010). Rapid biodegradation of SMX has been observed in activated sludge (AS) systems when sufficient sludge concentrations (>2.5 g L^{-1}) and HRTs (>6 h) were maintained (Yang et al., 2012). Additionally, microbiome diversity can be increased by extending HRT and SRT to influence food to microorganism (F/M) ratios. Longer SRTs combined with reduced F/M ratios under low HRT conditions can drive microorganisms to utilize alternative carbon sources (i.e. OMPs). This selective pressure could explain why some antibiotics are better removed in MBR systems with longer SRT and lower F/M ratio compared to conventional AS systems (Grandclément et al., 2017).

The redox conditions applied during biological processes significantly influence the active biotransformation pathways through the selection of electron acceptors (e.g. O_2, NO_3^-, SO_4^{2-} or CH_4) and shape microbial community composition (Alvarino et al., 2018; H.R. Branco et al., 2023). Aerobic conditions are often considered as more energetically favorable for the biotransformation of many OMPs due to the higher energy yield available to microorganisms (Ghattas et al., 2017; Wolff et al., 2018). However, for certain antibiotics, biotransformation may be more advantageous under anoxic than aerobic or anaerobic conditions. For example, SMX

and TMP exhibit better removal rates under anoxic and anaerobic conditions compared to aerobic environments (Alvarino et al., 2018; Burke et al., 2016). Despite this removal potential, research focusing specifically on anoxic conditions is scarce compared to other redox environments. Some studies have studied the combination of anoxic conditions with aerobic and anaerobic processes, demonstrating enhanced overall OMP removal (Alvarino et al., 2019; Arias et al., 2018). This improvement is likely due to the fact that multiple redox conditions support more diverse microbial communities. Such diversity increases the availability of metabolic pathways and expands the enzymatic pool, thereby enhancing the capacity for OMP removal.

Lastly, as water systems are inherently circular, the incomplete removal of antibiotics in WWTPs can lead to their accumulation and dissemination in the environment. This issue is particularly concerning when treated water is reused for purposes such as crop irrigation, where antibiotics can be taken up and accumulate in food crops, posing risks to human and ecological health(Krzeminski et al., 2019). Maximizing antibiotic removal through energy-efficient biological processes is therefore essential for mitigating their environmental spread.

1.3.2. Biotransformation of antibiotics during wastewater treatment

Biotransformation is the main removal mechanism for OMPs during biological wastewater treatment (Kennes-Veiga et al., 2022a; Oberoi et al., 2019). Biotransformation can be separated in two main pathways: metabolism and/or cometabolism (**Figure 1.4**). Microorganisms can degrade OMPs through metabolism, where OMPs serve as alternative growth substrate, or cometabolism, where OMP biotransformation relies on the presence of a primary substrate (Margot et al., 2015; Tran et al., 2013). Metabolic reactions occur when OMP concentrations support catabolic energy production and/or anabolic processes, such as cell maintenance, growth, and reproduction (Margot et al., 2015). In contrast, cometabolism involves the activation of non-specific enzymes or cofactors required for OMP biotransformation, which are induced by a primary growth substrate (Margot et al., 2015; Tran et al., 2013). Microorganisms involved in cometabolic processes must produce enzymes with broad substrate specificity, to ensure survival despite competition between OMPs and growth substrates (Benner et al., 2013).

Metabolic processes are often considered more advantageous, as they can result in complete mineralization of OMPs, whereas cometabolism may lead to the accumulation of transformation metabolites due to the absence of specific enzymes required for full biodegradation (Rios Miguel, 2022). However, higher antibiotic concentrations (mg L^{-1} range) would yield the necessary carbon for metabolic reactions, however, as antibiotics are specifically designed to inhibit bacterial growth and disrupt metabolic activity, high concentration can lead to microbial

death or stasis, which can cause toxicity and impede microbial growth as long as antibiotic activity persists (Stokes et al., 2019; Tran et al., 2013).

In biological wastewater treatment, OMP biotransformation predominantly occurs via cometabolism, driven by low-specificity enzymes involved in the metabolism of growth substrates (Kennes-Veiga, 2021). A key distinction between metabolic and cometabolic removal lies in gene regulation: during cometabolism, the genes involved in OMP transformation are not directly induced by the OMP, whereas in metabolism, the cell senses the OMP, expresses the necessary catabolic genes, and utilizes the OMP as a source of carbon or nitrogen (Men et al., 2017; Pérez-Pantoja et al., 2021; Rios Miguel, 2022). Importantly, both mechanisms can occur simultaneously in a process known as mixotrophy, where microorganisms degrade OMPs alongside other carbon sources (Kjeldal et al., 2014; Rios Miguel, 2022).

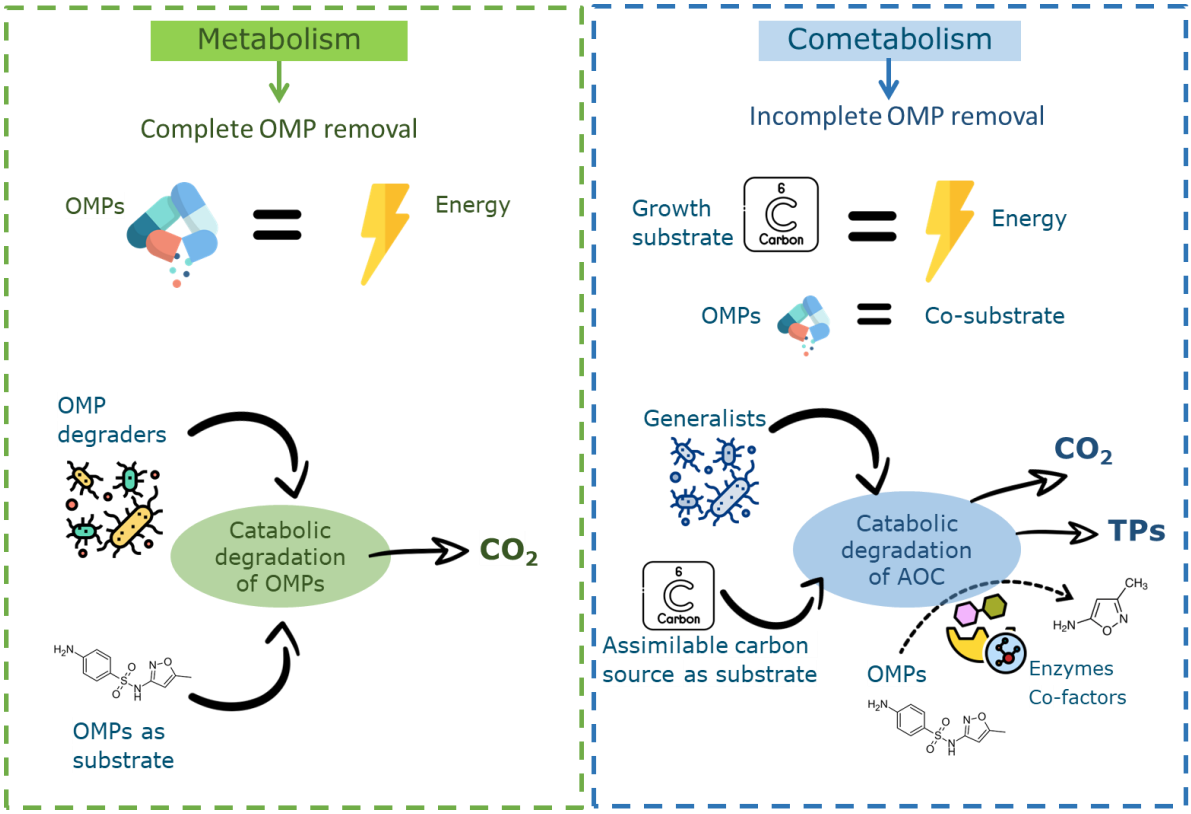


Figure 1.4. Micropollutant biotransformation by metabolism and cometabolism.

The Michaelis–Menten model, initially developed to describe enzyme-catalyzed reactions, offers a valuable conceptual and mathematical framework for understanding micropollutant biotransformation in microbial systems. By relating reaction rates to substrate concentrations, this model elucidates how degradation rates of OMPs can increase with rising substrate levels until a saturation point is reached (Schwarzenbach, 2005).

A common strategy to induce cometabolic reactions involves increasing the concentration of a growth substrate, as shown in heterotrophic and nitrifying activated sludge systems (Fernandez-Fontaina et al., 2012; Kennes-Veiga et al., 2021a) as well as in anaerobic processes (Alvarino et al., 2014). However, recent studies suggest that such cometabolic activity is not simply proportional to substrate load. Instead, it is confined to a specific substrate concentration window, bounded at the upper end by substrate saturation and at the lower end by an activation threshold (Kennes-Veiga et al., 2021b; Sheng et al., 2021). Consequently, cometabolic activity for OMPs would be apparent only within this concentration range, while concentrations above or below this window fail to enhance degradation.

Empirical evidence supports the existence of these thresholds. For instance, when substrate availability is sufficiently high, OMP removal rates can increase sharply (Fernandez-Fontaina et al., 2012; Kennes-Veiga et al., 2021a), demonstrating positive cometabolic tendencies. Identifying the lower activation threshold is more challenging, as recent findings show that even at very low concentrations (e.g., 0.1 μg L-1 SMX), metabolic responses are still triggered, enabling mineralization (Lopez Gordillo et al., 2024). However, the decline in biotransformation rate constants as pharmaceutical concentrations decrease from 10 to 0.5 μg L-1 further underscores the role of substrate thresholds in governing cometabolic processes (van Bergen et al., 2021). These collectively highlight the nuanced balance between substrate availability and microbial metabolic capacity, emphasizing the need to consider both upper and lower concentration thresholds when optimizing cometabolic OMP removal in wastewater treatment systems.

1.4. DENITRIFICATION PATHWAYS AND THEIR INFLUENCE ON ANTIBIOTIC BIOTRANSFORMATION

Denitrification, a critical process in the nitrogen cycle, is widely used for nitrogen removal in wastewater treatment systems. This section examines two primary denitrification pathways: heterotrophic denitrification and autotrophic denitrification via N-Damo (Nitrite-Dependent Anaerobic Methane Oxidation). Both pathways have similarities (i.e. expression of common enzymes such as nitric reductases and redox potential) but also their own unique microbial community compositions and enzymatic activities (i.e. expression of nitric oxide dismutase during N-Damo), which directly influence antibiotic biotransformation.

Heterotrophic denitrifiers typically consist of diverse bacterial taxa that rely on organic carbon sources and express a variety of reductase enzymes capable of degrading antibiotic molecules. In contrast, autotrophic denitrifiers involved in the N-Damo process are specialized microorganisms that utilize inorganic substrates, such as methane and nitrite, and possess distinct enzymatic systems that interact differently with antibiotic compounds (Lu et al., 2014a). By exploring these

differences in microbial communities and their enzymatic mechanisms, we can better understand how each denitrification pathway affects the degradation and transformation of antibiotics in both natural ecosystems and engineered wastewater treatment systems.

1.4.1. Heterotrophic Denitrification

Heterotrophic denitrification is the sequential reduction of nitrate or nitrite to nitrogen gas. In this respiratory process, nitrate and nitrite serve as electron acceptors in place of oxygen, enabling the metabolism of organic carbon (Hochstein, 1988). The process involves four stepwise reactions catalyzed by distinct nitrogen reductases: nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos) (**Figure 1.5**). The choice of carbon source used for denitrification in wastewater treatment significantly influences both the process yield and the composition of the microbial community (Lu et al., 2014b). Most denitrifying bacteria are facultative anaerobes, capable of utilizing ionic and gaseous nitrogen oxides as electron acceptors in the absence of oxygen. Diversity of such microbes, which reflects abundance of species, has effects on not only wastewater treatment performance but also stability of treatment systems (Miao and Liu, 2018). However, despite its widespread application in wastewater treatment, research on antibiotic biotransformation during heterotrophic denitrification is very scarce.

The type and availability of growth substrates, such as nitrogen and carbon sources, have been shown to significantly influence OMP removal during denitrifying processes (Polesel et al., 2017; Torresi et al., 2017a). The role of cometabolism under anoxic conditions, however, remains unclear. In Polesel et al., (2017), a positive relationship between increased denitrification activity and the biotransformation kinetics of sulfamethoxazole, erythromycin, and trimethoprim was reported, while in Martínez-Quintela et al., (2023), no correlation between denitrification activity and OMP biotransformation was found. Instead, shifts in microbial community composition were identified as more influential in OMP biotransformation than cometabolic interactions.

1.4.2. Nitrite-Dependent Anaerobic Methane Oxidation (N-Damo)

This microbially mediated process links the nitrogen and carbon cycles through the activity of specialized functional enzymes associated with NC10 bacteria and anaerobic methanotrophic archaea (ANME-2d) microbial consortia (Stein, 2020). During N-Damo, nitrate or nitrite is reduced to nitrogen gas without the formation of N_2O, while in the carbon pathway, methane is oxidized to CO_2. In **Figure 1.5**, N-Damo metabolic pathway is illustrated, where the nitrogen and carbon cycles are consumed simultaneously, each step triggered with a respective enzyme. The metalloenzymes present in N-Damo partially differ to conventional

heterotrophic denitrification, where special attention is given to the methanol dehydrogenase (MDH) which can be stimulated by using cerium and tus increase the overall microbial kinetics of the process (Versantvoort et al., 2018). The N-Damo process is primarily mediated by bacteria and archaea within the NC10 phylum, including bacteria from the family Candiatus *Methylomirabilis* (Ettwig et al., 2010) and archaea belonging to Candidatus *Methanoperedens* lineage (Haroon et al., 2013). While most members of the NC10 phylum remain uncultured, 16S rRNA gene sequencing has identified at least three bacterial species: Candidatus *Methylomirabilis oxyfera* (M. oxyfera), Candidatus *Methylomirabilis sinica* (M. sinica)(He et al., 2016) and Candidatus *Methylomirabilis lanthanidiphila* (Versantvoort et al., 2018). In contrast, for the archaeon involved in the N-Damo process, only a single species has been proposed: Candidatus *Methanoperedens nitroreducens* (Haroon et al., 2013)

Few studies have investigated the metabolic potential of N-Damo microorganisms for antibiotic removal. One of the first studies providing evidence of cometabolic biotransformation for several OMPs, including ROX, ERY and SMX, demonstrate that specific nitrite removal rates were positively correlated with specific OMP removal rates (Martínez-Quintela et al., 2021a). However, mechanisms underlying this correlation remain largely unclear. There is also limited information on the long-term stability and resilience of N-Damo communities in varied wastewater treatment settings. Addressing these gaps through comprehensive molecular and enzymatic studies is essential to harness the full potential of N-Damo microorganisms for effective antibiotic removal.

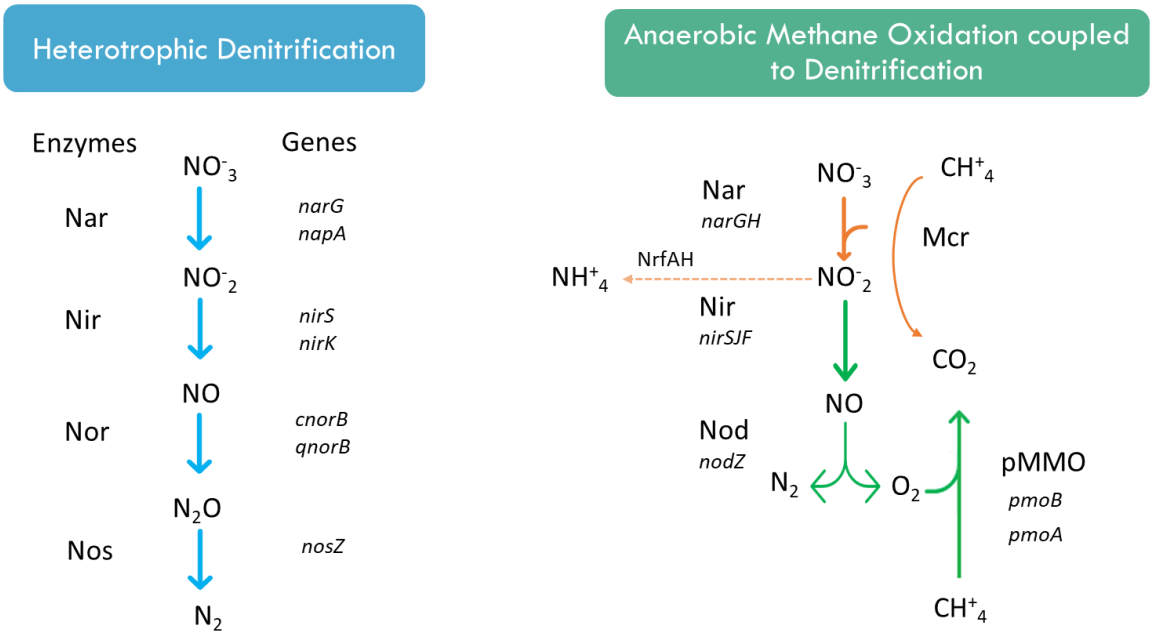


Figure 1.5. Denitrification pathways studied in this thesis.

1.5. RESEARCH OBJECTIVES

The main goal of this thesis is to understand antibiotic biotransformation mechanisms during denitrifying wastewater treatment to enhance existing biological treatment. This is planned through the following specific objectives:

I. Assess antibiotic cometabolic biotransformation during denitrifying processes during medium and high primary substrate loading rates.

II. Investigate microbiome dynamics and enzymatic responses due to increasing primary substrate loads and antibiotic exposure.

III. Understand the fate and biotransformation pathway of antibiotics and transformation metabolites during heterotrophic denitrification

This introduction establishes the scientific rationale and context for examining antibiotic biotransformation in anoxic wastewater treatment environments. The subsequent chapters present the methodological approaches (**Chapter 2**), and four experimental chapters addressing each research objective of this thesis.

In **Chapter 3**, we investigated whether increasing denitrifying activity in a lab-scale reactor could enhance the removal of selected antibiotics, spiked at μg L^{-1} concentrations, through a cometabolic biotransformation mechanism. This chapter also explores the influence of microbial community composition on antibiotic biotransformation under heterotrophic denitrifying conditions, providing a comprehensive understanding of the underlying microbial dynamics in response to increasing NLRs and antibiotic presence.

For **Chapter 4,** we aimed to metabolically enhance the N-Damo process to investigate whether high N-Damo activities can facilitate the biotransformation of a selected group of antibiotics during long-term operation. Similar to **Chapter 3,** this research seeks to elucidate the impact of primary substrates on the cometabolic biotransformation capabilities of N-Damo microorganisms. Additionally, this chapter provides insights into the microbiological implications of varying NLRs and the presence of antibiotics within a highly enriched N-Damo system.

Chapter 5 investigates the unique enzymatic activities of N-Damo microorganisms within the autotrophic denitrifying pathway. Building upon the findings of **Chapter 4**, this chapter assesses the highly enriched N-Damo community under elevated NLR exceeding 240 mg TN $L^{-1}d^{-1}$. The focus is specifically on metalloenzymes, which are analyzed using shotgun metaproteomics to elucidate their roles and contributions to the N-Damo process.

In **Chapter 6**, aims to elucidate the fate and removal mechanisms of two micropollutants with antimicrobial properties, sulfamethoxazole (^{14}C- SMX) and triclocarban (^{14}C- TCC), during heterotrophic denitrification process. This chapter investigates the degradation pathways and transformation metabolites of these

micropollutants to understand their biotransformation and ultimate fate within a conventional denitrification process.

Lastly, **Chapter 7** integrates the results and conclusions, highlighting the main outcomes of this doctoral thesis. The chapter concludes with future research perspectives, proposing strategies to enhance antibiotic removal during denitrification in biological wastewater treatment systems.

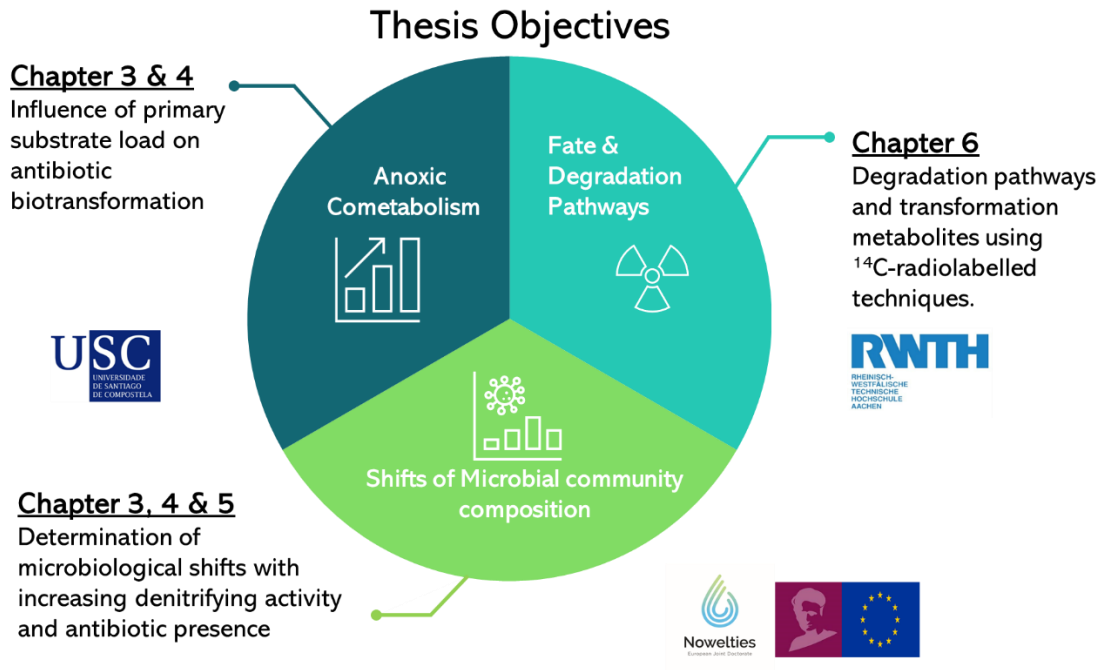


Figure 1.6. Schematic overview of the main thesis objectives and corresponding chapters

CHAPTER 2

Materials and Methods

Abstract

This chapter presents the methods used to investigate the biotransformation of antibiotics under denitrifying conditions in wastewater treatment systems.

Experimental strategies and set-up of lab-scale reactors operating under both conventional heterotrophic denitrification and the novel N-Damo (Nitrite-dependent Anaerobic Methane Oxidation) process are described. The chapter includes descriptions of the analytical techniques used to monitor denitrification activity in both the liquid and solid phases are provided. These include standard biochemical assays and spectroscopic methods. details the approaches for biomass characterization, including biomass concentration microbial community composition and metaproteomics. Lastly, the methodology used for on the radiolabeled experiments for the determination of fate is explained.

2.1. LIQUID PHASE

2.1.1. Nitrogen species and carbon source

The concentration of nitrogen species, including ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-), were quantified using spectrophotometric methods according to the standard methods (APHA, 2017). Prior to analysis, all samples were filtered through 0.45 μm membrane filters (Millipore) to remove solid particles and were measured immediately to ensure accuracy.

The carbon content in the samples was assessed through measurements of chemical oxygen demand (COD), total organic carbon (TOC), and total inorganic carbon (IC). COD concentrations were determined using a colorimetric method which consisting in a controlled digestion with potassium dichromate as oxidant. This method ensured that all oxidizable organic compounds reacted, reducing the dichromate ion ($Cr_2O_7^{2-}$) to chromic ion (Cr^{3+}), which was measured to determine COD (APHA, 2017).

TOC and IC concentrations were analyzed using a Shimadzu TOC-5000 analyzer. TOC was calculated indirectly as the difference between the total carbon (TC) and IC concentrations for each sample. The analyzer was equipped with an automated sampler (Shimadzu ASI-L) for efficient and precise processing of multiple samples(APHA, 2017).

2.1.2. Inorganic ions

Nitrite (NO_2^-), nitrate (NO_3^-), chloride (Cl^-), phosphate (PO_4^{3-}), sulphate (SO_4^{2-}), potassium (K^+), magnesium (Mg^{2+}) and calcium (Ca^{2+}) were quantified by ion chromatography (IC) with an Advanced Compact IC system (861, Metrohm) equipped with a CO_2 suppressor (MCS 853, Metrohm) and a sample processor/injector (838, Metrohm). Data acquisition and processing was performed using IC Net 2.3 software. Additionally, cerium (Ce), analyzed in **Chapter 4** and **Chapter 5** was quantified using inductively coupled plasma mass spectrometry (ICP-MS).

Triplicate samples were previously filtered through 0.45 μm to remove any particulate matter before analysis. Each sample was then injected with a volume of 20 μL into the IC system. For quantification, four to six calibration points in the range of 3-100 mg L-1 were prepared for each ion.

Anion Analysis: Anions were quantified using a Metrosep A column (Supp 5) with dimensions of 250 mm × 4.0 mm. The mobile phase consisted of a buffer solution containing 3.2 mM Na_2CO_3 and 1.0 mM $NaHCO_3$. The flow rate was maintained at 0.7 mL/min.

Cation Analysis: Cations were determined using a Metrosep C3 column, also with dimensions of 250 mm × 4.0 mm. The mobile phase for cation separation was 3.5 mM nitric acid. The flow rate for cation analysis was set to 1.0 mL/min.

2.2. GAS PHASE

The gas composition for N-damo reactors in chapter 4 was measured by gas chromatograph (Hewllet-Packrad, 5890 Series II) equipped with a Thermal Conductivity Detector (TCD) and a stainless-steel column (2 m length, 1/8" outer diameter) and filled with Porapack Q (mesh 80/100). Operational temperatures of the injector, column and detector were set at 110, 35 and 110 °C, respectively. Helium was used as carrier gas with a flow of 15 mL/min.

Quantification of target gases (CH_4, CO_2, N_2, and H_2S) was carried out using a standard gas mixture (CH_4: 66%; CO_2: 30%; N_2: 2%; H_2S: 2%) to establish response factors. The calibration procedure followed a response factor method, with CO_2 serving as the reference gas to ensure accurate quantification. Gas samples were collected in triplicates using 1 mL plastic syringes equipped with closing valves to prevent sample loss and contamination. Each gas sample extracted from the reactors headspace was injected into the gas chromatograph using the syringe, and measurements were conducted in triplicate

2.3. BIOMASS CHARACTERIZATION

2.3.1. Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS)

The amount of Total and Volatile Suspended Solids (TSS and VSS) in the reactor operation was done according to standard methods (APHA, 2017). Triplicate samples of 10 ml were taken from the moving liquid with a syringe after each antibiotic sampling campaign.

For TSS determination, samples were filtered onto pre-weighed glass fiber filters (GF/C, Whatman, USA), dried at 105 °C for 24 hours, and reweighed to calculate the TSS concentration. VSS was measured by igniting the dried filters at 550 °C for 1 hour to remove organic matter, followed by reweighing. The difference between TSS and VSS provided the inorganic suspended solids concentration. All measurements were conducted in triplicate to ensure analytical accuracy and reproducibility.

2.3.2. Molecular analysis

2.3.2.1. Microbial community composition

The microbial community composition was characterized by taxonomical assignments using 16S rRNA illumina sequencing and the MiDAS database. The inoculum and biomass extracted from both reactors were subject to DNA extraction using the Nucleospin Microbial DNA extraction kit (Machery-Nagel). During reactor

performance, triplicates of 5 mL of mixed liquor were extracted once the system reached steady state in Periods PI, PIII and PV. Samples were stored in -60 °C until DNA extraction. The V3-V4 hypervariable region of the 16 S rRNA gene from bacteria was amplified using Bakt_3 41 F (5′ CCT ACG GGN GGC WGC AG 3′) and Bakt_805R (5′ GAC TAC HVG GGT ATC TAA TCC 3′) (Herlemann et al., 2011). DNA metabarcoding analyses of the region in an Illumina NovaSeq PE250 platform, generating paired ends reads (2 x 250) by AllGenetics & Biology SL (www.allgenetics.eu).

Bioinformatic analyses were performed using the Microbial Genomics module (version 21.1) workflow of the CLC Genomics workbench (version 21.0.3). For the taxonomic assignment, raw sequences were filtered, trimmed and then clustered into Operational Taxonomic Units (OTUs) at 97% cut-off for sequence similarity and classified against the non-redundant version of the MiDAS database for taxonomical assignment (Dueholm et al., 2022) (https://www.midasfieldguide.org/guide). Only the most abundant bacterial OTUs (above 1 % of the total observed OTUs) were considered for further analysis.

2.3.2.2. Proteomic analysis

In **Chapter 5**, proteomic analyses were conducted to investigate the N-damo pathway, with a particular emphasis on metalloenzymes, to elucidate the enzymatic potential under conditions of high nitrite loading rates.

For this purpose, proteins were extracted from the active MBRs highly enriched with N-damo in (Kennes-Veiga et al., 2022b). Proteins were identified with shotgun metaproteomic approach by a tryptic digestion of the proteome as described in Zhang et al., (2013), and the resulting peptides were analyzed via nLC-MS/MS using a nanoElute system coupled to a TIMS-QTOF Pro mass spectrometer in data-dependent PASEF mode. Raw MS data were deposited in *ProteomeXchange* (PXD040475). Data processing was conducted with PEAKS Studio 10.6 using three databases: a custom *Methylomirabilis* database from UniProtKB/Swiss-Prot (Versantvoort et al., 2018, 2019; Kennes-Veiga et al., 2022) (Kennes-Veiga et al., 2022b; Versantvoort et al., 2019, 2018), a restricted reference database of selected genera (Blakeley-Ruiz and Kleiner, 2022), and a *Methanoperedens* reference set. Peptide taxonomic assignments were further explored using Unipept Desktop 3.0.

2.3.3. Specific denitrifying activity

Activity tests to determine the maximum biomass activity were conducted at the end each of the experimental periods for both denitrifying systems. For the heterotrophic denitrifying reactors, the protocol followed the method described by Buys et al., (2000). Briefly, mixed liquor from the reactor was extracted and washed in a phosphate buffer. The washed biomass was then placed in serum bottles, which were capped and spiked with a substrate. The pressure increases in the bottles,

caused by N_2 generation during the denitrification reaction, was measured until it stabilized. The headspace overpressure was monitored using a 0–5 psi pressure transducer (Centerpoint Electronics). The maximum biomass activity was determined by analyzing the steepest portion of the slope of N_2 gas production, following the ideal gas law.

For the N-damo reactors, the assay was performed directly inside the MBR. Once stable operation was achieved at the end of the experimental periods, the liquid feed was disconnected, and a controlled pulse of nitrite was introduced into the system. The degradation kinetics were then monitored until the nitrite was fully consumed, providing a direct measure of the biomass maximum activity under those specific operational conditions. The maximum activity was calculated by analyzing the steepest slope in the decline of nitrite concentration over time.

2.4. MICROPOLLUTANTS

During the reactor operation in **Chapter 3** and **Chapter 4**, a mixture of seven antibiotics was continuously fed at a final concentration of 5 μg L^{-1}: cephalexin (CFX), sulfamethoxazole (SMX), trimethoprim (TMP), ciprofloxacin (CIP), roxithromycin (ROX), azithromycin (AZY), and clarithromycin (CLA). For the batch experiments in **Chapter 6**, SMX and triclocarban (TCC) were individually evaluated. The physico-chemical characteristics of these compounds are summarized in **Table 2.1**. The selected antibiotics represent a range of properties, including varying modes of action, molecular structures, and environmental persistence. Their selection was guided by their occurrence in sewage and their designation as priority pollutants under the Water Framework Directive.

During each campaign, liquid samples were collected from both the feed and effluent streams on three separate days, ensuring at least one hydraulic retention time (HRT) interval with a new synthetic medium feed. Each sample, with a volume of 100 mL, underwent initial filtration through a 0.45 μm filter, followed by acidification to a pH 4.5. To enhance chelation of metal ions, Na2EDTA was added to a final concentration of 0,1% (g solute/g solution). The samples were then preconcentrated using a Solid Phase Extraction (SPE) based on an adapted protocol from Gros et al., (2013). The SPE protocol was performed manually using a vacuum pump using Oasis HLB cartridges (60 mg, 3 mL). Finally, analytes were eluted with 6 mL of pure methanol, evaporated to dryness using nitrogen gas stream for 30 minutes and subsequently reconstituted with 3 mL of methanol/water (10:90, v/v). Prepared samples were stored at 4 °C until further analysis.

Liquid chromatographic separations were carried out using an Agilent 6410B-QQQ LC/MS system. A C18 Zorbax eclipse XDB column (2,1x150mmx5μm) was used for the analysis. The mobile phase consisted of two solvents: Eluent A,

composed of 10 mM of formic acid/ammonium formate, and Eluent B, methanol. Each injection volume was 20 µl. The limit

Overall, the method recoveries in the liquid matrix ranged between 55-103% while the limits of quantification and detection ranged between 0.01 to 0.02 µg L-1, details are indicated in **table 2.2**.

Table 2.1. Physio-chemical characteristics of selected antibiotics

Group	Antibiotic	pK_a at 25 °C	Log K_{ow}	K_d anoxic sludge (L kg^{-1})	Solubility (mg L^{-1})	Molecular formula	Molecular weight (g mol^{-1})	Predominant species at pH=7-8
Macrolides	Clarithromycin	8.99	3.16	130	0.33	$C_{38}H_{69}NO_{13}$	748	Cation
	Azithromycin	8.74 and 9.45	4.02	130	2.37	$C_{38}H_{72}N_2O_{12}$	749	Cation
	Roxithromycin	9.17	2.1-2.8	173	0.02	$C_{41}H_{76}N_2O_{15}$	837	Cation
Cephalosporins	Cephalexin (1G)	5.2	-0.3454	0	297	$C_{16}H_{17}N_3O_4S$	347.4	Neutral
Sulfonamides	Sulfamethoxazole	1.85 and 5.70	0.5-0.9	40-162	610	$C_{10}H_{11}N_3O_3S$	253.28	Anion
Quinolones	Ciprofloxacin	6.09 and 8.63	0.28	1002	1.3	$C_{17}H_{18}FN_3O_3$	331.34	Zwitterion
Diamino-pyrimidines	Trimethoprim	3.23 and 7.2	0.9-1.4	14	400	$C_{14}H_{18}N_4O_3$	290.32	Zwitterion

Based on PubChem, Chemspider Chemicalize and Oberoi et al., (2019)

Table 2.2. Method recoveries, limits of quantification and limit of detection for the studied antibiotics

Antibiotics	Method recovery (%)	Quantification limit (µg L^{-1})	Detection limit (µg L^{-1})
Clarithromycin	70	0.5	0.02
Azithromycin	65	0.5	0.02
Roxithromycin	70	0.20	0.02
Cephalexin (1G)	100	0.5	0.01
Sulfamethoxazole	78	0.5	0.02
Ciprofloxacin	55	0.5	0.02
Trimethoprim	103	0.5	002

2.5. EXPERIMENTAL SET-UP

In this study, both heterotrophic and autotrophic denitrifying systems were investigated under continuous-flow (**Chapter 3**) and membrane bioreactor (MBR) configuration (**Chapter 4**) to test if enhancing denitrifying activity could improve the removal of selected antibiotics during denitrification. The primary aim was to assess if a cometabolic biotransformation mechanism contributed to antibiotic removal under anoxic conditions. To distinguish the effects of antibiotic exposure from those of increasing nitrogen loading rates (NLR), each system was operated in duplicate, with one reactor receiving an antibiotic feed at environmental concentrations (µ L-1) and the other serving as a control. Long-term operation of these systems involved a stepwise increase in NLR at defined intervals (**Figure 2.1**).

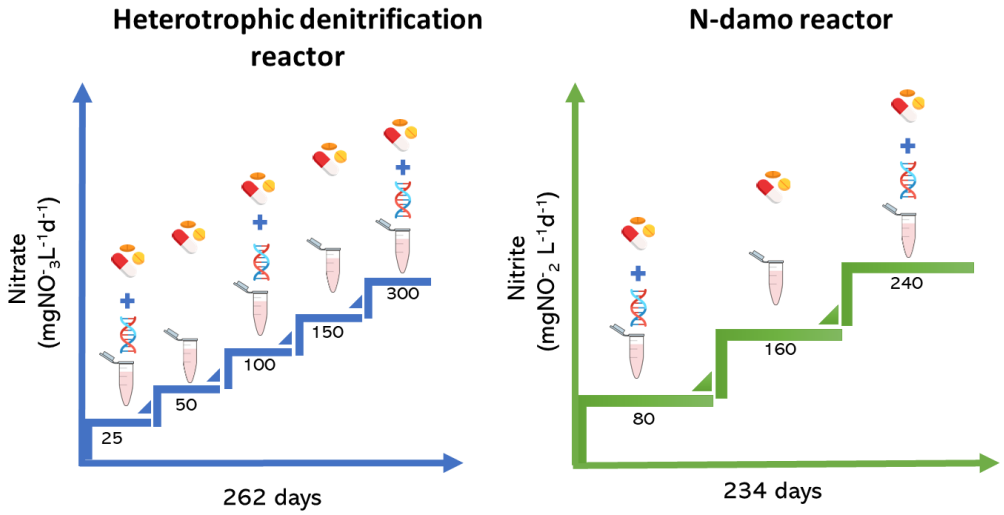


Figure 2.1. Comparison of experimental strategies across different denitrifying systems

Each system consisted of two reactors run in parallel under identical operational conditions, facilitating direct comparison between treatment scenarios. A summary of the differences in reactor type, inoculum, and nitrogen source across the systems is provided in **Table 2.2**. Before each sampling event, the reactors were stabilized at the new nitrogen load to ensure efficient nitrogen removal. Once steady-state conditions were stablished, samples were collected for micropollutant analysis (Section 2.4), and the biomass was characterized through volatile suspended solids (VSS) measurements and DNA extraction (Section 2.3). By following this systematic approach, it was possible to compare reactor performance, microbial community shifts, and antibiotic presence.

In addition to these core experiments, the denitrifying pathway of nitrite-dependent anaerobic methane oxidation (N-damo) was explored under high loads by proteomics (**Chapter 5**) to gain insight into microbial metabolism in this emerging process. Finally, the fate of antibiotics during heterotrophic denitrification, was examined in **Chapter 6** using ^{14}C labeled compounds in batch bottles to better understand the role of antibiotic biotransformation and transformation metabolites in the environment

Table 2.3. Operational parameter comparison between denitrifying reactors

Parameter	Heterotrophic denitrification	Autotrophic denitrification (N-damo)
Reactor type	Continuous-flow	Anaerobic MBR
Inoculum	Activated sludge WWTP	Methanogenic bioreactor
N source	NO^-_3	NO^-_2
Working volume	5 and 2 L	6.5 L
HRT	1 d	1 d
SRT	27 d	>240 d

2.5.1. Heterotrophic denitrifying reactors – Continuous flow

In **Chapter 3**, two continuous-flow denitrifying reactors, one with antibiotics and one without, were operated during long-term in an analogous way. For clarity, the reactor receiving antibiotics is referred to as Reactor A, while the antibiotic-free control reactor is referred to as Reactor Co. Reactor A had a working volume of 5 L coupled to a 2 L settler, and Reactor Co had a working volume of 2 L connected to a 1 L settler. **Figure 2.2** shows the experimental set-up of both reactors in operation.

Both systems were maintained at similar nitrogen loading rates (NLRs) while operating at a hydraulic retention time (HRT) of 1 d. Each reactor had a

recirculation system to return biomass from the settler back to the main compartment driven by a peristaltic pump at a recycle ratio of 10. Synthetic wastewater, prepared according to the recipe in **Table 2.3**, served as the influent. From day 18 onward, Reactor A was continuously supplemented with an antibiotic mixture at a final concentration of 5 µg L^{-1}. Over the course of the experiment, five distinct NLRs were tested, each corresponding to a defined operational period. These five periods, along with their respective NLRs, are summarized in **Table 2.4**.

Table 2.4. Composition of organic medium used as denitrifying feed

Heterotrophic denitrifying feed			
Macronutrients		**Trace solution**	
Compounds	Concentration [mg L^{-1}]	Compounds	Concentration [g L^{-1}]
$NaCH_3CO_2$	175[c]	$FeCl_3.6H_2O$	1.5
$NaNO_3$	25[c]	H_3BO_3	0.15
KH_2PO_4	12	$CuSO_4.5H2O$	0.03
Na_2HPO_4	24	KI	0.03
Trace solution	0.1	$ZnSO_4.7H_2O$	0.12
		$CoCl_2.6H_2O$	0.15
		$MnCl_2.4H_2O$	0.12

Based on (Suarez et al., 2010)

Table 2.5. Different experimental periods for heterotrophic reactor operation

Period	**Phase**	**Nitrate loading rate (mg N-NO^-_3 L^{-1} d^{-1})**	**Comments**
I	Start-up phase/ Low nitrate	25	Acclimation to denitrification and exposure to antibiotics
II	1st NLR increase	50	Progressive increase; stable operation for 3 weeks prior antibiotic sampling campaign
III	2nd NLR increase	100	
IV	3rd NLR increase	150	
V	Highest NLR	300	



Figure 2.2. Experimental set-up. Reactor A and Co in operation.

In **Chapter 6**, the 2L CSFR was operated under denitrifying conditions for 16 days to acclimate the inoculum before it was transferred into batch bottles for subsequent experiments.

2.5.2. Autotrophic denitrifying reactors – Anaerobic MBR

In **Chapter 4**, two membrane bioreactors (MBRs) were operated in parallel across three different experimental periods defined by progressive increases in nitrogen loading rates (NLRs) (**Figure 2.1**). Both reactors were fed with a synthetic wastewater, prepared as described in **Table 2.5**, and continuously supplied with methane in saturation through a 90:10% CH_4: CO_2 gas mixture. The gas flow rate was adjusted according to biomass activity and NLR requirements, with CO2 serving to regulate the pH.

To differentiate treatments, MBR1 served as a control, while MBR2 received an additional supply of antibiotic mixture at a final concentration of 5 μg L^{-1}. Throughout the operation, oxidation-reduction potential (ORP) was monitored in real-time using Hamilton Polyplast ORP BCN probes. These sensors were connected to a programmable logic controller (PLC, Micro 820, Allen-Bradley) for continuous data acquisition and process control. The hydraulic retention time (HRT) was

maintained at one day, and the temperature was kept at 28°C using a thermostatic water bath.

Table 2.6. Macronutrients and Trace Minerals Composition for N-damo

Compound	Amount	Units	Compound	Amount	Units
Fe	209.5	$\mu Fe.L^{-1}$	W	5.6	$\mu W.L^{-1}$
Zn	7.7	$\mu Zn.L^{-1}$	Mo	22.6	$\mu Mo.L^{-1}$
Co	14.9	$\mu Co.L^{-1}$	KH_2PO_4	5000	$\mu g.L^{-1}$
Mn	110.0	$\mu Mn.L^{-1}$	NH_4CL	15.300	$\mu g.L^{-1}$
Cu	63.8	$\mu Cu.L^{-1}$	$CaCl_2$	20000	$\mu g.L^{-1}$
Ni	11.8	$\mu Ni.L^{-1}$	$MgCl_26H2O$	33000	$\mu g.L^{-1}$
B	1.2	$\mu B.L^{-1}$	$MgSO_4$	5000	$\mu g.L^{-1}$
Ce	4.5	$\mu Ce.L^{-1}$	Na_2EDTA	14000	$\mu g.L^{-1}$
Se	9.5	$\mu Se.L^{-1}$			

Based on (Arias et al., 2022; Ettwig et al., 2009; Quiton-Tapia et al., 2023)

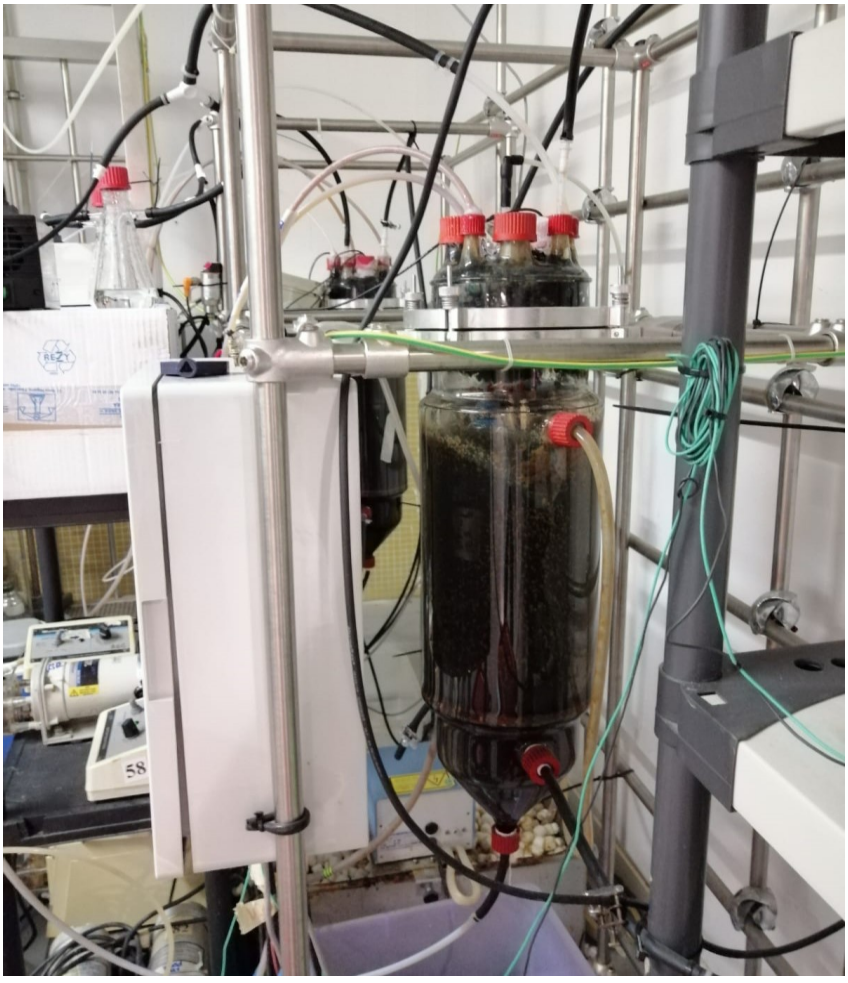


Figure 2.3 Experimental set-up for N-damo reactors.

2.5.3. Batch incubation tests

In **Chapter 6**, antibiotic fate was determined during heterotrophic denitrification. For this purpose, fresh activated sludge was collected as inoculum and acclimated to denitrifying conditions using a continuous-flow reactor described in previous section 2.2.2. Following acclimation, two set of batch experiments were conducted in 250 mL batch bottles with a gas trap (**Figure2.3.),** using the acclimated biomass to evaluate the degradation and fate of TCC and SMX during a 14 days incubation under denitrifying conditions.

Labeled compounds were spiked into the batch bottles, enabling complete mass balance assessments of ^{14}C. Systematic analysis was conducted to quantify ^{14}C distribution in the liquid, solids, and any $^{14}CO_2$ produced during the incubation period. Triclocarban was labeled at the Chlorophenyl ring-^{14}C(U) whereas, sulfamethoxazole was labeled at Phenyl ring-U-^{14}C. In brief, the fate of selected antibiotics, radioactivity was measured across four different fractions: the water phase which would constitute the available fraction, the extractable fraction which incorporates and extraction with $CaCl_2$ and three consecutive extractions with methanol (MeOH), the non-extractable residues (NER), and mineralization as explained in **Figure 2.3**. Details on the extraction procedure are specified in **Chapter 6.**

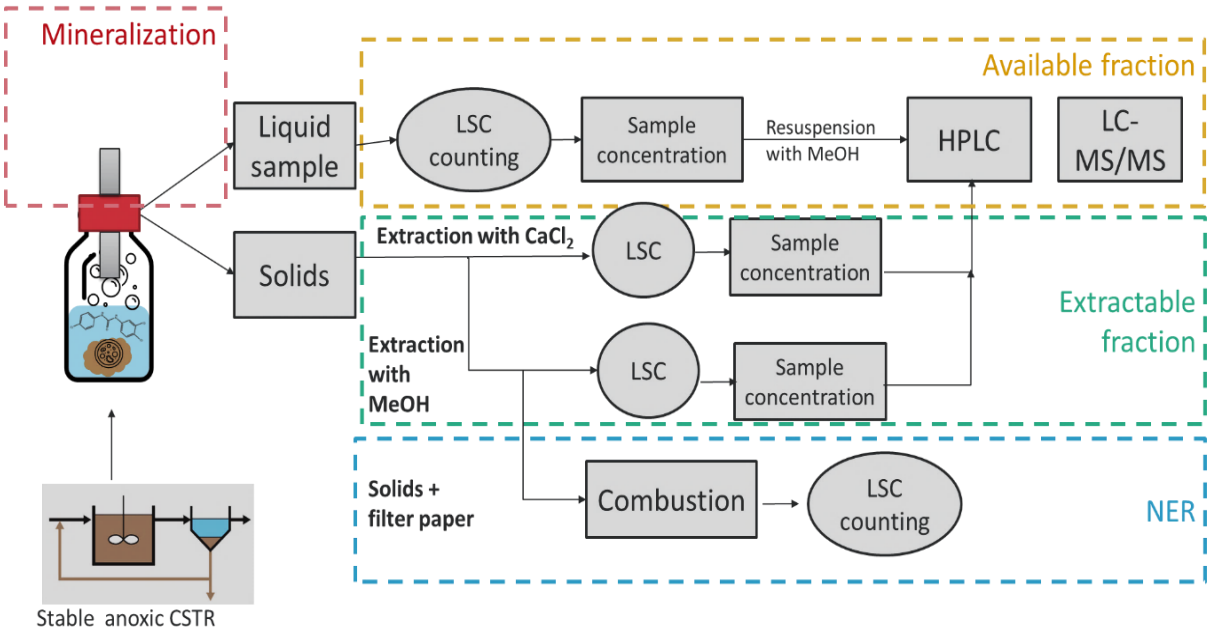


Figure 2.4. Sample processing and extraction procedure for batch bottle

The radioactivity present in each fraction of the extraction procedure (including water phase, sequential extractions, the soda lime and glass wool) was determined using a Liquid Scintillation Counter (LSC). For this analysis, 1 mL aliquots from each fraction were mixed with a liquid scintillation cocktail (Perkin Elmer). The

international unit of radioactivity is the Becquerel. One becquerel (Bq) is defined as the activity of a quantity of radioactive material in which one nucleus decays per second. Radioactivity was normalized to the sample volume to express results as dpm/mL, thus providing a direct measure of the radioactivity concentration within the sample.

1 Bq = 60 dpm (disintegrations per minute)

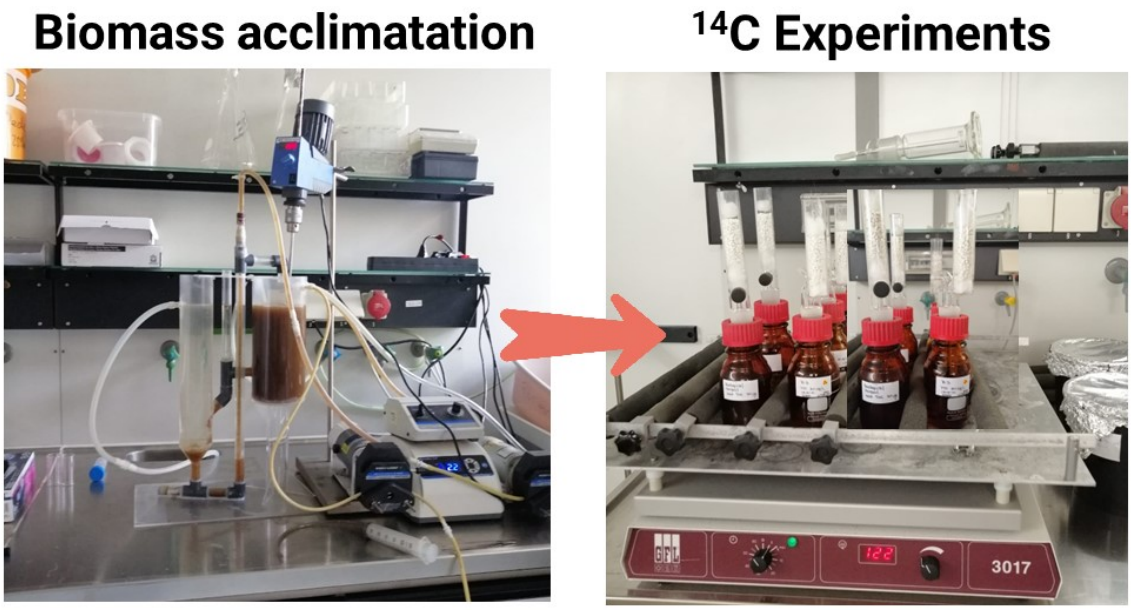


Figure 2.5. Experimental set-up for determining antibiotic fate using ^{14}C labelled techniques

Because the test systems were sealed with a CO_2 trap and the formation of volatile organic compounds was not anticipated, no loss of applied radioactivity (AR) was expected during the incubation period. If both the amount of AR and the recovered radioactivity were accurately quantified, the recovered AR should not exceed 100%. Hence, total AR recovery functions as a key quality criterion in radiolabeled compound degradation studies and should ideally fall within 90% to 110% as recommended by OECD Guideline for Testing Chemicals 307.

Finally, further analysis of the samples to identify potential transformation metabolites was conducted using radio-HPLC. These analyses were performed using an Agilent 1260 Infinity II system using a reverse column Zorbax Eclipse XDB-C18 (4.6 x 150 mm) coupled to a radioactive detector (Raytest Ramona liquid cell detector, Elysia, Belgium).

CHAPTER 3

Microbial Community Shifts and Cometabolic Limitations in Heterotrophic Denitrification

Quiton-Tapia, Silvana; Balboa, Sabela; Suarez, Sonia; Omil, Francisco. Microbial Community Shifts and Cometabolic Limitations in Heterotrophic Denitrification. *In preparation.*

Abstract

Microbial communities are engines for all biotechnological processes and significant changes in their structure can impact system performance. Despite the widespread application of heterotrophic denitrification in wastewater treatment, its biotransformation mechanisms are not fully understood. This study investigates the potential of increasing Nitrogen Loading Rates (NLRs) to enhance antibiotic removal through a cometabolic mechanism and examines how shifts in microbial community structure during long-term heterotrophic denitrification influence removal efficiencies. DNA metabarcoding analyses revealed antibiotic exposure alone (5 µg L-1) influenced the microbial community composition, leading to notable structural shifts. Further, the results suggest that taxa within the order Rhizobiales played an active role in OMP biotransformation. However, these taxa were outcompeted at high NLRs, resulting in diminished antibiotic removal efficiencies. Interestingly, our findings indicate that under denitrifying conditions, antibiotic biotransformation is governed by microbial community structure and diversity rather than denitrifying activity, with removal efficiency deviating from an expected cometabolic trend.

3.1. INTRODUCTION

Current wastewater treatment plants (WWTPs) face the dual challenge of achieving the European Union's goal of a zero-pollution and energy-neutral environment by 2050 (Council of the EU, 2024). Among the key biological processes in WWTPs, conventional heterotrophic denitrification remains crucial for effective nitrogen removal. Interestingly, emerging evidence suggests that denitrifying systems can also biodegrade a wide range of organic micropollutants (OMPs) (Martínez-Quintela et al., 2023; Quiton-Tapia et al., 2023; Suarez et al., 2010). In addition, anoxic biotransformation can exhibit equal or superior efficiency compared to other redox conditions for the degradation of certain antibiotics, such as sulfamethoxazole (Alvarino et al., 2018; Burke et al., 2014). However, the efficiency of OMP removal is highly dependent upon the operational parameters governing denitrification processes (Alvarino et al., 2018). Optimizing these parameters is therefore critical to enhancing the potential of denitrifying systems for micropollutant biodegradation in WWTPs.

The type of primary substrate (i.e. carbon source and nitrogen source) and its availability, expressed as Nitrogen Loading Rate (NLR) have been shown to significantly influence OMP removal during denitrifying processes (Polesel et al., 2017; Torresi et al., 2017a). Studies suggest a linear correlation between NLR and the removal of several pharmaceuticals, including antibiotics, which points to cometabolic biotransformation as the main OMP removal mechanism during autotrophic nitrogen removal (Fernandez-Fontaina et al., 2014; Su et al., 2021). Similar cometabolic trends have been observed under aerobic conditions, where increasing the organic loading rate enhanced OMP removal by aerobic heterotrophs (Kennes-Veiga et al., 2021a). Given that most denitrifiers are facultative, acting as de facto aerobic heterotrophs when oxygen is present, it is plausible that anoxic heterotrophs could similarly exhibit improved OMP biotransformation kinetics with higher substrate availability.

For most OMPs, biotransformation mechanisms (cometabolic or catabolic) during heterotrophic denitrification remain poorly understood. One study showed a strong correlation between denitrification activity and biotransformation kinetics of Sulfamethoxazole, Erythromycin and Trimethoprim, pointing to cometabolic biodegradation as a likely pathway (Polesel et al., 2017). However, recent findings suggest that microbial community composition may play a more decisive role in OMP biotransformation than previously assumed cometabolic interactions (Martínez-Quintela et al., 2023).

Denitrifying communities harbor a diverse array of functional taxa, with denitrifying bacteria estimated to represent 10-15% of the bacterial population in the environment (Ambus and Zechmeister-Boltenstern, 2007; Casella and Payne, 1996). Moreover, recent phylogenetic analyses, have revealed a higher diversity and

abundance of denitrifying microorganisms in the environment than previously recognized (Wei et al., 2015). This diversity suggests that denitrifying communities may exhibit varying OMP biodegradation capacities, driven by differences in community structure, diversity, and functional potential. For instance, denitrifying moving bed biofilm reactors (MBBRs) achieved Trimethoprim removal efficiencies of 23% ± 26 (Ooi et al., 2018) to 50%±20 (Torresi et al., 2017a), highlighting the impact of microbial population differences on the reactor performance. Additionally, different species within a microbial community might exhibit varying tolerance to OMPs. Some species may be more tolerant to pollutants, while others may be more sensitive and disappearing (Desiante et al., 2021; Tlili et al., 2016).

Changes in microbial community composition can affect both microbiome functionality and redundancy, ultimately influencing OMP biotransformation (Desiante et al., 2022; Helbling et al., 2012b). Functional redundancy, where multiple microbial taxa perform similar roles, is essential for maintaining stable ecosystem functions under stress. On the other side, richer and more diverse microbial communities provide a wider array of metabolic pathways and enzymes, facilitating the biochemical reactions required for the degradation of a variety of OMPs (Drewes et al., 2014; Johnson et al., 2015). Therefore, higher taxonomic and functional diversity increases the likelihood that microbial consortia possess the specific traits needed to metabolize different antibiotics.

Reactor functionality can be evaluated by examining the relationships among taxonomic richness, reactor performance, specific denitrifying activity, and OMP removal. In this context, functional profiling of microbial communities using PICRUSt analysis, based on 16S rRNA gene sequencing, can provide valuable insights into changes in functional genes associated with the denitrification process (Yan et al., 2019). PICRUSt (Phylogenetic investigation of communities by reconstruction of unobserved states) is a technique that employs evolutionary modeling to predict metagenomes from 16S rRNA data, using a reference genome database to infer functional potential of microbial communities (Langille et al., 2013).

Based on these evidences, we aimed to investigate whether increasing denitrifying activity in a lab-scale reactor could enhance the removal of selected antibiotics spiked at $\mu g\, L^{-1}$ concentrations through a cometabolic biotransformation mechanism. Additionally, the study sought to elucidate how operational conditions (by the increase in NLR) impacted the microbiome in terms of the dynamics of the community composition and its functionality during long-term reactor operation. By addressing these interconnected objectives, our study aims to provide a comprehensive understanding of the influence of microbial community composition on antibiotic biotransformation under denitrifying conditions. Understanding microbial community dynamics in response to NLR and

its functional implications for antibiotic biodegradation may lead to further optimization of biological wastewater treatment.

3.2. MATERIAL AND METHODS

3.2.1. Reactor configuration and operation

Two continuous-flow denitrifying reactors, with and without antibiotics were operated long-term (262 days) in an analogous way. From now onwards, named Reactor A (with antibiotics) and Reactor Co (without antibiotics used as control). Nitrate as sodium nitrate and COD as sodium acetate were used as primary substrates to promote denitrification. Reactor A had a working volume of 5 L coupled to a 2 L settler, and Reactor Co had a working volume of 2 L connected to a 1 L settler. Both reactor operation was controlled to have similar NLRs while maintaining a Hydraulic Retention Time (HRT) of 1 d. Each reactor had a recirculation system to return biomass from the settler back to the main compartment driven by a peristaltic pump at a recycle ratio of 10. An antibiotic mix at a final concentration of 5 μg L^{-1} was continuously spiked to Reactor A from day 18 onwards.

The biomass inoculum was taken from a conventional activated sludge reactor of a wastewater treatment plant (WWTP) from a city in northwest of Spain. The initial biomass was sieved and bubbled for 24 hours to reduce possible recalcitrant compounds before inoculation. Then, both reactors were inoculated at the same time with a biomass concentration of approximately 1.5 g volatile suspended solids (VSS) L^{-1} and operated at ambient temperature in a range of 20 – 23 °C.

Anoxic conditions were applied to promote heterotrophic denitrification. Synthetic wastewater was prepared with sodium nitrate as nitrogen source (electron acceptor) and a mixture of sodium acetate and acetic acid as carbon source (electron donor) to maintain a neutral pH (7-8.5). Phosphate buffer and trace elements were included to promote the denitrification process, as described in Suarez et al., (2010). The NLR was progressively increased in five different experimental periods (from 24 to 285 mg N- NO_3^- L^{-1} day^{-1}) while maintaining a COD/N ratio of 6 – 7 to avoid any COD limitation.

3.2.2. Organic micropollutants

Seven antibiotics commonly present in WWTPs were continuously fed to Reactor A at a final concentration of 5 μg L^{-1} with a peristaltic pump. The selected compounds spiked were: Cephalexin (CFX), Sulfamethoxazole (SMX), Trimethoprim (TMP), Ciprofloxacin (CIP), Roxythromycin (ROX), Azythromycin (AZY) and Clarythromycin (CLA). Fresh stock solutions were prepared in HPLC-grade methanol (except for CFX) stored at -20 °C during a maximum of three months. CFX stocks were prepared in MilliQ water and divided into different aliquots, which were

used with only one freeze-thaw cycle to ensure stability of the compound as indicated in Gros et al., (2013). Due to its persistent detection throughout the reactor operation, CIP was excluded from further analysis to focus on compounds that were biotransformed.

3.2.3. Analytical methods

3.2.3.1. Conventional parameters

The growth substrates, nitrate and carbon acetate, were measured weekly in both reactors. Nitrate was determined by a spectrophotometric method (at 200 and 275nm) while the carbon source was determined by performing a colorimetric method as described in the standard methods (APHA, 2000). Samples were measured in triplicates and diluted according to their concentration. Biomass concentration was measured weekly by taking 10 mL of mixed liquor from both reactors to determine MTSS and MVSS concentrations according to the standard methods (APHA, 2000). Additionally, pH, temperature, dissolved oxygen and redox potential were measured every time a sample was extracted from the reactors using Hach HQ40d multiparameter using a temperature and redox probe.

3.2.3.2. Organic micropollutant analysis

Five antibiotic sampling campaigns were conducted during steady-state operation of Reactor A, corresponding to each experimental period. During each campaign, liquid samples were collected from both the feed and effluent streams on three separate days, ensuring at least one hydraulic retention time (HRT) interval with a new synthetic medium feed. Each sample, with a volume of 100 mL, underwent initial filtration through a 0.45 µm filter, followed by acidification to a pH 4.5. To enhance chelation of metal ions, Na_2EDTA was added to a final concentration of 0,1% (g solute/g solution). The samples were then preconcentrated using a Solid Phase Extraction (SPE) based on an adapted protocol fromGros et al., (2013). The SPE protocol was performed manually using a vacuum pump using Oasis HLB cartridges (60 mg, 3 mL). Finally, analytes were eluted with 6 mL of pure methanol, evaporated to dryness using nitrogen gas stream for 30 minutes and subsequently reconstituted with 3 mL of methanol/water (10:90, v/v). Prepared samples were stored at 4 °C until further analysis.

Liquid chromatographic separations were carried out using an Agilent 6410B-QQQ LC/MS system. A C18 Zorbax eclipse XDB column (2,1x150mmx5µm) was used for the analysis. The mobile phase consisted of two solvents: Eluent A, composed of 10 mM of formic acid/ammonium formate, and Eluent B, methanol. Each injection volume was 20 µl.

3.2.4. Microbial community composition

The microbial community composition was characterized by taxonomical assignments using 16S rRNA illumina sequencing and the MiDAS database. The inoculum and biomass extracted from both reactors were subject to DNA extraction using the Nucleospin Microbial DNA extraction kit (Machery-Nagel). During reactor performance, triplicates of 5 mL of mixed liquor were extracted once the system reached steady state in Periods PI, PIII and PV. Samples were stored in -60 °C until DNA extraction. The V3-V4 hypervariable region of the 16 S rRNA gene from bacteria was amplified using Bakt_3 41 F (5′ CCT ACG GGN GGC WGC AG 3′) and Bakt_805R (5′ GAC TAC HVG GGT ATC TAA TCC 3′) (Herlemann et al., 2011). DNA metabarcoding analyses of the region in an Illumina NovaSeq PE250 platform, generating paired ends reads (2 x 250) by AllGenetics & Biology SL (www.allgenetics.eu).

Bioinformatic analyses were performed using the Microbial Genomics module (version 21.1) workflow of the CLC Genomics workbench (version 21.0.3). For the taxonomic assignment, raw sequences were filtered, trimmed and then clustered into Operational Taxonomic Units (OTUs) at 97% cut-off for sequence similarity and classified against the non-redundant version of the MiDAS database for taxonomical assignment (Dueholm et al., 2022) (https://www.midasfieldguide.org/guide). Only the most abundant bacterial OTUs (above 1 % of the total observed OTUs) were considered for further analysis.

3.2.5. PICRUSt analysis

The PICRUSt analysis was performed by using 16S rRNA information to predict the functional profiling of microbial communities present (Langille et al., 2013). The function prediction was applied to standardize the OTU abundance table by PICRUSt according to the MiDAS database. Enzymes were categorized according to their Enzyme Commission (EC) numbers, which delineate six primary classes based on the type of reaction catalyzed: 1. Oxidoreductases, 2. Transferases, 3. Hydrolases, 4. Lyases, 5. Isomerases, and 6. Ligases (McDonald and Tipton, 2014). Specifically, our analysis concentrated on the Oxidoreductases class, particularly EC subclass 1.7. This subclass comprises enzymes that oxidize nitrogenous compounds, aligning them with the primary enzymes involved in denitrification: nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (NOS) (Hochstein, 1988). Initially, enzyme reads were classified into these six major classes based on their respective EC numbers. Subsequently, we focused on enzymes associated with the denitrification process, examining enzymatic predictions related to the four critical gene clusters. This analysis spanned three experimental periods PI, PIII, and PV in both Reactor A and Reactor Co.

3.3. RESULTS AND DISCUSSION

3.3.1. Reactor performance

Denitrification was assessed in terms of NLR (volumetric denitrification) and biomass activity (specific denitrification) as summarized in **Table 3.1**. Both reactors had similar NLRs, achieving a maximum value of 277.9 and 307.6 mg N-NO^{-}_{3} L^{-1} d^{-1} for Reactor A and Co, respectively. However, differences in specific denitrification activity emerged from PIII onwards. Such variation was attributed to variations in biomass concentrations between reactors. Nonetheless, specific denitrification activity increased consistently with the rising NLR, reaching the highest activity in PV (**Table 3.1**). Similarly, the redox potential ranged between -50 mV till -264 mV for both Reactor A and Reactor Co, with the highest redox potentials observed during PV.

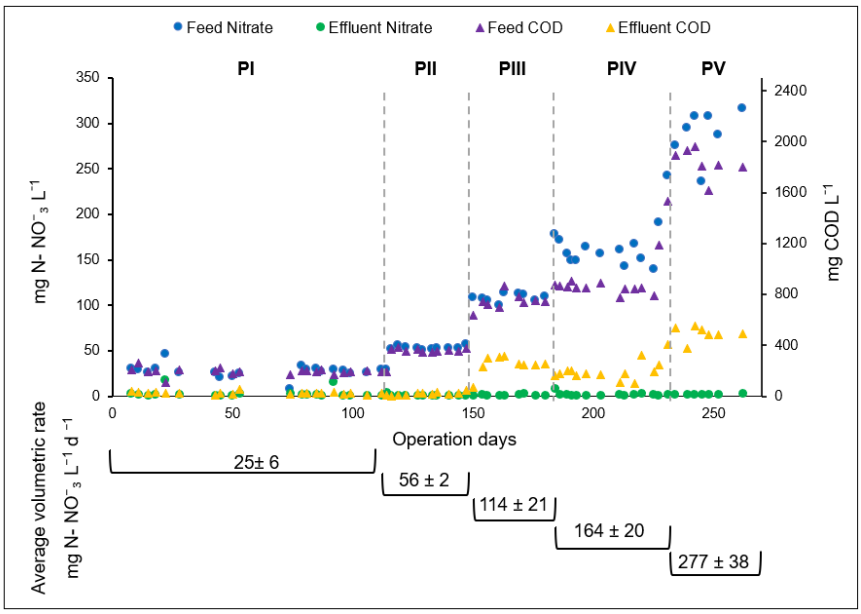


Figure 3.1. Reactor performance for Reactor A**.** Nitrate concentration (Y left axis) and COD concentration (Y right axis) in the feed and effluent. (n=3) during the operation days (X axis). Below, average volumetric rates in each experimental period with standard deviations.

Table 3.1. Reactor A and Co performance. Average values for volumetric and specific nitrate loads and biomass concentration in each experimental period.

Periods	**Volumetric denitrification (mg N- NO^{-}_{3} L^{-1} day^{-1})**		**Biomass concentration (g MLVSS L^{-1})**		**Specific denitrification (mg N- NO^{-}_{3} g VSS^{-1} day^{-1})**	
	Reactor A	Reactor Co	Reactor A	Reactor Co	Reactor RA	Reactor Co

PI	24.7	25.0	1.55	1.2	17.1	20.7
PII	56.1	53.6	2.0	1.5	25.5	37.3
PIII	114.2	117.6	2.5	1.5	47.9	81.5
PIV	164.5	181.7	2.4	1.9	72.2	100.7
PV	277.9	307.6	2.2	1.8	125.2	168.2

Overall, the reactors showed a stable and efficient operation until period IV. However, during the increase in NLR in period V, a reduced settling efficiency of the biomass in the mixed liquor was observed (i.e., longer settling time). This could be due to a change in equilibrium of in the population of filamentous and non-floc-forming bacteria according to previous results and macroscopic observations in these reactors. So, high substrate concentrations have shown to promote the overgrowth of filamentous bacteria during denitrification (Henriet et al., 2017; Tiwari et al., 2017). In both reactors' operation, but especially in reactor A, it became apparent that the high NLR during period V was affecting the biomass structure (i.e., increase in filamentous bacteria) from a macroscopic perspective. Further information on the microbiological shifts observed are discussed in section 3.3.2.

3.3.2. Dynamics on the denitrifying community composition, diversity and richness

The inoculum exhibited a highly diverse and rich starting community, as evidenced by both the community composition (**Figure 3.2**) and the alpha diversity index presented in **Table 3.2**. Both Chao and Shannon indices were similarly high in the inoculum and during PI of both reactors but declined progressively in the later periods. This decrease in diversity during PIII and PV suggested a shift towards less diverse community dominated by a few species (reduced evenness).

Metabarcoding analyses revealed that microorganisms from the order *Burkholderiales* dominated the community throughout most of the operation, except during PIII of Reactor A. Other notable orders present in the inoculum included *Rhizobiales* (7%), *Chloroflexales* (8%), *Chitonophagales* (5%), and *Rhodobacterales* (2%). A significant microbial shift occurred from the inoculum to PI, as anticipated, driven by the acclimatization to denitrifying conditions. These conditions exerted selective pressure on the initial microbial community, favoring microorganisms involved in nitrate removal and leading to a decline in other groups, such as nitrifiers, that were originally present in the inoculum.

Microbial shifts differed between Reactor A and Reactor Co. In Reactor A, *Rhizobiales* increased to 19% becoming the second most abundant taxa after *Burkholderiales* (51%) instead of *Chloroflexales* which reduced in abundance. Conversely, in Reactor Co the abundance of *Rhizobiales* declined to 3%, accompanied by slight increases in *Rhodobacterales* (6%) and *Flavobacteriales* (7%). These differences were amplified over time, particularly during PIII, where

antibiotics likely exerted selective pressure. This was most evident in Reactor A, where *Rhizobiales* became more prevalent and *Chloroflexales* were uniquely present. These results suggest that the presence of antibiotics selectively influenced long-term microbial community structures between the reactors, driving distinct compositional dynamics between the reactors.

Bacteria affiliated with *Rhizobiales* and *Burkolderiales* are known to degrade OMPs (Phan et al., 2016). Genera within *Rhizobiales* have been implicated in the transformation pathways of compounds like Caffeine and Atrazine (Zhou et al., 2022) which contain heterocyclic structures similar to those found in trimethoprim and sulfamethoxazole. *Chloroflexales* are known for their ability to utilize a wide range of electron donors that enhances their adaptability to variable environmental conditions (Freches and Fradinho, 2024). Additionally, *Chloroflexales* are effective phenolic degraders (Sánchez-González et al., 2019), a trait that may contribute to the breakdown of phenolic structures present in the antibiotics in Reactor A. Although *Rhizobiales* and *Burkholderiales* were in the inoculum which had previous exposure to various OMPs as it came from an activated sludge plant, their abundance shifted under specific NLR conditions and antibiotic exposure (**Figure 3.2**).

The increasing NLRs led to significant changes in microbial community composition. However, resilient taxa, such as *Burkholderiales*, maintained a high prevalence across all operational periods, suggesting their robustness in handling increasing nutrient loads. In Reactor Co, the relative abundance of *Burkholderiales* increased over time, rising from 64% in Phase I (PI) to 85% and 86% in Phases III (PIII) and V (PV), respectively. In contrast, the abundance of *Rhizobiales* displayed more dynamic changes in Reactor A, with an increase to 72% in PIII, followed by a sharp decline to 1% in PV. In Reactor Co, however, *Rhizobiales* represented 8% of the microbial community in PI, but decreased to 2% and 4% in PIII and PV, respectively.

Despite the microbial shifts observed in both reactors, nitrogen removal efficiency remained unaffected. The stable reactor performance, despite changes in community structure, may be explained by the functional redundancy within microbial community. In line with this, previous studies have shown that shifts in bacterial communities structure in anoxic conditions did not affect nitrogen removal (Phan et al., 2016). Furthermore, microbial communities in both reactors exhibited a decrease in diversity (Chao index) and became more even (Shannon index) with increasing NLR (**Table 3.2**). This decline in diversity and richness suggested that fewer species are able to grow at high NLR. Given that both reactors operated simultaneously at the same NLR, the observed differences in microbial composition were likely influenced by the presence of antibiotics.

Table 3.2. Diversity index comparison depicting the start of the operation (Inoculum) and its evolution in reactor A and Co over period I, III and V

Sample	Period	Simpson	Shannon	Chao
Inoculum	Start	0.98	6.57	310.01
	PI	0.95	5.86	373.47
	PIII	0.83	4.26	321.75
Reactor A	PV	0.71	3.16	151.74
	PI	0.91	5.01	341.26
	PIII	0.82	3.84	188.57
Reactor Co	PV	0.82	3.51	110.32

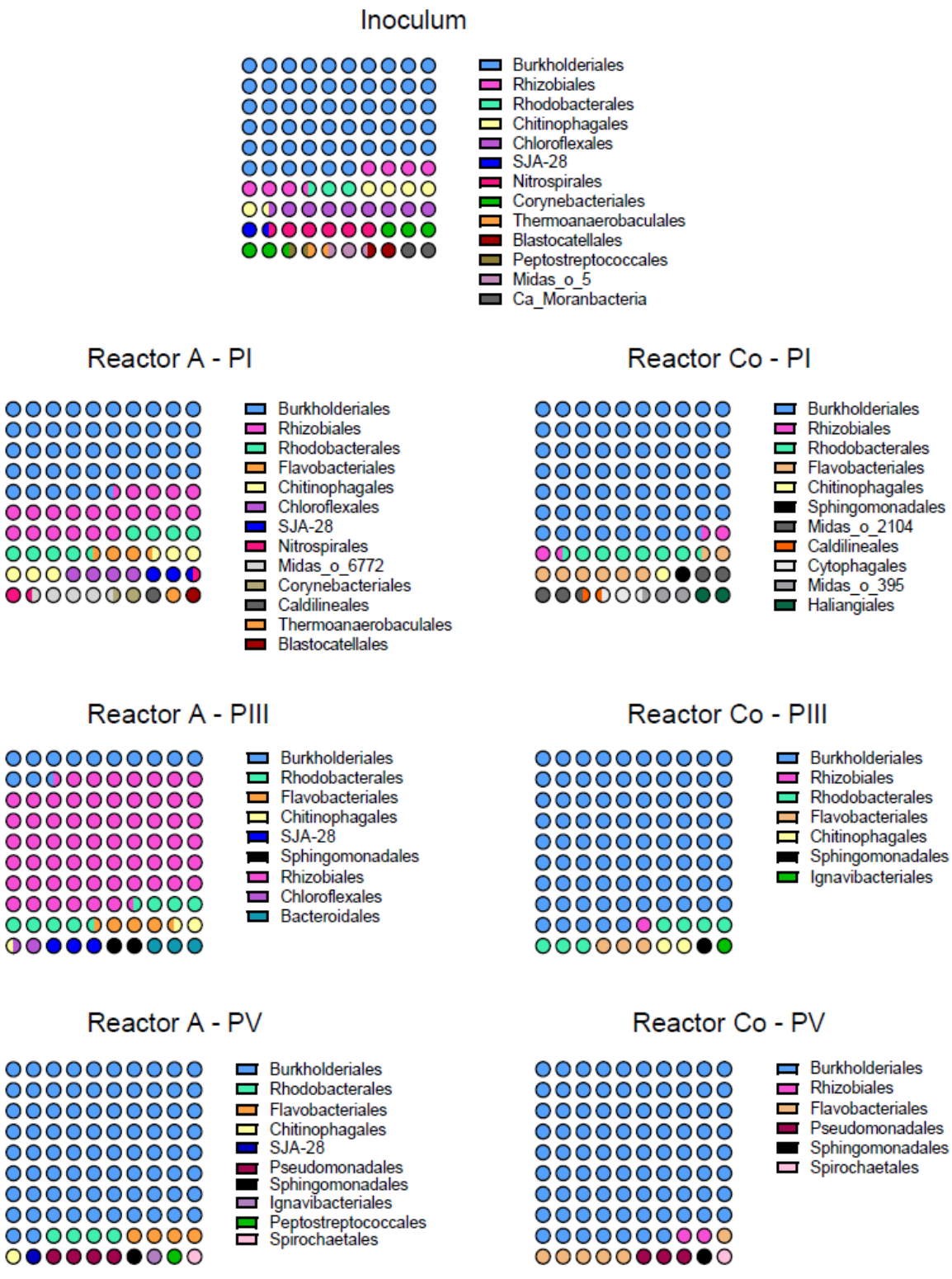


Figure 3.2. Microbial community composition at order level of the Inoculum and for Reactor A and Co in PI, PIII and PV. Relative abundances above 1% are visualized as parts of a whole, where each circle represents a fraction of the total.

3.2. Removal of antibiotics

For all selected antibiotics, PIII consistently exhibited the highest removal efficiencies, closely followed by PII (**Figure 3.3**). A progressive increase in removal efficiencies from PI to PIII was observed, subsequently declining in PIV and PV, with PV recording the significantly lower removals for TMP, AYZ, CLA and ROX. In the case of SMX, the removal efficiency increased from 58±8% in PI to 70±7% during the rest of the reactor operation. These SMX removal efficiencies are significantly higher than those reported in previous studies under nitrate reducing conditions, which achieved only 36% (Su et al., 2023). Similarly, CFX was almost completely removed independently of NLR. Also, high removals (>80%) for CFX were consistently observed across different NLRs during autotrophic denitrification (Quiton-Tapia et al., 2023); highlighting its biodegradability under anoxic conditions.

Specific antibiotic removal was significantly lower for TMP, CFX, CLA and ROX for PIV and PV compared to previous periods (**Figure 3.3**). TMP and CFX removals were similar during PI to III but subsequent declined in PIV and PV. CLA exhibited the highest removal during PIII, which declined significantly during later phases. Similarly, ROX removal was highest in PIII, followed by reduced efficiencies in PIV and PV. In contrast, SMX removal showed a more stable trend, with moderate variation across the experimental periods, while AZY removal exhibited relatively low and consistent values throughout the whole operation. These trends suggest that higher NLR may have negatively impacted the antibiotic removal. This was further supported by the negative correlation (r^2 = 0.9246) between specific antibiotic removal and specific denitrifying activity, as shown in **Figure 3.4**. To highlight the trends observed in **Figure 3.3**, the average antibiotic removals for each period were computed, reinforcing the relationship between declining removal efficiencies and increased NLR.

Contrary to the initial hypothesis, increasing Nitrogen Loading Rate (NLR) did not promote cometabolic activity. A critical factor to consider when interpreting these results is the reactor design and its capacity to retain biomass. Previous studies demonstrating cometabolic activity during denitrification utilized a three-stage Moving Bed Biofilm Reactor (MBBR) system, which allowed for tiered substrate gradients and enabled distinct biofilm communities to adapt to varied environmental conditions (Polesel et al., 2017) In contrast, our study employed a single continuous stirred reactor with a recirculation pump, which may have been insufficient to avoid biomass loss and promote adequate microbial adaptability, as discussed in Section 3.3.1 (Reactor Performance). Similarly, a recent study using a single continuous flow reactor also failed to observe enhanced specific OMP removal with increasing primary metabolic activity during heterotrophic denitrification. Instead, shifts in microbial community composition were identified as the primary

factor influencing OMP removal differences (Martínez-Quintela et al., 2023). While cometabolic removal of OMPs is recognized as a major mechanism, limitations exist, including substrate concentration thresholds. Below a critical level, cometabolism is not activated, and at excessively high biodegradable substrate concentrations, competitive inhibition may hinder OMP biodegradation (Kennes-Veiga et al., 2021a; Li et al., 2012).

In summary, the results suggest that increasing NLR did not enhance cometabolic activity for antibiotic removal, with reactor design and microbial community dynamics playing critical roles in shaping removal efficiencies. The negative correlation between specific denitrifying activity and antibiotic removal highlights the influence of microbial adaptability and biomass retention. The next section will examine enzymatic abundance and functional dynamics to better understand the molecular factors driving these removal patterns.

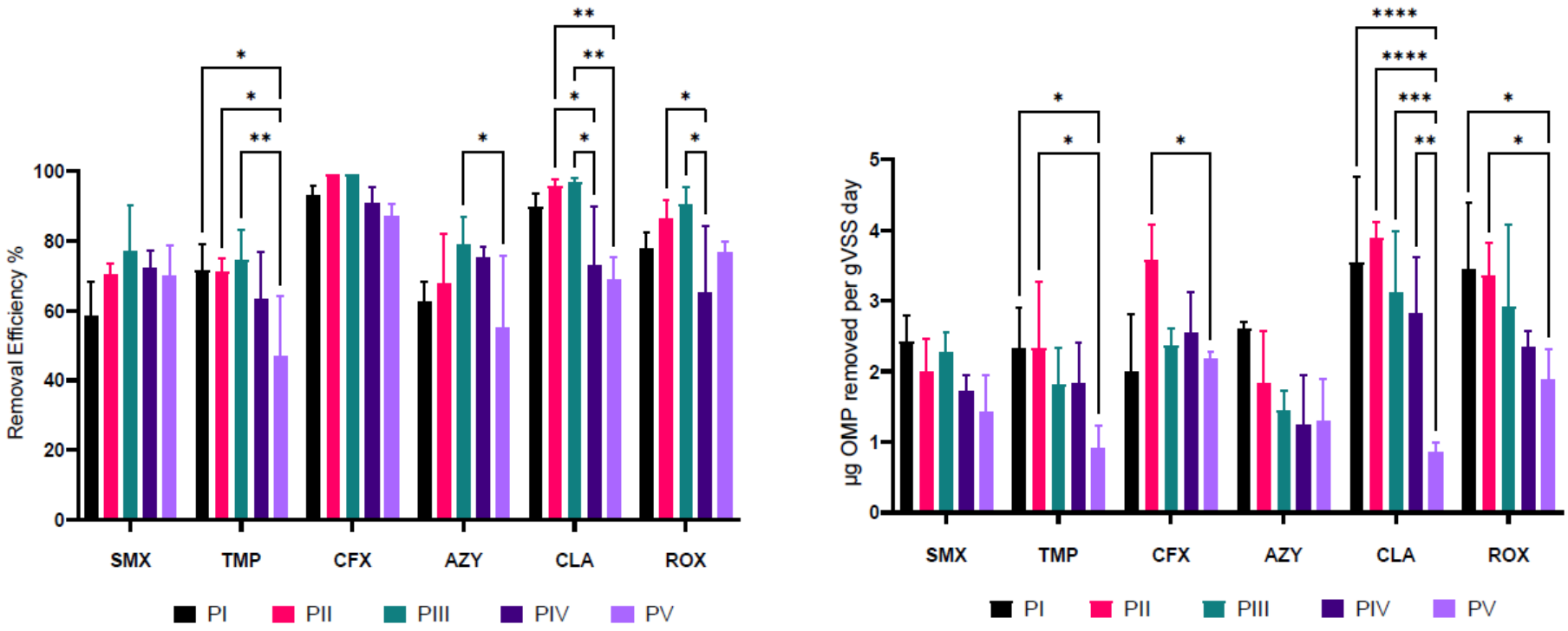


Figure 3.3. Antibiotic removal efficiency (on the left) and specific antibiotic removal (on the right) for all experimental periods (PI - PV). Error bars show standard variation (n=3). The asterisks highlight those antibiotics whose removal are significantly different among periods (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001) by a One-way Anova and Tukey's multiple comparison test.

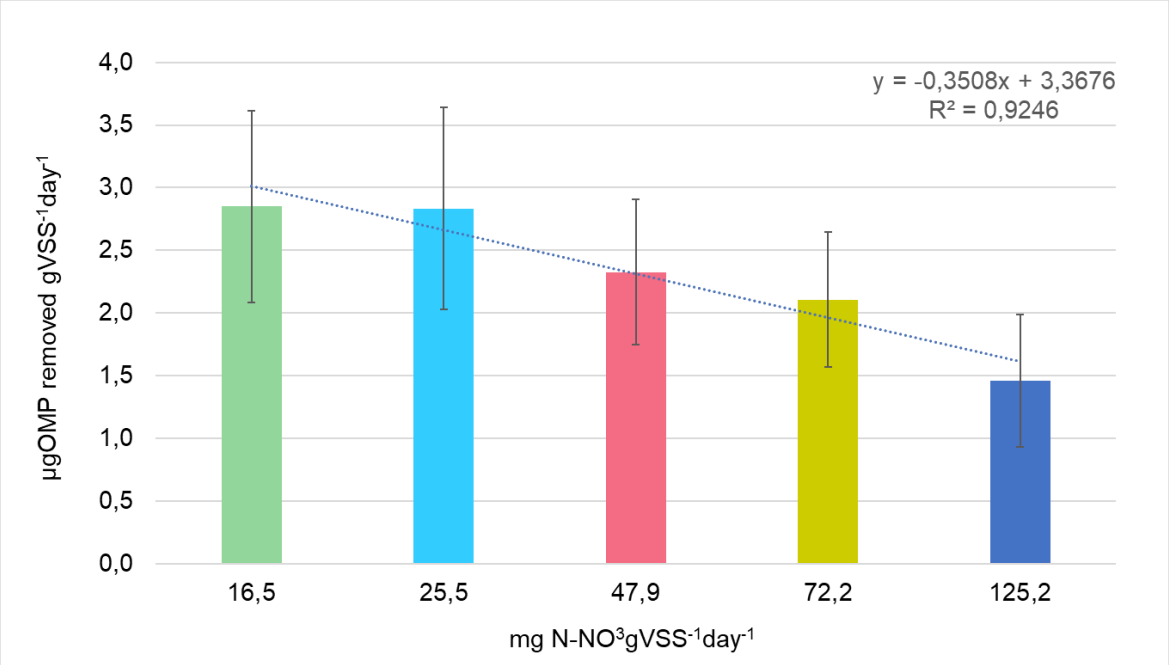


Figure 3.4. Mean specific OMP removal at specific denitrifying rate

3.3.3. Enzymatic Abundance and Functional Dynamics in Denitrification

Oxidoreductases, transferases, and hydrolases were the most abundant enzymes across both reactors, as depicted in **Figure 3.5**. In Reactor A, enzyme abundance peaked in PI and PIII before a sharp decline in PV. Similarly, Reactor Co showed the highest enzyme expression in PIII and reduction in PV. Given the similar operational conditions of the reactors, the increased enzyme abundance in PI in Reactor A versus Reactor Co, is likely a result of antibiotic exposure as a cell can response to stress by activating survival pathways for protection (Fulda et al., 2010). Notably, PV in both Reactor A and Reactor Co showed a marked decrease in enzyme reads compared to earlier phases, despite exhibiting the highest denitrifying activity. This decline in functionality correlates with a decrease in microbial diversity and richness, as discussed in Section 3.3.2.

Multiple studies have highlighted the important capacity of oxidoreductases and hydrolases to assist in OMP biodegradation (Bilal et al., 2019; Krah et al., 2016; Liang et al., 2024). Specifically, certain hydrolases (E.C. 3.5.2.6), capable of cleaving the β-lactam ring to biotransform Cephalexin (Reis et al., 2020), were consistently found throughout the study, with their highest expression occurring in PIII, aligning with the peak of Cephalexin removal, as shown in **Figure 3.5**. Conversely, esterases, which can break down the macrocyclic lactone ring found in macrolides Cephalexin (Reis et al., 2020) were less abundant in PV, possibly explaining the reduced removal efficiency observed during this phase. This pattern of enzymatic activity and

antibiotic removal underscores the intricate relationship between enzyme presence, reactor functionality, and microbial community dynamics.

Predicted NAR and NIR enzymes were significantly more abundant than NOR and NOS across all periods and in both reactors (**Figure 3.5**). The predominance of NAR and NIR can be explained by two factors: a) NAR and NIR are more highly expressed than NOR and NOS, as denitrifying capabilities are widespread across the bacterial domain, yet fewer populations encode the complete denitrification gene set (Lu et al., 2014b). Analysis of complete genomes of sequenced denitrifying bacteria indicated that about one third lack the NOS gene which results in an incomplete denitrification, which supports the greater abundance of NAR and NIR (Philippot et al., 2011). b) There may also be biases in enzymatic detection that favor NAR and NIR, as PICRUSt predictions are more accurate for well-characterized genes, such as nirK, which has more reference genomes than nosZ. This assertion is supported by a study that showed a higher correlation between predicted and qPCR-measured nirK abundance (r^2 = 0.92) compared to nosZ (r^2 = 0.70)(Agrawal et al., 2019).

Our results indicate a reduced overall reactor functionality with increasing NRL but an increased abundance of denitrifying genes, particularly NOR and NOS. The contrast between the reduced enzymatic activity and the highest denitrifying activity in PV highlights a functional trade-off, where microbial communities may optimize specific pathways, such as denitrification, at the expense of overall enzymatic diversity and activity.

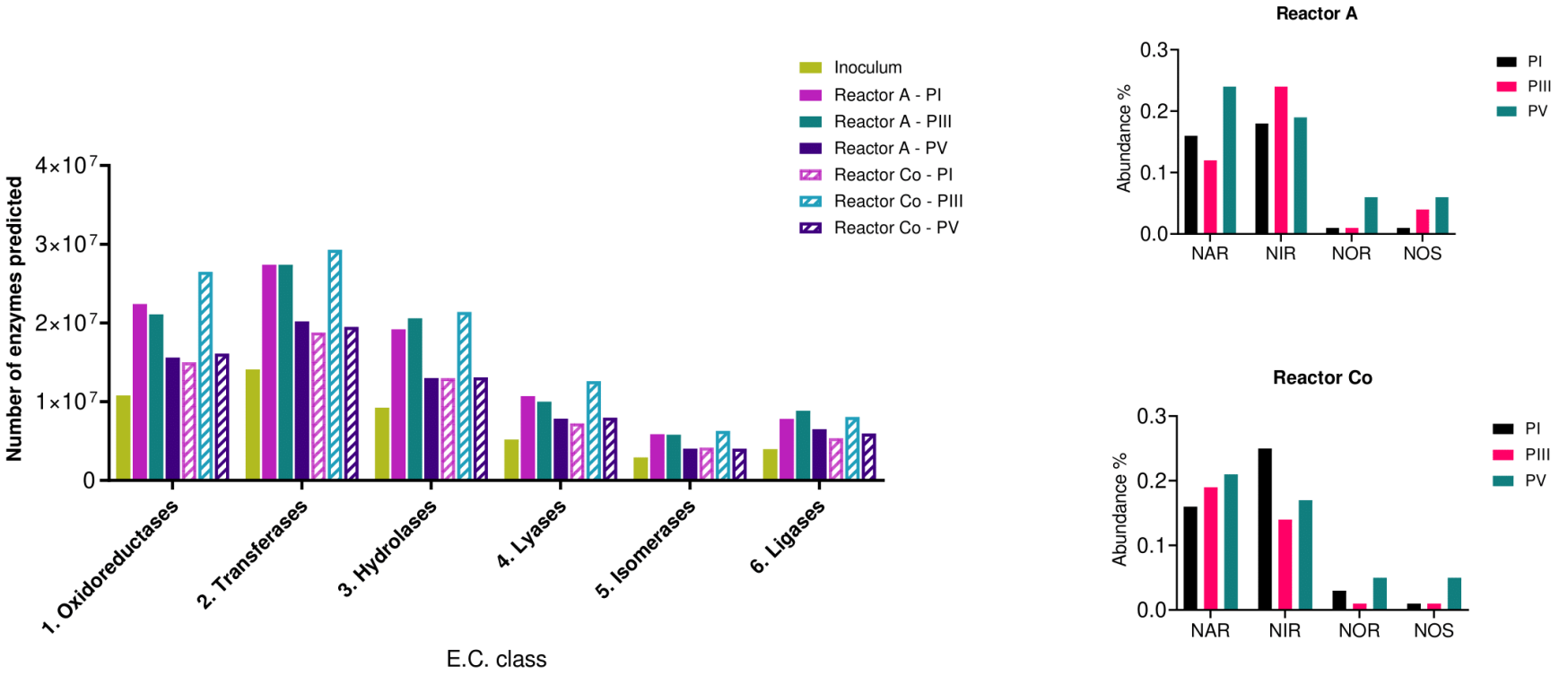


Figure 3.5. Predicted enzyme reads and abundance in Reactor A and Reactor Co across experimental periods PI, PII and PV. Classification based on the PICRUSt database and the BRENDA Comprehensive Enzyme Information

3.4. CONCLUSIONS

Our findings highlight the dynamic interplay between reactor performance, microbial composition and functionality under denitrifying conditions. The main outcomes of the study are summarized as follows:

- A progressive increase in the NLR enhanced denitrifying activity but caused significant microbial shifts in microbial community structure and abundance. High NLR favored fast-growing denitrifiers, reducing the prevalence of specialized taxa involved in antibiotic biotransformation.
- Functional and taxonomic richness of the microbial community were critical determinants for antibiotic biotransformation. Denitrification activity negatively correlated with antibiotic biotransformation, emphasizing the role of community composition over cometabolic trends.
- DNA metabarcoding analyses suggested that there were functional taxa involved in OMP biotransformation within the order *Rhizobiales*; however, these taxa were outcompeted at high NLRs. Antibiotic exposure caused shifts in microbial community composition, becoming more pronounced in long-term operation (after 100 days in PIII).

Altogether, our results reveal functional trade-offs between maintaining high NLRs for enhanced denitrification and preserving metabolic functions such as antibiotic biotransformation. Understanding and balancing these trade-offs is essential for optimizing reactor performance under varying operational conditions.

Looking forward, studies on the removal of antibiotics should not only address the parent compounds but also delve into the transformation metabolites and residual antibiotic activity. This expanded scope would provide a more detailed understanding of the effects that these factors exert on microbial communities at the functional level and optimize treatment processes effectively.

CHAPTER 4

How efficiently does a metabolically enhanced system with denitrifying anaerobic methane oxidizing microorganisms remove antibiotics?

*Part of this chapter has been published in:

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Abstract

In this work, the novel N-damo (Nitrite dependent anaerobic methane oxidation) process was investigated at high biomass activities for its potential to remove simultaneously nitrite and methane, as well as selected antibiotics commonly found in sewage in trace amounts. For this purpose, two MBRs were operated at three high nitrite loading rates (NLRs), namely 76 ± 9.9, 161.5 ± 11.4 and 215.2 ± 24.2 mg N-$NO^{-}2$ L-1 d-1, at long-term operation. The MBRs performance achieved a significantly high nitrite removal activity for an N-damo process (specific denitrifying activity of up to 540 mg N-$NO^{-}2$ g-1 VSS d-1), even comparable to heterotrophic denitrification values. In this study, we have implemented a novel operational strategy that sets our work apart from previous studies with similar bioreactors. Specifically, we have introduced Cerium as a trace element in the feeding medium, which serves as a key differentiating factor. It allowed maintaining a stable reactor operation at high NLRs. Microbial community composition evidenced that both MBRs were dominated with N-damo bacteria (67 to 87% relative abundance in period III and I, respectively). However, a decrease in functional N-damo bacteria (*Candidatus Methylomirabilis*) abundance was observed during the increase in biomass activity and concentration, concomitantly with an increase of the other minor families (*Hypomicrobiaceae* and *Xanthobacteraceae*). Most of the selected antibiotics showed high biotransformation such as sulfamethoxazole, trimethoprim, cefalexin and azithromycin, whereas others such as roxithromycin and clarithromycin were only partially degraded (20-35%). On the contrary, ciprofloxacin showed almost no removal. Despite the metabolic enhancement, no apparent increase on the antibiotic removal was observed throughout the operation, suggesting that microbiological composition was of greater influence than its primary metabolic activity on the removal of antibiotics.

4.1. INTRODUCTION

Nitrite dependent anaerobic methane oxidation (N-damo) is a microbial process with promising technological features for sustainable nitrogen removal in wastewater treatment (van Kessel et al., 2018). The particularity of the N-damo process is the capability to remove methane and nitrogen simultaneously without the emission of nitrous oxide as an intermediate (Equation 1). As a result, two important Greenhouse Gases (GHGs) (i.e. methane and nitrous oxide), are mitigated during this process (Stein, 2020). The nitrite removal is catalyzed by bacteria associated to the NC10 phylum, identified with the genus *Candidatus Methylomirabilis* (Ettwig et al., 2010). This technology is particularly relevant for wastewater treatment plants (WWTPs) based on anaerobic processes, because they produce effluents with high dissolved methane concentrations, specially at low to moderate temperatures (Sánchez et al., 2016).

Equation. 1 $3CH_4 + 8NO^-_2 + 8H^+ \rightarrow 3CO_2 + 4N_2 + 10H_2O$ ($\Delta G°$ -928 kJ mol^{-1} CH_4)

The challenge is achieving stable application of N-damo processes at relevant activities. Previous studies reported low (Chang et al., 2021) and even decreasing values in long-term reactor operation with no proven reason (Kampman et al., 2012), mainly explained by the low doubling times of N-damo microorganisms (up to 2 weeks) (Ettwig et al., 2010), as well as oxygen and nitrite inhibition (Arias et al., 2022; Luesken et al., 2011). Previous enrichment experiments showed specific nitrite activity as low as 2 mg N g^{-1} VSS d^{-1} (He et al., 2015b). Recent N-damo works using MBRs showed a significative increase in activities with values between 64 (Arias et al., 2022) and 95 mg N g^{-1} VSS d^{-1} (Allegue et al., 2018).

Despite the technological advancements on the N-damo process, the reported specific activities are far below those corresponding to heterotrophic denitrification and anammox biomass (Lotti et al., 2015; Metcalf & Eddy et al., 2014). This fact might limit, among others, the practical development of N-damo bioreactors to treat wastewater One strategy for increasing the capacity of N-damo bioreactors, could be the modification of the feed medium by adding trace amounts of lanthanides such as Cerium (Ce), as suggested by some authors (Guerrero-Cruz et al., 2019; Versantvoort et al., 2018).

In addition to the removal capacity of nitrogen and methane, new WWTPs also need to consider the removal efficiency of Organic Micropollutants (OMPs). The OMP biotransformation potential is determined by the specialized microbial community present in biological systems (Wolff et al., 2018). This biotransformation mechanism can be performed by a metabolic or cometabolic process, although the latter was more often reported due to the relatively low OMP concentrations in the environment (Tran et al., 2013).

Previous research has shown that the increase in primary substrate is an effective operational strategy to enhance OMP removal, as higher biomass activities correlate with OMP biotransformation (Alvarino et al., 2014; Kennes-Veiga et al., 2021; Martínez-Quintela et al., 2021). Accordingly, the N-damo process has shown evidence of cometabolic biotransformation of several OMPs, including antibiotics (Martínez-Quintela, et al., 2021). In the latter research, a cometabolic removal was suggested by the correlation between increasing specific nitrite removal rates and OMP removal, with a maximum nitrite removal rate of 59 mg $N.L^{-1}d^{-1}$. However, the relationship between primary metabolism and cometabolic OMP removal is complex and not always follows a positive correlation, as it is suggested that cometabolism has a lower (activation) and upper limit (saturation) (Sheng et al., 2021). In this sense, it is still unclear if a cometabolic tendency is still observed during high N-damo activities.

The aim of this work was to metabolically enhance the N-damo process to reveal if high and steady N-damo activities can promote the biotransformation of a selected group of antibiotics during long-term operation. To this end, we progressively increased the Nitrogen Loading Rate (NLR) and adapted the micronutrients content by the addition of Cerium. Our results provide insights on the influence of primary substrate on the potential biotransformation capacity of N-damo microorganisms, as well as understanding the microbiological implications of the effect of NLRs and antibiotic presence by evaluating shifts on the microbial community composition.

4.2. MATERIALS AND METHODS

4.2.1. Reactor configuration and continuous-flow operation

Two analogous MBRs, MBR1 and MBR2, each with 10 L capacity and a working volume 6.65 L, were operated in parallel at stable conditions in a similar configuration as described in Allegue et al., (2018). Both MBRs had a submerged hollow-fiber ultrafiltration membrane module (Puron) with a 0.5 m^2 surface and a pore size of 0.03 μm. The membrane cycle operation consisted of 7 min of permeation and 30s of relaxation. The system continuously measured transmembrane pressures using a pressure sensor PN2069. No back flushing was performed during the reactor operation, but frequent biomass resuspension with nitrogen gas was performed to avoid biomass accumulation in the membrane. Additionally, the Oxidation-Reduction Potential (ORP) inside each MBR were measured in real-time by using a redox probe Hamilton Polyplast ORP BCN. All sensors were connected to a programable PLC Micro 820 (Allen-Bradley) for real-time control. The HRT was maintained at ~1 day and the temperature was fixed at 28°C using a thermostatic water bath.

The inoculum used had been subject to previous N-damo enrichment strategies for more than 3 years (Allegue et al., 2018; Arias et al., 2022; Martínez-Quintela et al., 2021). It was analyzed through 16S rRNA metabarcoding sequencing that evidenced a maximum abundance of N-damo phylum of around 30-50%, mainly represented by the *Methylomirabilaceae* family.

The system was fed with synthetic wastewater containing sodium nitrite as nitrogen source and a mixture of macronutrients and trace compounds according to Arias et al., (2022). Cerium (Ce) was included as trace element to promote the growth of N-damo bacteria (Versantvoort et al., 2018) and other methanotrophic microorganisms associated to N-damo cultures (Pol et al., 2014). Methane was fed continuously in saturation as a gas mixture CH_4 and CO_2 90:10%. The gas volume was adjusted in function of the biomass activity and NLR as the CO_2 was used for pH control. The pH was measured in the feed and permeate daily using a Hach pH probe. The gas was recirculated from bottom to the top using a mini laboratory blower N86 KT 18 (Laboport) for mixing purposes and prevention of membrane fouling. The gas volume was quantified by using a Mili GasCounter MGC-1V3.3 PMMA (Ritter).

4.2.2. Operational strategy

The two MBRs were operated in parallel during three experimental periods defined by the applied NLRs: 70 (PI), 140 (PII) and 220 (PIII) mg $N\text{-}NO^{-}_2 L^{-1}$. The only difference was that a continuous mix of antibiotics was fed only to MBR2. Thus, MBR1 was used as a microbial control to evaluate separately the influence of increasing the NLR in the microbial composition. Once the system reached a steady state, samples were taken to evaluate the antibiotic removal in MBR2 as well as the microbial composition in MBR1 and MBR2.

4.2.3. Selected OMPs

A mixture of seven OMP at a final concentration of 5 μg L^{-1} was fed to MBR2. Compounds were selected based on their occurrence in sewage, persistence in the environment and their high priority to monitor by the European legislation (Watch List, WFD 2000/60/EC). The selected substances correspond to four classes of antibiotics: cephalexin (CFX) as a representative of the group of cephalosporins; sulfamethoxazole (SMX) a widely used sulfonamide, usually combined with trimethoprim (TMP); ciprofloxacin (CIP) as a member of fluoroquinolones and the macrolides roxithromycin (ROX), azithromycin (AZY) and clarithromycin (CLA).

4.2.4. Analytical methods

Nitrogen species were determined by spectrophotometric measurements. Solids were only measured extracting mixed liquor biomass directly from the MBRs as no solids were found in the permeate due to the membrane retention Biomass concentration was measured by extracting 10 mL of mixed liquor from the MBRs. Total suspended solids (TSS) were quantified by weighting the solid fraction after

0.45 µm filtration using a glass fiber filter. Consequently, volatile suspended solids (VSS) accounted for the remaining solids after the muffle oven at 550º C for 30 min as indicated in the standard methods (APHA, 2000).

Total organic carbon (TOC) was measured following the method 5310 from the Standard Methods (APHA, 2000), using a Shimazu TOC-L CSN. The method measures TOC in an indirect way by determining the difference between Total Carbon (CT) and Inorganic Carbon (CI). The trace element composition was measured in each experimental period in the liquid feed and permeate using inductively coupled plasma mass spectrometry (ICP-MS).

The gas volume fed to each reactor was quantified using a Mili GasCounter MGC-1V3.3 PMMA (Ritter). The system also had an online Ultramat 23 gas analyzer (Siemens) to detect the presence of nitrous oxide. Additionally, the gas composition (nitrogen gas, carbon dioxide and nitrous oxide) was determined periodically by using a gas chromatograph (HP 5890 Series II).

Antibiotic removal efficiencies were determined by analyzing their concentrations in the aqueous phase of the influent and permeate from MBR2. Sorption was not considered as a relevant removal mechanism due to the low lipophilicity of the compounds selected. Three antibiotic sampling campaigns were performed for each experimental period (n=9). All replicates were analyzed for data quality control according to average values and standard deviation, outliers were excluded from further analysis.

All samples were collected in amber glass bottles and immediately filtered at 0.45 µm with an initial volume of 200 ml. Afterwards, samples were preconcentrated using Solid Phase Extraction (SPE) as indicated in Gros et al. (2013) and later quantified by using a liquid chromatograph Agilent 6410B-QQQ LC/MS. The column used was C18 Zorbax eclipse XDB 2,1x150mmx5µm. The solvents for Phase A were 10 mM of formic acid/ ammonium formate and for Phase B: Methanol. Each injection used 20 µl of sample.

4.2.5. Microbiological Analysis

Biomass samples were taken from the MBRs during steady operation, after resuspension with nitrogen gas. Subsequently, genomic DNA was extracted by triplicate using the Nucleospin Microbial DNA extraction kit (Machery-Nagel). The replicas from each sample were pooled together after quantification, and quality control using Nanodrop and Qubit fluorometer (Thermo Fisher Scientific Waltham, MA, USA). The V3-V4 hypervariable region for Bacteria was amplified using Bakt_341F (5’ CCT ACG GGN GGC WGC AG 3’) and Bakt_805R (5’ GAC TAC HVG GGT ATC TAA TCC 3’). DNA metabarcoding analyses of the region were carried out in an Illumina NovaSeq PE250 platform, generating paired ends reads (2 x 250) by All Genetics & Biology SL (www.allgenetics.eu).

Bioinformatic analyses were performed using the Microbial Genomics module (version 21.1) workflow of the CLC Genomics workbench (version 21.0.3). Raw sequences were filtered and then clustered into Operational Taxonomic Units (OTUs) at 97% cutoff for sequence similarity and classified against the non-redundant version SILVA SSU reference taxonomy (release 132; http://www.arb-silva.de). Only the most abundant bacterial OTUs (above 1 % of the total observed OTUs) were considered for further analysis.

4.2.6. Activity assays

The maximum activity of the biomass was determined by a single "in situ" batch assay once the reactor reached a stable operation in each of the three experimental periods (PI, PII and PIII). The liquid feed was disconnected, and a nitrite pulse was added to the reactors. Degradation kinetics were followed with time until there was no nitrite concentration left.

4.3. RESULTS AND DISCUSSION

4.3.1. MBRs performance

Two anoxic MBR reactors were operated in an analogous way for 234 days divided in three experimental periods defined by different nitrite loading rate (NLR) (**Table 4.1**). The operation was characterized by a stable hydraulic performance in both reactors and robustness of the system to reach and maintain the different high NLRs. The HRT for MBR1 was 1.04 ± 0.18 d and for MBR2 was 1.04 ± 0.08 d. Transmembrane pressure was below an average of -39 mbar for both MBRs, but particularly lower for MBR2. The membrane permeability was approximately 35 and 15 L m^{-2} h^{-1} bar^{-1} for MBR1 and MBR2, respectively. Moreover, the system efficiently removed all nitrite fed, reaching NRRs up to 220 mg N- NO_2^- L^{-1} d^{-1} for period III (**Table 4.1**). Overall, the average nitrite concentrations in permeates were of 0.9 ± 2.0 and 0.6 ± 1.6 mg N-NO_2^- L^{-1} for MBR1 and MBR2, respectively.

Additionally, nitrate and ammonium concentrations were quantified in both influent and permeate. Ammonium was fed as a macronutrient with average concentrations of 3.27 ± 0.61 and 3.20 ± 0.66 mg N-NH+4 L^{-1} for MBR1 and MBR2 respectively. On the contrary, nitrate was not purposely fed to the system but was found present in concentrations of 3.69 ± 1.71 mg N-NO^-_3 L^{-1} for MBR1 and 2.66 ± 1.68 mg N-NO^-_3 L^{-1} for MBR2. The presence of nitrate was probably due to the oxidation of nitrite with the remaining oxygen left in the feeding bags after the purging of dissolved oxygen with nitrogen gas. TOC concentrations in the feed had an average value of 3.81 ± 2.42 mg TOC L^{-1} and 3.73 ± 1.70 mg TOC L^{-1} for MBR1 and MBR2 respectively, limiting heterotrophic denitrification. As for pH, the system was kept very stable with average values in the permeate of 7.76 ± 0.31 and 7.63 ± 0.35 for MBR1 and MBR2, respectively.

Table 4.1. Different experimental periods and corresponding NLRs for MBRs operation.

Period	Phase	Reactor operation (days)	Nitrite Loading Rate* (mg N-NO^-_2 L^{-1} d^{-1})	
			MBR1	MBR2
I	Initial load	0-136	68.78±10.24	70.31±10.01
II	Medium load	137-185	162.5±9.6	161.5±11.4
III	High load	186-234	220.1±26.3	215.2±24.2

*Average values for the whole period are shown from which standard deviation values were calculated after the reactor reached the desired NLR, excluding the days that the load was progressively being increased (n=3).

Period I (PI) (days 0-136) included the start-up of the system with a progressive increase of the NLR until a steady operation at 74.7 ± 8.5 and 76.0 ± 9.9 mg NO^-_2-N·L^{-1} for MBR1 and MBR2, respectively (in approximate 20 days). The MBRs steady operation was sustained until day 63, when the antibiotic mixture was added in MBR2 while maintaining analogous operation with MBR1. Then, both MBRs operation was maintained for another 58 days to ensure the biomass acclimation to antibiotics in MBR2 before the first antibiotic campaign (days 122 to 136). The antibiotic mixture did not show any impact on the reactor performance by means of primary metabolism. During the first days of both MBRs operation, sodium carbonate (in a range of 250-300 mg L^{-1}) was included in the feeding medium to avoid acidification of the bioreactor due to absorption of CO_2 present in the methane-carbon dioxide gas mixture fed to the system. Later, when the denitrifying activity could avoid the acidification of the bioreactor, sodium carbonate addition was discontinued.

Period II (PII) (days 137-185) incorporated the first increase of the nitrite load with a 2.18-fold increase in the NLR within two weeks (going from 79 to 172 mg N-NO^-_2 L^{-1} d^{-1}). The maximum biomass concentration in this stage reached 2.98 and 1.49 g VSS L^{-1} for MBR1 and MBR2, respectively. The increase in biomass concentration accounted for less than 0.12 g VSS for both reactors.

Period III (PIII) (days 186-234) refers to the highest NLR applied. The system swiftly sustained a fast and progressive increase of 60 mg N-NO^-_2 L^{-1} d^{-1} in only 8 days reaching an average NLR of 220.07 ± 26.27 mg N L^{-1} d^{-1} for MBR1 and 215.21 ± 24.22 mg N L^{-1} d^{-1} for MBR2 (**Figure 4.1**). The steady efficiency of the reactor during the increase in NLR shows the system capacity to adjust to higher nitrite loads. The increase in NLR also had an influence in the biomass concentration. During this period, the biomass in MBR2 doubled, reaching a final concentration of 2.47 g MLVSS from the 1.49 g MLVSS in PII.

The ORP measurements evidenced reducing conditions with average values of - 347.7 ± 135 mV for MBR1 and -197.5±109.5 mV for MBR2. The ORP deviation in both MBRs correlated with abundance and depletion of nitrite and nitrous oxide in the system. The real time values were also dependent on the cycle feed, showing higher values when the reactor was actively receiving the feed. Therefore, redox measurements in real time were used as a nitrite surrogate parameter to quickly identify the system capacity when nitrite was increased.

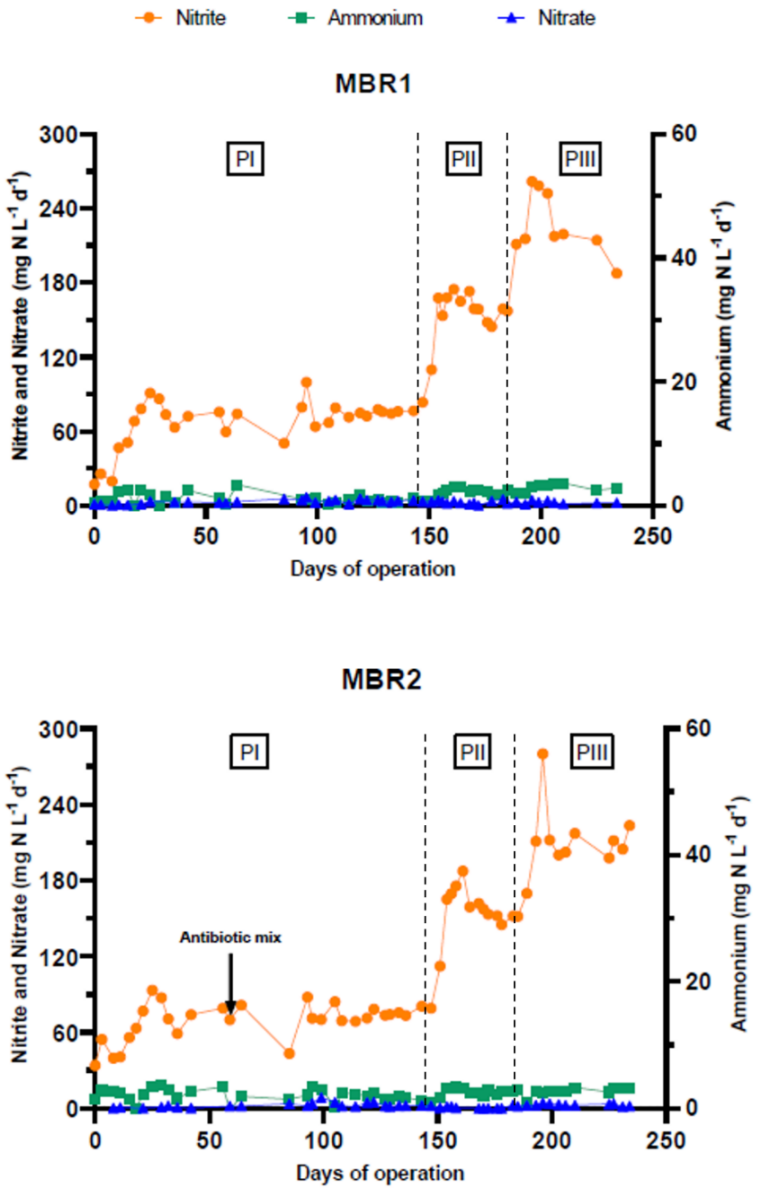


Figure 4.1. Volumetric nitrogen rates in MBR1 (above) and MBR2 (below) during the reactor operation. In the left Y axis, nitrite removal as in mg N- NO^-_2 L^{-1} d^{-1} and nitrate removal as in mg N-NO^-_3 L^{-1} d^{-1} are shown while ammonium removal is displayed in the right axis as mg N-NH^+_4 L^{-1} d^{-1} (n = 3). Experimental periods are separated by dashed vertical lines.

The removal rates achieved are similar as those evidenced by Arias et al., (2022) and higher than those evidenced in similar MBRs (Allegue et al., 2018; Martínez-Quintela et al., 2021) which supports the assertion of the importance of media composition, in this work by the addition of the lanthanide Cerium, which we link to the observed boosted reactor efficiency. Accordingly, the ICP-MS measurements evidenced that Cerium was completely removed in all experimental periods from the initial concentration fed to the system. These results agree with previous research that suggests that methanotrophic and methylotrophic microorganisms, present in N-damo enrich cultures, can employ Ce instead of Calcium as cofactor and ultimately, enhance microbial kinetics (Guerrero-Cruz et al., 2019).

4.3.1.1. Biomass growth

The apparent doubling time (T_d) and yield (Y) were determined as key physiological properties of the N-damo culture in the highly enriched MBRs. For the T_d quantification, biomass concentration was monitored during the complete operation in both MBRs. The evolution of biomass growth is depicted for MBR2 from an initial concentration of 1.10 g MLVSS.L^{-1} up to a final biomass concentration of 3.08 g MLVSS L^{-1} after an exponential growth in PII and PIII.

The T_d was calculated considering the values for the biomass growth from days 189 till 234 (PII and PIII), which showed the highest biomass increase during the reactor operation. For this purpose, the reaction rate (B) was estimated using a linear equation for an exponential growth model (Equation 4.2), from which the intercept (B) was used to calculate the doubling time (Equation 4.3). The calculated T_d was 60.3 days; however, this value depicts only an apparent biomass growth in the reactor and does not reflect the physiologically capacity of the N-damo microorganisms as growth substrates could have been a limiting factor during the reactor operation. Microbial growth rate is influenced by substrate limitation and higher growth rates are observed in a resource rich environment (Lipson, 2015). In our study, the permeate in MBR2 had a negligible average nitrite concentration of 0.35 ± 0.5 mg NO^{-}_2 L^{-1} in the corresponding days (day 189-234), which suggests a nitrite limitation that could impact the growth kinetics and consequently the T_d.

Eq 4.2. Linear equation for exponential growth $Y = Ae^{Bx}$

Eq 4.3. Doubling time $T_d = {\ln (2)}/{B}$

The quantification of the biomass refers mainly to suspended flocs in the mixed liquor of the MBRs. However, the quantification does not include some remaining biomass adhered to the glass surface inside the MBRs as biofilms. Nevertheless, the excluded biomass fraction was not significant, and efforts were done to resuspend all the available biomass inside the MBRs continuously (i.e.,

Methane recirculation during the operation and nitrogen gas resuspension previous sampling to detach biofilm formation in the glass walls).

4.3.1.2. Biomass Yield

The yield estimation (Y) considered the values from the last 70 days (PII and PIII) of MBR2 operation corresponding to the log phase (days 164 to 234) for quantifying the amount of biomass gained versus the amount of nitrite denitrified in the system (Equation 4.4). The biomass gained was 1.76 g MLVSS L-1 and the nitrite removed accounted for 14.8 g N L^{-1} considering an average NLR of 211 mg N-NO^{-}_{2} $L^{-1}d^{-1}$which resulted in an estimated yield of 0.12 g VSS g N^{-1} denitrified.

Eq 4.4. $$Yield\ \frac{(gBiomass)}{(gN\ denitrified)} = \frac{grBiomass\ earned}{(days*NLR)}$$

Table 4.2. Comparison of yield estimation with available literature (units recalculated)

Source	Yield (g VSS g N^{-1})
This study	0.12
Guerrero-Cruz et al., 2019	0.13[a]
Winkler et al., 2015	0.11[b]

a. Assuming a stoichiometric relation 3/8 C:N during N-damo and a 50%C of the molecular biomass formula $C_5H_8O_{2.3}N_{1.1}$

b. Assuming a relation of 1.42 g COD g^{-1} VSS and 1.14 g COD g^{-1} N

The calculated Y is similar to the estimated in previous N-damo studies (Guerrero-Cruz et al., 2019; Winkler et al., 2015) (**Table 4.2**). The Y represents a rough estimation of the process performance, contributing to the scarce knowledge on basic N-damo process parameters corresponding to high-rate operation. Generally, microorganisms that have high substrate consumption capacities have lower yields as there is usually a compensation regarding yield and substrate consumption (Ni et al., 2017). In this sense, the relative low yield of autotrophic N-damo compared to other heterotrophic microorganisms (i.e., the Y in heterotrophic denitrifiers is above 185 g VSS g N^{-1}(Ni et al., 2017)) result in lower sludge production and consequently, longer SRTs would be needed for its growth.

4.3.1.3. Evolution of the off-gas phase

The concentration of nitrous oxide, nitrogen, carbon dioxide and methane in the off gas were continuously measured by gas chromatography. The measurements showed that N_2O was absent most of the time during the reactor operation in both MBRs. In MBR1, the concentration of N_2O was zero on 42 out of the 52 measurements performed in the reactor in the whole operation. In fact, in the first 203 days the average concentration was 0.02 mg N-N_2O L^{-1}, and N_2O was present in

only three specific points with an average concentration of 19.88 mg N-N_2O L^{-1} during the last 30 operational days. In the case of MBR2, N_2O emissions were present only in the first 64 days of operation with an average concentration of 0.88 ± 0.9 mg N-N_2O L^{-1}. In the rest of the operation, N_2O emissions wero cero for 34 out of 37 measurements.

The specific measurements in which N_2O was found in both MBRs, corresponded to minor operational perturbances and to the presence of nitrite in the permeate. The few isolated peaks of N_2O identified corresponded to those moments in which there was a slight nitrite build up in the bioreactors, with concentrations below 3 mg N-NO^{-2} L^{-1}. Considering the N-damo metabolism does not include N_2O as a process intermediate nor encodes the nitrous oxide reductase, the presence of trace concentrations of N_2O suggests the coexistence of conventional heterotrophic bacteria which includes N_2O as intermediate step as previously mentioned in similar reactor operation (Allegue et al., 2018; Arias et al., 2022). Moreover, it is also presumed that the specific medium composition used in this work, which has a reduction of salts (namely Ca, P and K) and the addition of EDTA (which enhances the Cu and Fe bioavailability), also favoured the low/none N_2O emissions as previously demonstrated by Arias et al., (2022).

The values for methane, as it was fed in saturation, remained similar throughout the reactor operation accounting for about 80-87% of the biogas composition. The latter was influenced by the production of N_2 and presence of CO_2 that was about 5%.

4.3.1.4. Activity assays

Activity assays showed a high potential for nitrogen removal, with a maximum volumetric rate of 1332 mg N $L^{-1}d^{-1}$ and a specific nitrite removal rate as high as 540 mg N $gVSS^{-1}$ d^{-1} for PIII (**Table 4.3**). These values suggest that the N-damo process has the capability to remove nitrite at a practical useful rate, even comparable to the values observed by heterotrophic denitrification in full-scale installations (up to 420 mg N g^{-1} VSS d^{-1}); or even higher than other autotrophic process like Anammox (200 mg N g^{-1} VSS d^{-1}) (Lotti et al., 2015; Metcalf & Eddy et al., 2014). The maximum specific activity achieved in this study represent a step forward in the technological development of this process compared with previous values, which ranged from 64.13 mg N g^{-1} VSS d^{-1} (Arias et al., 2022), 95.53 mg N g^{-1} VSS d^{-1}(Allegue et al., 2018) to 186.3 mg N g^{-1} VSS d^{-1} (Martínez-Quintela et al., 2021). Other studies working with different technical approaches have reported considerable lower values, below 7 mg N g^{-1} VSS d^{-1} (Chang et al., 2021; He et al., 2015a; Hu et al., 2019).

The activity assays showed a maximum volumetric rate of 4-6-fold higher than the apparent volumetric rate measured during the continuous reactor operation (**Table 4.3**). The maximum activity values evidenced in this work are

amongst the highest reported in literature, which in turn, supports the relevance of N-damo process for a successful technological application at large scale. Similarly, other studies using nitrite dependent N-damo enrichments have shown high volumetric rates up to 285.7 mg N g^{-1} L^{-1} d^{-1} (Arias et al., 2022). Moreover, enrichments combining Anammox, N-damo bacteria and N-damo archaea have shown values up to 330 mg N L^{-1} d^{-1} for nitrite removal (N-damo bacteria) and up to 684 for nitrate removal (N-damo archaea) (Cai et al., 2015).

Table 4.3. Maximum activity tests result for all experimental periods.

Period	Activity Assays*			Continuous Reactor Operation		
	Susp. biomass	Maximum activity	Maximum volumetric rate	Apparent volumetric rate	Average nitrite in permeate	Suspended biomass
	g VSS L^{-1}	mg N-NO^-_2 g^{-1} VSS d^{-1}	mg N-NO^-_2 L^{-1} d^{-1}	mg N-NO^-_2 L^{-1} d^{-1}	mg N-NO^-_2 L^{-1} d^{-1}	g VSS L^{-1}
I	1.32	245	323	75.84±10.01	1.01±2.10	1.27±0.10
II	1.38	486	672	161.48±11.41	0.11±0.02	1.40±0.07
III	2.47	540	1332	215.21±24.22	0.38±0.50	2.27±0.48

*Activity assays were performed in MBR2.

4.3.2. Microbiological analysis

To understand the microbial community structure to the reactor functionality, the taxonomic analysis is discussed at a family level for a clearer comparison with previous work using similar reactors.

Relative abundances, showed that the N-damo population, represented by the *Methylomirabilaceae* family, dominated the microbial community during all reactor operation in both MBRs showing a relative abundance of up to 87 % (**Figure 4.3**). The N-damo bacteria, corroborates that the enrichment strategy (medium composition and reactor operation) was efficient in cultivating N-damo bacteria. The N-damo bacteria population achieved for both MBRs surpasses previous enrichments strategies using a similar MBRs where a maximum abundance of 57 % was evidenced (Arias et al., 2022). The incorporation of the lanthanide Cerium in the feeding composition is hypothesized to have boosted the growth of the N-damo culture and influenced the adaptation of the microbial community to high NLRs. Similarly, previous studies have supplemented their NC10 enrichment culture with the lanthanide Cerium to release a potential growth limitations successfully (Versantvoort et al., 2018). This is mainly because many methylotrophs can use Cerium instead of Calcium as cofactor for the activation of the enzyme methanol dehydrogenase (MDH) (Guerrero-Cruz et al., 2019). This is particularly relevant, as

it is suggested that lanthanides can provide superior catalytic properties for the respective enzymatic function (Pol et al., 2014).

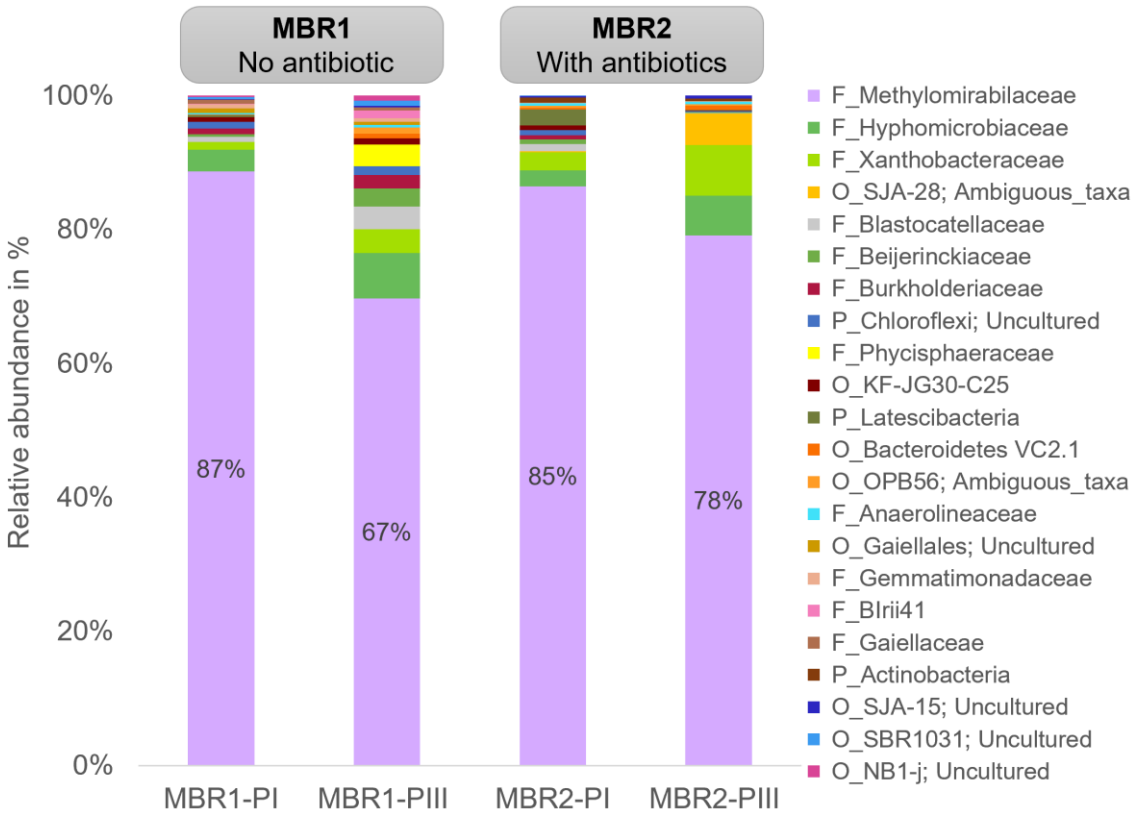


Figure 4.2. Microbial communities are present in both MBR (MBR1 and MBR2) at the beginning (PI) and end of operation (PIII). The legend shows the respective taxonomic classification with the first letter: P_phylum, O_order, and F_family. Only taxon with relative abundances over 1% are represented. The shades of the same color identify microorganisms from the same order.

Interestingly, the N-damo population, although predominant, suffered a significant decrease with the increase in NLR, this trend was observed in both MBRs. In Period III, the family *Methylomirabilaceae* decreased from 87 % to 67 % and from 85 % to 78 % of relative abundance in MBR1 and MBR2, respectively (**Figure 4.2**). The decrease of the predominant N-damo family was concomitant with the increase in abundance of minor species present in both reactors. Particularly, microorganisms from the Order Rizhobiales depicted by the families *Hyphomicrobiaceae* and *Xanthobacteraceae* increased their abundance in both MBRs (**Figure 4.2**). Specifically in the case of MBR2, microorganisms from the order SJA-28 were distinctly favored in period III.

Microorganisms belonging to the family *Xanthobacteraceae* are known to be facultative chemolithoautotrophy with hydrogen and/or reduced sulfur compounds and to have the hability to fix N_2 (Oren, 2014). Moreover, some family representatives have been involved in the cometabolic degradation of different

OMPs chlorinated alkanes (Ensign et al., 1992) and antibiotics, particularly SMX (Yang et al., 2020) which suggest potential biotechnological applications. In fact, several species (*Xanthobacter autotrophicus, X. flavus, Labrys methylaminiphilus* and others) have been shown resistant to antibiotics such as ampicillin, chloramphenicol, erythromycin, penicillin and tetracycline (Oren, 2014).

The increase of the *Hyphomicrobiaceae family* in both MBRs at higher NLRs (**Figure 4.2**) can be explained by the simultaneous decrease in N-damo population and the respective loss of reverse methanogenenic activity. Members from the *Hyphomicrobiaceae* family (belonging to the *Hyphomicrobium* genus) have been characterized as methylotrophs involved in methanol-fed denitrification systems (Rissanen et al., 2016). Similarly, another study has shown that *Hyphomicrobiaceae* is a key bacterial population involved in methane-dependent denitrification (Osaka et al., 2008). Therefore, the loss of N-damo population would provide less substrate competition for the methane oxidation and thus potentially provide an opportunity for other methanotrophs to thrive.

Previous N-damo studies at low NLR, also identified a significant presence of microorganisms from the order SJA-28 that showed opposite temporal behavior to the *Methylomirabiliaceae* family (Allegue et al., 2018). In the former study, as the MBRs were increasing in NLRs and in simultaneous the abundance of N-damo bacteria, microorganisms from SJA-28 declined, which suggested an alternating relationship between both microorganisms. In this study, NLRs were distinctly higher and reactor efficiency was not altered, however, we evidenced the same opposing tendency as SJA-28 abundance increased with the decreased in N-damo. Based on all the above, it appears that both groups of microorganisms have an intrinsic relationship that ultimately impacts the community structure. However, it remains unclear why only MBR2 showed a significative increase of the order SJA-28 and MBR1did not show a negligible presence despite having the same NLRs and similar initial microbial compositions (**Figure 4.2**). In this sense, although SJA-28 is a relatively unknown taxon, it is possible that the increase might be related with the presence of antibiotics.

Regarding the influence of antibiotic presence, the similarity in the microbial composition of both in PI for both MBRs suggests that microbial shifts were not immediate but rather visible after long-term exposure to environmental concentrations of antibiotics (**Figure 4.3**). At the end of the reactor operation (PIII), Shannon diversity index increased for MBR1, but decreased for MBR2 despite having similar NRLs (**Figure 4.1**). These results suggest two possible assertions, firstly, higher NLRs during N-damo demand for more diversity in the microbial community structure to be able to maintain reactor efficiency. And secondly, the presence of antibiotics causes an impact on the diversity of the community composition, especially observed at long-term operation (MBR1-PIII vs. MBR2-PIII).

4.3.3. Removal of antibiotics

Based on the overall extent of their biotransformation, the selected antibiotics can be classified in three trends due to their average removals across the operation: i) highly biodegradable: TMP (83 % ± 7.9), CFX (82 % ± 2.1), SMX (75 % ± 2.1) and AZY (86 % ± 9.1); ii) moderately biodegradable: ROX (36 % ± 6.0) and CLA (24 % ± 15.4); and iii) persistent CIP (0%) (**Figure 4.3**).

For the highly biodegradable compounds, two behaviors were observed, i) high and stable removal, which was independent of the changes of operational conditions and microbial dynamic composition (TMP and CFX) and ii) compounds with wider variations in their removal as occurred with SMX and AZY (**Figure 4.4**).

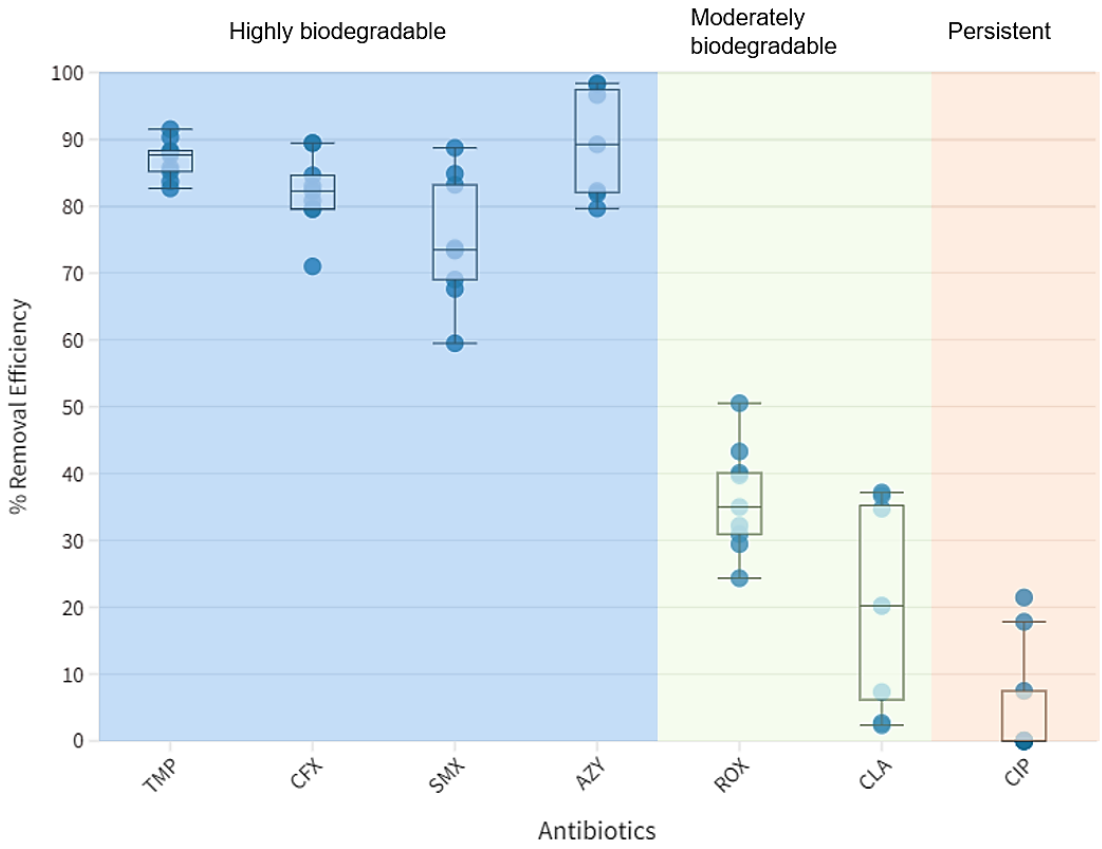


Figure 4.3. Overall removal efficiencies of selected antibiotics. Each compound was measured in MBR2 in all three experimental periods in triplicates when MBR2 (n=9, except for CIP that only included positive removals). Error bars show standard variations. The colored background helps identify the three removal tendencies observed: Highly biodegradable, moderately biodegradable, and persistent.

CFX was efficiently removed in all operation periods with an average removal of 82 ± 6%. Although the information about its removal under anoxic conditions is scarce, previous works operating with other conditions (aerobic activated sludges, concentrations of 100 µg CFX L^{-1}) reported almost total biodegradation (97%), with a biodegradation constant rate (k_{biol}) of 0.36 h^{-1}(Li and Zhang, 2010). CFX, as most

β-lactams, is easily biodegraded during biological wastewater treatment due the cleavage of its unstable β-lactam ring by the activity of β-lactamases, a group of enzymes widely excreted in wastewater microbiomes (Liu et al., 2021; Oberoi et al., 2019).

In the case of TMP, efficiencies obtained in this work were quite high (87±3%) and stable. Previous works in anoxic conditions, showed significantly lower values, with removal efficiencies below 20% (Martínez-Quintela et al., 2021; Suarez et al., 2010). The increased removal efficiency in our work could be explained by the significantly higher biomass activities used compared to the other authors.

Similarly, the removal efficiencies obtained for SMX were high during all operation but particularly in PIII (85-90%), which showed a significative increase with respect PI and PII (**Figure 4.4**). These results are quite different from those obtained in conventional heterotrophic denitrification, where SMX was considered persistent, with removals below 26% (Suárez et al., 2010). Other MBBRs studies using methanol/ethanol dosing as carbon source reported biodegradability of SMX up 50%, operating at a very low SMX concentration 0.04 μg L^{-1} (Torresi et al., 2017). Previous N-damo studies indicated SMX could be removed up to 62% (Martínez-Quintela et al., 2020). In our study, the maximum specific removal rate was 2.2 μg SMX g^{-1} VSS d^{-1} reached in period I (at 76 mg N L^{-1} d^{-1}). In a similar manner, Martínez-Quintela et al., (2020), reached a maximum specific removal of 1.8 μg SMX g^{-1} VSS d^{-1} at 50 mg N L^{-1} d^{-1}. Despite the significative increase in average removal in PIII with PII and PI, there was no increase in specific SMX removal rate. These results suggest that the growth of biomass was not directly functional for SMX removal but rather to the nitrite removal.

With regards to the macrolide AZY, the information about its fate in anoxic conditions is scarce. Our results show a high biodegradability (75-97%) (**Figure 4.3**), but with a decreasing tendency in the long-term operation (**Figure 4.4**). Similarly, as with SMX, the specific removal of AZY was not enhanced as the biomass activity increased. In PI, the average specific removal was 3.45 μg AZY g^{-1} VSS d^{-1} and in PIII, this value dropped until 1.70 μg AZY g^{-1} VSS d^{-1}. Previous works under aerobic conditions using environmental relevant concentrations from 0.1 to 13 μg L^{-1} report removal efficiencies around 50% using activated sludge (Abegglen et al., 2009; Blair et al., 2015) or MBRs (Abegglen et al., 2009).

For these four highly biodegradable OMPs, a positive tendency between antibiotic removal and NRL were evidenced mainly for SMX (**Figure 4.4**). However, decreasing tendencies were observed when considering the specific antibiotic removal and the specific denitrifying activity for SMX and AZY. Several explanations have been given to explain this different behavior respect to the cometabolic tendency of OMPs during N-damo at lower NLR (Martínez-Quintela et al., 2021). One of them is referred to the HRT used, 1 d in this work, which can be high enough to

hide the cometabolic effect showing the same removal for operational periods (Kennes-Veiga et al., 2021). On the other hand, the exponential increase in biomass concentration observed in PII and PIII could suggest that the microbial growth was especially linked to microorganisms involved in N removal more than the species related to OMP biotransformation, which finally caused a shift in the microbial community composition. Another factor that should be considered is the difficulty for an accurate quantification of the biomass present as biofilm in the system (only suspended has been measured), which might underestimate real biomass concentrations inside the reactors.

Besides these considerations, other factors, such as the evolution of the microbial composition in the MBRs should be considered. In this way, microbiological analysis revealed a 7% reduction of the N-Damo culture in MBR2 (relative abundance of *Methylomirabilaceae* family (**Figure 4.2**), along with the increase of NLR applied during the reactor operation. The negative trend between biotransformation and specific biomass activity (especially observed in AZY) might be explained by a reduction in the functional population involved in the biotransformation of both SMX and AZY. In the same manner, previous research links the potential biotransformation capacity to the presence of the key enzyme methane monooxygenase (MMO) (present in N-damo) which is suggested to be capable of target OMP functional groups (Jiang et al., 2010; Wang et al., 2022).

In the case of moderately biotransformed antibiotics, the macrolides ROX and CLA, results showed hardly any increase in removal efficiency nor specific OMP removal despite the increase in NLRs (i.e., N-damo activity) (**Figure 4.4**). The moderate removal of ROX was also evidenced by Suarez et al. (2010) during heterotrophic denitrification, where the removal of ROX accounted for less than 20% (using 20 μg L^{-1} as initial concentration). On the other hand, CLA was removed between 4 up to 42% with a decline in efficiency in period II. Although there are no information in anoxic conditions, previous research has also shown moderate removal of CLA (30 – 55 %) during aerobic biological removal ($C_{initial}$ =14 μg L^{-1}) (Escolà Casas et al., 2015). From these results, it appears that in both systems the maximum kinetics capacity (i.e., k_{biol}) for the biotransformation of both compounds has been achieved, which can be explained either by the achievement of the saturation level at these concentrations or because of thermodynamic constraints (chemical equilibrium or enzymatic reversibility) (Gonzalez-gil et al., 2018; Kennes-Veiga et al., 2022; Sheng et al., 2021).

CIP was persistent throughout all the reactor operation. Similarly, Dorival-García et al., (2013), evidenced less than 5% removal of CIP ($C_{initial}$ = 500 μg L^{-1}), suggesting a recalcitrant behavior of CIP in anoxic conditions. Even though the presence of antibiotics did not show any inhibitory effects in this study, CIP was also found to be recalcitrant to biodegradation in water and soil samples while also

strongly inhibiting the microbial activities (Girardi, 2011). The persistence of CIP to biodegradation could be explained by its zwitterionic behavior, which suggests that biodegradation is a secondary removal route and CIP is mainly removed by sorption (Dorival-García et al., 2013).

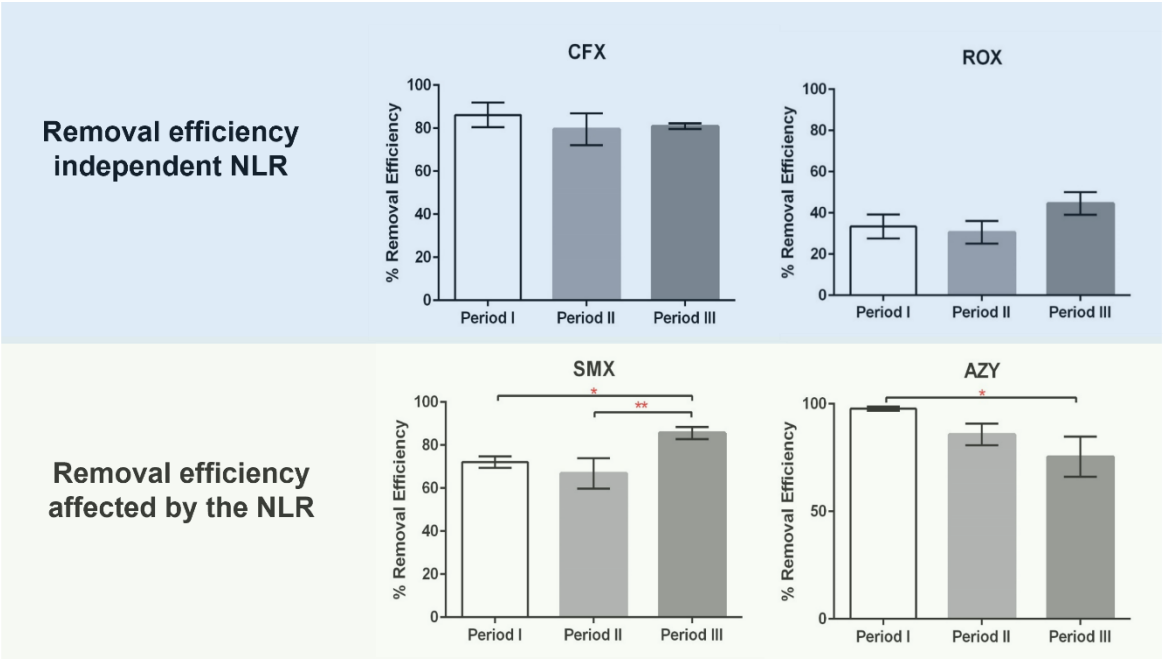


Figure 4.4. Average removal efficiency in each period. Above, CFX and ROX as compounds which were not affected by the NLR. Below, SMX and AZY as compounds who were affected with the NLR. The asterisks highlight those antibiotics whose removal efficiencies are significantly different among periods ($p \leq 0.05$) by a One-way Anova and Tukey's multiple comparison test. Error bars show standard variation (n=3).

Although most of the selected antibiotics were highly removed in the N-damo MBR system, the specific OMP removal did not correlate with the specific denitrifying activity. For most compounds, higher NLRs did not significantly enhance antibiotic biotransformation, at least when operating at 1 d of HRT. Microbiological factors, such as differences in taxonomic composition, including the abundance of N-damo bacteria as functional microorganisms might be having more influence on the biotransformation mechanism of the selected compounds.

Previous research has evidenced shifts in microbial community composition associated solely by the presence of OMPs even at environmental relevant concentrations (Li et al., 2020; Zou et al., 2021). For example, the presence of 50 μg L^{-1} of CIP has been linked to the decrease of proteobacteria from 44% to 36% (Li et al., 2020). Other authors evidenced that denitrifying bacteria are particularly sensitive to trace concentrations of CIP or similar fluoroquinolones (even at ng L^{-1} level) (Yi et al., 2017; Zou et al., 2021).

Combining all results, it appears that the microbials shifts happening in the MBRs have a greater impact on antibiotic biotransformation than the primary metabolism associated to the NLRs. The negative tendencies of specific antibiotic removal observed (namely on SMX and AZY) might be explained by the decline of the functional N-damo bacteria, associated to the M*ethylomirabilaceae* family.

4.4. CONCLUSIONS

A stable and long-term MBR operation with high NRRs (up to mg 220 $N\text{-}NO^{-}_{2}\ L^{-1}$) was achieved. Moreover, N-damo activity evidenced an even higher nitrite removal potential of up to 540 mg $N\text{-}NO^{-}_{2}\ g^{-1}$ VSS d^{-1} during the maximum activity assays performed. This values, jointly with the reactor stability and robustness to high nitrogen load increase, constitutes an important progress for its technological applicability.

The *Methylomirabiliaceae* family dominated the microbial community with a relative abundance of up to 87 % during stable reactor operation. However, it appears that high NLRs seem to favour the growth of minor species present in the community (namely from the order Rizhobiales).

Most of the antibiotics were biotransformed except for CIP that showed persistent behavior. CFX, TMP, SMX and AZY were highly biotransformed in the average range of 74-89% and in the case of ROX and CLA, a moderate biotransformation with average values of 20 to 36% across all NLRs was observed. The increase in biomass activity (in PII and PIII) did not enhance the removal efficiency, as highest removals were observed in PI. Moreover, it seems that the shifts in microbial community composition due to the different NLRs appears to have a greater impact for the removal of the studied antibiotics.

Combining all evidence, it appears that N-damo microorganisms seem to be functionally involved in the biotransformation of the selected antibiotics, and their decrease seems to affect the removal efficiency of the system, especially in the case of AZY.

CHAPTER 5

Enzymatic Underpinnings of N-Damo Systems Revealed by Metaproteomics

*Part of this chapter has been published in:

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Abstract

Nitrite-driven anaerobic methane oxidation (N-damo) is a promising biological process to achieve carbon-neutral wastewater treatment solutions, aligned with the sustainable development goals. In this study, we investigated the enzymatic activities in a membrane bioreactor highly enriched in N-damo bacteria and operated at high nitrogen removal rates. Metaproteomic analyses, with a special focus on metalloenzymes, revealed the complete enzymatic route of N-damo including their unique nitric oxide dismutases. The relative protein abundance evidenced that "*Ca. Methylomirabilis lanthanidiphila"* was the predominant N-damo specie, attributed to the induction of its lanthanide-binding methanol dehydrogenase in the presence of supplemented cerium. Metaproteomics also disclosed the activity of the accompanying taxa in denitrification, methylotrophy and methanotrophy. The most abundant functional metalloenzymes from this community require copper, iron, and cerium as metal cofactors which was correlated with the metal consumptions in the bioreactor. This study highlights the usefulness of metaproteomics for evaluating the enzymatic activities in engineered systems to optimize microbial management.

5.1. INTRODUCTION

Methane (CH_4) and nitrous oxide (N_2O) are two powerful greenhouse gases (GHGs) produced as microbial metabolism byproducts (Glass and Orphan, 2012; Stein, 2020). During biological wastewater treatment, CH_4 is the final product of anaerobic digestion, whereas N_2O is an intermediate product of heterotrophic denitrification of nitrite/nitrate to nitrogen gas (N_2) (Kampschreur et al., 2009). Mitigation of GHGs is critical for new wastewater treatment technologies adhering to sustainable goals (Ren et al., 2020).

Nitrite-driven anaerobic methane oxidation (N-damo) is a singular microbial process capable of removing CH_4 during a simultaneous denitrification without producing N_2O as intermediate (Raghoebarsing et al., 2006). The only N-damo bacterial genus described to date is "*Candidatus Methylomirabilis*", and belongs to the NC10 phylum (Ettwig et al., 2008). Four "*Ca. Methylomirabilis*" species have been identified: "*Ca.* M. oxyfera" (Ettwig et al., 2010), "*Ca.* M. sinica" (He et al., 2016), "*Ca.* M. limnetica" (Graf et al., 2018) and "*Ca.* M. lanthanidiphila" (Versantvoort et al., 2018). Additionally, one genus of N-damo archaea was also described (i.e.: "*Ca.* Methanoperedens", ANME-2d clade) and shown to couple reverse methanogenesis to the use of nitrate as terminal electron acceptor (Haroon et al., 2013)

N-damo microorganisms are very attractive for sustainable wastewater treatment, and their biotechnological applications have demonstrated its efficiency to remove nitrogen in the form of nitrate (Cai et al., 2015) or nitrite (Arias et al., 2022) up to competitive removal rates (e.g. : 285.7 mg N - NO^{-}_{2} - $L^{-1}d^{-1}$) (Arias et al., 2022). However, despite previous research efforts, full-scale implementation of the N-damo remains a challenge due their slow growth (Ettwig et al., 2010), low biomass activities (Chang et al., 2021), strict anaerobic requirements and need for sufficient CH_4 availability (Chen et al., 2023).

A model for the methane activation and nitrite reduction metabolic pathways in "*Ca. Methylomirabilis lanthanidiphila*" was fully described in (Versantvoort et al., 2019, 2018). Their key reactions are catalyzed by specific enzymes that rely on the metal ion availability (Reimann et al., 2015) as was shown before in other microorganisms relying on respiratory chains that couple electron flow and proton translocation across their membranes (Budhraja et al., 2021, 2019). Accordingly, a successful operational strategy to increase the N-damo activity was adjusting the mineral composition on the influent by increasing the bioavailability of trace metals which ultimately contributed to higher removal rates and a more stable operation (Arias et al., 2022; He et al., 2015; Reimann et al., 2015). Particularly, the dosage of Cu, Fe and Mo in adequate concentrations showed to significantly stimulate nitrite removal rates (NRR) (Arias et al., 2018; He et al., 2015; Jiang et al., 2018a) by inducing the expression of functionally relevant genes (Jiang et al., 2018b; Reimann et al., 2015; Versantvoort et al., 2018). Additionally, the presence of lanthanides,

such as cerium, have been shown to promote the activity of N-damo bacteria (Keltjens et al., 2014) particularly of "*Candidatus* Methylomirabilis lanthanidiphila" (Guerrero-Cruz et al., 2019; Versantvoort et al., 2018).

N-damo species have not been isolated so far and previous works were done in enrichment cultures around 14% (J. Wang et al., 2019) to a maximum of 50% (Allegue et al., 2018) relative abundance of "*Ca. Methylomirabilis*". Other species might be outcompeting with N-damo for methane, nitrogen, and other components of the medium such as trace metals, therefore it is of great interest to find a tool to assess the activities of all the members of the community.

Mass spectrometry based metaproteomic approaches have emerged as potent tools to discover the activities of specific microorganisms in a mixed community (Zhang et al., 2013).. The technical advances achieved in the last decades have allowed the identification of thousands of proteins from complex samples at once. Protein expressions can be more directly related to microbial activities than gene detections (metagenomics)(Blakeley-Ruiz and Kleiner, 2022)or mRNA levels (metatranscriptomics). The latter comparison is especially relevant as mRNA levels do not always reveal the real bacterial activities(Wang et al., 2016). The relevance of metaproteomics to calculate the protein contribution of each member of the community was recently highlighted by Kleikamp et al., (2021) and Kleiner et al., (2017), and has been successfully applied in highly complex communities as aerobic activated sludge samples in Azizan et al., (2020) and Kennes-Veiga et al., (2022). The usefulness of metaproteomics to study enriched microbial communities in engineered systems is recently attracting biological wastewater researcher community (Kouba et al., 2022). In Wang et al., (2021), he authors showed how total annamox relative abundances and total protein expressions remained stable with temperature while the key anammox proteins for nitrogen metabolism had a lower expression at 15 °C compared to 30ºC.

The aim of the present study was to evaluate the enzymatic activities of the different members of a highly enriched N-damo microbial community in a MBR during stable operation at a nitrite loading rate (NLR) >240 mg TN L^{-1}. d^{-1} with a special focus on metalloenzymes. For this purpose, shotgun metaproteomics were applied to the proteome of "*Ca. Methylomirabilis*" and its accompanying taxa. The corresponding results were related to metal concentrations in the reactor to assess their usefulness for process optimization stimulating the preferring N-damo pathways.

5.2. MATERIAL AND METHODS

5.2.1. Reactor operation

The MBR system operated for about 350 days with a progressive increase in the nitrite loading rate (NLR). The MBR, previously described in Allegue et al.,

(2018), consisted in a 10 L reactor with a working volume of 6.65 L (**Figure 5.1**). Real time measurements for water level, gas volume flow, redox, and membrane performance (TMP) were performed by in-situ sensors and collected by programable PLC Micro 820 (Allen-Bradley) connected to a computer. The hydraulic retention time (HRT) was fixed in 1 day and temperature at 28°C (using a thermostatic water bath).

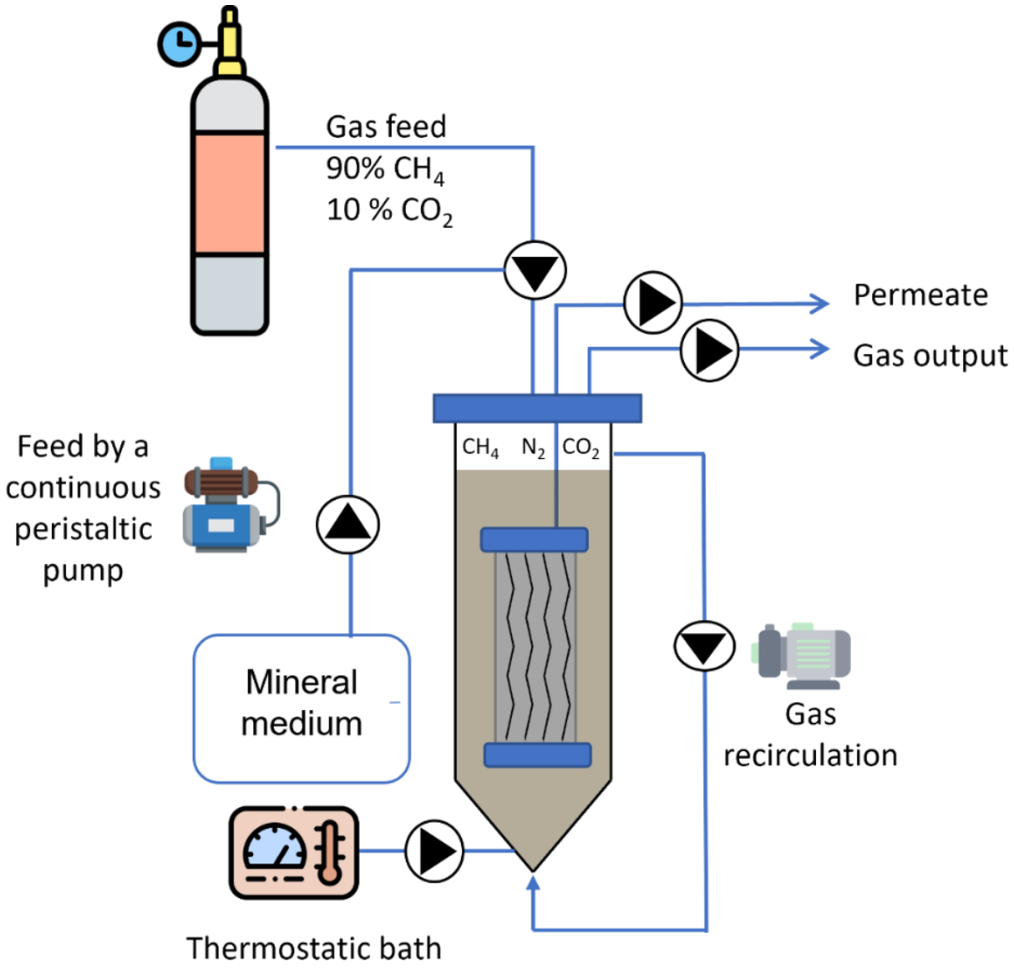


Figure 5.1. Schematic representation of the N-damo enriched MBR reactor.

The MBR was operated under anaerobic conditions. The liquid feed consisted of nitrogen as sodium nitrite in a nitrogen removal rate of 70 mg TNL^{-1} d^{-1} up to 240 mg TN L^{-1} d^{-1}. The feed also included a mix of trace metals and other medium constituents as described in Arias et al., (2022), with the sole difference that Cerium was included. All metals were fed in a continuous concentration throughout the reactor operation. In brief, relevant metals included in the medium were iron, copper, zinc, molybdenum, nickel, and cerium among others. The MBR gas feed consisted of a mixture of 90 % CH_4 to maintain the liquid saturated and 10% CO_2 to buffer the increase of pH due to denitrification.

5.2.2. Reactor performance monitoring

Nitrogen species (nitrite, nitrate and ammonia) were measured following spectrophotometric standard methods (APHA, 2000). Biomass concentration was quantified by means of total suspended solids (TSS) and volatile suspended solids (VSS). The redox potential and pH were measured daily to monitor the adequate functioning of the system. Biogas measurements were performed by gas chromatography using a 1 mL syringe to sample the outflow gas of the system. The gas volume fed to the system was measured using Mili GasCounter MGC-1V3.3 PMMA (Ritter). Total organic carbon (TOC) was measured in the feed and permeate weekly by following standard methods (APHA, 2000) in a Shimazu TOC-L CSN.

Trace metal quantification was done by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). At the end of the reactor operation, when the reactor reached maximum performance, two samples taken in duplicates were extracted from the feed and permeate. Samples were extracted during stable reactor operation at days 300 and 335 for sample 1 and 2, respectively. Samples were then immediately filtered at 0.45 μm and frozen at –20 °C until analysis. The metals selected for analysis were those who were supplemented in the feeding medium: Mn, Fe, Co, Ni, Cu, Zn, Se, Mo, W and Ce.

5.2.3. Proteomic analysis

5.2.3.1. Biomass collection

The two sampling campaigns (Sample 1 and 2) were done during steady state of the MBR and are shown in **Figure 5.2**. First, bioreactor content was nitrogen-bubbled for 10 minutes to suspend biofilms and membrane-attached microorganisms. Afterwards, liquid aliquots containing the suspended biomass were taken separately from the upper and the bottom part of the bioreactor and mixed 1:1 v/v to a final 10 mL volume in duplicate samples. Samples were collected minimizing the oxygen exposure and frozen immediately at 20 °C. This whole procedure was repeated in three different time-points separated for at least three natural days for each sampling campaign. Proteins were extracted from each sample separately. The resulting 6 protein extracts for each sampling campaign were then pooled prior to MS/MS injection.

5.2.3.2. Protein extraction

The protocol for protein extraction was described in (Kennes-Veiga et al., 2022). Briefly, cells were centrifuged at 6000 rpm, washed twice with PBS buffer, digested at 90 ºC, 20 minutes in 1% SDS Tris-HCl extraction buffer and broken by bead beating. Centrifugation at 3000 rpm was subsequently applied to remove cell debris and glass beads. Proteins were then precipitated with acetone at -20ºC and resuspended in molecular grade water. Protein concentration was estimated by the

BCA assay and the protein quality of the samples was checked by SDS-PAGE electrophoresis as detailed in (Kennes-Veiga et al, 2022).

5.2.3.3. nLC-MS/MS analyses

Proteins were identified with a shotgun metaproteomic approach after "in-solution tryptic digestion" of the proteome samples (Zhang et al., 2013).. For this, samples were reduced, alkylated, trypsin-digested, acidified, desalted, vacuum-dried and reconstituted in water with 2% acetonitrile (ACN) and 0.1% formic acid (FA) (Kennes-Veiga et al., 2022). Aliquots of the obtained peptide mixtures (200 ng) were analyzed in a nanoElute (Bruker) nano-flow liquid chromatograph (LC) equipped with a C-18 reversed-phase column coupled to a high-resolution TIMS-QTOF (timsTOF Pro, Bruker) with a CaptiveSpray ion source (Bruker). After ESI ionization, peptides were analyzed in data-dependent mode with the Parallel Accumulation–Serial Fragmentation (PASEF) enabled. These analyses were done at the Proteomics Platform-Proteored-ISCIII from the Biomedicine Research Institute of A Coruña (INIBIC). The mass spectrometry proteomics data was deposited in the ProteomeXchange Consortium via the PRIDE[40] partner repository with the dataset identifier PXD040475 and 10.6019/PXD040475. During the review process, the data can be accessed via a reviewer account (Username: reviewer_pxd040475@ebi.ac.uk; Password: yiKi2ZCV).

5.2.3.4. Protein data analyses

Mass spectrometry raw files were processed with PEAKS Studio 10.6 build 20201221 (Bioinformatics Solutions Inc.) in three different rounds of analyses. First, data were interrogated against a self-constructed database containing all UniProtKB/Swiss-Prot (release 2022_03) protein sequences corresponding to *Methylomirabilis*. It contained a total of 3012 entries belong to the *M. lanthanidiphila*, 3125 to *M. oxyfera* and 2413 to *M limnetica*. There is no protein sequences deposited corresponding to *M. sinica*. The gene callings starting with MELA_ from UniProtKB/Swiss-Prot database correspond to the genomic sequences from *M. lanthanidiphila* functionally annotated as described in Versantvoort et al., (2019) and (2018). In the present study the protein names presented through the text were corrected according to this paper.

As a second approach, we applied a restricted reference database, as described in Blakeley-Ruiz and Kleiner, (2022) including all available protein sequences belonging to the genera *Methylomirabilis, Hyphomicrobium, Denitratisoma, Ignavibacterium, Methylosinus, Rhodoplanes, Gaiella* and *Mehtylocystis* from the NCBI protein public database (September 2022). Those taxa were selected according to previous DNA metabarcoding sequencing of the 16S rRNA gene from the MBR bioreactor (data not shown). Last, we did a specific search against UniProt entries of the N-damo archaea *Methanoperedens* (Sept. 2022). The

webtool Unipept Desktop 3.0. was used for analyses of the peptides identified on the MS/MS analyses.

5.3. RESULTS AND DISCUSSION

5.3.1. Reactor performance

The MBR system highly enriched with N-damo (60 % relative abundance of *Methylomirabilis*, according to 16S rRNA gene sequencing, data not shown), operated for 346 days. The operation was characterized by a robust and stable reactor performance to reach and maintain the different high NLRs. During the MBR operation, the NLR was progressively increased from an initial concentration of 10 mg TN $L^{-1}d^{-1}$ to a maximum value of 269 mg TN $L^{-1}d^{-1}$ on day 199 (**Figure 5.2**). The MBR operation sustained the increasing NLR with an efficient performance, as evidenced by the increase between day 147 and 156, from 88 to 176 mg TN L^{-1} d^{-1}in the TN volumetric removal. Samples 1 and 2 were taken at the end of the operation at a stable NLR of 246 ± 5 mg TN $L^{-1}d^{-1}$ during a period of 35 days in between samples (corresponding to the operation days 300 till 335). The NLR accounted mainly for $N\text{-}NO^{-}_{2}$, which was the limiting substrate. Overall, the average nitrite concentrations in permeates were of 0.9 ± 2.0 mg $N\text{-}NO_2^-$ L^{-1}. Traces of nitrogen as nitrate and ammonium were also detected in the feed, as the medium contained ammonium chloride (Arias et al., 2022) and some nitrite possibly oxidazed to nitrate with the remaining oxygen in the system. Nevertheless, nitrate and ammonium removal rates had an average removal rate of 3 ± 1.4 mg $N\text{-}NO_3^-$ L^{-1} d^{-1} and 1.9 ± 1.1 mg $N\text{-}NH_4^+$ L^{-1} d^{-1}, respectively.

The pH was maintained in neutral range (average 7.8 ±0.3) by modifying the inflow of the gas mixture (90 %CH_4/ 10%CO_2). The average redox potential measurements were - 347.7 ± 135 mV. As methane was fed to the reactor in saturation, relatively stable biogas composition was maintained with values of 83% CH_4, 13% N_2 and less than 0.02% of N_2O. The presence of trace concentrations of N_2O corresponded with temporal perturbances in the MBR performance.

TOC concentrations in the feed and permeate of a system showed an average value of 3.7 ± 1.7 mg TOC L^{-1} and 4.6 ± 4.7 mg TOC L^{-1}, respectively. The slight increase in the permeate TOC concentration was mainly due to four measurements with significative higher TOC concentration in the permeate, on days 0, 105, 206, and 210, which ranged between 11.8 and 16.7 mg TOC L-1. This increase could be attributed to biomass death or by-products of methane oxidation.

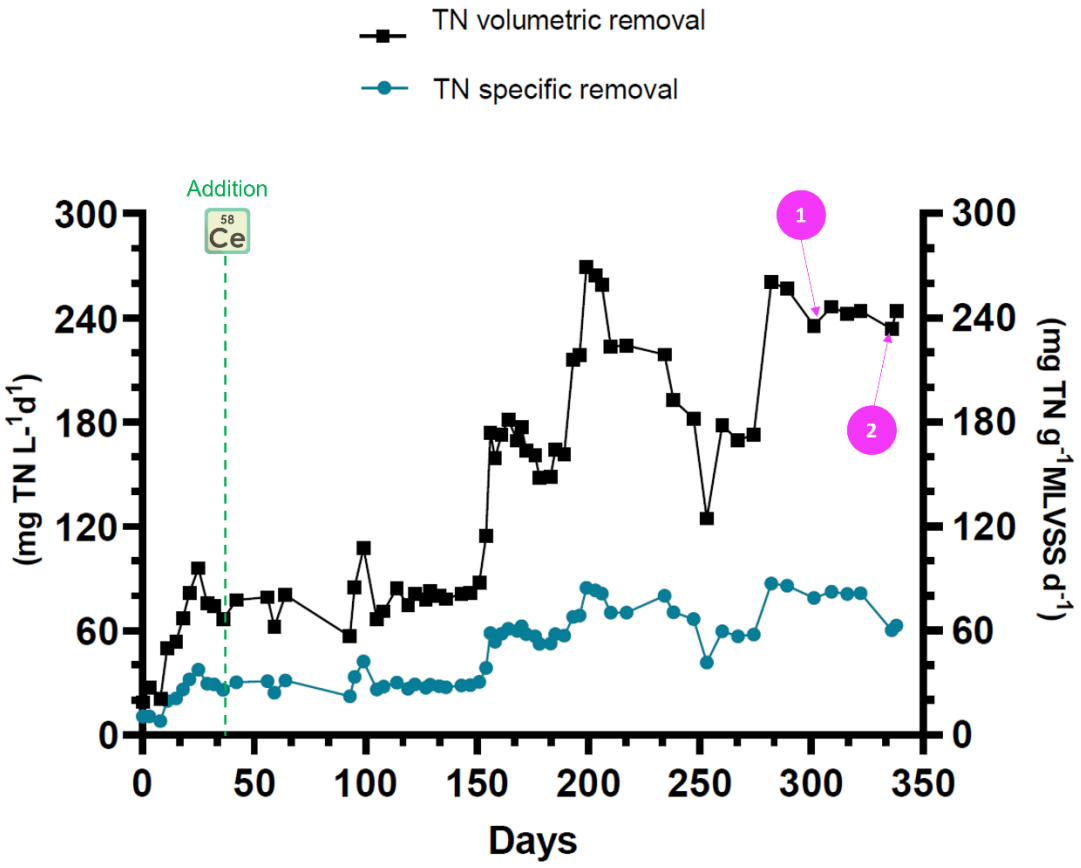


Figure. 5.2. TN in volumetric (by flow fed to the system) and specific (by biomass concentration) removal in the MBR. In circles, 1 and 2 correspond to both sampling campaigns for metal quantification and proteomic analyses. Nitrogen removal was 99–100 % during the period between sampling campaigns 1 and 2.

5.3.2. Metal consumption

The trace metal concentration was maintained in the feed during the whole reactor operation. Cerium was added in the mineral feed on day 33 onwards in a constant concentration of 4.5 μg L^{-1} and it was completely consumed across the operation. The other metals were consumed to different extents (**Figure 5.3**). In the case of Fe, Cu and Zn a significant consumption was observed (>10%) while other metals like Mn, Se, Co, W, Ni and Mo did not show a significative difference (<10%) between the feed and permeate concentrations.

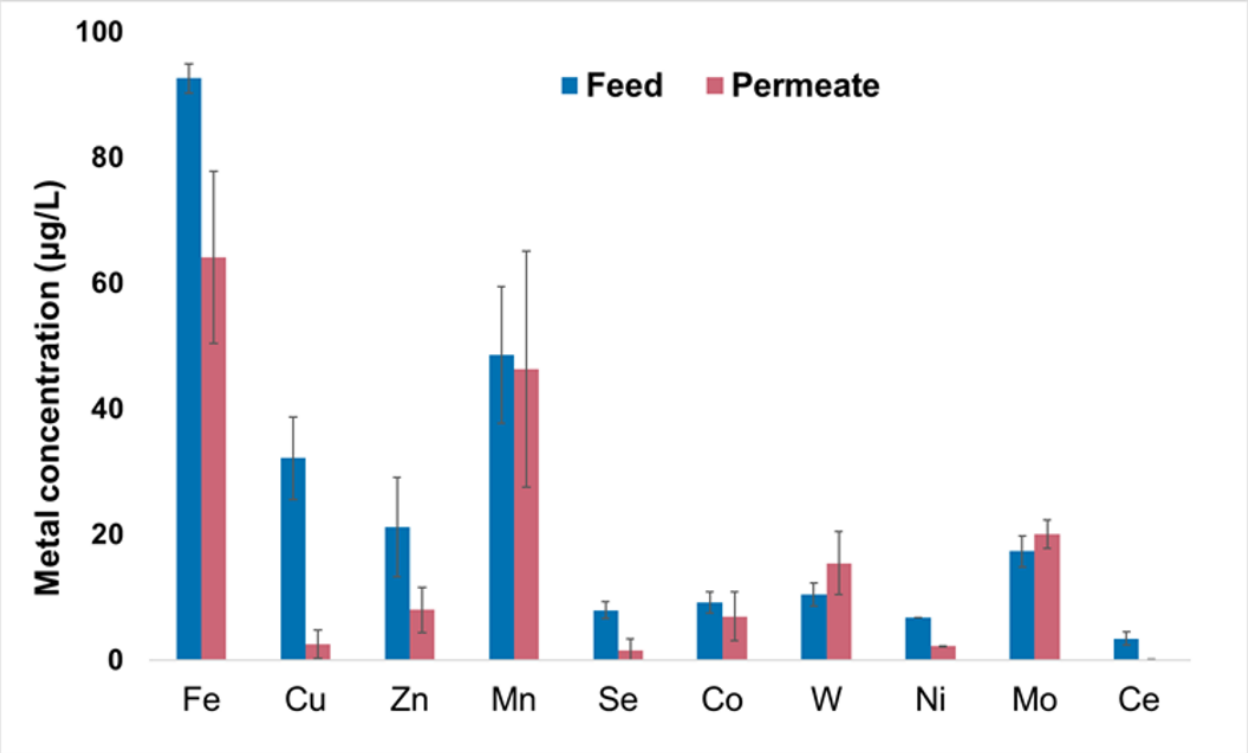


Figure. 5.3. Trace metal concentrations in the feed and permeate of the MBR (n = 3).

5.3.3. Methylomirabilis

5.3.3.1. Strains differential abundance

The six protein extracts corresponding to each sampling campaign on the bioreactor were obtained and pooled prior its nLC-MS/MS injection. A total of 1262 and 1365 proteins belonging to the genus *Methylomirabilis* were identified in PEAKS analysis and 567 and 651 of them with 2 or more unique peptides in samples 1 and 2, respectively. The aminoacid sequences of all peptides detected in this analysis (over 1500 per sample) were cleaned and subsequently analyzed by Unipept 3.0. for treeview visualization of the taxonomies (**Figure. 5.4**). Our proteomic results indicate a strong predominance of *M. lanthanidiphila* over the other two *Methylomirabilis* species at the two sampling campaigns (**Figure 5.4**), which can be attributed to the operational conditions, specially to cerium supplementation, and not the number of sequences from each of them available in the database used in this study.

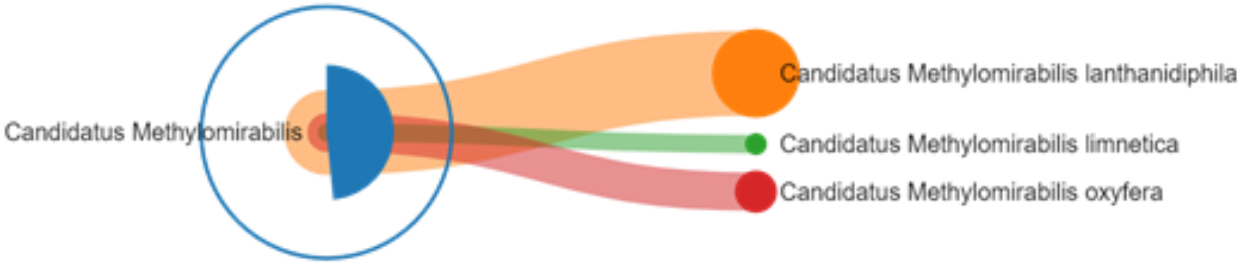


Figure. 5.4. Treeview visualization of the relative abundance of each *Methylomirabilis* strain on the N-damo enriched MBR reactor metaproteome during steady state (on days 300 and 335) at high-nitrogen loading rate (246 ± 5 mg TN L^{-1} d^{-1}).

5.3.3.2. Protein functional categorization

The percentage of peptides belonging to each Enzyme Commission and Gene Onthology (molecular function) category was calculated with Unipept 3.0. Regarding EC classification the most abundantly expressed category is DNA-directed RNA polymerases followed by metalloendopeptidases and RuBisCo, which is the major player on carbon fixation from CO_2 into organic biomass via the Calvin–Benson–Basham pathway. Nitrite reductase and methane monooxygenase activities are found at the 7th and 18th position, respectively. Regarding Gene Ontology, the most abundant categories were ATP binding, metal ion binding and DNA binding. In both categorizations it is clear how the variety and availability of metals in the environment is crucial for bacterial fitness and is reflected at the proteome level. In this sense, it can be seen how the categories metal ion binding, magnesium ion binding, zinc ion binding, heme binding, metalloendopeptidases, iron-sulfur cluster binding, calcium ion binding and molybdenum cofactor binding are among the most abundant ones

5.3.3.3. Methane oxidation

Methylomirabilis first oxidize methane to methanol by the copper-dependent membrane-bound enzyme: particulate methane monooxygenase (pMMO), which consists of three subunits. The PmoA and PmoC cross the membrane while PmoB has two transmembrane helices and a soluble β-barrel motif which forms a hole for substrate entry and product exit (Ross et al., 2019). The three subunits were detected in our bioreactor samples. The *Methylomirabilis* genomes described so far do not encode for the counterpart soluble methane monooxygenases (sMMO), in contrast to most methanotrophs(Versantvoort et al., 2018).

The next step in this route is methanol oxidation to formaldehyde. There are two known groups of methanol dehydrogenases (MDH), the canonical MxaFI type, which carries calcium as essential cofactor and the more recently discovered XoxF-type, which binds lanthanides in the active site (Keltjens et al., 2014). The most

abundantly expressed MDH from *Methylomirabilis* in our bioreactor belongs to the XoXF type and specifically to *M. lanthanidiphila (*MELA_00916). The other one detected belong to the MxaF type and to *M. oxyfera* (D5MI98|D5MI98_9BACT). Interestingly, regarding the sequence analyses described in Versantvoort et al., (2018), the genome of *M. lanthanidiphila* encodes for a single XoxF type MDH while the cluster encoding for the calcium-MDH is absent. In contrast, *M. oxyfera* genome is redundant encoding for canonical MxaFI MDHs and a XoxF type one (Wu et al., 2015). This difference relates with the advantage of *M. lanthanidiphila* over *M. oxyfera* when growing in the presence of cerium. A specific aspartate residue was found to play a crucial role in the rare-earth element interaction with the active site of Xox type MDH (Keltjens et al., 2014). From genomic analyses 'Ca. *M. limnetica'* is also most likely dependent on lanthanides to produce an active MDH, yet in our experiments this strain was detected in a much lower relative abundance than the other two. The reason for this difference remains unknown to us.

Formaldehyde can be oxidized to formate by XoxF-MDH, but *Methylomirabilis* species also encode for two specific systems to oxidize it. In the first one the 5,6,7,8-tetra-hydromethanopterin pathway (H_4MPT) act as the C1 carrier, while in the second, this role is taken by the 5,6,7,8-tetrahydrofolate (H_4F). Formaldehyde activating enzyme, methylene-H4MPT dehydrogenase and methenyl-dH4MPT cyclohydrolase are the three enzymes responsible for the sequential formation of 5-formyl-H_4MPT from formaldehyde in the first described route and were accordingly found expressed. In the H_4F pathway, a methenyl-H_4F-cyclohydrolase and formyl-H4F lyase are the actors and were also found expressed. The subsequent formate oxidation system consisting of FdhA and FdhB. In our study, the FdhA subunit was detected. In FDHs, the cofactor is coordinated to either molybdenum (Mo-bisPGD) or tungsten (W-bisPGD) at which the metal additionally binds either cysteine or selenocysteine (Reimann et al., 2015).

Methylomirabilis are autotrophic, and in contrast to other methanotrophs fix carbon by the Calvin-Benson-Bassham (CBB) cycle instead of the serine pathway or ribulose monophosphate (RuMP) cycle (Ettwig et al., 2010). The main step in this pathway is catalyzed by the RuBisCo enzyme and consists of the carboxylation of ribulose-1,5-bisphosphate. From all RuBisCo types found in nature, the one encoded by *Methylomirabilis* belongs to type I (Versantvoort et al., 2019).

In regards of electron transfer, *M. lanthanidiphila* encodes for multiple cytochrome bc1 complexes, which is not uncommon in nature, as these complexes can be inserted into any type of respiratory chain mediated by quinones. Accordingly, we detected peptides belonging to cytochrome biogenesis proteins in our analyses (i.e: cytochrome c-type biogenesis protein CycK, CcmF and CcmE).

5.3.3.4. Metabolism of nitrogenous compounds

N-damo bacteria reduce nitrite into nitric oxide by a cytochrome cd1 type nitrite reductase (Cd1-NIR), using copper as metal cofactor. The next step is performed by their unique NO dismutase (No-d) and consists of the dismutation of two molecules of nitric oxide into molecular oxygen and nitrogen gas. As detailed in Versantvort, et al, 2019, this reaction might be the limiting step in *Methylomirabilis* metabolism, though it its thermodynamically feasible. No-d are homologous to nitric oxide reductases but present amino acid substitutions in the catalytic sites, quinol binding sites and proton channel that impede NO reduction to N_2O. Additionally, *Methylomirabilis* encode canonical quinol-dependent nitric oxide reductases (NOR), preventing NO accumulation, that was also found expressed. NOR are also electrogenic enzymes, transferring electrons from the ferrous irons for NO conversion coupled to ATP production.

N-damo bacteria encode for a single F_1F_0 ATP synthase to harvest the proton-motive force (pmf) produced by the various respiratory complexes and form ATP. Several ATPase subunits were detected in our shotgun analyses: specifically: α, β, γ, B and C.

In our work, by in solution shotgun analyses we were able to identify all enzymes involved in the N and C cycle from *Methylomirabilis*. The full list of identifications can be found in Supplementary excel file. A schematic representation of the enzymatic pathway for the simultaneous nitrite reduction and oxygen-dependent methane oxidation "*Ca.* Methylomirabilis", the number of peptides identified for each enzyme and their corresponding metal cofactor is shown in **Figure 5.5.**

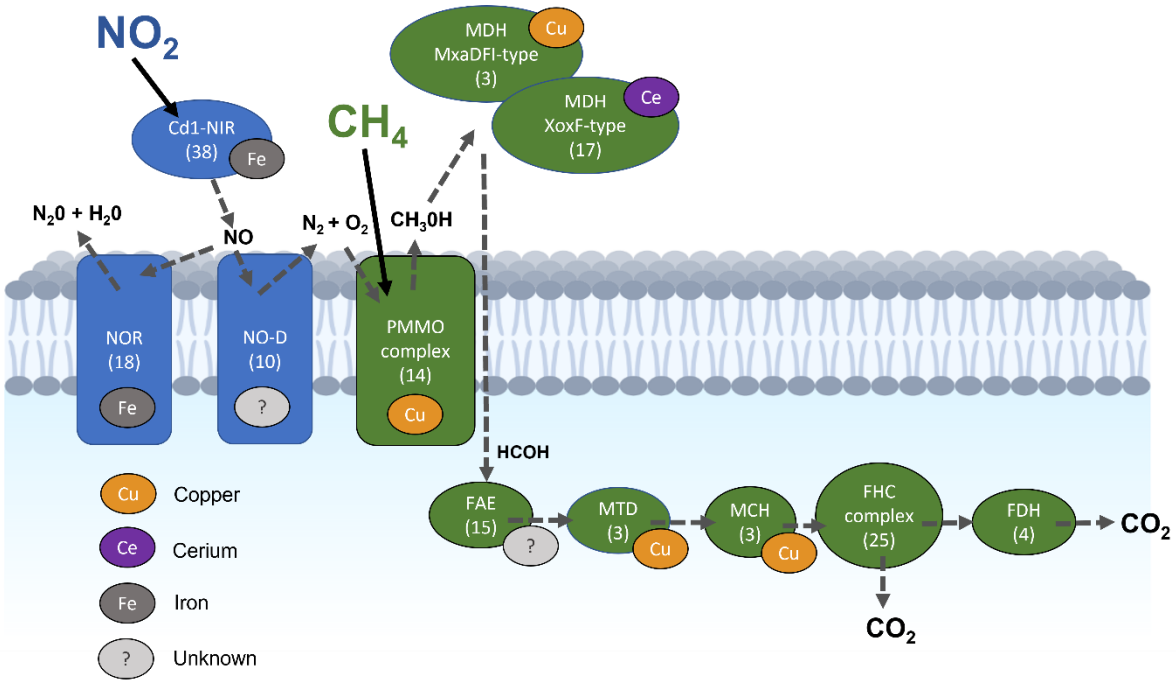


Figure. 5.5. Scheme of the anaerobic metabolism of N-damo bacteria ("*Ca.* Methylomirabilis") showing the enzymatic routes for nitrite reduction and subsequent dismutation of NO into N2 and O2 by their unique NO dismutases. The generated oxygen is used for methane oxidation initiated by their methane monooxygenases. Enzymes from the nitrite reduction route are represented in blue colour while those from the methane activation route are represented in green. For each enzyme we show the number of total peptides (in brackets) detected in this study and their corresponding metal cofactor (in circles). Adapted from the model described in (Versantvoort et al., 2019).

5.3.3.5. Other physiological features from Methylomirabilis

Metaproteomics allowed us to detect other aspects from *Methylomirabilis* lifestyle not directly linked with the removal of target pollutants, but with its general physiology. Regarding oxidative stress defense, three iron-containing superoxide dismutases (MELA_02229 from *M. lanthanidiphila* and DAMO_0395 from *M. oxyfera* and CLG94_07065 from *M. limnetica)* were expressed. We speculate they serve as protection mechanism in case of transitional periods of oxidative stress if intracellular oxygen from Nod activity is not consumed, e.g., in methane or copper deficit. Methane-utilizing bacteria typically flourish in nature at the aerobic-anaerobic interface, as they require the oxygen for the methane activation, so from a biological point of view they should have a defence against oxidative stress. Interestingly, the expression of the *M. oxyfera* superoxide dismutase (DAMO_0395) was also detected in Hanson and Madsen, (2015), in environmental subsurface waters. Superoxide dismutases have also been shown to have a role in biofilm formation and bacterial morphology in other anaerobes (Najmuldeen et al., 2019).

Twitching motility plays a key role in bacterial biofilm formation and was confirmed by the detection of the twitching motility protein (PilT) and Type IV pilus assembly protein (TapB) (Cursino et al., 2009).. Interestingly, we detected the cell shape-determining protein MreB, which was often associated to non-spherical shape bacteria in previous literature (White and Gober, 2012). However, mela_0085 which was found in Gambelli et al., (2021), as the main S-layer protein from *M. lanthanidiphila* and involved in their cell shape was not detected in our study.

Considering metal intake and its transport, the following proteins were also expressed: TonB-denpendent receptor, TonB-dependent outer membrane receptor for cobalamin and iron transport, iron-sulfur cluster carrier protein, putative heavy metal efflux pump CzcB family, molybdopterin molybdenum transferase, magnesium, and cobalt efflux protein CorC, putative molybdopterin biosynthesis protein moeB (modular protein) and molybdenum cofactor biosynthesis protein B.

5.3.4. Activities of the other members of the microbial community

To investigate the individual proteins related to metabolic functions active on the predominant bacterial genera present in the MBR we performed a second metaproteomic analysis using a restricted reference database, based on previous 16S rRNA sequencing analyses. House-keeping related proteins (e.g., ribosomal related proteins, chaperons and chaperonins or elongation factors) were therefore not considered here.

The high relative abundance of cytochrome cd1-type nitrite reductases identified from *Denitratisoma* indicate how members of this genus were playing the role of denitrifiers in our bioreactor and probably competing with *Methylomirabilis* for this main substrate. In previous studies this genus was also found to transform nitrite in simultaneous anammox denitrification (SAD) bioreactors and to be an endogenous denitrifier, specifically degrading the decaying bacteria usually existing in annamox enrichments (Ma et al., 2017; Nie et al., 2019; D. Wang et al., 2019).

Ignavibacterium was involved in denitrification, as we detected the Sec-dependent nitrous oxide reductase catalyzing the final step of this route, the reduction of nitrous oxide to dinitrogen and activating the acetate by the acetate CoA ligase. Even though members of *Ignavibacterium (i.e: I. album)* harbor genes encoding respiratory nitrite reductases and nitric oxide reductases (Liu et al., 2012), those were not detected.

As expected, both *Methylosinus* and *Methylocystis* were acting as methanotrophs in regards of their abundantly expressed methane monooxygenases pMMO and periplasmic pyrroloquinoline quinone (PQQ)-dependent, methanol dehydrogenases (calcium-containing). Both genera belong to the Alphaproteobacteria (previously known as Type II methanotrophs) in which the pMMO is housed on the intracytoplasmic membranes (Kalyuzhnaya et al., 2019).

Last, methylotrophy is the main activity detected for *Hyphomicrobium*, regarding its expression of (PQQ)-dependent methanol dehydrogenases, 5-6-7-8-tetrahydromethanopterin-hydrolyase and formaldehyde-activating enzyme (FAD). *Rhodoplanes* role in the community could not be clarified as only chaperonins, elongation factors and adenosyl homocysteinase were detected.

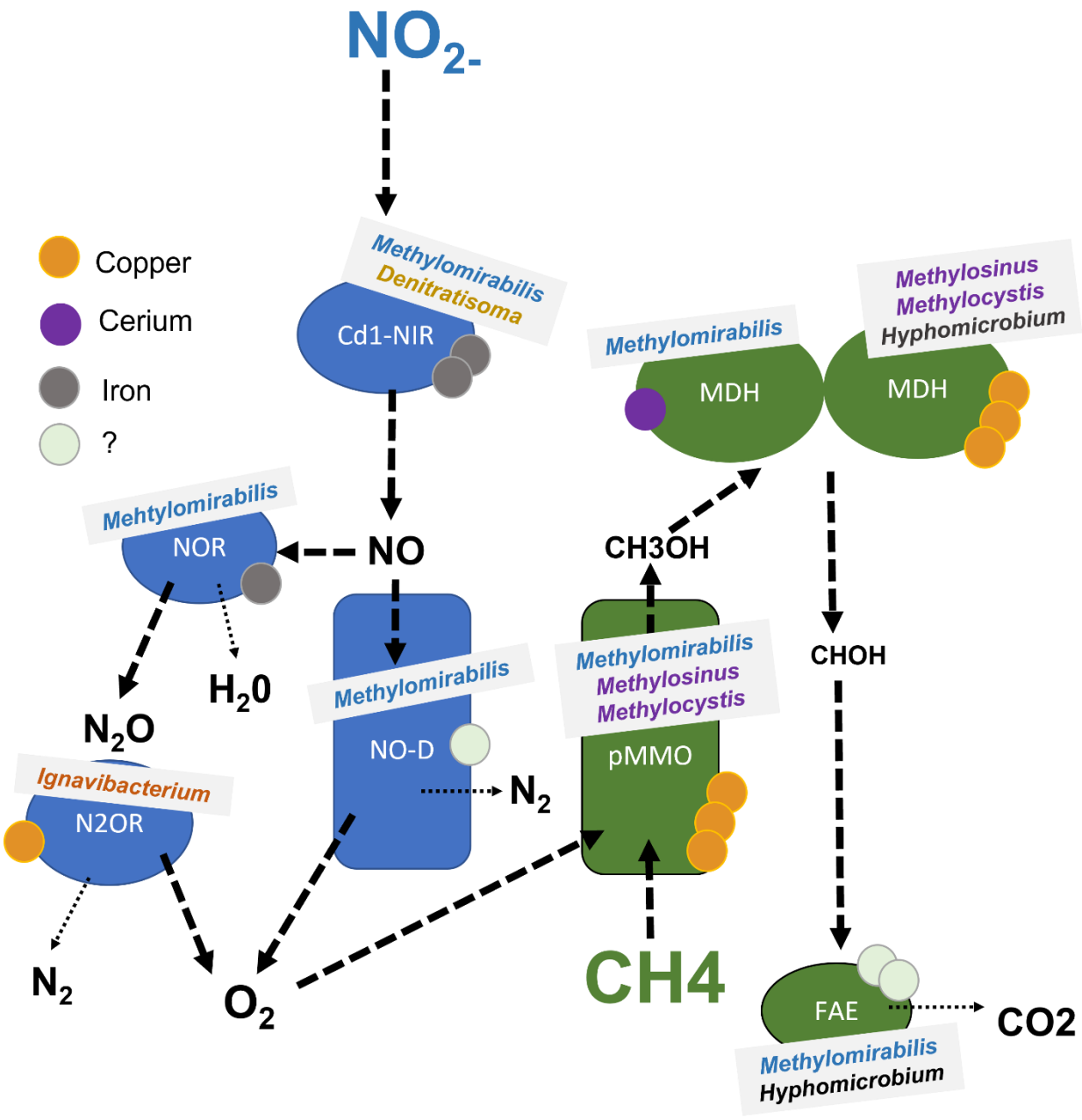


Figure. 5.6. Metalloenzymes from the members of the N-damo enriched MBR community involved in the nitrogen and methane cycle according to metaproteomic analyses.

5.3.5. Nitrate consumption

The trace amounts of nitrate detected in the influent were consumed along the operation of the bioreactor resulting in a removal rate of 3±1.4 mg $N\text{-}NO_3^-$ L^{-1} d^{-1}. N-damo archaea (Ca. *Methanoperedens*) have been previously shown to reduce nitrate to nitrite (Haroon et al., 2013). To investigate its presence and potential nitrate consumption we specifically searched for its proteins in a third round of analyses. Proteins with at least 2 total peptides detected were considered, although the sequence coverage was very low (below 10%), regarding the interest on N-damo archaea for this study. The low number of identifications (4) manifests the low abundance of *Methanoperedens* in the community. The most abundantly detected protein was an ATP dependent chaperone (ClpB) followed by the nitrogen regulatory protein P-II (one of the most widely distributed families of signal transductors in nature) and a DNA-directed RNA polymerase subunit. ClpB is a disaggregase known to inhibit and reverse protein aggregation during stress survival (upon reactive oxidative species, pH and osmolarity changes) but also during normal physiological conditions (Alam et al., 2021).

Interestingly, the nitrate reductases identified in this study belong to *Methylomirabilis* and not to *Methanoperedens, Hyphomicrobium or Ignavibacterium* (also harboring nitrate reductases on their genomes). These were specifically the nitrate reductase catalytic subunit, Nar (identified with 9 peptides), periplasmic nitrate reductase subunit alpha, NapA (identified with 5 peptides) and nitrate reductase subunit beta, NapB (identified with 2 peptides). So far it couldn't be predicted from sequence analyses neither by biochemical evidence whether the nitrate reductases from *Methylomirabilis* function as nitrite-oxidizing or nitrate-reducing protein complexes. Previous enrichment cultures containing *Methylomirabilis* only fed with nitrite (no nitrate) were found to be expressing the three systems to reduce nitrate to nitrite that its genome harbor (Versantvoort et al., 2019).

5.4. CONCLUSIONS

Iron and copper were among the most consumed metals, as they are cofactors for the enzymes: nitrite reductases, nitric oxide reductases, MDH and pMMO. The predominance of "Ca. *Methylomirabilis lanthanidiphila*" over other "Ca. *Methylomirabilis*" strains is attributed to the addition of cerium inducing the expression of its Xox-F-type MDH with affinity with lanthanides. To promote N-damo activity, the metal composition in the feed could be enhanced by increasing concentrations of cerium and copper, as these metals were almost fully consumed. This study exemplifies the usefulness of metaproteomics to help on decision making towards promoting preferred microbial activities during biotechnological processes.

CHAPTER 6

Physico-Chemical Drivers of Antibiotic Fate and Transformation Pathways

Quiton-Tapia, Silvana; Suarez, Sonia; Omil, Francisco; Schäffer, Andreas. Physico-Chemical Drivers of Antibiotic Fate and Transformation Pathways. *In preparation.*

Abstract

The increasing presence of antimicrobial micropollutants in wastewater poses significant environmental risks, including the development of antibiotic resistance and disruption of aquatic ecosystems. Despite their prevalence, the specific behaviors and removal mechanisms of these compounds during denitrification processes remain inadequately understood, particularly regarding the influence of their physico-chemical properties and production of transformation metabolites. This study addresses these gaps by investigating the fate and removal mechanisms of two antimicrobials, sulfamethoxazole (^{14}C-SMX) and triclocarban (^{14}C-TCC), during heterotrophic denitrification using ^{14}C-labeled techniques in fed-batch experiments. Results indicate that chemical structure and physico-chemical properties critically determine pollutant behavior. ^{14}C-TCC, due to its hydrophobicity, exhibited strong sorption to sludge (>59% after 14 days) but showed no significant mineralization. Conversely, ^{14}C-SMX, highly polar and predominantly anionic (>98% at pH >7.5), demonstrated minimal sorption (~2%) and rapid biotransformation of the parent compound (~80% within one day). However, mineralization was limited as SMX was converted into more polar and resistant metabolites that accumulated in the aqueous phase. These findings highlight the importance of considering both parent compounds and their transformation products when evaluating treatment efficiency as well as including important factors such as hydrophobicity, polarity, and bioavailability, to optimize treatment processes and minimize the persistence of harmful micropollutants in the water cycle.

6.1. INTRODUCTION

Antibiotics have become emerging environmental contaminants because of their extensive use, persistence, and their role in promoting the spread of antibiotic resistance, particularly in natural water systems (Carvalho and Santos, 2016). Once in the environment, these chemicals are subject to complex biotic and abiotic transformations, which influence their persistence, bioavailability, and ecological impact (Fenner et al., 2013). The hydrophilic nature of many antibiotics and their ability to ionize into charged forms including cations, zwitterions, and anions further complicates their environmental behavior and fate (Harrower et al., 2021). These ionized states are critical in determining their mobility, interactions with environmental matrices, and bioavailability, thereby shaping their persistence and removal in both natural and engineered systems (Baalbaki et al., 2017).

Bioavailability, a critical determinant of biodegradability, often acts as a limiting step in the complete removal of antibiotics from environmental systems (Ortega-Calvo et al., 2020). Compounds that become inaccessible to microorganisms, due to entrapment within soil pores, sequestration in voids, or chemical binding, cannot undergo biodegradation. Such entrapped and bound residues are collectively classified as non-extractable residues (NER). These residues, which may include parent compounds or their transformation products (TPs), play a significant role in the long-term environmental persistence of antibiotics (Schäffer et al., 2018). So, NER fractions are non-bioaccessible and non-bioavailable for microbial biodegradation. Their quantification requires the use of isotope labeled compounds such as ^{14}C and ^{13}C, which provide precise tools for tracing and characterizing these fractions (Kästner et al., 2014; Schäffer et al., 2018).

Biological wastewater treatment systems represent a primary barrier to antibiotic dissemination into the environment, with biodegradation and sorption being the main removal pathways (Rios-Miguel et al., 2023). However, these removal mechanisms differ significantly under different redox conditions (i.e. aerobic, anoxic and anaerobic) which alter the efficiency of antibiotic biotransformation and sorption (Oberoi et al., 2019). Even for antibiotics deemed biodegradable, such as sulfonamides, removal efficiencies in full-scale activated sludge systems often range from 50% to 90%, with incomplete removal of parent compounds frequently observed (Göbel et al., 2007a). For instance, despite their inherent polarity, antibiotics such as sulfamethoxazole (SMX) can undergo sorption in significant amounts. SMX has been detected in biological sludge at concentrations up to 17 μg/kg dry weight, evidencing high sorption potential (Li et al., 2013). Charge-mediated interactions explain sulfonamides fate, as they can cationic, neutral, or anionic depending on the environmental pH (Zhao et al., 2018). On the other hand, Triclocarban (TCC),an halogenated antibacterial, has a low solubility and a high octanol-water coefficient and consequently it mainly partitions onto

solids during conventional wastewater treatment processes(WWTP) (Lozano et al., 2013). It has been reported that about 13.1 ± 0.9 μg d^{-1} dry weight end up in the primary sludge from WWTP. Additionally, it was found that only a small fraction of TCC (~18%) was biodegraded during a nitrification-denitrification process and that the majority of the TCC (~79%) was transferred to the solid during wastewater treatment (Lozano et al., 2013). Several authors have suggested that sorption affinity of TCC limits its biodegradation (Al-Rajab et al., 2009; Snyder et al., 2010; Ying et al., 2007).

Despite advancements in understanding antibiotic removal processes, significant knowledge gaps remain concerning relative contributions of biotransformation, sorption, and mineralization, particularly under different redox conditions and very specially anoxic environments. Additionally, the fate and potential impacts of transformation intermediates remain poorly understood (Oberoi et al., 2019). For instance, research using ^{14}C-SMX under denitrifying conditions highlighted the formation of metabolites within 190 hours of incubation, accounting for 5.9% of the total radioactivity (Alvarino et al., 2016). In contrast, studies involving ^{14}C-TCC revealed low mineralization rates, with yields never exceeding 4% of the applied radioactivity of ^{14}C-TCC when evaluating its fate on wastewater biosolids (Al-Rajab et al., 2009; Snyder et al., 2010). In addition, ^{14}C-TCC readily formed NER reaching a maximum of 15% of the applied radioactivity after 49 days of incubation (Al-Rajab et al., 2009). These studies indicated that the bioavailability of ^{14}C-TCC is a limiting factor in its biodegradation.

To address the aforementioned gaps, the main aim of this study is to elucidate the fate and removal mechanisms of two micropollutants with antimicrobial properties, sulfamethoxazole (^{14}C- SMX) and triclocarban (^{14}C- TCC), during heterotrophic denitrification process. This chapter investigates the degradation pathways and transformation metabolites of these micropollutants to understand their biotransformation and ultimate fate within a conventional denitrification process.To achieve this, two set of batch experiments were performed with each antibiotic, and complete mass balances were calculated by using ^{14}C -labelled techniques. Our purpose was to investigate the main removal mechanisms of antibiotics with different molecular structures under fed-batch anoxic conditions.

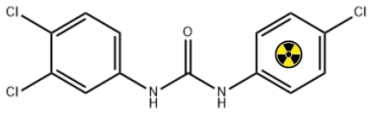
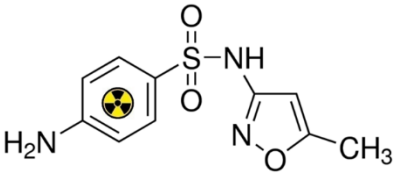
6.2. MATERIALS AND METHODS

6.2.1. Labelled compounds

Labelled Sulfamethoxazole [phenyl ring-U–^{14}C] and Triclocarban [chlorophenyl ring-^{14}C(U)] were chosen for our study based on their different physicochemical characteristics (**Table 6.1**). The compounds were spiked into fed-batch bottles at day 0, each with a desired starting concentration of 30 μgL^{-1}. The

applied radioactivity levels were 5.11x10^6 dpm L^{-1} for TCC and 5.4x10^6 dpm L^{-1} for SMX.

Table 6.1. Physicochemical properties for selected antibiotics

	LABELLED COMPOUNDS	
Physicochemical characteristics	**Triclocarban (TCC)**	**Sulfamethoxazole (SMX)**
Location of the ^{14}C	Chlorophenyl ring-^{14}C(U)	Phenyl ring-U-^{14}C
Molecular structure		
Specific activity	30 mCi/mmol	20.19 mCi/mmol
Chemical formula	$C_{13}H_9Cl_3N_2O$	$C_{10}H_{11}N_3O_3S$
Molecular weight ^{14}C	316.56	254.04
Radiochemical Purity	96.2%	99.3%
Log Kow	4.3-4.9	0.5-0.9
Kd (Lkg^{-1})	25704	104-162
pKa	12.7	pKa$_1$ 1.85(cation) pKa$_2$ 5.70 (anion)
Log P	5.7	0.79
Water solubility (mg/mL) at 25^{0}C	0.0029	0.459
LD50 in mice (mg/kg)	2100	2300

Data from Drugbank, Pubchem and Chemspider and (Nguyen et al., 2018; Yun et al., 2020)

6.2.2. Inoculum extraction and acclimation to denitrifying conditions

Fresh biological sludge was collected from a conventional activated sludge reactor at a wastewater treatment plant (WWTP) in Aachen, Germany, and used as

inoculum. This inoculum was acclimated to denitrifying conditions over 16 days in a Continuous Stirred Tank Reactor (CSTR) fed with medium for denitrification as described in Chapter 3. The CSTR, with a working volume of 2 L and connected to a 1 L settler, was operated to have an Hydraulic Retention Time (HRT) of 1 d with peristatic pumps. The CSTR was operated at a constant nitrate loading rate of 50 mg $NO^{-}_{3}L^{-1}day^{-1}$, with a mix of sodium acetate and acetic acid as carbon sources in a ratio COD/N 6:1. A recirculation system was established from the settler back to the main compartment, with recycle ratio of 10. Biomass concentration was maintained at 2.5 g $MVSSL^{-1}$ (Mixed Volatile Suspended Solids). The performance of the CSTR was monitored twice a week by measuring nitrate and COD levels in the influent and effluent to ensure efficient nitrate removal and adequate COD availability.

6.2.3. Biodegradation tests

Two set of batch experiments were conducted to evaluate the degradation and fate of TCC and SMX during a 14-day incubation under denitrifying conditions. Each set included duplicate biotic bottles with hot compound (^{14}C-SMX and ^{14}C-TCC) spiked at 30 µg L^{-1} as starting concentration. For control, abiotic bottles in each sets were prepared using a inoculum that had been sterilized inoculum by autoclaving twice.

The inoculum of these experiments was sourced from the denitrifying CSTR, during stable operation. For each bottle, 50 mL of moving liquor from the denitrifying CSTRs was rinsed three times with phosphate buffer to remove all nitrogen present, then centrifuged. The resulting sludge was added to each batch bottle to achieve a biomass concentration of 1 $gMVSS^{-1}$. The bottles were filled with 125 mL of mineral medium, serving as synthetic wastewater (see **Figure 6.1**).

To monitor mineralization, CO_2 traps were installed in the flasks, consisting of soda lime pellets and paraffin-coated glass wool to absorb any produced $^{14}CO_2$ and organic volatile, respectively. The bottles were incubated at 20^0C and shaken at 120 RMP in the dark, and sampled destructively in duplicates on days 0, 1, 3, 4, 7, 9, 11, and 14 following the procedure outlined in section 6.1.4. After each sampling, the remaining bottles were supplied again with nitrate (50 mg NO_3^- L^{-1} day^{-1}) and a mixture of sodium acetate and acetic acid (COD/N ratio of 6:1) to prevent substrate depletion. When necessary, samples were acidified with HCl to maintain neutral pH (7-8.5).

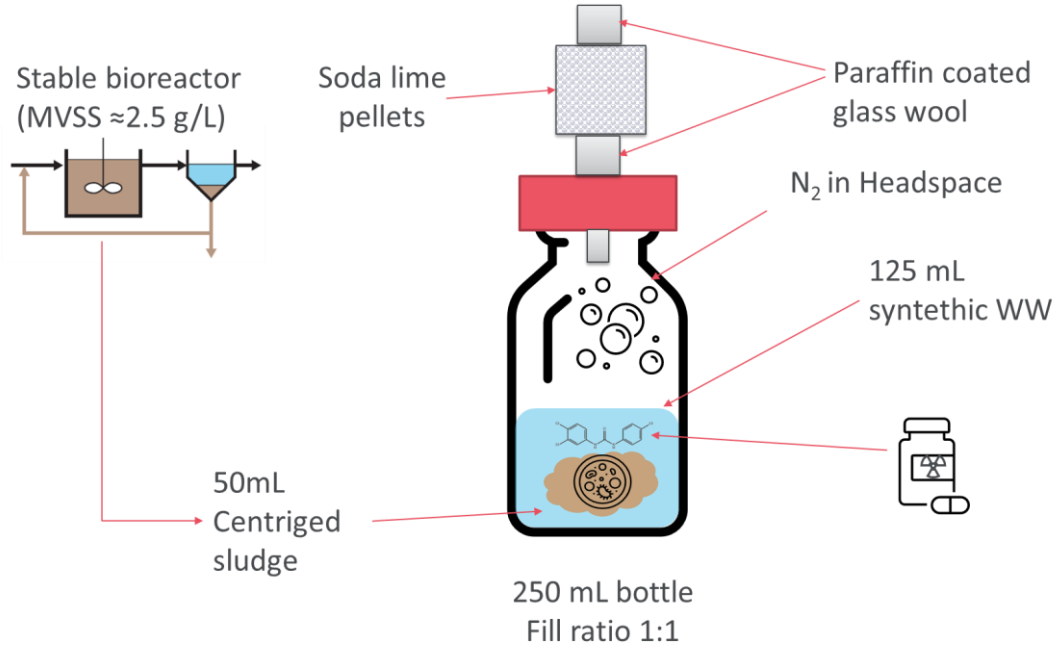


Figure 6.1. Experimental design and batch bottle composition

6.2.4. Sampling processing and extraction procedure

To ensure a comprehensive mass balance to determine the fate of selected antibiotics, radioactivity was measured across four different fractions: the water phase which would constitute the available fraction, in the solid phase, the extractable fraction which incorporates and extraction with $CaCl_2$ and three consecutive extractions with methanol (MeOH) as well as the non-extractable residues (NER), in the gaseous phase, the mineralization (**Figure 6.2**). This approach ensured a comprehensive mass balance to track the fate of antibiotics during biotransformation. The total ^{14}C was determined by summing the radioactivity from the water phase, extractable fractions, NER combustion and soda lime. The radioactivity recovery was calculated to set up a complete mass balance.

6.2.4.1. Mineralization

To quantify mineralization, soda lime pellets of each bottle were dissolved in 25% HCl (VWR, 37%), releasing $^{14}CO_2$. The released gas was channeled through a gentle N_2 stream into 20 mL polyethylene vials containing an alkaline liquid scintillation cocktail (Oxysolve C-400, Zinsser Analytic, Frankfurt a.M., Germany). The ^{14}C content was measured using a liquid scintillation counter (LSC Hidex 300/600 SL, Finland). Additionally, glass wool used to capture residual $^{14}CO_2$ was immersed in n-hexane, and aliquots were analyzed by LSC on the same day to prevent solvent evaporation. Glass wool was measured but results showed no ^{14}C captured in this material, indicating as compounds were not volatile and any mineralized gas was captured by the soda limes.

6.2.4.2. Available fraction (water phase)

The water phase from the experimental bottles was filtered twice through 0.45 µm cellulose filters to separate particulates and colloids. Triplicate aliquots of the filtered water were analyzed by LSC. The filtered water was then lyophilized and subsequently resuspended in 1.5 mL of methanol and stored at -20°C until radio-HPLC analysis.

6.2.4.3. Extractable fraction

To determine the extractable fraction, sludge retained on the filters underwent a series of sequential extractions using two different solvents. The process began with an extraction using 0.01 M $CaCl_2$ solution, applying approximately 80 mL of the solution to the sludge. Following the initial extraction, three successive methanol (MeOH) extractions were conducted with an approximate total volume of 200 mL. All extractions were carried out on an orbital shaker operating at 170 RPM for a duration of 15 minutes to facilitate efficient solute transfer from the solid phase to the liquid phase.

After each extraction, three aliquots were taken to measure the radioactivity with LSC. Methanol extracts were then subjected to rotary evaporation at 35 mbar and a water bath temperature of 40°C to remove the solvent. The $CaCl_2$ extracts were lyophilized similar to the water phase. Both concentrated extracts were reconstituted in 1.5 mL of methanol and stored in HPLC vials under -20°C until radio-HPLC analysis.

6.2.4.4. Non-extractable residues (NER)

The non-extractable residues (NER) were quantified by combusting the filter papers containing sludge that had previously undergone sequential extractions with $CaCl_2$ and MeOH. This combustion process was conducted using a biological oxidizer OX-501 (Zinsser Analytic, Frankfurt a.M., Germany). During combustion, the resulting $^{14}CO_2$ was captured in an alkaline LSC cocktail (Oxysolve-C 400, Zinsser Analytic) within LSC vials (20 mL, polyethylene, PerkinElmer). To ensure accurate quantification of the ^{14}C content, cellulose combusted in the oxidizer as a blank control. The ^{14}C content was then measured using the Hidex 300/600 SL liquid scintillation counter.

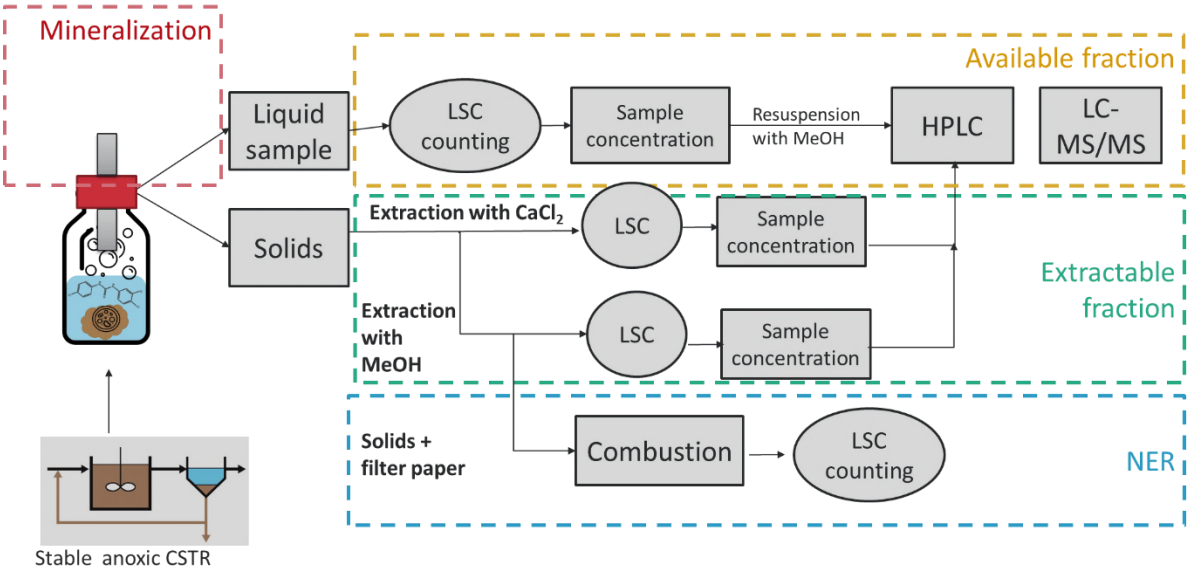


Figure 6.2. Sample processing and extraction procedure for batch bottle

6.2.5. Radioactive quantification

The radioactivity present in each fraction of the extraction procedure (including water phase, sequential extractions, the soda lime and glass wool) was determined using a Liquid Scintillation Counter (LSC). For this analysis, 1 mL aliquots from each fraction were mixed with a liquid scintillation cocktail (Perkin Elmer). Further analysis of the methanol-resuspended fractions from the liquid phase was conducted using high-performance liquid chromatography (HPLC). This was performed on an Agilent 1260 Infinity II system using a reversed phase column Zorbax Eclipse XDB-C18 (4.6 x 150 mm) coupled to a radioactive detector (Raytest Ramona liquid cell detector, Elysia, Belgium). The mobile phases consisted of two eluents, MiliQ water acidified with 0.1% formic acid as eluent A and methanol with 0.1% formic acid as eluent B . There was a gradient change in ratio of eluent A and B with a flow rate of 0.9 mL min^{-1}.

6.2.6. Determination of half-lives (DT_{50})

The half-life (DT_{50}) of the parent compound SMX was calculated based on the radioactivity data obtained from the liquid scintillation counting (LSC) and radio-high-performance liquid chromatography (radio-HPLC) analyses. These calculations were performed using the decline of applied radioactivity, expressed in percentage, from the water phase fractions measured with the HPLC. For these calculations, the software CAKE (Computer Assisted Kinetic Evaluation, Tessella, Version 3.7) was used. The selection of the model used to determine the DT_{50} was selected by the quality of the fit, as recommended by FOCUS, (2006). Mineralized and volatile fractions were excluded from this analysis because they were not expected to contain the parent substances. Non-extractable fractions may contain small amounts of the parent substance(Hennecke et al., 2023); but since no further

analysis was performed to characterize these residues, the fraction was also excluded to calculate half-lives .

6.3. RESULTS AND DISCUSSION

6.3.1. Mass balances

The total recovery of applied radioactivity for ^{14}C-SMX ranged between 93 to 106% while the recovery for ^{14}C-TCC was above 91%, except for the last sample which showed a 79% recovery. Losses of applied radioactivity of TCC might result by the hydrophobic nature of the molecule, which can complicate the pooling of its entire concentration. Nevertheless, the total recoveries for both compounds fall within the optimal range of 90% to 110% as indicated by the (OECD Guideline for Testing Chemicals 301.) for conducting degradation studies with radiolabeled compounds.

The distribution of the applied radioactivity was different for ^{14}C-TCC and ^{14}C-SMX highlighting different environmental fates (**Figure 6.3**). In the case of ^{14}C-TCC, the majority of the compound was present in the MeOH extractable fraction (71 ± 8%) and the water phase only represented 18 ± 4%. This result is in line with previous studies that showed the high affinity of TCC for sorption (Lozano et al., 2013; Wang et al., 2020). Conversely, ^{14}C-SMX predominantly remained in the water phase (87 ± 3%) aligning with its polar nature and high occurrence in aquatic environments (Harrower et al., 2021).

The applied radioactivity in the water phase remained relatively stable throughout the extraction process in both sets of experiments for ^{14}C-SMX and ^{14}C-TCC. This stability is attributed to the buffering capacity of the solid fractions, which regulate the release of contaminants into the water phase (available fraction). The biofilms and solid fractions act as reservoirs, gradually releasing the pollutants as the available fraction is consumed. This phenomenon is driven by the chemical activity gradients between the solid and aqueous phases. For instance, as the concentration of dissolved contaminant molecules decreases in the available fraction, contaminants partitioned onto the solid fractions are spontaneously released to re-establish equilibrium. This diffusive uptake continues until a state of partitioning equilibrium is achieved (Smith, 2020; Smith et al., 2012) .

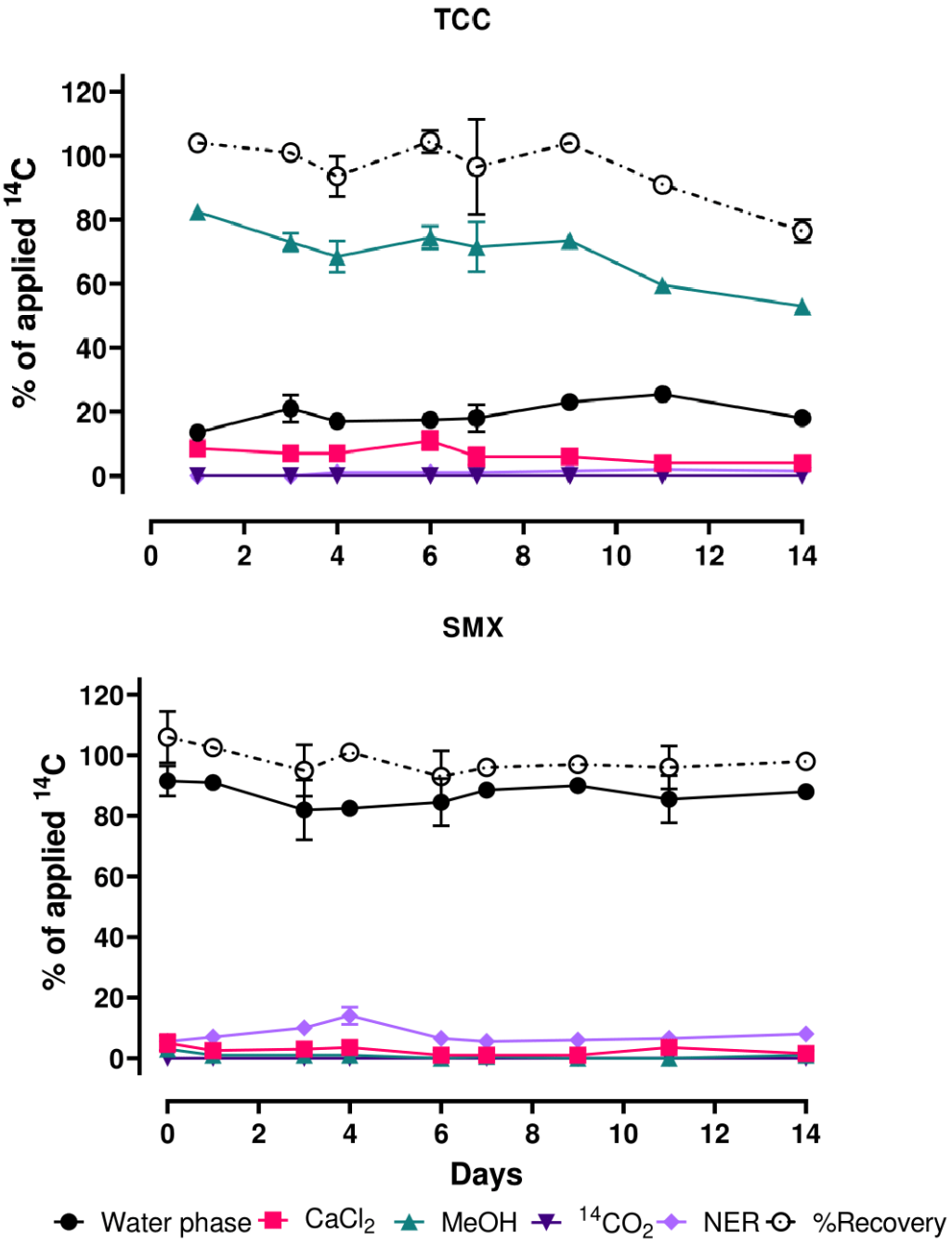


Figure 6.3. Distribution of applied radioactivity of TCC (above) and SMX (below) in the water phase, $CaCl_2$ and MeOH extractable residues, NER and mineralization in the biodegradation tests during 14 incubation tests.

6.3.2. Mineralization of ^{14}C-TCC and ^{14}C-SMX

No mineralization of ^{14}C-TCC was observed during the first 6 days of incubation, suggesting a lag-phase in microbial activity under denitrifying conditions (**Figure 6.4**). Mineralization increased only after day 8 and continued with a minimal

upward trend until the end of the experiment. However, in our study, mineralization of TCC was minimal as the maximum $^{14}CO_2$ captured on day 14 only accounted for 0.02% of the applied radioactivity. The slow mineralization rate may indicate an inhibitory effect of TCC on microbial processes, as previous studies have demonstrated that exposure of traces of TCC (within the 0 to 100 µg L^{-1} range) inhibited denitrification in sequencing batch reactor systems by reducing nitrogen removal rates (Wang et al., 2020). Our findings align with the slow TCC biodegradation kinetics previously reported that indicated half-life values exceeding 165 days in agricultural soils when applied at environmental relevant concentrations (50 ng L^{-1}) (Thelusmond et al., 2018). Furthermore, fate modelling studies, predicted TCC's half-life of 108 days (Ying et al., 2007). Authors have suggested that TCC biodegradation is limited by its molecular sorption affinity. These findings collectively emphasize TCC's environmental stability and its persistence.

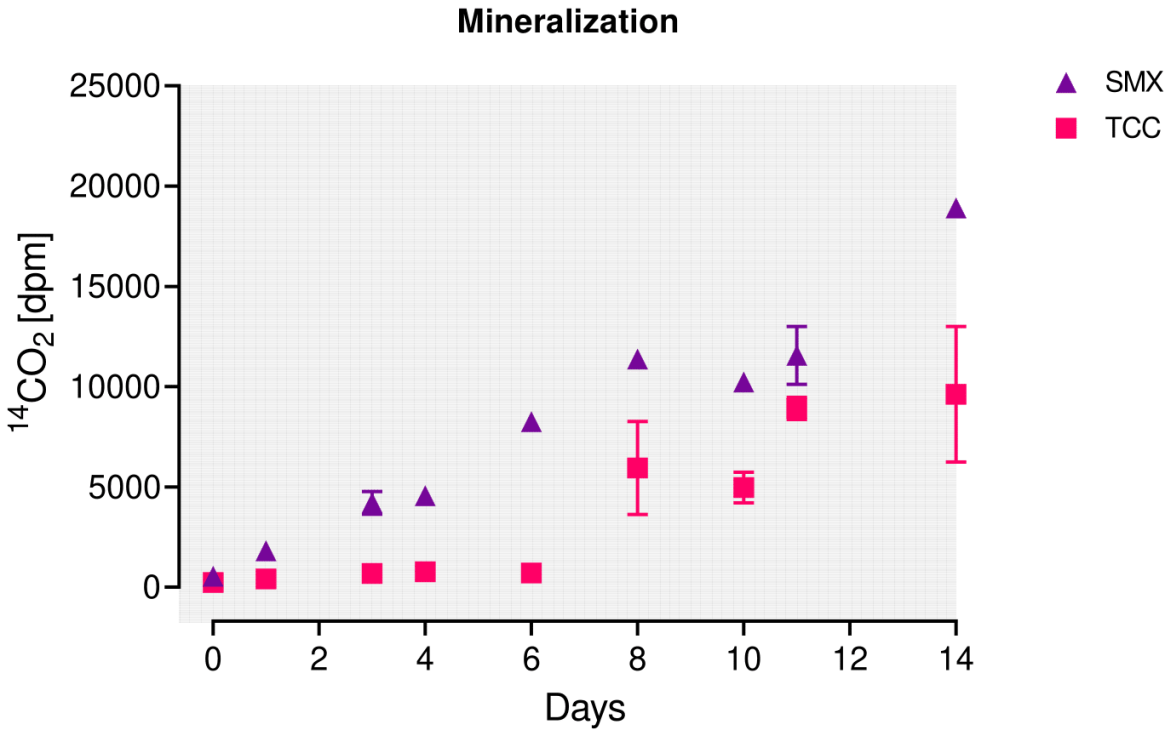


Figure 6.4. Mineralization of SMX and TCC during denitrification.

In the case of ^{14}C-SMX, mineralization started immediately from day 1 (**Figure 6.4**). Previous studies have also shown immediate SMX biotransformation of the ^{14}C-SMX parent compound during heterotrophic denitrification without any lag-phase (Alvarino et al., 2016). The maximum mineralization after 14 days, represented 0.4% of the applied radioactivity. The mineralization rate was faster in the first day 3.38 x 10^{-4} % day^{-1} and followed a first order kinetics until day 6, when the rates stabilized between day 8 and 11 to increase again at day 14. Similarly,

mineralization rates of SMX have shown to follow first order kinetics in pure cultures, and SMX were able to be metabolized and mineralized at concentrations as low as 0.1 μg L^{-1} (Lopez Gordillo et al., 2024). It is possible that the increase of mineralization rate in day 14 is due to the release of SMX from the solid fraction to balance the concentration gradient between both phases.

6.3.3. Extractable residues of ^{14}C-TCC and ^{14}C-SMX

For ^{14}C-TCC, the extractable residues, measured through $CaCl_2$ and MeOH extracts, exhibited a more pronounced decrease from day 10 onwards (**Figure 6.5**) compared to the earlier days, which displayed a relatively steady trend. This marked decrease may be explained by two potential mechanisms. Firstly, it could result from a concentration gradient driven by the increased mineralization observed from day 8 onwards (**Figure 6.4**). As mineralization progresses, the redistribution of ^{14}C-TCC between phases could reduce the extractable fraction. Secondly, the reduction in extractable residues might be attributed to the physical effects of shear forces generated by constant mixing (120 RPM) during the incubation of the batch experiments. These forces could facilitate the desorption or release of ^{14}C-TCC from solid fractions, thereby reducing its extractable pool. Accordingly, and increase in ^{14}C-TCC was observed in the water phase, suggesting a remobilization of the extractable fraction to the water phase.

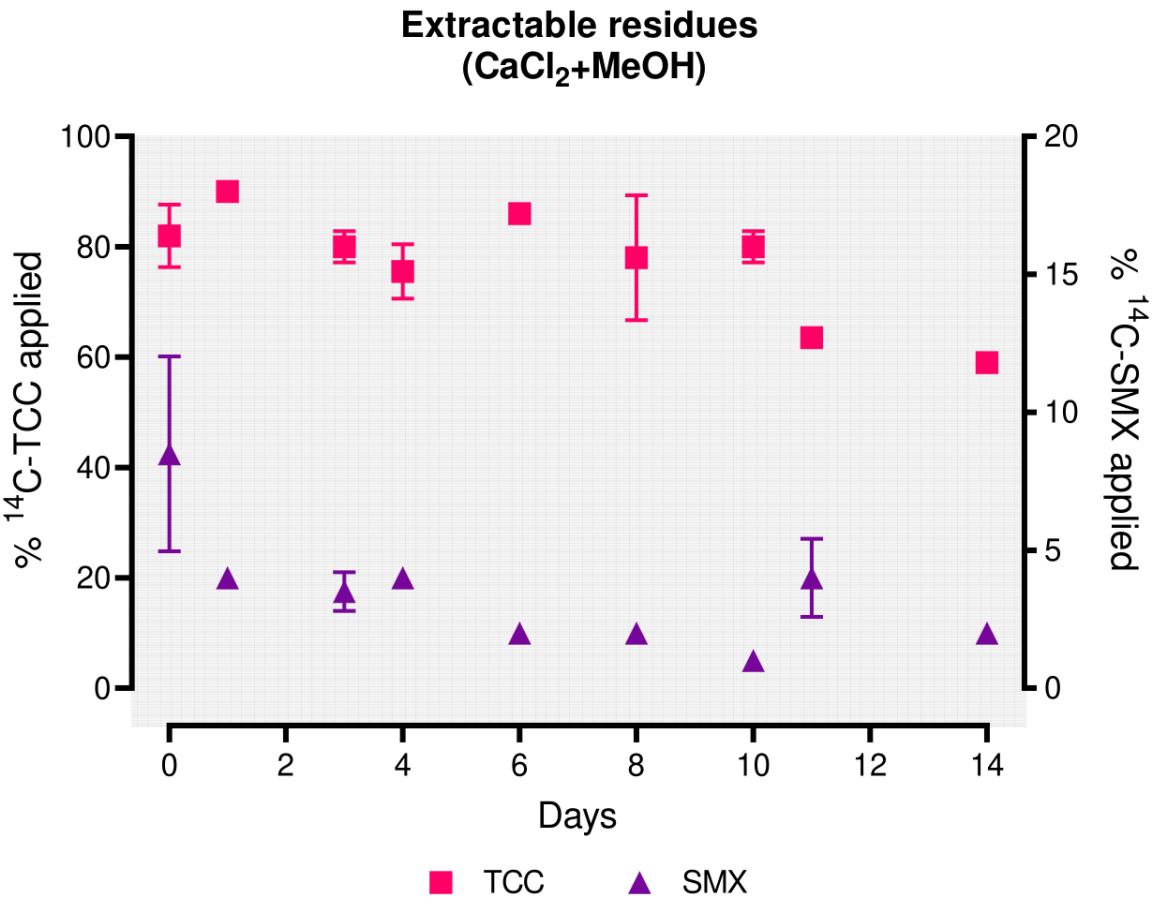


Figure 6.5. Extractable residues for SMX and TCC

In the case of SMX, the extractable fraction decreased from day 0 (8 ± 2%) to day 1(4 ± 0.1%), indicating a rapid initial remobilization of extractable residues to the aqueous phase. After this initial phase, the extractable fraction stabilized for the remaining of the incubation period (**Figure 6.5**). This trend can be attributed to the influence of environmental pH on the ionization state of SMX. At a pH 6, SMX is present as 41 % anion and 60% neutral species, whereas at a pH of 8 it is almost entirely (99%) anionic (Tran et al., 2016). During the experiment, the pH in the bottle increased due to the denitrifying activity. The increased pH likely affected the mobility and sorption behavior of SMX. This is because molecules in their ionized form tend to be more soluble because charged species interact less strongly with nonpolar phases like organic matter or hydrophobic surfaces. This highlights the critical dependence of the mobility and sorption behavior of ionizable organic compounds, such as zwitterionic SMX, on environmental pH (Sigmund et al., 2022). The higher mobility and bioavailability of SMX in aquatic systems may facilitate its biodegradability, however, it can also increase its dispersion to the environment. If SMX's capacity to promote antibiotic resistance (Yan et al., 2022).

6.3.4. Non -extractable residues (NER) of ^{14}C-TCC and ^{14}C-SMX

NER are environmentally relevant in fate studies because all chemicals that enter the environment form NER in solid matrices in varying amounts (Schäffer et al., 2018). In our study, NER from ^{14}C-TCC were formed in minimum amounts, with a slight increase over the incubation period. The maximum value was recorded on day 11 which represented only 2 ± 0.2% of the applied radioactivity. However, it is possible that this fraction may continue to increase over time, as previous studies have shown that TCC becomes less bioavailable with prolonged exposure (Snyder et al., 2010). Furthermore, previous studies indicated that the type of solid matrix significantly impacts both the removal rate of ^{14}C-TCC and the production of NER. For example, NER formation from ^{14}C-TCC happen immediately in dewatered biosolids, whereas NER formation was limited in liquid municipal biosolids, likely due to the higher moisture content that increased TCC's availability (Al-Rajab et al., 2009).

In the case of ^{14}C-SMX, a noticeable increase in NER was observed until day 4, with a highest value accounting for 14 ± 3% of the applied radioactivity. However, from day 6 till day 11 NER levels stabilized around 6-7%, and gained a slight increase on day 14 up to 8 ± 0.4 % of the applied radioactivity. These fluctuations suggest remobilization of residues, possibly driven by either chemical gradients in the solid-phase equilibrium or by shear forces during incubation, as discussed previously. The results imply that the part of NER that decreased, was predominantly type I, characterized by residues that are physically entrapped and have the potential to remobilize. The remobilization of antibiotic residues is of particular concern as it can potentially contribute to the spread of antibiotic resistance.

NER fractions can consist of both parent compounds and their TPs (Kästner et al., 2016). TPs may be transported to or sequestered within solid matrices due to their differing sorption affinities compared to the parent compounds, as demonstrated in previously studies, specifically for sulfonamides (Ma et al., 2021). It is a common practice to use octanol-water partitioning coefficient (K_{ow}) as an indicator of sorption affinity. However, hydrophobicity alone does not fully explain sorption affinity for ionizable organic compounds, as most key interactions for charged compounds are not driven by hydrophobicity but rather by ionization speciation as well as sorbent surface charge and the chemical composition of the solution (Sigmund et al., 2022). Therefore, understanding the dynamics of extractable residues is crucial for evaluating the fate and environmental impact of antibiotics, including parent compounds and their metabolites. Parent ^{14}C-SMX and its residues are further examined in the following section.

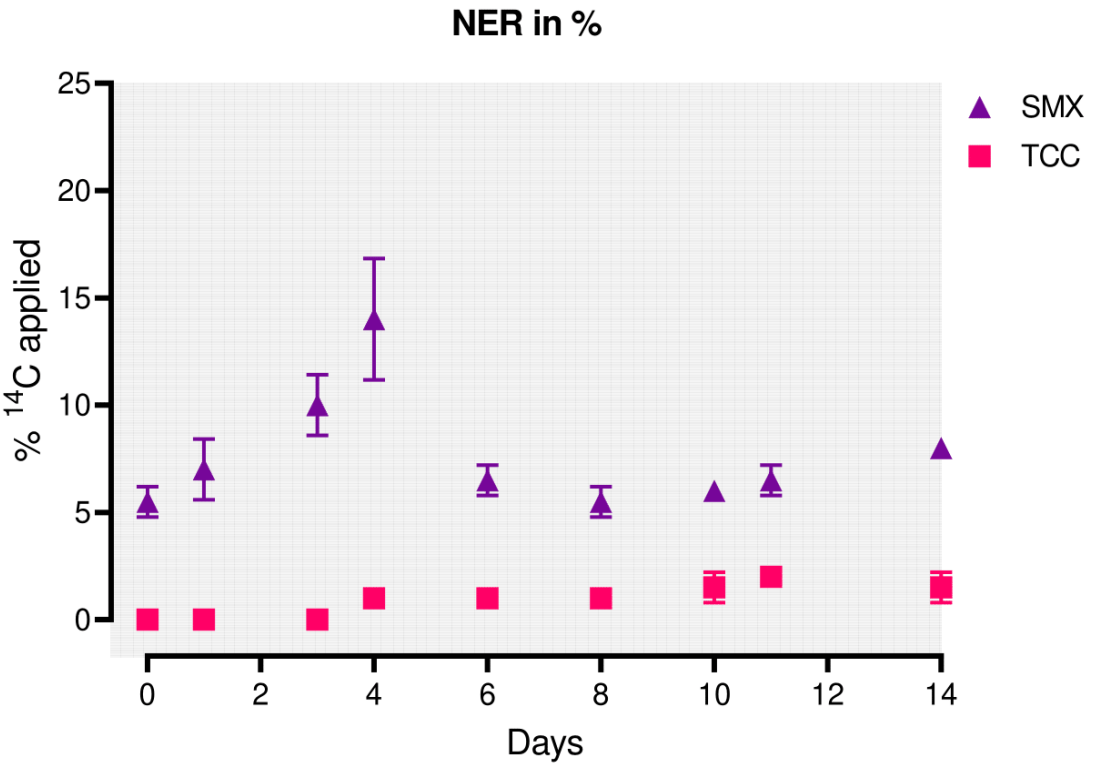


Figure 6.6. Non-extractable residues of SMX and TCC

6.3.5. Changes in Parent SMX concentration and transformation metabolite

Despite limited mineralization, significant biotransformation of ^{14}C-SMX was observed, as evidenced by the appearance of transformation metabolite(s). Radio-HPLC results indicated a progressive disappearance of the parent ^{14}C-SMX over time (RT=6.9 min) and the simultaneous emergence of either a single transformation metabolite or a mixture of more polar metabolites (RT= 2 min) (**Figure 6.6**). Previous studies, have evidenced rapid biotransformation of ^{14}C-SMX during heterotrophic denitrification within the first 8 days (190 h) of batch incubation (Alvarino et al., 2016). Those authors noted the formation of two new peaks in their radio-chromatograms that eluted earlier (2.3 and 8.4 min) compared to the parent SMX signal (~9min) suggesting these metabolites have a more polar structure. In alignment with these findings, our study identified a peak at 2 min RT that progressively increased, accounting for 87% of the total signal, compared to the remaining 13% from the parent ^{14}C-SMX , as determined by the radio-HPLC analysis.

Previous research investigating sulfamethoxazole (SMX) biotransformation in activated sludge have identified extensive biotransformation capabilities, with more than 11 transformation products (TPs) appearing within just one day (Achermann et al., 2018). These findings align with our results, where a newly formed TP was observed within hours after the initial SMX addition, indicating rapid microbial adaptation and transformation under our experimental conditions.

Notably, the TP eluted approximately 4.1 minutes earlier than the parent SMX, suggesting a higher polarity relative to the parent compound (**Figure 6.7**). Based on the early retention time (ca. 2 minutes) of this peak almost at the void volume of the HPLC column, it could comprise a mixture of polar degradation products with little chance to elucidate their structures.

Multiple studies on SMX biotransformation pathways have reported that bacterial consortia in wastewater treatment environments have the capacity to break the sulfonamide bond (S-N), followed by partial or complete mineralization of the resulting TPs (Nunes et al., 2020). After the cleavage of the S-N bond, multiple TPs that keep the phenyl ring intact have been reported, which are benzoquinone imine (BQI) that has shown to further transformed into 1,2,4-trihydroxybenzene (THB), presumably via hydroquinone (HQ), another very polar metabolite. Based on previous detected TPs, the signal of the labelled phenyl and the change in polarity, a putative degradation pathway and possible TPs are presented in **Figure 6.8**. Nevertheless, further analysis would be needed to confirm the identity of this metabolite.

Moreover, the observed build-up of this TP over time suggests either a more persistent behavior compared to the parent compound, SMX (**Figure 6.9**) or the increasing accumulation of a mixture of polar degradates (**Figure 6.10**). Our results indicate that SMX biotransformation was only partial, as the phenyl ring-U-^{14}C structure remained largely unchanged. This is evidenced by low mineralization rates and LSC measurements in both the aqueous phase and extractable fractions. Similar results have been obtained under heterotrophic denitrification, were SMX was partially metabolized, resulting in the accumulation of the isoxazole ring in its metabolites (Alvarino et al., 2016).

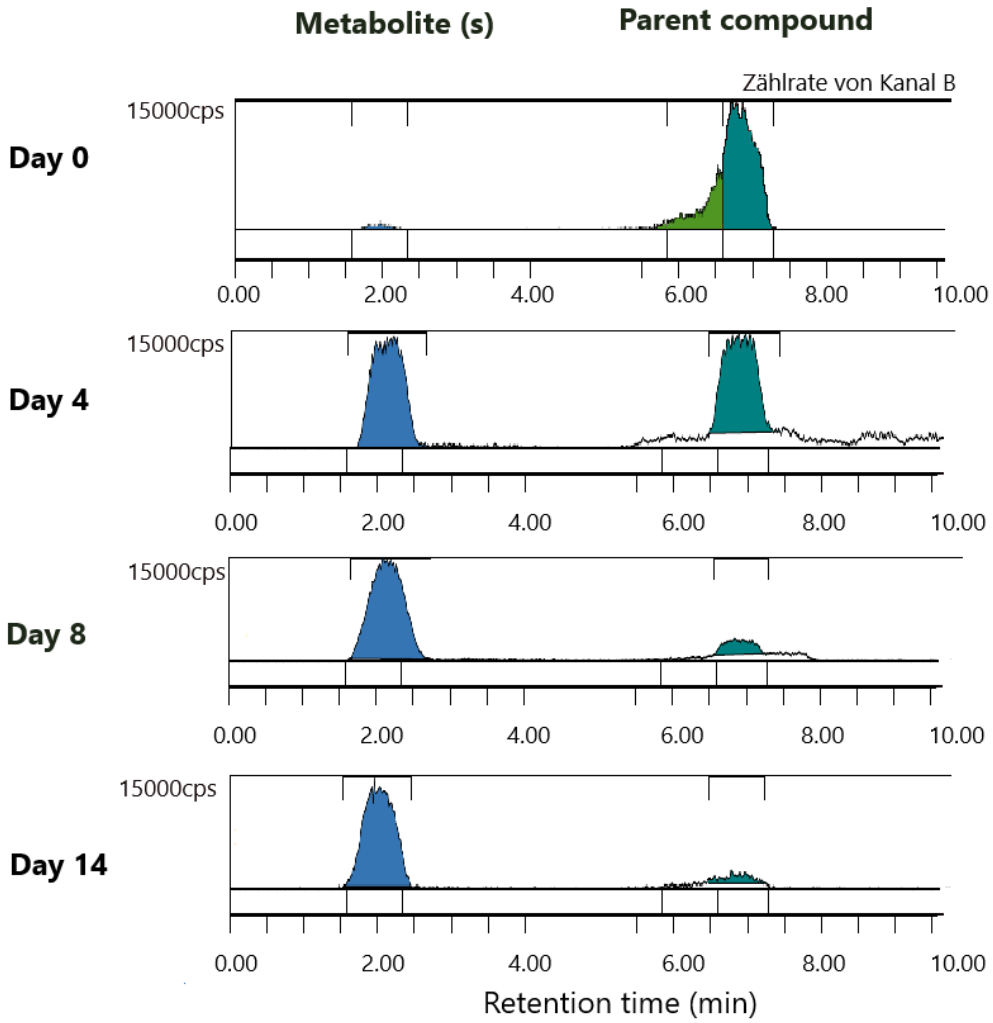


Figure 6.7. Chromatogram of Parent SMX and transformation metabolite on day 0, 4, 8 and 14. Signals represent peak areas across the retention time of the radio-HPLC quantification method.

Modifications of sulfonamides through single-step transformation reactions may not reduce their toxicity; in fact, certain TPs can exhibit toxicity levels equal or greater than that of the parent compound (Majewsky et al., 2014; Nunes et al., 2020). Furthermore, these TPs may retain antibiotic activity. For instance, sulfonamides metabolites modified at the *para*-amino position have shown to still exhibit antibiotic activity (Achermann et al., 2018; Majewsky et al., 2014). These converging lines of evidence underscore the environmental relevance of characterizing not only parent compounds but also their TPs, specifically of those that appear to be more polar (mobile) and persistent.

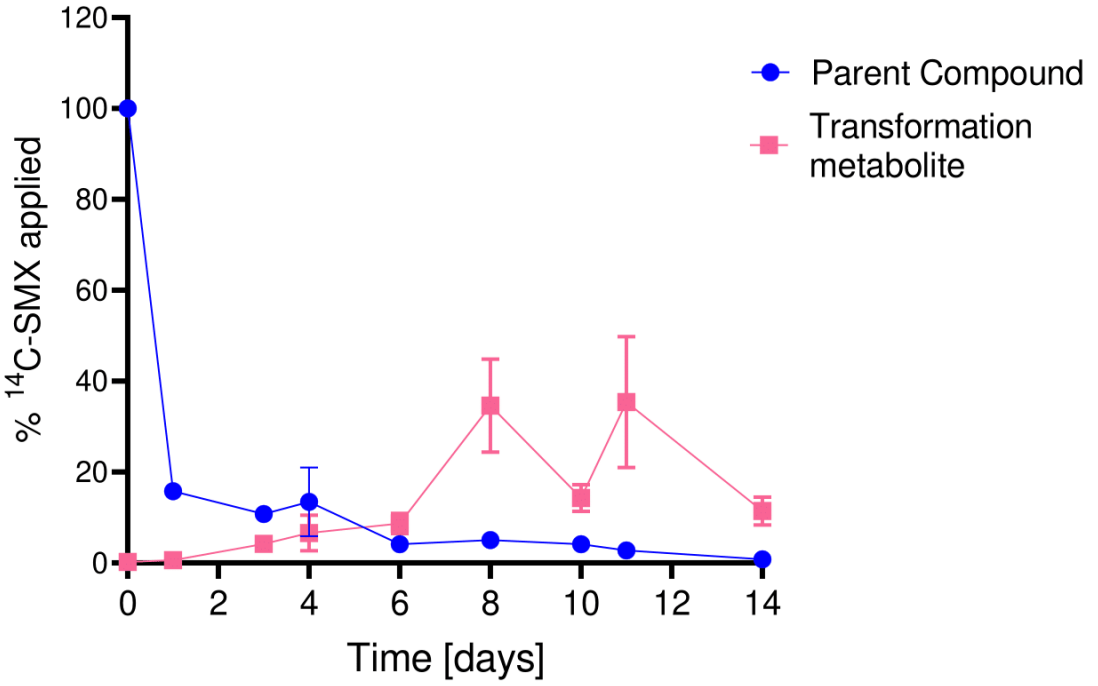


Figure 6.8. Temporal variation in radioactivity of the parent compound and its metabolite over a 14-day period. Error bars indicate standard deviations.

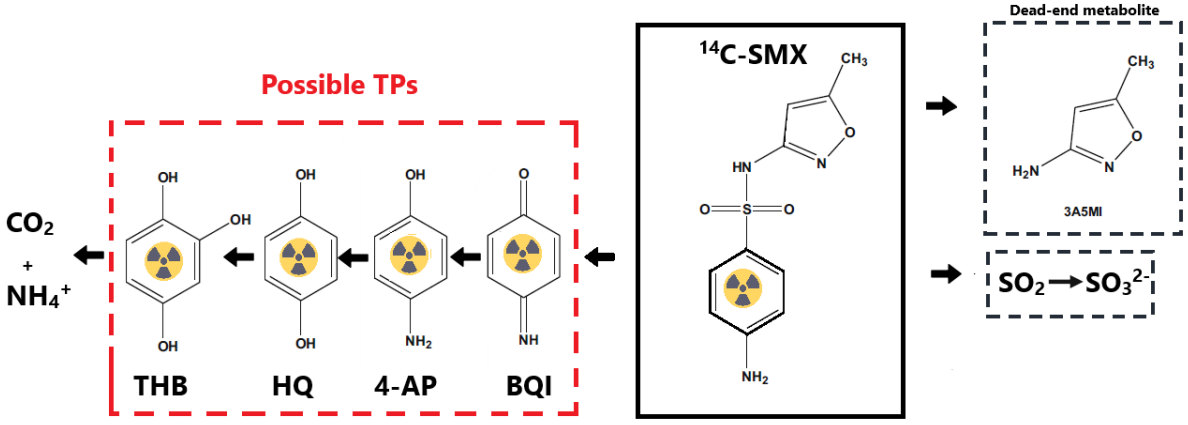


Figure 6.10. Putative Sulfamethoxazole degradation pathway and possible transformation products (in the red box) based on the radioactivity signal of the aromatic ring and the polarity observed in the radio-HPLC. Adapted from Nunes et al., (2020).

Contrary to the fate of SMX, the analysis of TCC samples using the radioactive detector of the HPLC did not reveal any additional peaks. Combined with the observed low mineralization, these findings indicate that no biotransformation

occurred during the 14-day incubation under heterotrophic denitrification conditions.

6.3.6. Determination of half-life (DT_{50}) of SMX during denitrification

Under denitrifying conditions, SMX underwent rapid biotransformation when acclimated activated sludge was used as the inoculum. The estimated half-life (DT50) of the parent compound was approximately 0.23 days (5.52 hours), as determined by monitoring the decline in applied radioactivity in $\%^{14}C$ in the aqueous phase (the nominal SMX concentration was defined as 100%). A Dual First-Order in Parallel (DFOP) model best fit the observed data, indicating that SMX likely comprises two fractions with distinct transformation rates. Detailed model parameters are presented in **Table 6.2**, and the corresponding model fit is illustrated in **Figure 6.10.**

Compared to previously reported data, the removal observed in our study is notably faster. SMX has shown to be readily transformed under both aerobic and anaerobic conditions, yet degradation rates vary widely. For instance, in water–sediment tests, DT_{50} values range from 8.5 to 17.2 days (Radke et al., 2009), while in soil batch studies SMX half-lives were reported from 1 to 11 days (Baumgarten et al., 2011; Lin and Gan, 2011). The faster degradation rate in our experiments can be explained by the high bioavailability of SMX compared to that if a sorbing matrix reduces the bioavailability. However, longer half-lives attributed to the formation of reversible metabolites during denitrifying conditions were also recorded (Ma et al., 2021); ranging from 69 ± 25 days in anoxic tests to 12 ± 11 days in oxic conditions. These results highlight the influence of environmental conditions and microbial communities on SMX degradation dynamics.

Table 6.2. Model outputs for SMX DT_{50} determination in the water phase

Values in the water phase	DFOP model output
DT_{50}	0.23 days
DT_{90}	2.69 days
k1 [DT_{50}]	0.178
k2 [DT_{50}]	4.11
Error model	6.23 %
Efficiency [Obs v Pred]	r^2=0.9985

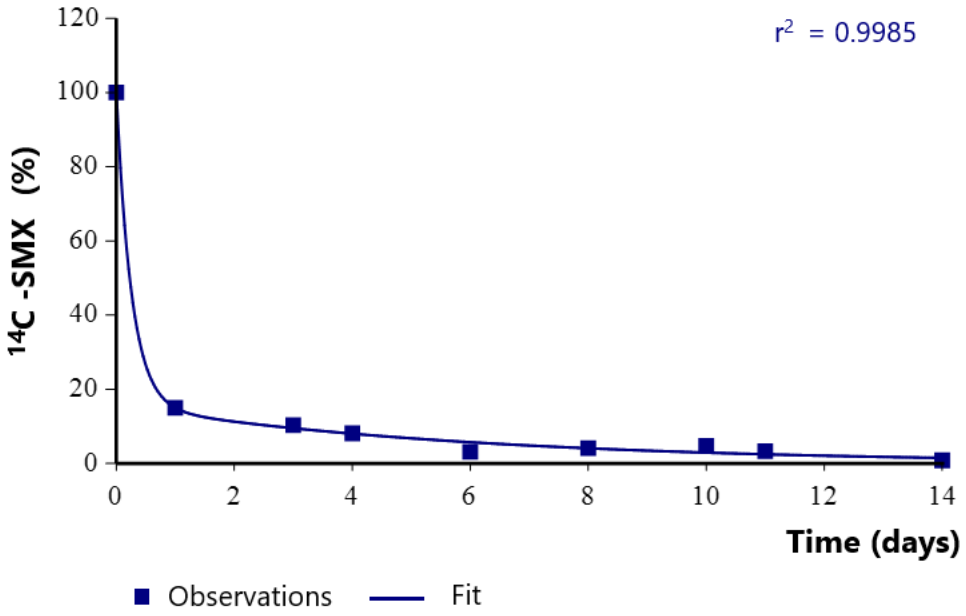


Figure 6.10. Model fit for the half-life of parent SMX during this study

6.4. CONCLUSIONS

The fate of radiolabeled SMX and TCC was determined under denitrifying conditions in terms of biotransformation, mineralization and sorption leading to the following key outcomes:

- TCC showed a persistent behavior under denitrifying conditions. Mass balanced showed that TCC was mainly present in the extractable fraction which likely prevented its bioavailability and subsequent biotransformation.
- SMX was partially biotransformed, resulting in the accumulation of a more polar and persistent transformation product or a mixture if very polar metabolites, This finding evidences that focusing solely on the removal of the parent compound is insufficient. Instead, we emphasize the need to integrate comprehensive fate studies that characterize biotransformation pathways, particularly through the identification of TPs and the assessment of their antibiotic activity.
- Extractable fractions for both SMX and TCC declined over time, suggesting a remobilization of the residues likely due to chemical gradient differences but also shear forced during mixing. The remobilization of these residues was more pronounced in the case of TCC for which most of applied radioactivity was found in the extractable fraction .

- NER fractions were also remobilized during our experiments , specifically in the case of SMX. The dynamic behavior of extractable and non-extractable residues plays a critical role in assessing the environmental impact of antibiotics and their derivatives.

Our results highlight the critical need to consider both parent compounds and their transformation products along with non-extractable residues when evaluating antibiotic fate, as these persistent and potentially bioactive metabolites and remobilized fractions could pose ongoing ecological and health concerns.

CHAPTER 7

General discussion

7.1. MAIN THESIS GOAL AND RESEARCH GAPS

The growing concern over the prevalence of antibiotics in wastewater has become a significant global environmental and public health issue (Huemer et al., 2020; Jiang et al., 2024). Antibiotics can disrupt aquatic ecosystems, bioaccumulate in human and animal tissues, and contribute to long-term health effects(Felis et al., 2020). Critically, antibiotic resistance has emerged as a leading cause of mortality worldwide (IHME Institute for Health Metrics and Evaluation, 2024). WWTPs serve as essential barriers to prevent the dispersion of these chemicals; however, incomplete removal renders them significant point sources of a wide range of micropollutants, including antibiotics (Bürgmann et al., 2018).

In response, new regulations are enforcing stricter water quality standards to mitigate the environmental impact of OMPs. The revised EU Urban Wastewater Treatment Directive, aligned with the EU's Zero-Pollution Action Plan, mandates tertiary nutrient removal in WWTPs serving populations of 150,000 or more by 2039. By 2045, a quaternary treatment step targeting micropollutant removal will also become mandatory for these large-scale plants (Directive 91/271/EEC, 2024). These treatments must comply with energy neutrality targets, favoring processes that minimize energy demands (European Commission, 2019). These regulatory pressures emphasize the urgent need for research to optimize biological treatment processes for enhanced micropollutant biotransformation. In this sense, to improve existing nitrogen removal bioprocesses to maximize antibiotic removal is of great relevance. Leveraging existing technologies (i.e. activated sludge) and exploring new sustainable bioprocesses (i.e. N-Damo), offers a sustainable and energy-efficient approach for reducing micropollutant loads in wastewater.

Biological nutrient treatment, primarily through activated sludge processes, is a core component of wastewater treatment plants (WWTPs) and the primary site for most biotransformation mechanisms. In activated sludge systems, both nitrification and denitrification processes play a key role in biological nutrient removal. While significant research has focused on antibiotic biotransformation under aerobic (nitrifying) conditions, far less is known about their fate under anoxic (denitrifying) conditions. The mechanisms and extent of antibiotic biotransformation during denitrification remain unexplored, with considerably fewer studies available compared to aerobic treatment processes.

Therefore, the central theme of this doctoral thesis was to explore antibiotic biotransformation under denitrifying processes for wastewater treatment. The work focuses on three key aspects: (1) anoxic cometabolic biotransformation, (2) shifts in microbiome composition dynamics and enzymatic activity, and (3) the fate and transformation metabolites of antibiotics during biotransformation, as outlined in **Section 1.5** of the introduction of this thesis**.** The research gaps and the

corresponding experimental set-up designed to address them are depicted in **Table 7.1.**

Table 7.1. Overview of research gaps tackled in this thesis and experimental set-up

Research Gaps	Denitrification Type	Reactor Type	NLR (mg N L^{-1} d^{-1})	Operational details	Thesis Chapter
Anoxic cometabolism & microbiome dynamics	Heterotrophic	Continuous flow	25-300	C/N ratio 6:1 Acetate as e- donor	2
	Autotrophic	Anaerobic MBR	80-240	CH_4 as e- donor	3
Enzymatic activity at high and steady N- Damo process	Autotrophic	Anaerobic MBR	240	Cerium supplementation	4
Antibiotic fate of and degradation pathways	Heterotrophic	Continuous flow & Batch	50	Radiolabeled compounds SMX and TCC	5

7.2. COMPARISON OF ANTIBIOTIC REMOVAL EFFICIENCIES BETWEEN DENITRIFYING PROCESSES

Significant differences in antibiotic removal efficiencies were observed between the N-Damo and heterotrophic denitrification systems, highlighting the influence of the type of denitrification pathway on micropollutant biodegradation. **Figure 7.1** compares these removal efficiencies under similar specific denitrifying activities. Clarithromycin (CLA) and roxithromycin (ROX) were highly removed during heterotrophic denitrification, with efficiencies exceeding 90%, significantly outperforming the N-Damo system, where removals ranged between 25% and 40%. In contrast, azithromycin (AZY) was effectively removed in both systems, with higher efficiency observed in N-Damo (98%) compared to the heterotrophic system (74%).

The observed differences in removal efficiencies may be attributed to variations in microbial community composition and associated enzymatic pathways, which differ between the two processes. Notably, the removal efficiencies for CLA, ROX, and AZY in these experimental systems far exceeded typical removal

rates reported for full-scale WWTPs, which average 33% for CLA, 17% for ROX, and 39% for AZY ROX (Göbel et al., 2007b; Margot et al., 2015).

For sulfamethoxazole (SMX) and trimethoprim (TMP), removal efficiencies were similar in both systems, as indicated by their proximity to the diagonal in **Figure 7.1**. TMP removal efficiencies exceeded 75%, significantly higher than the average 47 ± 26% reported for full-scale WWTPs (Luo et al., 2014). SMX removal efficiencies (72–77%) were consistent with full-scale averages of 64 ± 20% (Luo et al., 2014b).Both systems exhibited limited removal of ciprofloxacin (CIP), underscoring its recalcitrance under the tested conditions.

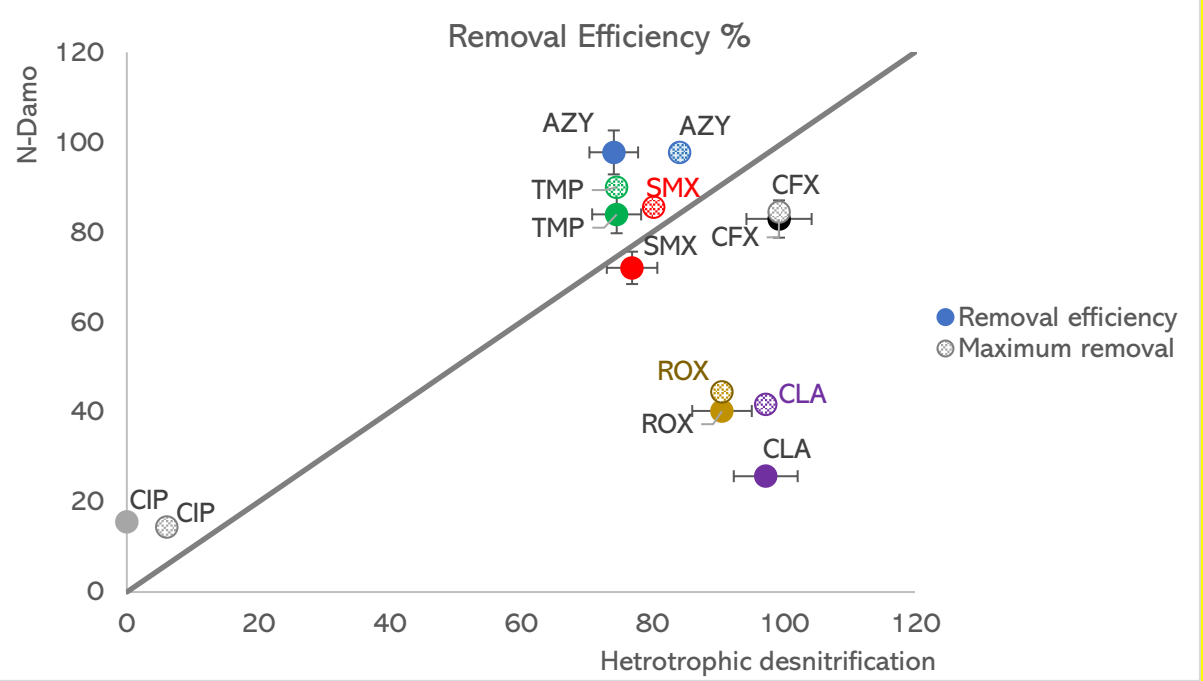


Figure 7.1. Removal efficiency in N-Damo (PI) and heterotrophic denitrification (PIII) during similar denitrification activities. For N-Damo, Y axis show values for Period I (mg 59.7±2.8 N- NO^{-}_{2} g VSS·d^{-1}) (see **Chapter 4**). For the heterotrophic denitrification, X axis depict values in Period III (56.4 ± 2.4 mg N- NO^{-}_{3} g VSS·d^{-1}) (see **Chapter 3**). The diagonal line represents equal removal efficiencies for both processes.

Interestingly, the maximum removal values, represented by the patterned circles in **Figure 7.1**, closely align with the antibiotic removal efficiencies observed during **PI** (mg 59.7±2.8 N- NO^{-}_{2} g VSS·d^{-1}) for the N-Damo system and **PIII** (56.4 ± 2.4 mg N- NO^{-}_{3} g VSS·d^{-1}) for the heterotrophic reactors. These findings suggest that antibiotic removal efficiency may have reached a kinetic plateau at these denitrifying activities, with further increases in substrate load yielding negligible improvements in removal performance.

7.3. COMETABOLIC BIOTRANSFORMATION DURING DENITRIFYING PROCESSES

7.3.1. High specific denitrifying activities reached

Reactor performance in both **Chapter 3** and **4,** consistently achieved complete nitrogen removal and maintained stable, efficient performance over long-term operation. However, despite an observed increase in metabolic activity, this did not translate into enhanced antibiotic removal. These findings suggest that although the systems operated at medium to high denitrification rates, the microbial community's biotransformation capacity may have already reached its upper limit. As a result, no further co-metabolic degradation of antibiotics could be detected under the tested conditions.

Figure 7.2 compares the maximum denitrification activities achieved in these lab-scale reactors with those typically applied in full-scale WWTPs. The comparison emphasizes the high-rates applied in this thesis. In particular, for **Chapter 4**, where the elevated denitrification activities N-Damo microorganisms, coupled with the reactor's stability and resilience under increased nitrogen loads, represent a significant advancement towards practical, full-scale applications.

For context, **Table 7.2** provides an overview of previous N-Damo enrichment strategies and their corresponding specific denitrification rates (SDNR). Despite the promising metabolic performance, no substantial improvement in antibiotic removal was observed. This outcome underscores the importance of microbial community composition over metabolic activity alone in governing antibiotic degradation. It suggests that future efforts to enhance micropollutant removal should focus on selecting and cultivating specialized microbial consortia, rather than relying solely on increased metabolic rates, to achieve more effective removal of antibiotics in wastewater treatment systems.

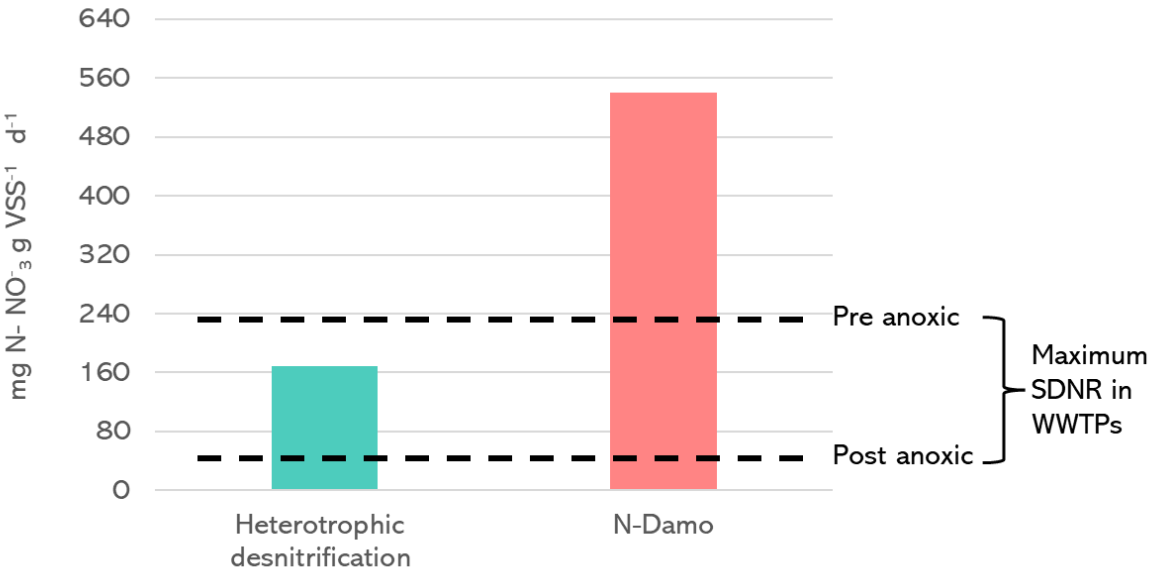


Figure 7.2. Maximum specific denitrification rates (SDNR) applied in the lab-scale denitrifying reactors in Chapter 3 and N-Damo MBR reactors in Chapter 4. Dotted lines indicate maximum SDNR applied in full-scale WWTPs for pre and post anoxic reactors according to Metcalf & Eddy et al., (2014).

Table 7.2. Overview of the nitrite removal rates reported in literature for N-Damo bacteria and typical values for full-scale heterotrophic denitrification and Anammox

Reference	**Maximum activity (mg N-NO^{-}_{2} g^{-1}VSS d^{-1})**
N-Damo, Kampman et al., (2012)	47
N-Damo, Allegue et al., (2018)	95
N-Damo, Martínez-Quintela et al., (2021)	186
N-Damo, Chang et al., (2021)	>5
Heterotrophic desnitrification, typical full-scale treatment(Metcalf & Eddy et al., 2014)	250
Autotrophic desnitrification with Anammox (Capodaglio et al., 2014)	200
N-Damo, this thesis (**Chapter 4**)	540

7.3.2. Kinetic plateau for antibiotic biotransformation

We hypothesized that the lack of observable cometabolic tendencies in **Chapter 3** was due to the high denitrification rates applied, which likely positioned OMP removal near the upper saturation limit, leaving little room for enhancement. Typically, OMP biotransformation is modeled as a first-order kinetic process derived from Michaelis–Menten theory (Schwarzenbach, 2005), as discussed in **section 1.3.2.** of the introduction. Under saturating substrate conditions (S ≫ Ks), further increases in substrate concentration have little impact on removal efficiency, as metabolic activity is already approaching its maximum capacity. However, applying a strictly linear approximation may overlook key aspects of cometabolism, namely the necessity of both an upper saturation limit and a lower activation threshold for growth substrates(Kennes-Veiga, et al., 2021; Sheng et al., 2021).

These insights underscore the limitations of relying solely on a linear Michaelis–Menten model for predicting OMP biotransformation kinetics, as noted by van Bergen et al., (2021, 2024). Accounting for both upper and lower substrate thresholds refine predictive models and emphasizes the importance of understanding microbial metabolic capacity when evaluating OMP removal efficiency (**Figure 7.3**).

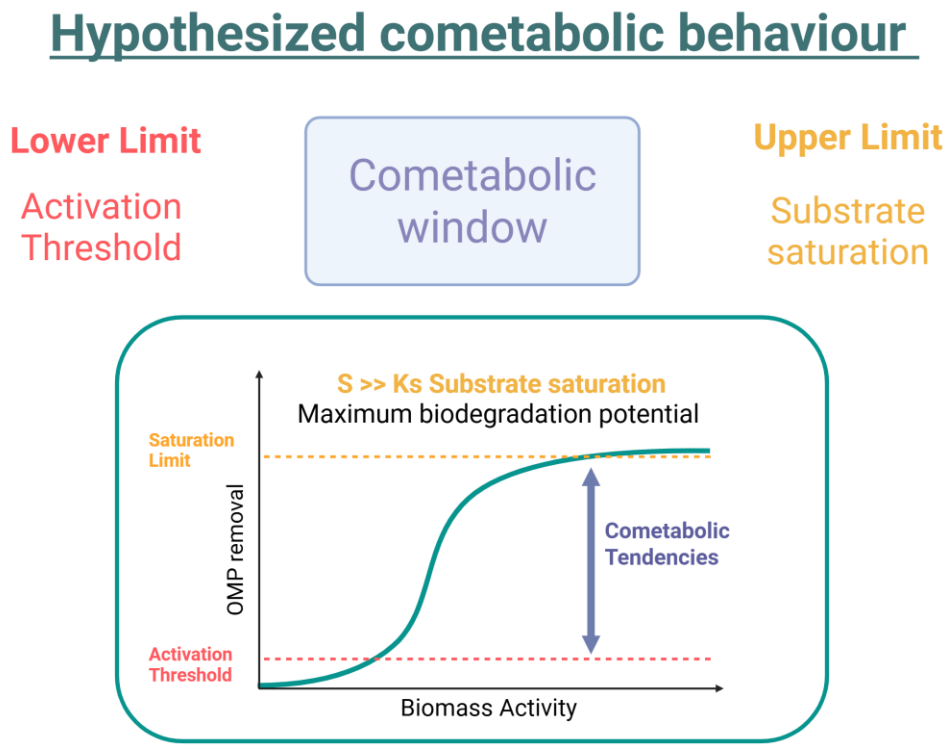


Figure 7.3 . Hypothesed cometabolic behaviour for OMPs with upper and lower subtrate threshold for observable cometabolic tendencies.

To examine differences in antibiotic biotransformation under distinct denitrification pathways, heterotrophic desnitrification and N-Damo, we compared specific antibiotic removal at similar NLRs corresponding to PI (mg 59.7±2.8 N- NO^{-}_{2} g VSS·d^{-1}) in N-Damo (**Chapter 4**) and PIII (56.4 ± 2.4 mg N- NO^{-}_{3} g VSS·d^{-1}) in heterotrophic desnitrification (**Chapter 3**) (**Figure 7.4**). Under N-Damo conditions, the removal of certain antibiotics, such as AZY and TMP, was more pronounced than under heterotrophic denitrification. Conversely, under heterotrophic denitrification, a different group of antibiotics (CFX, ROX, and CLA) demonstrated enhanced removal. Similarities were observed for SMX, which was equally removed in both anoxic systems and for CIP, that remained recalcitrant under both conditions.

These results highlight that antibiotic biotransformation is not a uniform process and is instead influenced by the specific enzymatic capabilities and ecological niches present within each microbial community. By leveraging multiple metabolic pathways and diverse microbial consortia, wastewater treatment systems can broaden their contaminant removal profiles. Integrating various microbiological processes into a single configuration may thus improve overall OMP removal efficiency, as a more extensive enzymatic repertoire can enhance the degradation of a wide range of antibiotic compounds.

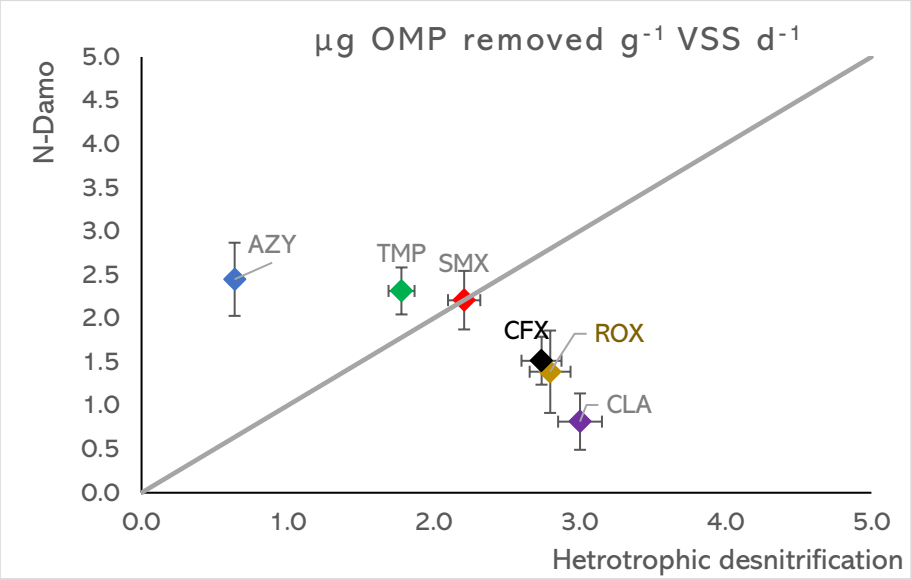


Figure 7.4. Specific antibiotic removal in N-Damo and heterotrophic denitrification during similar denitrification activities. For N-Damo, Y axis show values for Period I (mg 59.7±2.8 N- NO^{-}_{2} g VSS·d^{-1}) (see **Chapter 4**). For the heterotrophic denitrification, X axis show values for Period III (56.4 ± 2.4 mg N- NO^{-}_{3} g VSS·d^{-1}) (see **Chapter 3**). The diagonal line represents equal specific OMP removal for both processes.

7.4. MICROBIAL SHIFTS UNDER VARIABLE SUBSTRATE LOADS AND ANTIBIOTIC PRESENCE

In addition to the observed variations in removal efficiency and antibiotic-specific degradation among different metabolic pathways (**section 7.1** and **7.2**), shifts in the microbial community exerted a more substantial influence on antibiotic biotransformation than the reactor's overall denitrifying activity in both **Chapter 3** and **4**. In both systems, microbial richness decreased as denitrification rates increased (**Table 7.3**)., but this reduction in diversity was more pronounced under heterotrophic denitrification, which initially exhibited a highly diverse community. The decline in diversity and richness suggested a specialization of the microbiome towards the primary substrate and a trade-off with the decline of functional redundancy that affected antibiotic biotransformation.

Moreover, the mere presence of antibiotics prompted alterations in the heterotrophic denitrifying community structure, whereas the N-Damo community, highly enriched with *Ca. Methalomyrabilis* remained largely unaffected by antibiotic addition. These findings indicate that certain minor heterotrophic taxa are more susceptible to antibiotic exposure than Ca. Methalomyrabilis. Notably, the relative abundance of *Ca. Methalomyrabilis* correlated with antibiotic removal, suggesting its functional involvement in biotransformation processes. Previous studies, conducted at lower NLRs, also reported a cometabolic relationship between N-Damo communities and various pharmaceuticals (Martínez-Quintela et al., 2021b).

Similarly, in **Chapter 3**, an increase in the order *Rhizobiales* was positively associated with the removal of all studied antibiotics, consistent with earlier reports on their biotransformation capabilities (Phan et al., 2016; Zhou et al., 2022). Collectively, these compositional shifts underscore the pivotal role of specific microbial taxa in antibiotic biotransformation. Preserving or enhancing key microbial groups, such as *Ca. Methalomyrabilis* and *Rhizobiales*, may therefore represent a crucial strategy for optimizing antibiotic removal in denitrifying treatment systems.

Table 7.3. Chao diversity differences between reactors exposed to antibiotics in different NLR in both denitrification systems.

Type of denitrification	Chao	mg N-NO^-_2 $L^{-1}d^{-1}$	mg N- NO^-_3 $L^{-1}d^{-1}$
N-Damo	130,77	70,31 ± 10	
	110,95	215,2 ± 24	
Heterotrophic	310,01		25± 6
	321,75		114± 20
	151,74		277±38

Beyond the influence of NLRs on microbial community composition, enzymatic responses also varied as shown by PICRUSt analysis in **Chapter 3**. Under high NLR conditions, the abundance of key respiratory enzymes, nitrate reductase, nitric reductase, nitric oxide reductase, and nitrous oxide reductase, increased, reflecting the enhanced denitrification activity. However, this result was expected due to the intensified respiration required at elevated nitrogen loads. More notably, as the rates increased, the overall enzymatic abundance decreased, coinciding with a reduction in community richness and diversity, and ultimately diminishing functional redundancy.

Changes in medium composition, particularly through trace additions of cerium, further influenced microbial community structure, enzymatic responses, and reactor performance. In **Chapter 5**, we demonstrated that high and sustained reactor activities can be achieved by continuously supplying 4.5 μg L^{-1} of cerium. Metaproteomic analyses revealed that *Ca. Methylomirabilis lanthanidiphila* dominated under these conditions, likely due to the induction of its lanthanide-binding methanol dehydrogenase in the presence of cerium. However, under high denitrification activity, *Ca Methylomirabilis* abundance decreased and instead showed greater abundance of accompanying taxa involved in denitrification, methylotrophy, and methanotrophy. These shifts indicate increased competition for the primary substrate, potentially affecting both nitrogen and micropollutant removal.

Collectively, these findings underscore how operational conditions, such as NLR and trace metal supplementation. shape microbial community composition, enzymatic potential, and process performance. Understanding these dynamics is essential for strategically tuning reactor conditions to meet specific treatment goals, whether emphasizing high-rate nitrogen removal or enhancing the removal of antibiotics.

7.5. FATE OF ANTIBIOTICS AND TRANSFORMATION METABOLITES DURING HETEROTROPHIC DENITRIFICATION

The fate of selected compounds, SMX and TCC, was primarily determined by the chemical structure and physico-chemical properties of the OMPs. The mineralized fraction, sorption and the ^{14}C respective recovery are depicted in **Table 7.2.**

7.5.1. Sorption and remobilization

TCC showed significant sorption (>59%) after 14 days, evidencing its hydrophobic nature promoted strong interactions with sludge particles. In contrast, SMX displayed minimal sorption (2%), likely due to its high polarity and predominant anionic ion speciation (>98% above pH 7.5) during heterotrophic denitrification.

Over time, both SMX and TCC exhibited gradual remobilization of their extractable and non-extractable residues into the aqueous phase. This remobilization was likely driven by chemical gradients and mixing-induced shear forces, which disrupt the interactions between OMPs and sludge particles, leading to their release back into the water column.

7.5.2. Bioavailability, biotransformation and mineralization

The bioavailability of the molecule influences its biotransformation. The high sorption affinity of TCC likely limited its biotransformation as the molecule needs to be accessible for the microorganisms. TCC showed no discernible mineralization nor transformation. On the contrary, parent SMX achieved rapid removal of up to 80% within the first day. This removal efficiency aligned closely with previously reported values of 73 ± 2% during heterotrophic denitrification (**Chapter 3**) and approximately 72% for N-Damo processes (**Chapter 4**). The partial biotransformation of SMX was enabled by its higher bioavailability, resulting in a faster dissipation of the parent SMX ($DT_{50} \approx 0.23$ days) than previously documented. However, this rapid biotransformation converted SMX into one or a mixture of more polar metabolites, which accumulated in the aqueous phase rather than being fully mineralized. This indicates that while the initial removal of parent SMX was effective, complete degradation remained limited, necessitating further treatment.

Table 7.4. Fate of radiolabeled compound SMX and TCC after 14 days of incubation during heterotrophic denitrification.

OMPs	Physico-chemical characteristics	Mineralization (% ^{14}C)	Sorption (% ^{14}C)		% ^{14}C Recovery
			Extractable	*NER*	
^{14}C-SMX	Polar charged anionic	0.4	2	7.9	98
^{14}C-TCC	Hydrophobic neutral	0.2	59.2	1.5	79

Overall, **Chapter 6** reinforced the importance of considering factors as both parent compounds and their transformation products, hydrophobicity, polarity, and bioavailability, to optimize treatment processes and minimize the persistence of harmful micropollutants in the water cycle.

CHAPTER 8

Conclusions and Future Research Perspectives

8.1. MAIN OUTCOMES OF THIS THESIS

The main goal of this thesis was to understand antibiotic biotransformation mechanisms during denitrifying wastewater treatment to enhance existing biological treatment. To achieve this, three primary objectives were pursued: (i) assessment of cometabolic biotransformation during denitrification (**Chapters 3** and **4**), (ii) investigation of microbiome and enzymatic responses (**Chapters 3, 4,** and **5**), and (iii) characterization of antibiotic fate and biotransformation pathways (**Chapter 6**). The principal findings emerging from these investigations can be summarized as follows:

To investigate the biotransformation potential of denitrifying systems, two different anoxic processes were analyzed: the widely used conventional heterotrophic denitrification in **Chapter 3**, and the innovative autotrophic denitrification process N-Damo in **Chapter 4**. In both systems, increasing biomass activity did not enhance antibiotic removal efficiencies, indicating a lack of cometabolic tendencies. However, high NLR, significantly influenced microbial community composition, diversity, and richness. During heterotrophic denitrification, the initially highly diverse microbiome, suffered a significant decrease in diversity as higher NLRs selected for a more specialized microbial community. In the N-Damo system, while overall richness slightly decreased at high NLRs, the dominance of *Ca. Methylomirabilis*, initially comprising over 85% of the community, declined by 20%. This was accompanied by an increased abundance of taxa involved in denitrification, methylotrophy, and methanotrophy. This shift is likely explained by the increasing endogenous respiration associated with the very high rates applied.

In **Chapter 5,** we investigated the enzymatic activity of N-Damo, building on the significant microbial shifts observed in **Chapter 4** and the high denitrifying activity measured (540 mg N $gVSS^{-1}$ d^{-1}). Special emphasis was placed on the role of metalloenzymes in response to cerium supplementation in the N-Damo medium. Metaproteomic analysis revealed that "*Ca. Methylomirabilis lanthanidiphila"* was the predominant N-Damo species, attributed to the induction of its lanthanide-binding methanol dehydrogenase facilitated by cerium addition. These findings confirmed our hypothesis that high N-Damo activity can be achieved by adjusting the metal composition in the feed, offering valuable insights for optimizing treatment processes and enhancing pollutant removal efficiency. Additionally, the study identified accompanying taxa involved in denitrification, methylotrophy and methanotrophy, which likely competed with *Ca. Methylomirabilis* for the primary substrate.

In **Chapter 6,** we aimed to complement our understanding of antibiotic biotransformation by studying the fate and transformation metabolites of radiolabeled sulfamethoxazole (SMX) and triclocarban (TCC). The results

emphasized the role of chemical structure and physico-chemical properties in determining the fate of organic micropollutants (OMPs). TCC, a neutral hydrophobic molecule, was primarily sorbed and predominantly detected in the extractable fraction. In contrast, SMX, a highly polar zwitterion, underwent partial biotransformation, resulting in the accumulation of a more polar and persistent transformation product or a mixture of highly polar metabolites, primarily observed in the aqueous phase. Furthermore, both chemicals exhibited partial remobilization of extractable and non-extractable back to the aqueous phase. These findings highlight the complex interactions governing OMP fate and transformation in wastewater treatment systems.

8.2. FUTURE RESEARCH PERSPECTIVES

8.2.1. Enhancing Sustainability of Denitrification Processes

Heterotrophic denitrifying systems require the addition of an external carbon source, which can increase operational costs. In **Chapter 3,** we used sodium acetate as carbon source in a C/N ratio 6:1 to ensure sufficient carbon availability. In **Chapter 4** we investigated autotrophic denitrification as a more sustainable alternative; however, this process remains in the early stages of development and is not yet ready for full-scale implementation.

To advance the sustainability of denitrification in wastewater treatment, future studies should explore the utilization of low-cost and renewable natural organic substrates such as rice straw, rice husk as external solid carbon sources (Cai et al., 2024; Jia et al., 2019). These agricultural biomasses are not only economically viable but also contribute to water circularity by repurposing residual materials. Additionally, diversifying carbon sources can stimulate a broader enzymatic repertoire within the denitrifying microbial communities, thereby enhancing the breakdown of a wider array of OMPs. Additionally, leveraging these renewable substrates reduces the dependency on fossil-based carbon sources, aligning denitrification practices with sustainable and circular economy principles.

8.2.2. Diversifying Carbon Sources to Improve OMP removal

In this thesis we focused on the nitrogen route of denitrification. However, the impact of external carbon dose has shown to yield better results on OMP removal, as methanol-dosed MBBR showed higher K_{biol} than an ethanol-dosed MBBR for 9 out of 22 studied compounds (Torresi et al., 2017b). Here, the integration of humic acids as alternative carbon sources in denitrification presents a promising avenue for enhancing micropollutant removal. Heterotrophs can benefit from humic acid-like structures as they possess a variety of functional groups such as carboxyl and phenolic groups that facilitate interactions with diverse contaminants. Acting as structural analogues, humic acids can promote the removal of pharmaceuticals, pesticides, and endocrine-disrupting compounds through

mechanisms including adsorption, complexation, and redox interactions (Drewes et al., 2014; Li et al., 2012; Luo et al., 2019). Furthermore, humic acids can function as electron shuttles, enhancing electron transfer processes and thereby stimulating microbial activity and biodegradation pathways. This dual role not only improves the efficiency of micropollutant degradation but also supports the resilience and adaptability of denitrifying microbial communities.

8.2.3. Elucidating Metabolic Pathways and Functional Microorganisms

In **Chapter 3** and **4** 16S rRNA sequencing was used to analyze microbial community composition and its changes in response to antibiotic presence and increasing NLRs. In **Chapter 5,** metaproteomics was employed to investigate the N-Damo pathway and identify key metalloenzymes involved.

A critical frontier in advancing denitrification-based wastewater treatment lies in elucidating metabolic pathways and identifying functional microorganisms responsible for the concurrent reduction of nitrate and micropollutant degradation. Integrating comprehensive microbiome analyses with multi-omics approaches—such as genomics, transcriptomics, proteomics, and metabolomics—offers a powerful means to unravel the complex biological processes underlying denitrification. By mapping metabolic networks and pinpointing the genetic and enzymatic determinants of micropollutant biotransformation, researchers can identify biomarkers or genetic traits linked to high degradation rates. This knowledge will enable the engineering or selective enrichment of efficient microbial strains, ultimately enhancing treatment performance and system stability.

8.2.4. Identification of Transformation Products

Future research should prioritize not only the removal of parent antibiotic compounds during denitrification but also the comprehensive identification and characterization of their transformation metabolites. In **Chapter 3** and **4**, we did not analyzed any transformation products (TPs) resulting from the reactor operation. In **Chapter 6**, we evidenced the formation of TPs evidencing that removal of parent compound is insufficient. Analyzing such metabolites, would help to assess if these by-products may retain residual antibiotic activity or exhibit unexpected toxicity, thereby posing additional environmental risks. Additionally, assessing the antimicrobial efficacy of these metabolites will provide insights into their ecological impact. This holistic approach will ensure that denitrification processes are optimized not only for the degradation of target micropollutants but also for the mitigation of potential secondary contamination, thereby advancing the field toward more effective and safer wastewater treatment solutions.

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Zou, H., He, J., Guan, X., Zhang, Y., Deng, L., Li, Y., Liu, F., 2021. Microbial responses underlying the denitrification kinetic shifting exposed to ng/L- and μg/L-level lomefloxacin in groundwater. Journal of Hazardous Materials 417. https://doi.org/10.1016/j.jhazmat.2021.126093

Appendix A
Published Arcticles & Licences

Title: How efficiently does a metabolically enhanced system with denitrifying anaerobic methane oxidizing microorganisms remove antibiotics?

Authors: Silvana Quiton-Tapia, Sabela Balboa, Francisco Omil, Juan Manuel Garrido, Sonia Suarez

CRETUS Institute. Department of Chemical Engineering, School of Engineering, Universidade de Santiago de Compostela, Rúa Lope Gómez de Marzoa, E-15782, Santiago de Compostela, Spain

Journal: Environmental Pollution Volume 334, 1 October 2023, 122033.

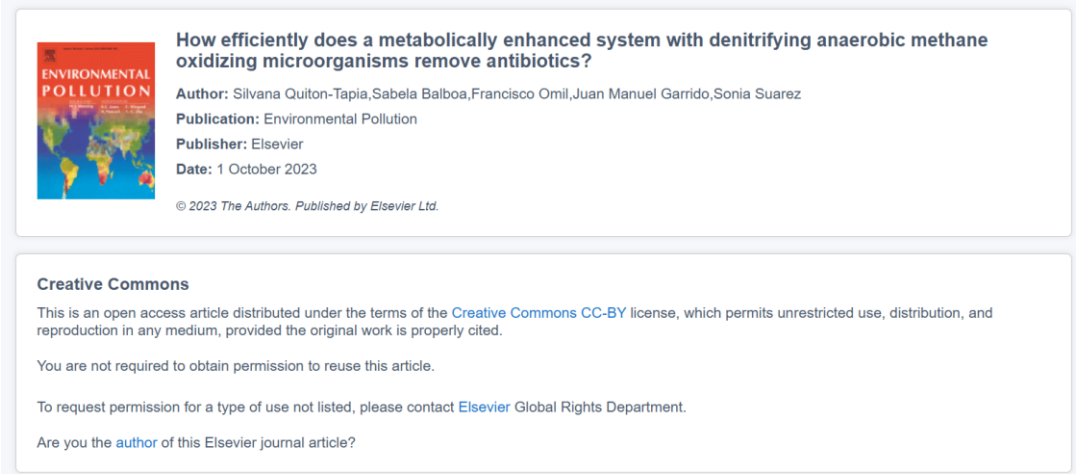
Rank from Journal of Citation Reports: **Q1** Highest percentile 98% 3/133 Toxicology

DOI: https://doi.org/10.1016/j.envpol.2023.122033

Author Contribution (CRediT): Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - Original Draft, Writing - Review & Editing, and Visualization.

Chapter reproducing the article content: Chapter 4

Rights and access: The article is Open Access. The author is allowed to include the content of the article in this thesis without requesting further permissions.



Title: Metalloenzymes play major roles to achieve high-rate nitrogen removal in N-damo communities: Lessons from metaproteomics

Autrors: Silvana Quiton-Tapia, Alba Trueba-Santiso,Juan M. Garrido, Sonia Suarez, Francisco Omil.

Journal: Bioresource Technology Volume 385, October 2023, 129476
Rank from Journal of Citation Reports: **Q1** Highest percentile 97% 6/197 Environmental Engineering

DOI: **https://doi.org/10.1016/j.biortech.2023.129476**

Author Contribution (CRediT): Methodology, Investigation, Data curation, Writing – review & editing.

Chapter reproducing the article content: Chapter 5

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Appendix B
Conference Proceedings

Oral presentations

Quiton-Tapia,S.; Balboa,S.; Suarez, S.; Omil, F. *Biotransformation of antibiotic micropollutants in a high-rate anoxic autotrophic reactor enriched with N-Damo.* 12th Micropol & Ecohazard Conference 2022. Santiago de Compostela, Spain, June 2022.

Quiton-Tapia,S.; Balboa,S.; Suarez, S.; Omil, F. *Evaluating antibiotic biotransformation during denitrification by heterotrophic and nitrite-driven anaerobic methane oxidation bacteria.* NOWELTIES' final conference. New Materials and Inventive Waste Water Treatment Technologies. Harnessing Resources Effectively through Innovation. Dubrovnik, Croatia, May 2022.

Quiton-Tapia, S., Martínez-Quintela, M., Suárez, S., Omil, F., Garrido, J.M. *Cerium as trace element to enhance nitrite-dependent anaerobic methane oxidation processes*. 5th IWASpecialized International Conference "Ecotechnologies for Wastewater treatment (EcoSTP)". Milan, Italy, June 2021.

CURRICULUM VITAE

Silvana Inés Quiton Tapia



Oost-Breukelderweg 92-5, 6721 PE

+31 658795520

silvana.quitontapia@wur.nl /silvanaquiton@hotmail.com

https://www.linkedin.com/in/silvanaquiton/

https://nowelties.eu/people/silvana-quiton-tapia/

Sex Female | Date of birth 19/04/1988 | Nationality Bolivian

WORK EXPERIENCE

October 2022 to today

Postdoc Researcher

University of Wageningen University & Research Centre, (Wageningen – The Netherlands).

Environmental Technology Department

Research focus: How DOC quality affects micropollutant biodegradation in drinking water aquifers?

- PARAFAC modelling for DOC characterization
- Micropollutant biodegradation tests using groundwater microorganisms

September 2019 to September 2022

PhD candidate (MSCA fellow)

University of Santiago de Compostela (Santiago de Compostela, Spain)

European Joint Doctorate NOWELTIES (Innovative Training Network from Horizon 2020)

Understanding biotransformation mechanisms of organic micropollutants during anoxic biological wastewater treatment.

- Study of cometabolic biotransformation of antibiotics in different denitrifying conditions (Heterotrophic denitrification and N-Damo cultures
- Operation of different type of reactors (MBRs, CFST)
- Insights of microbial community composition and evolution using16S rRNA gene amplicon sequencing.
- Identification and quantification of functional denitrifying genes using qPCR.

January 2019 to July 2019

Environmental Specialist

Empresa Boliviana de Alimentos EBA (La Paz- Bolivia).

Environmental management of 21 food plants with different production lines (dairy, honey, sweeteners, almonds, and quinoa) across Bolivia, responsibilities include:

- Supervise and provide technical support to wastewater treatment plants.
- Management of industrial safety and occupational health.
- Project planning.

April 2018 to September 2018

Research Internship

IHE-Delft Institute for Water Education (Delft –The Netherlands)

Independent researcher studying innovative biological treatments.

- Bioreactors set-up and operation for wastewater treatment analysis.
- Biological ammonium removal using photobioreactors with different algae and Anammox bacteria.

Grade: 8/10.

September 2017 to March 2019

Master Thesis

Environmental Technology Department, Wageningen University & Research Centre (Wageningen – The Netherlands).

Removal of micropollutants from industrial wastewater using constructed wetlands.

Topic: "The influence of biocides and salt on the biodegradation process in constructed wetlands".

Grade: 8/10.

September 2015 – July 2016

Environmental Engineer

Department of Wastewater and Solid Waste Management, Cochabamba Municipality (Cochabamba –Bolivia)

- Design, coordination, and assistance in the public management of solid and liquid waste.
- Project design, management, and supervision of related projects.
- Environmental quality of municipal wastewater treatment plant.

December 2014 – August 2015

Technical supervisor- Leachate treatment plant

TERSA S.A. operating La Paz city landfill (La Paz –Bolivia)

Operation and quality control of the leachate treatment plant. The job implied understanding of anaerobic biologic process for removal of pollutants. Duties included:

- Sampling and Laboratory analysis (DBO, DQO, Nitrogen, SST).
- Project elaboration and execution on site.
- Troubleshooting and improvement measures.

EDUCATION

2019-2022

PhD in Environmental Chemistry and Environmental Engineering

Joint PhD program between University of Santiago de Compostela (host institution) and RWTH Aachen University (mobility institution).
Expected submission by the end of 2022 (October)

2016 -2018

MSc. Environmental Sciences track Environmental Technology

Wageningen University & Research Centre (Wageningen – The Netherlands).

Thesis: "The impact of biocides on biodegradation in constructed wetlands" Grade: 8/10.

Courses included:

- Biological Process for Resource recovery
- Physical and Chemical Processes for Water Treatment and Reuse
- Renewable Energy: Sources, Technology & Applications

2005-2012

BSc. Environmental Engineering

Thesis: "Implementation and evaluation of the removal of physical and chemical parameters according to two species (Scirpus californicus y Typha dominguensis) in the constructed wetlands of the Granja Modelo Pairumani". Grade: 92/100.

PERSONAL SKILLS

Mother tongue(s) Spanish

Other language(s)	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	C2	C2	C1	C1	C2
Galician	C1	C1	B1	A1	A2
French	A2	A1	A1	A1	A1

Levels: A1/A2: Basic user - B1/B2: Independent user - C1/C2 Proficient user
Common European Framework of Reference for Languages

Communication skills Proficient writing and communication skills with interdisciplinary team.

Organisational / managerial skills Coordination and connection with professionals at different levels of influence, especially during my work at the municipality I had to relate with onsite workers and also executive-level authorities that included executive meetings with the Mayor himself.

Laboratory skills

- Operation of column equipment: HLPC, Radio-HPLC, LC-MS/MS for micropollutant analysis.
- Chromatography: GC, TLC
- Molecular techniques: DNA, RNA manipulation and extraction, PCR and qPCR, microbial community composition by16S rRNA gene sequencing
- Reactor operation: MBR, CFST
- Batch experiments: microcosm experiments for micropollutant degradation

ACADEMIC RECORD

Publications : Articles in peer-reviewed journals

Wagner, T., Helmus, R., **Quiton Tapia**,S, Rijnaarts, Pim de Voogt, Alette A.M. Langenhoff, John R. Parsons. (2020). *Non-target screening reveals the mechanisms responsible for the antagonistic inhibiting effect of the biocides DBNPA and glutaraldehyde on benzoic acid biodegradation.* Journal of Hazardous Materials. https://doi.org/10.1016/j.jhazmat.2019.121661

Quiton-Tapia *Silvana; Balboa, Sabela; Omil, Francisco; Garrido, Juan Manuel; and Suarez, Sonia. How efficiently does a metabolically enhanced system with denitrifying anaerobic methane oxidizing microorganisms remove antibiotics?.(2023) Environmental Pollution 334, 12203 https://doi.org/10.1016/j.envpol.2023.122033*

Quiton-Tapia*, Silvana; Trueba-Santiso, Alba; Garrido, Juan Manuel; Suarez, Sonia; and Omil, Francisco. Metalloenzymes play major roles to achieve high-rate nitrogen removal in N-damo communities: Lessons from metaproteomics.(2023) Bioresource Technology 385, 129476. https://doi.org/10.1016/j.biortech.2023.129476*

Conference proceedings: *Cerium as trace element to enhance nitrite-dependent anaerobic methane oxidation processes.*

Quiton-Tapia, S., Martinez-Quintela, M., Trueba-Santiso, A., Suarez, S., Omil, F., and Garrido, J.M.

Oral presentation EcoSTP IWA Ecotechnologies for Wastewater Treatment. 23th June 2021.Milan, Italy.

Biotransformation of antibiotic micropollutants in a high-rate anoxic autotrophic reactor enriched with N-Damo **Quiton-Tapia, S.**, A., Suarez, S., Garrido, J.M., and Omil, F.

Oral presentation Micropol & Ecohazard Conference IWA. 6-10th June 2022. Santiago de Compostela, Spain.

Specialized Courses:

Management Of Micropollutants In The Urban Water Cycle. Summer course. (5 -9 July, 2021)Organized by INRAE. Narbonne, France.

Advanced technologies for biological treatment of wastewater and design of hybrid systems and Advanced oxidation and electrochemical processes (April 2021) Organized by NOWELTIES. Virtual mode.

Introductory Training School for cutting-edge technologies for wastewater treatment and resource recovery. (7 -11th October 2019) Organized by NOWELTIES. Santiago de Compostela, Spain

HONORS AND AWARDS

Second prize for Best poster presentation Aquaconsoil conference 2023. Prague Czech Republic. Marie Sklodowska-Curie fellowship for doctorate studies (2019-2022).

Scholarship recipient for postgraduate studies “100 Scholarships in Science, Technology and Heath”

Awarded by the Ministry of Education of Bolivia (2016)

Second best average for the bachelor Environmental Engineering (2013) Awarded by the Universidad Católica Boliviana- San Pablo, Cochabamba-Bolivia

