Ph.D. Thesis

University of Limoges
École Doctorale Sciences et Ingéniere
E2Lim, UR 24 133

Universidad Pablo de Olavide
Biotecnología, Ingeniería y Tecnología Química

A thesis submitted to University of Limoges and Universidad Pablo de Olavide
in partial fulfillment of the requirements of the double degree of
Doctor of Philosophy
Eau, Sol, Environnement & Biotecnología, Ingeniería y Tecnología Química

On June 14, 2024

Presented and defensed by
Eric CAROCA SEPÚLVEDA

Characterization of the labile metal fraction in complex matrices containing microbes

Thesis supervisor: Gilles GUIBAUD, Rémy BUZIER, Stéphane SIMON,
Fernando FERMOSO and Soraya ZAHEDI

JURY :

President of jury	
M. Emmanuel GUILLON	Professeur des universités Université de Reims Champagne-Ardenne
Reviewers	
M. Eric VAN HULLEBUSCH	Professeur des universités, Université Paris Cité - Institut de Physique du Globe de Paris
M. Bagdhad OUDDANE	Professeur des universités Université de Lille
Examiners	
Mme. Véronique DELUCHAT	Professeure des universités Université de Limoges

This research was conducted within European Union's Horizon 2020 Marie Sklodowska-Curie grant (Nº 861088): M2ex “Exploiting metal-microbe applications to expand the circular economy”

Once upon a time, a cock wished to play the dog. But he had no luck because everyone recognized him right away (...) on the other hand, the dog that wished to play the cock was never recognized.

The Bald Soprano, Eugene Ionesco

Acknowledgements

At the end of this stage, which has been a great challenge at the professional and personal level, I want to thank the people and institutions that have been part of this process.

First of all, I want to thank European Union's Horizon 2020 Marie Skłodowska-Curie Innovative Training Network for the funding and the members of the project M2ex "Exploiting metal-microbe applications to expand the circular economy" for the opportunity of this European Joint Doctorate and the organization of the workshops and training activities during these three years.

To my supervisors at UNILIM, Rémy Buzier, Stephane Simon, and Gilles Guibaud, and at the UPO-CSIC, Fernando Fermoso, and Soraya Zahedi for their dedication, mentorship, and guidance during this period. I also want to include Jimmy Roussel and his team during my stay at the Luxembourg Institute of Science and Technology. My gratitude to the technical and administrative members who helped me develop the experiments and solve all paperwork problems. Lourdes, Karine, Emanuelle and Patrice at UNILIM, Victor at UPO-CSIC and Ana at the M2ex project.

During this period I met wonderful people and had the opportunity to meet again friends from the past, I thank all of them for their sympathy, the conversations, and the laughter we shared. To all 13 ESRs, with their different backgrounds, cultures, and points of view. To Alexandra and Nicolas, for the memorable moments. New and old friends at UPO-CSIC. Juan, Ángeles, Rafael, Paco, Ramón, Guillermo, and Africa, it was a pleasure to see you again, and the new ones, Elena, Manuel, Merchi, the neighbor Abigail, María, and Nicoló. I also want to include Susan and Kris for all the adventures we had in Andalusia. I cannot leave out the amazing people with whom I shared the theatre workshop: “la lumínica” María and all the souls trapped in the Hotel Strange, thank you for all your energy, teachings, support, and stories.

Finally, I extend my gratitude to my family, to my mother, my brother, and my sisters. To my cousins, uncles, and grandmothers. Thank you for your love from the other side of the ocean. Special thanks to my life friends Sandiaza and Vely, and my college friend Jeni, for being my confidants, your invaluable distraction and always having a supportive phrase.

To all, thank you very much.

Abstract

Trace elements (TE) are necessary for anaerobic digestion (AD) since many are essential in the metabolic pathways of microorganisms. However, depending on their concentration, they can also have inhibitory or toxic effects. The digestate, a by-product of AD, can be used as fertilizer but the presence of TE could impede its use due to potential contamination in soil and crops. TE can be present under different species amongst which only the bioavailable ones are responsible for the biological response. Diffusive gradients in thin films (DGT) is a passive sampling technique that quantifies the labile fraction, which can be related to the bioavailable fraction. DGT has been studied in waterbodies, sediments, and solids systems, but its application to anaerobic digestates is not straightforward and the methodology must be carefully adapted. The main objectives of this research were to analyze the impact of the presence of a trace element, Cobalt (Co), in the operation of an anerobic reactor and to evaluate the feasibility of using the DGT technique in anaerobic systems: in digestates and also directly inside a bioreactor.

Firstly, the effect of continue addition of different concentrations of Co in an upflow anaerobic sludge bed reactor (UASB) was studied. UASB performance was monitored at three different positions along the reactor: bottom and top sludge, and liquid zone. Co concentration for solid (granules), liquid soluble, and extracellular polymeric substances (EPS) were measured in these samples. Biogas production was affected positively for Co dose lower and equal to 50 µM of Co and negatively for Co dose higher than 100 µM of Co. A general decrease in EPS was observed due to Co dose, which compromised the granular stability. No strong differences were observed in Co profile along the depth in the reactor for solid and liquid soluble fractions. The solid Co concentration was around 1000 times higher than the liquid concentrations, indicating a high accumulation of Co in the granules. However, the Co accumulated was found as inert species.

In a second stage, Chelex, Zirconia, and Thiol DGT devices were deployed in two different digestates to evaluate the applicability of the passive sampling technique. Time-series deployments were performed in presence and absence of air to determine whether maintaining anaerobic conditions is mandatory. A theoretically expected linear accumulation profile was observed for some TE only (Fe, Mn, Ni or As(III)), depending on the digestate, whereas other elements were detected without a consistent trend over time (Al, As, and Cu). The labile fraction of Co, Cu, Fe and Mn demonstrated changes over a 72 h period when deployments occurred in the presence of air.

In the third stage, DGT devices were deployed inside the UASB under Co stress to assess the feasibility of using DGT directly inside a reactor. Biogas accumulation was observed inside the DGT, which invalidated the device. Different deployment configurations were evaluated to limit the phenomenon. The valid deployment was restricted to a perpendicular flow for a maximum of 4 h in the liquid zone, and 8 h in the sludge zone. Additionally, repeatability problems were observed, which indicates the presence of unidentified problems still to be solved.

DGT thus appears applicable to evaluate the labile fraction of some TE in digestates but requires careful verification. Consequently, it is recommended to conduct time-series deployments to identify only the elements displaying a linear accumulation trend. Anaerobic conditions should be maintained during deployment to avoid significant shifts in the labile fraction over time. The direct deployment of DGT in an UASB induced some problems and limitations still to be solved.

French

Les éléments traces sont nécessaires à la digestion anaérobie car plusieurs d'entre eux jouent un rôle essentiel dans les voies métaboliques des micro-organismes. Cependant, leur concentration peut également avoir des effets inhibiteurs ou toxiques. Le digestat, un sous-produit de la digestion anaérobie, peut être utilisé comme engrais, mais la présence d'éléments traces pourrait en limiter l'utilisation en raison d'une contamination potentielle du sol et des cultures. Ces éléments traces peuvent être présents sous différentes formes chimiques et seule la fraction biodisponible est responsable de la réponse biologique. La technique de gradients de diffusion en couches minces (DGT) est une méthode d'échantillonnage passif qui permet de quantifier la fraction labile, pouvant être reliée à la fraction biodisponible. Elle a été étudiée pour les eaux, les sédiments et les sols. Son application aux digestats anaérobies pourrait s'avérer pertinente mais la méthodologie doit être soigneusement adaptée.

Les principaux objectifs de cette recherche étaient d'analyser l'impact de la présence d'un élément trace, le cobalt (Co), sur le fonctionnement d'un réacteur anaérobie et d'évaluer la faisabilité de l'utilisation de la technique DGT dans le contexte de la digestion anaérobie : dans les digestats et directement à l'intérieur d'un bioréacteur.

Dans un premier temps, l'effet de l'ajout continu de différentes concentrations de Co dans un réacteur à lit de boues anaérobies ascendant (UASB) a été étudié. Les performances de l'UASB ont été suivies à trois positions différentes le long du réacteur : boues de fond, boue de surface et zone liquide. Les concentrations en Co des fractions solides (granules), liquide soluble et substances polymériques extracellulaires (EPS) ont été mesurées dans ces échantillons. La production de biogaz a été affectée positivement pour des doses de Co inférieures ou égales à 50 µM et négativement pour des doses supérieures à 100 µM. Une diminution générale des EPS a été observée en raison de la dose de Co, ce qui a compromis la stabilité des granules. Aucune différence significative n'a été observée dans le profil de Co en fonction de la profondeur dans le réacteur pour les fractions solide et liquide soluble. La concentration en Co particulaire était environ 1000 fois plus élevée que les concentrations solubles, indiquant une forte accumulation de Co dans les granules. Cependant, le Co accumulé s'est avéré être sous des formes inertes.

Dans une deuxième étape, des dispositifs DGT de type Chelex, Zirconium et Thiol ont été déployés dans deux digestats différents pour évaluer l'applicabilité de la technique d'échantillonnage passif. Des déploiements de durées variables ont été réalisés en présence et en absence d'air afin de déterminer si le maintien de conditions anaérobies était nécessaire. Le profil d'accumulation linéaire théoriquement attendu n'a été observé que pour certains éléments traces (Fe, Mn, Ni ou As(III)), selon le digestat, tandis que d'autres éléments ont été détectés sans tendance cohérente (Al, As et Cu). La fraction labile de Co, Cu, Fe et Mn a montré des variations sur une période de 72 heures lorsque les déploiements ont eu lieu en présence d'air.

Dans une troisième étape, des dispositifs DGT ont été déployés à l'intérieur de l'UASB supplémenté en Co pour évaluer la faisabilité de l'utilisation des échantillonneurs DGT directement à l'intérieur d'un réacteur. Une accumulation de biogaz a été observée à l'intérieur des dispositifs DGT, ce qui a invalidé leur utilisation. Différentes configurations de déploiement

ont été évaluées pour limiter ce phénomène. Un déploiement considéré valide a été obtenu pour un flux perpendiculaire pendant une durée maximale de 4 h dans la zone liquide et de 8 h dans les boues. De plus, des problèmes de reproductibilité ont été observés, ce qui indique la présence de problèmes non identifiés qui doivent encore être résolus.

La DGT semble donc applicable pour évaluer la fraction labile de certains éléments traces dans les digestats, mais nécessite une vérification minutieuse. Par conséquent, il est recommandé d'effectuer des déploiements de durées variables pour identifier uniquement les éléments présentant une tendance d'accumulation linéaire. Les conditions anaérobies doivent être maintenues pendant le déploiement pour éviter des variations importantes de la fraction labile dans le temps. Le déploiement direct des échantillonneurs DGT dans un UASB a induit des problèmes qui doivent encore être résolus.

Spanish

Los elementos traza (TE) son necesarios para la digestión anaerobia (AD) ya que muchos son esenciales en las rutas metabólicas de los microorganismos. Sin embargo, dependiendo de su concentración, también pueden tener efectos inhibidores o tóxicos. El digestato, un subproducto de la AD, puede ser utilizado como fertilizante, pero la presencia de TE podrían impedir su uso debido a la posible contaminación en el suelo y los cultivos. Los TE pueden estar presentes en diferentes especies entre las cuales solo las biodisponibles son responsables de la respuesta biológica. Los gradientes de difusión en películas delgadas (Difuisive Gradient in Thin films, DGT) es una técnica de muestreo pasivo que cuantifica la fracción lábil, que puede estar relacionada con la fracción biodisponible. DGT ha sido estudiado en cuerpos de agua, sedimentos y sistemas sólidos, pero su aplicación en digestatos anaerobios no es sencilla y la metodología debe adaptarse cuidadosamente. Los principales objetivos de esta investigación fueron analizar el impacto de la presencia de un TE, cobalto (Co), en el funcionamiento de un reactor anaerobio y evaluar la viabilidad de utilizar la técnica DGT en sistemas anaerobios: en digestatos y también directamente dentro de un biorreactor.

En primer lugar, se estudió el efecto de adición continua de diferentes concentraciones de Co, en un reactor de lecho anaerobio de flujo ascendente (Upflow Anaerobic Sludge Bed, UASB). El reactor UASB fue monitoreado en tres posiciones diferentes a lo largo del reactor: lodo inferior y superior, y zona líquida. En estas muestras se midió la concentración de Co para sólidos (gránulos), líquidos solubles y sustancias poliméricas extracelulares (Extracellular Polymeric Substances, EPS). La producción de biogás se vio afectada positivamente para las dosis de Co inferior e igual a 50 µM de Co y negativa para las dosis de Co superior a 100 µM de Co. Se observó una disminución general de los EPS debido a la dosis de Co, que comprometió la estabilidad granular. No se observaron fuertes diferencias en el perfil de Co a lo largo de la profundidad en el reactor para fracciones solubles sólidas y líquidas. La concentración de Co sólido fue alrededor de 1000 veces mayor que las concentraciones de líquido, lo que indica una alta acumulación de Co en los gránulos. Sin embargo, el Co acumulado se encontró como especie inerte.

En una segunda etapa, se evaluaron dispositivos DGT Chelex, Zirconia y Thiol en dos digestatos anaerobios diferentes para evaluar la aplicabilidad de la técnica de muestreo pasivo. Se evaluaron despliegues de series de tiempo en presencia y ausencia de aire para determinar si es obligatorio mantener las condiciones anaerobias. Se observó el perfil de acumulación lineal teóricamente esperado solo para algunos TE (Fe, Mn, Ni o As(III)), dependiendo del digestato, mientras que otros elementos se detectaron sin una tendencia constante a lo largo del tiempo (Al, As y Cu). La fracción lábil de Co, Cu, Fe y Mn demostró cambios durante un período de 72 horas cuando se produjeron despliegues en presencia de aire.

En la tercera etapa, los dispositivos DGT fueron desplegados dentro del reactor UASB bajo estrés de Co para evaluar la viabilidad de usar DGT directamente dentro de un reactor. Se observó acumulación de biogás dentro de los DGT, lo que invalidó el dispositivo. Se evaluaron diferentes configuraciones de implementación para limitar el fenómeno. El despliegue válido se restringió a un flujo perpendicular durante un máximo de 4 h en la zona líquida y 8 h en la

zona de lodo. Además, se observó un problema de repetibilidad, lo que indica la presencia de un problema no identificado aún por resolver.

Por lo tanto, DGT parece aplicable para evaluar la fracción lábil de algunos TE en digestatos, pero requiere una verificación cuidadosa. En consecuencia, se recomienda realizar despliegues en series de tiempo para identificar solo los elementos que muestran una tendencia de acumulación lineal. Las condiciones anaerobias deben mantenerse durante el despliegue para evitar cambios significativos en la fracción lábil a lo largo del tiempo. El despliegue directo de la DGT en una UASB provocó algunos problemas y limitaciones que aún no se han resuelto.

Rights

Table of Contents

List of Figures

List of Tables

Introduction

Waste generation, both solid and liquid, is a problem that currently affects humanity since if not treated, waste has a negative environmental impact. These impacts include pollution to soil, aquifer systems and the atmosphere. For example, generation of leachate with pollutants that percolate the soil, greenhouse gas emissions due to the decomposition of organic matter and eutrophication of rivers and lakes by the discharge of nitrogen and phosphorus into them. Solid waste generation in 2022 was estimated at 2.24 billion tons. Among these, 33% is disposed of in open dumps, 37% is treated in landfills and only 14% is recycled. The rest is treated by incineration or composting. Moreover, an increase to 3.88 billion tons is expected in 2050 (Kaza et al., 2018). Similarly, it is estimated that 380 billion m^3 of wastewater are produced annually worldwide (IPCC, 2023), where about 58% of the household wastewater were treated in 2022 (United Nations Department of Economic and Social Affairs, 2023). An increase to 471 billion m^3 by 2030 and 574 billion m^3 by 2050 is expected in wastewater generation (European Investment Bank & Environment and Natural Resources Department, 2022).

At the same time, energy consumption has been increasing. In 2022, the global energy consumption was estimated at 644 exajoules, where 77% was covered by fossil sources (oil, coal, and natural gas) and 20% by different renewable sources. Additionally, global energy consumption continues increasing, around 1% to 2% per year (Energy Institute, 2023). The highest percentage of energy being obtained through non-renewable fuels has caused a substantial increase in the emission of CO_2 and other polluting gases such as NO_x and SO_x, which has led to serious environmental problems such as global warming (IPCC, 2018). That is why in recent decades the use of new sources of energy has been sought and enhanced, which are renewable and non-polluting. Obtaining energy through waste brings various advantages: i) the negative impact of uncontrolled waste disposal is avoided and ii) it can be a renewable and clean source of energy.

Among the options for obtaining energy from waste, anaerobic digestion (AD) appears as a sustainable option for the treatment of solid and liquid organic waste. Biogas from AD is known to be the most cost-effective technology and environmentally sound compared to other biofuels. The energy gain/input ratio in AD was estimated at 29 MJ/MJ, which outcompetes in energy efficiency the other technologies for energy production from biomass (Paudel et al., 2017). In AD process, the organic matter is decomposed by a consortium of microorganisms in no free oxygen conditions. The main product, called biogas, is a mixture of gases composed mainly of CH_4 and CO_2 which can be used as fuel. An additional by-product is the digestate, a semi solid remnant containing non-degraded matter and biomass. Given its nitrogen and phosphorus content, the digestate has the potential to be used as fertilizer, although the content of microorganisms, metals and other pollutants may induce difficulties.

Although the AD is a consolidated and widely studied technology, there are still several challenges that must be solved: the low digestibility of certain wastes, the increase of biogas productivity and the understanding of the biochemical interactions between the different substances present in the system, in particular the quantification and description of biochemical reactions involving trace elements. For a correct performance of the AD, the presence of certain trace elements is required, such as Fe, Co, and Ni. Trace elements are necessary for proper cellular functioning and some of these are essential on the metabolic pathways that convert organic matter to the biogas. However, in high concentrations, these trace elements can have an inhibitory or toxic effect. The evaluation of trace elements levels is therefore necessary to understand and estimate the performance of the AD system. However, several factors make the recommended (or optimal) concentration not unique to the AD process. One of the most relevant factors to consider is the chemical speciation of trace elements. Indeed, in an AD system, trace elements can be found in different species, for example as free ions, complexes, precipitates, among others. Within all these species, only the bioavailable species are those that have a biological effect.

Various analytical techniques have been used to approximate the bioavailable fraction of trace elements in anaerobic digestion, such as the determination of the liquid fraction, the free ionic fraction or some specific fractions obtained through sequenced extractions. However, all these techniques represent a "potentially bioavailable" fraction which may not include all possible bioavailable species or including more species than only the bioavailable ones. In addition, many of these techniques in their methodology can induce errors in quantification. The presence of air or the addition of certain reagents can change the equilibrium between species and modify the composition of the bioavailable fraction, so a technique is required that minimizes the alterations in the system to be investigated. In addition, the bioavailable fraction is usually found in orders of concentration that are not reached by these techniques, showing a lack of sensitivity. A passive in-situ sampling technique, the Diffusive Gradient in Thin Films (DGT), has appeared as an alternative to approximate the bioavailable fraction. DGT quantifies the labile fraction, which has been shown to be more related to the bioavailable fraction than other fractions. Initially used for watercourses, the use of DGT has been expanded to soils, sediments and recently to waste matrices. DGT technique has been previously studied in AD samples, but currently the number of studies is limited and more data is needed to reliably apply this technique in the complex AD matrix.

In this thesis two main objectives are evaluated. The first objective is the analysis of the impact of the presence of a trace element, Cobalt (Co), in an anaerobic reactor type UASB. Generally, studies focus on some specific response, such as biogas production, but this thesis will evaluate the overall impact, i.e., on several responses. More specifically, different fractions of Co are characterized along the reactor for each dose, in parallel of the characterization of the residual organic matter quantity and quality. The second objective is the evaluation of the effectiveness of the DGT technique for the determination of the labile fraction in digestates, as well as the evaluation of the requirement of oxygen-free conditions for its deployment. Additionally, the effectiveness and applicability of DGT is tested *in-situ* in an anaerobic reactor.

In this way, the thesis is structured in different chapters, as follows:

Chapter I: A literature review is presented. Participation of trace elements in AD is described, as well the recommended interval concentration found in literature. The diverse fractionation techniques that has been applied in AD system are indicated, and the main results discussed. Finally, the DGT technique is described. the components, the fundaments, and the studied were DGT was applied in digestates are presented.

Chapter II: The impact of Cobalt on the AD process is evaluated in a UASB reactor. Several responses, including biogas productivity, organic matter removal, and extracellular polymeric substances concentration are selected and related to the different fractions of Co measured.

Six continuous doses of Co are evaluated, covering recommended and possibly inhibitory concentrations. The Co profiles are described along the reactor through three fractions: total, total liquid and soluble liquid.

Chapter III: The validity of the DGT technique is examined in two different digestates. Three types of DGTs are used to encompass cationic metals, oxyanions and reduced metalloid species. In parallel, air exposition is assessed, to determine whether maintaining anaerobic conditions during DGT deployment is required.

Chapter IV: *In situ* application of the DGT technique in AD reactor is explored. DGT devices are deployed during the operation of the Chapter 1 UASB reactor at the same time when the samples for Co concentration are taken. The problems and limitations observed during the use of the DGT in this AD reactor are presented, and the results of the validation of the DGT technique are commented.

References

Energy Institute. (2023). *Statistical Review of World Energy* (72nd edition).

European Investment Bank & Environment and Natural Resources Department. (2022). *Wastewater as a resource: May 2022.* Publications Office. https://data.europa.eu/doi/10.2867/31206

IPCC. (2018). *Global Warming of 1.5°C. An IPCC Special Report on the impacts of global warming of 1.5°C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change, sustainable development, and efforts to eradicate poverty* (In Press).

IPCC. (2023). *Climate Change 2022 – Impacts, Adaptation and Vulnerability: Working Group II Contribution to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change* (1st ed.). Cambridge University Press. https://doi.org/10.1017/9781009325844

Kaza, S., Yao, L. C., Bhada-Tata, P., & Van Woerden, F. (2018). *What a Waste 2.0: A Global Snapshot of Solid Waste Management to 2050*. Washington, DC: World Bank. https://doi.org/10.1596/978-1-4648-1329-0

Paudel, S. R., Banjara, S. P., Choi, O. K., Park, K. Y., Kim, Y. M., & Lee, J. W. (2017). Pretreatment of agricultural biomass for anaerobic digestion: Current state and challenges. *Bioresource Technology*, *245*, 1194–1205. https://doi.org/10.1016/j.biortech.2017.08.182

United Nations Department of Economic and Social Affairs. (2023). *The Sustainable Development Goals Report 2023: Special Edition*. United Nations. https://doi.org/10.18356/9789210024914

Chapter I. Background

I.1. Trace Metals in Anaerobic Digestion

I.1.1. Anaerobic Digestion

Anaerobic digestion (AD) is becoming a key method for both waste reduction and recovery of renewable fuel and other valuable co-products (Chen & Cheng, 2005). A wide variety of organic waste can be treated by AD, both liquid and solid, household and industrial. AD process is recognized as a versatile technology that has already established itself as an environmentally friendly and economically advantageous technology for organic waste treatment and management. The benefits of the AD include high resilience to high organic loading rates, low sludge yield, low energy consumption, and the potential for energy neutralization through biogas production with a high energy gain/input ratio, estimated at 29 MJ MJ^{-1} (Cecchi & Cavinato, 2015; Paudel et al., 2017; X. Zhang et al., 2024). The first AD reactors date back to the late 19th century (Metcalf & Eddy, 2014) and the existing number of AD plants in Europe have increased over the last two decades, with 6507 AD plants in 2009 to 18843 AD plants and 1067 biomethane plants in 2021 (European Biogas Association, 2022).

AD is a complex process by which microorganisms degrade complex organic matter and produce biogas and requires strict anaerobic conditions (oxidation reduction potential < -200 mV). As a result of this process, the organic matter is transformed into a gas mix composed mostly of CH_4 and CO_2, with a small fraction of H_2, NH_3, and H_2S. The non-degraded organic matter and biomass remain in a semisolid phase called digestate, which is rich in N and P, as well as metals. The AD of organic matter follows 4 steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. In the hydrolysis step, insoluble organic material and high molecular weight compounds such as lipids, polysaccharides, proteins, and nucleic acids, are hydrolyzed by hydrolytic enzymes into soluble monomers (e.g. saccharides, amino acids, and fatty acids). In the acidogenic step, the monomers are further decomposed into short-chain volatile fatty acids (VFA) through the function of acidogenic (or fermentative) bacteria. Other by-products are formed as ammonia (NH_3), CO_2, and H_2S. In the acetogenesis step, organic acids with more than 2-carbon molecules and alcohols will eventually be decomposed by acetogens into acetic acid as well as CO_2 and H_2. Finally, in the methanogenesis stage, methane is produced by 2 groups of methanogenic bacteria: the acetogenotrophic group which splits acetate into CH_4 and CO_2, and the hydrogenotrophic group which uses H_2 and CO_2 to produce CH_4 (Appels et al., 2008; X. Zhang et al., 2024).

Various parameters must be considered for a correct performance of AD: temperature, pH, alkalinity, presence of VFA, adequate proportion of macronutrients (mainly carbon, nitrogen and phosphorus), as well as the presence of certain trace elements (Appels et al., 2008; Fermoso et al., 2019b). Similarly, certain operational parameters must be considered so as not to impair the performance of AD, such as solid retention time, hydraulic retention time, the organic loading rate, mixing and substrate limitation (Bajpai, 2017). The effects on AD of temperature, pH, presence of VFA and nutrient ratio have been extensively studied. In fact they are basic parameters when maximization of the production of biogas or some specific byproduct is expected. For example, pH and presence of VFA have been studied in production of biohydrogen through AD, suppressing methanogenesis (Özen Daş et al., 2024), or in AD split in 2 stages (Phan et al., 2023). The nutrient ratio is analyzed in co-digestion processes, where two or more different substrates are mixed and degraded through AD (Esposito et al., 2012). Similarly, operational parameters have been extensively studied and have given rise to bioreactors such as upflow anaerobic sludge bed (UASB) and expanded granular sludge bed (EGSB) which operate at high organic charges and low retention time (Metcalf & Eddy, 2014). Although AD is a well-studied process and consolidated technology, the study of the effect of trace elements on AD performance is a recent and developing area.

I.1.2. Role of trace elements in anaerobic digestion

Trace elements play a significant role in microbial activity. The participation of trace elements is multiple and diverse within the activity of microorganisms. As can be seen in Table I.1, trace elements are involved in different metabolic activities, participating in enzyme synthesis, being co-factors and enzyme activators. Additionally, they can inhibit the metabolism and influence the effect of other elements and substances. Some enzymes (or group of enzyme) are associated with more of one trace element. Iron, nickel, and selenium are associated with Carbon monoxide dehydrogenase (CODH). The synthesis of Acetyl-CoA is supported by iron, molybdenum, and selenium. Hydrogenases involve a high number of different trace elements: cobalt, iron, molybdenum, nickel, and selenium.

Table I.1: Trace elements and their role in microorganisms (Matheri et al., 2021; Schattauer et al., 2011)

Element	Role and General functions (microorganisms)
Boron	cofactor of enzymes
Chromium	metabolism of the glucose
Cobalt	activator to the metallic enzyme present in corrinoids inhibit metabolism required for synthesis of vitamin B_{12} (cyanocbalamin) activates carboxylpeptidase participation in mechanism of CODH[a]
Copper	metallic enzyme activator present in hydrogenase and SODM[b] in methanogens, in anaerobic and clostridic facultative metabolism inhibition chelates other substances and reduces their toxicity act as a pigment
Iron	enhance redox property act as an electron acceptor in cytochromes (Fe^{3+}) synthesis of catalase, peroxidase, acotinase, and Acetyl-CoA essential in ferredoxin hydrogenase, in CODH[a] and FDH[c]
Magnesium	enzyme activator for number of kinases and phosphotransferase
Manganese	activates isocitric dehydrogenase and malic enzymes of bacteria stabilizes methyltransferase in methane producing bacteria enhancement to the redox reaction often interchangeable with magnesium in kinase reactions co-factor to the specific enzymes

Molybdenum	present in FDH[c] and hydrogenases synthesis of Acetil-CoA inhibitor to the sulphate reducing bacteria co-factor to various flavinous enzymes
Nickel	synthesis of co-enzymes factor F430, CH3-CoM reductase co-factor of urease stabilizes the DNA, RNA present in CODH[a] and hydrogenases
Selenium	participated in hydrogenases, CODH[a], and Acetil-CoA. FDH[c] in methane producing bacteria and clostridia
Zinc	act as an activator to the metallic enzyme cell growth stimulator inhibit metabolism and exacerbate the toxic effects of other trace elements hydrogenase in methane producing and sulphate reducing bacteria activity of carbonic anhydrase and carboxylpeptidase A cofactor of RNA- and DNA-polimerase present in FDH[c] present in SODM[b]

a CODH: carbon monoxide dehydrogenase
b SODM: superoxide dismutase
c FDH: formate dehydrogenase

Regarding the AD process, the degradation and transformation of nutrients and metabolic products in each of the stages is performed by enzymes, which may require certain trace elements for their activation and operation. The degradation of polysaccharides in the hydrolysis stage in AD is mostly performed by extracellular enzymes, with no trace element requierements. The involved microorganisms require basic set of trace elements for their cellular metabolism, usually in redox process. Co, Fe, Ni and Zn are typically involved in these metalloenzymes (Garuti et al., 2018). Then sugars are fermented in the acedogenesis stage producing low molecular weight products, such as VFA, ethanol, CO_2 and H_2. The electron-transport mechanisms used by the bacteria in this stage depend on particular trace elements to keep them functional. Usually, Fe-dependent hydrogenases and cytochromes mediating electron transport are involved in these pathways (Lehninger et al., 2003).

In the final stage of AD (methanogenesis) acetate, CO_2 or methyl-compounds are converted to methane. Around 2/3 of the CH_4 is produced by oxidation of acetate, and 1/3 by reduction of CO_2 with electrons from H_2, and conversion from compounds with methyl groups, such as methanol, methylamines, and dimethylsulfide. Thus, three pathways can be found in methanogenesis: acetoclastic (acetate oxidation), hydrogenotropic (CO_2 reduction) and methylotropic (C1 conversion). The three pathways differ in the enzymes used to generate methyl-tetrahydromethano(sarcina)pterin, but converge in the last two steps for CH_4 generation (Glass & Orphan, 2012). Figure I.1 shows the enzymes involved in each methanogenic pathway indicating the major trace element requirements. Many enzymes involved in all the methanogenic pathways are metalloenzyme, i.e, enzymes that require the presence of trace elements (Zerkle, 2005). The requirements of trace elements in each step are presented in Glass & Orphan (2012), and the exact requirement may differ depending of the pathway. However, the general trend is similar in order of abundance: Fe, Ni and Co, Mo and/or W, and Zn. Fe is present in all the pathways, almost every metalloenzyme contains Fe,

Fe_2S_2, Fe_3S_4, or Fe_4S_4 clusters. In hydrogenotropic pathway the first enzyme, formylmethanofuran dehydrogenase, can bind up to 9 Fe_4S_4 clusters binding 8 to polyferredoxin and 9 to a Mo or W-pterin (Vorholt et al., 1996). The four different types of Ni–Fe hydrogenases involved in hydrogenotropic pathway all contain Fe. They contain 3 Fe_4S_4 clusters and a Ni–Fe active site (Thauer, 2011), and additionally polyferredoxin subunits with 6, 10, or 14 Fe_4S_4 clusters can be present (Tersteegen & Hedderich, 1999). Continuing the hydrogenotropic pathway, a group of Ni-Fe hydrogenase that contains 4 Fe_4S_4 clusters, reduces coenzyme F420 with H_2 (Fox et al., 1987). In Ni-limited conditions, some hydrogenases can be substituted by Ni-free Fe hydrogenase (Afting et al., 1998). If the electron source is formate instead of H_2, Ni-Fe hydrogenases are replaced by formate dehydrogenase, which contains 1 Mo/W-pterin cofactor and 2 Zn atoms (Schauer & Ferry, 1986). In acetoclastic pathway, the enzyme Carbon monoxide dehydrogenase/acetyl-CoA synthase is the enzyme that requires most amount of trace elements, with 5 Fe_4S_4 clusters, 1 $NiFeS_4$ cluster and 1 reduced 2×[Fe_4S_4]ferredoxin (Glass & Orphan, 2012).

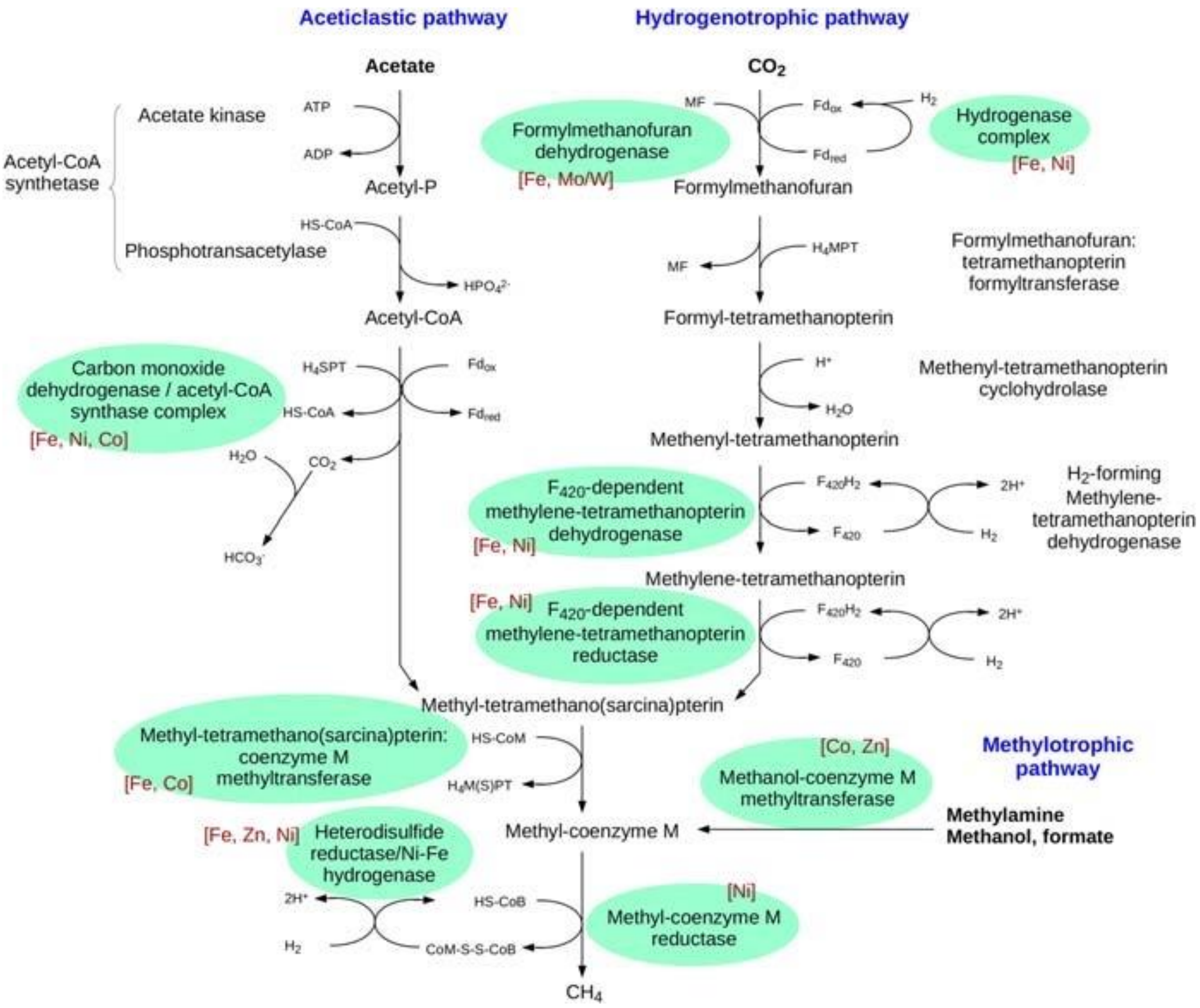


Figure I.1: Scheme of the three methanogenic pathways indicating enzymes involved and trace elements requirements (Fermoso et al., 2019b)

Both the aceticlastic and hydrogenotrophic pathways converge at a common enzymatic pathway, where the involved enzymes are described hereafter. Methyl-tetramethano(sarcina)pterion-coenzyme M methyltransferase is used for all methanogens. This enzyme contains 8 Fe atoms and 2 cobamide factors (containing 1 Co atom each) (Gärtner et al., 1993). A group of coenzyme M methyltransferase is also required in this step, some of them containing Co (Thauer, 1998). For the methyllotropic pathway, the coenzyme M methyltransferase contains 1 Zn atom and 1 cobalamin, which contains 1 Co (Hagemeier et al., 2006). Heterodisulfide reductase involves a group of 5 subunits depending on the presence or absence of cytochromes in the archea. These subunits have a different number of Fe_4S_4 clusters and structural Zn, and form a tight complex with the Ni–Fe hydrogenase, which in turn contains clusters of Fe_2S_2, Fe_4S_4 and Fe_3S_4 (Glass & Orphan, 2012). Methyl coenzyme M reductase catalyzes the final step in methanogenesis, which contains 2 coenzyme F430 Ni tetrapyrroles (Ermler et al., 1997).

In parallel to the AD pathways, reducing sulphate bacteria, which are naturally found in the AD consortium of microorganisms, perform the reduction of sulphur compounds to H_2S. The tetraheme cytochrome c3 represents the constitutive enzyme group in elemental sulfur reduction, which contains a [4Fe–4S] center. Additionally, these enzyme are dependent of hydrogenases that contain Fe, Ni-Fe, and Ni-Fe-Se (Glass et al., 2014).

I.1.3. Trace elements requirements in Anaerobic Digestion

Trace elements are essential for AD microorganisms, they are needed in the metabolic pathways that involved the degradation of organic substrate to biogas, and activities related to other cellular metabolisms and mantainement. However, the observed effect depends of the concentration of the trace element. The characteristic curve of the effect of a trace element according the concentration is shown in Figure I.2. For low concentrations, trace elements have a stimulating effect, then an optimal value is reached, and finally, at higher concentration, an inhibitory or toxic effect is detected.

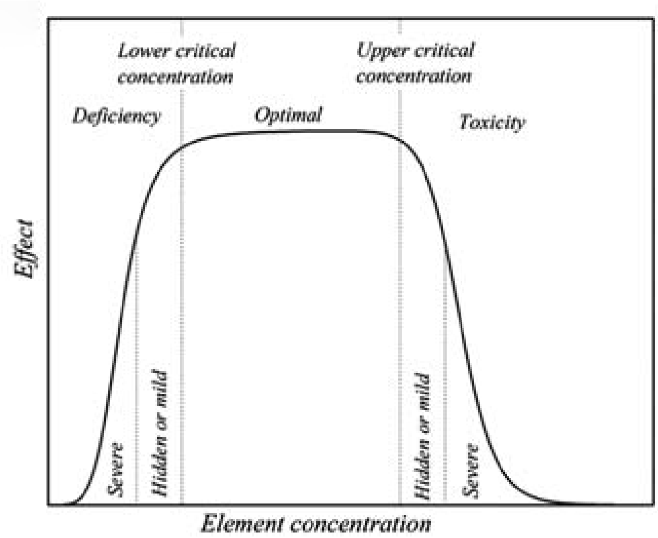


Figure I.2: Characteristic curve of effect for an esential trace element (Fermoso et al., 2019a)

Therefore, many studies have been conducted in AD to determine recommended or optimal concentrations that improve the production of biogas/methane or some intermediate metabolites of interest. The grouped range of 24 studies on different trace element in AD are presented in Figure I.3. These results are extracted from 4 compilations: (Schattauer et al., 2011) (n=8), (Garuti et al., 2018) (n=3), (Weinrich & Nelles, 2021) (n=12) and (Lindorfer et al., 2012) (n=1, compilation of monitoring of 680 anaerobic digesters).

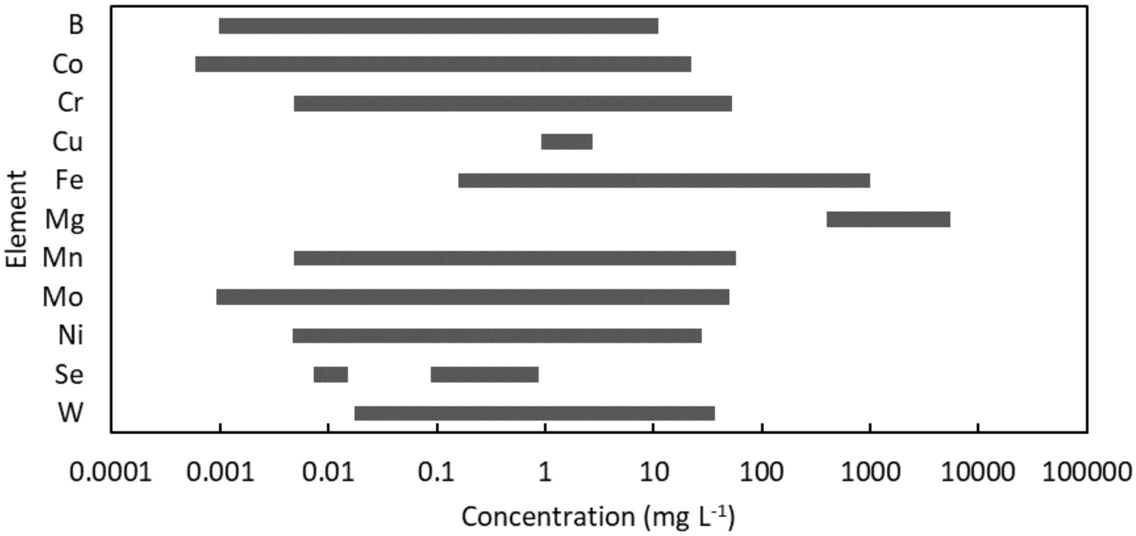


Figure I.3: Recommended values for optimal concentrations of trace elements in Anaerobic Digestion (Garuti et al., 2018; Lindorfer et al., 2012; Schattauer et al., 2011; Weinrich & Nelles, 2021)

The main trace elements studied correspond to those elements that have direct participation in methanogenesis, Ni, Co, Fe, Mo and W with 20, 18, 14, 17 and 8 studies focused on each element, respectively. Se has also been widely considered, with 12 studies, this trace element is related to certain hydrogenases involved in the generation of methane (Schattauer et al., 2011). Studies that consider any of the remaining trace elements (i.e, B, Ca, Cr, Cu, Mg, Mn and S) do not exceed 5.

It is complicated to establish general statements about the effect of different trace elements in AD, as well as the synergistic or antagonistic effect (Chen et al., 2008). The optimal trace element concentration for a pure culture can inhibit another species (Oleszkiewicz & Sharma, 1990). It has been observed a better enhancement in biogas generation when a trace element mixture is dosed (Facchin et al., 2013; Vintiloiu et al., 2012), but as well, a null effect with trace element mixtures, compared to an improvement when a single trace element was dosed (Zitomer et al., 2008). The recommended range of AD concentration differs for each trace element (Figure I.3), with some elements recommended at concentrations higher than 1 mg L^{-1}, such as Ca, Cu, and Mg, while for others recommended concentration is less than 1 mg L^{-1}, such as Se. However, for most elements, the range goes from the order of ug L^{-1} to about 100 mg L^{-1}. Only Fe covers an upper limit, reaching the order of units g L^{-1} . The amplitude of the ranges may vary from the same order of magnitude, for example for Cu, with a range from 0.9 to 1.8 mg L^{-1}, to elements where the difference between the minimum and maximum value is 40000 times, as for Co, with limits of 0.0006 and 23 mg L^{-1}.

Due to these ranges, the definition of stimulatory and inhibitory concentrations of trace elements for AD becomes uncertain. However, it should be noted that AD is a complex process and various factors influence its performance and may explain these discrepancies. Among these factors, one is the original consortium of microorganisms present in the inoculum, as well as its adaptation and tolerance to the presence of trace elements. The composition of methanogenic archaea is more sensitive to nutrient deficiency than fermentative bacteria (Feng et al., 2010). For example, the presence of Fe, Cu, and Ni increases the concentration of acetoclastic methanogens (Karlsson et al., 2012). Similarly, feedstock composition influences the adaptability and availability of microorganisms to trace elements. For example, in lignocellulosic agricultural waste, the lignin and cellulose network is a barrier that reduces the accessibility of nutrients for use in AD (Sánchez, 2009). Type of digester and its geometry, and operating parameters affect the mass transfer process and thus affect the distribution and availability of trace elements within the digester (Hamdi, 1991), being able to observe an apparent recommended requirement of trace element in the system. Additionally, sampling and analytical methods, as well as the chemical speciation and bioavailability of trace elements affect the optimal range of concentration of trace elements (Garuti et al., 2018). Usually, the reported value corresponds to the total or soluble concentration of the trace element in the digester. However, the fraction that is used by microorganisms, the fraction called bioavailable, is generally far lower.

I.1.4. Chemical species of trace elements in anaerobic digestion

According to the International Union of Pure and Applied Chemistry (IUPAC), a chemical species is defined as “a specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure” (Templeton et al., 2000). Chemical speciation refers to the distribution of different chemical species of an element in a system. The chemical speciation of trace elements in AD is a complex and multifaceted issue that involves metal chemistry, physical interactions of metals and solids, and microbiology (Fermoso et al., 2015). Figure I.4 shows the main routes and interactions between the different species in an AD system. Trace elements enter the anaerobic digester via substrate, material abrasion of the equipment, or additives, usually in free ionic or bound forms, which are expected to be easily bioavailable. The speciation of trace elements is determined by the environmental conditions: pH, redox potential, kinetics of precipitation by sulfide, carbonate, and phosphate, and kinetics of chelation by organic ligands, synthesized by microorganisms (Oleszkiewicz & Sharma, 1990).

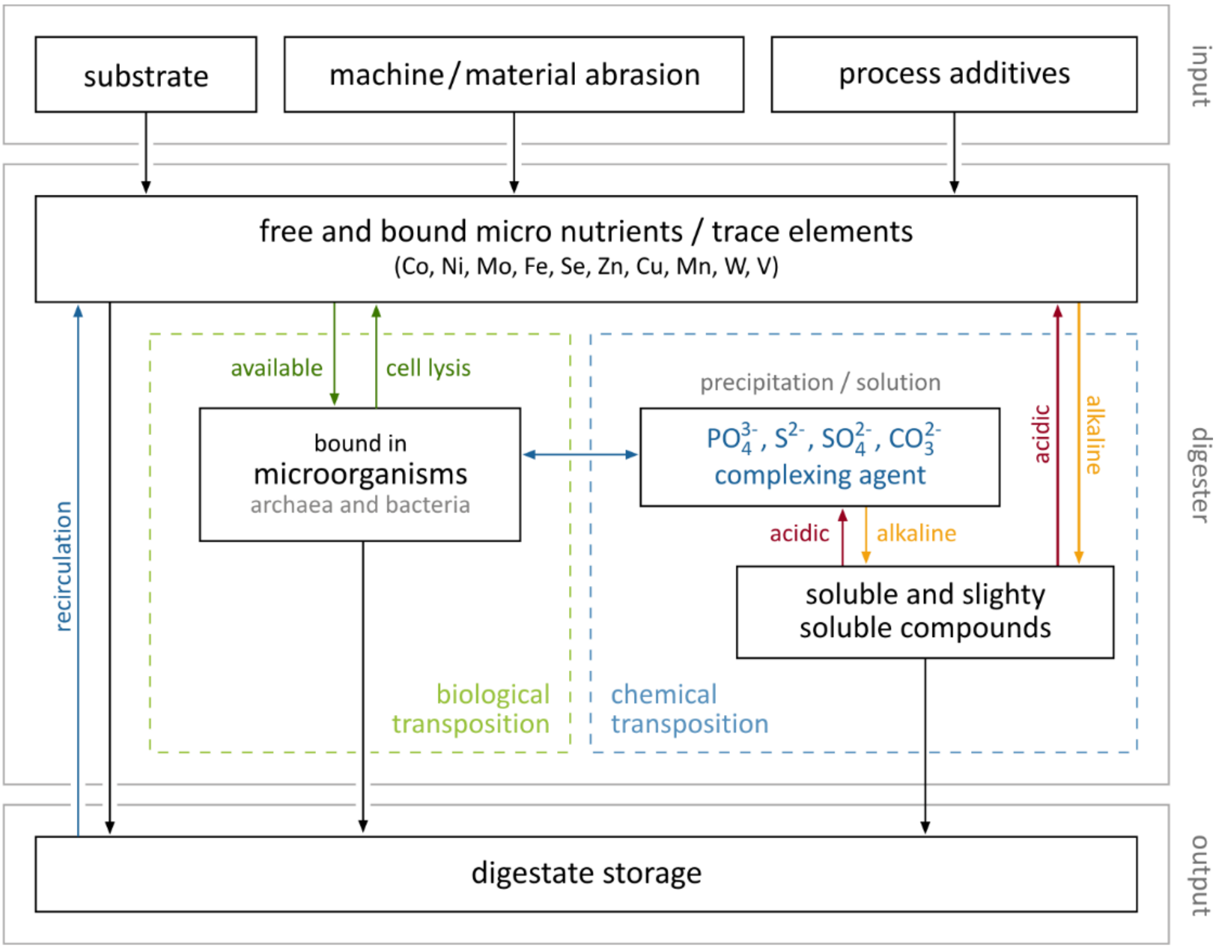


Figure I.4: Evolution of trace elements in an AD system (Weinrich & Nelles, 2021)

It is expected that trace elements are present in different chemical species in an AD system, due to the dynamics of multiple chemical and biological reactions. Then it is common to find a metal in different forms, such as precipitates, complex, etc. Only some of these forms can be used by/or affect the microorganisms, which constitute the bioavailable fraction. The bioavailable forms represent only a fraction of the total trace element content in the system, the total trace element concentration cannot be used to predict stimulatory or inhibitory response (Oleszkiewicz & Sharma, 1990). It has been assumed that the soluble fraction may be more useful to describe the response in AD compared to the total fraction. Studies have been performed in defined growth media for pure methanogenic cultures to determine the optimal soluble concentration for some trace elements (Glass & Orphan, 2012) (Figure I.5). The resulting values were close to the minimum value of the recommended range concentration for total trace elements.

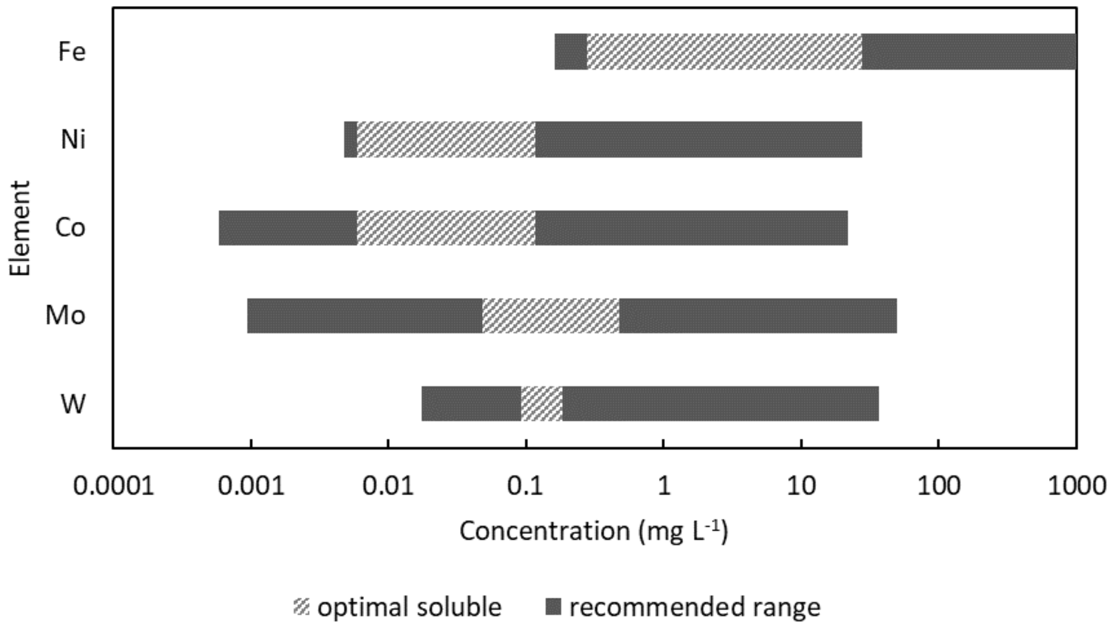


Figure I.5: Optimal soluble concentration values in studies of growth of specific methanogens (Glass & Orphan, 2012). Minimum and maximum value of recommended concentration are included (Garuti et al., 2018; Lindorfer et al., 2012; Schattauer et al., 2011; Weinrich & Nelles, 2021)

Free metal species are generally considered bioavailable, and soluble complexes are often not bioavailable, a decrease in polarity implies a decrease in sorption affinity (Oleszkiewicz & Sharma, 1990). However some small complexes have shown to be bioavailable (Takashima, 2018) as Co-EDTA complex (Fermoso et al., 2008). Thus, bioavailable species are not strictly chemically defined and their quantification would provide important information. Understanding the bioavailable fraction of trace elements is crucial for monitoring anaerobic digestion processes and assessing the specific nutrient requirements.

I.2. Fractionation techniques of trace elements in anaerobic digestates

Identification of the different trace element fractions in an AD system is relevant to determine the effect associate to specific element forms. For example, while many sulfur and carbonates precipitates are inert, free ions have potential biological effect. Fractionation techniques of trace elements involve analytical methods to separate and identify different elements classes, generally grouped according to certain common physical and or chemical properties (Gleyzes et al., 2002). Initially, these techniques were designed for soils and sediments (Filgueiras et al., 2002), but their application has been extended to AD samples (Hullebusch et al., 2005), as anaerobic sludges and digestates. In this section, metal fractions characterization techniques that have been applied to AD system are presented.

I.2.1. Total fraction

The total fraction represents the total amount of the trace metal and includes all the species of the trace element in the system. This fraction allows the identification of the different trace elements present in the AD system.

I.2.1.1. Principle

The determination of the total fraction in AD samples implies a destructive analysis. The matrix is decomposed by acid digestion in a microwave in order to completely release the metal. HNO_3, HCl or aqua regia (HNO_3–HCl 1:3 vol/vol mixture) are used as strong acids, in minor cases HF is used (Du Laing, 2010). H_2O_2 can be added in a previous step to help the organic matter destruction. Then, the metal concentration in the digested solution is measured by a suitable instrumental analysis technique. The most used techniques are absorption spectrometry techniques : flame atomic absorption spectrometry (F-AAS), graphite furnace absorption spectrometry (GF-AAS), thermoelectric atomic absorption spectrometry (ET-AAS), hydride generation-AAS (HG-AAS) and cold vapour-AAS (CV-AAS) ; and inductively coupled plasma techniques: inductively coupled plasma-atomic emission spectrometry (ICP-AES) and inductively coupled plasma-mass spectrometry (ICP-MS) (Du Laing, 2010).

I.2.1.2. Applicability and limitations

This fraction is simple to obtain and is part of the basic characterization when metals are studied in AD systems. Additionally, it is used as a reference value for validation of other fractionation techniques, where the sum of all the fractions is equal to the total fraction (Van Der Veen et al., 2007). Its main limitation is the lack of information regarding the different chemical species present since only the total concentration of the trace element can be obtained.

I.2.1.3. Application in digestates

The quantification of the total fraction of some trace elements in the AD studies found in the literature responds to their objectives, meaning that not all studies have the same trace elements selected. For example, Zn, Cu, and As are studied in pig manure digestate due to these elements are part of the animal diet (Jin & Chang, 2011; F. Zhang et al., 2012). The main and most common elements present in these studies are indicated in this section. Also, these studies do not present homogeneity in the concentration units used and do not provide the information to carry out the necessary conversion. Therefore, the most frequently used unit of concentration (mg kg^{-1} TS) was selected for this analysis. Nevertheless, discarded studies (Dąbrowska, 2012; Huang et al., 2018; Jin & Chang, 2011; Thanh et al., 2017; Q. Wang et al., 2021) followed the same trends that are presented in this review.

The main elements selected and quantified are Zn, Cu, Ni, Pb, Cr, As, and Cd. Those elements have been selected for: i) being the main metals found in the substrates used in AD (Dąbrowska, 2012; Legros et al., 2017; Zheng et al., 2021), ii) compliance with the proposed local standards for land application of sludge (Dacera & Babel, 2013) and iii) ecological risk assessment, due to their toxicity and therefore potential pollution to the surrounding environment if they are not managed properly (Q. Zhang et al., 2016). Additionally, some of these elements, such as Zn and Cu, are necessary for the AD process (González-Suárez et al., 2020) and are essential micronutrients for plants (Jin & Chang, 2011) so they are quantified to evaluate the performance of anaerobic digestion or the possibility of using the digestate as a fertilizer.

In all the studies, the same the same decreasing order in the elements was observed, so they can be grouped in the same analysis (Table I.2). Similarly to Figure I.3, Table I.2 shows the strong variability between the element and among the studies (for a given element). However, Table I.2 shows the characterization of trace elements in digestates meanwhile Figure I.3 shows the recommended concentration of trace elements in different types of AD systems. The high variability in the total metal concentration in all trace elements in the digestates can be explained that even if it is a digestate of the same type, the complexity, and heterogeneity of the sludge source affects the metal content (Dacera & Babel, 2013). It is highlighted that digestate from WWTP was the group with the most variability in the concentration, for example, Cu concentration fluctuated between 207 to 5727 mg kg^{-1} TS, and this could be due to the diversity of origin of the effluents treated in the WWTP.

Table I.2: Total fraction (mg kg^{-1} TS) of principal elements studied in diverse digestates

Element	mean	std	RSD (%)	min	median	max	n
Fe	7010	4707	67	365	9840	12100	7
Zn	2116	1874	89	13	1631	6580	14
Cu	1137	1724	152	3.1	487	5727	15
Cr	214	218	102	1.9	109	530	8
Pb	137	135	99	1.1	115	420	8
Ni	118	128	109	2.2	65	339	10
Cd	40	95	237	0.1	1.7	233	6
Co	17	13	79	1.4	24	25	3
As	9						1

Data for creation of this table extracted from: (Cestonaro do Amaral et al., 2014; Dacera & Babel, 2013; Dong et al., 2013; Fernández-Rodríguez et al., 2021; Flyhammar, 1997; Hanay et al., 2008; Legros et al., 2017; Marcato et al., 2009; Ortner et al., 2014; Seruga, 2021; T. Wang et al., 2020; Zdeb et al., 2020; Q. Zhang et al., 2016).

I.2.2. Soluble fraction

Soluble fraction include all the trace element species that are present in the liquid phase after centrifugation and /or filtration of the sample. This fraction is easy to obtain and is closer to bioavailable fraction compared to total fraction, but it is still too general covering species that may not be bioavailable and it is not a valid approximation.

I.2.2.1. Principle

Soluble fraction is obtained by subjecting the sample to a separation process, either by only centrifugation (Zheng et al., 2021; Zhu et al., 2014) or centrifugation and filtration (Jin & Chang, 2011; Micolucci et al., 2016) separating the liquid phase from the solid. Centrifugation conditions should be selected to prevent cell lysis and thereby release the metals from the intracellular medium to the liquid phase. An acid digestion is required to ensure the release of all trace elements from possible particles. Analytical instruments are the same as for the total fraction.

I.2.2.2. Applicability and limitations

To avoid any changes in the different fractions care must be taken during sampling, mainly to avoid air exposure of the anaerobic sample, which can induce changes by oxidizing certain species, such as Fe species (Holleman et al., 2001). Likewise, separation of the liquid and solid phases should be done as soon as possible after sampling, to avoid any interaction between the components of the solid and liquid phases after sampling.

I.2.2.3. Application in digestates

The quantification of trace elements in the soluble fraction was not recurrent in the characterization studies of digestates, 47% of the collected studies have determined this fraction. Similarly to the total fraction, the studies present heterogeneity around the selected trace elements as well as the concentration units used. The most frequently studied trace elements were selected and those results with concentrations in mg kg^{-1} TS are presented in a representative form, although the other studies (Cestonaro do Amaral et al., 2014; Jin & Chang, 2011; Liu et al., 2019; Zheng et al., 2020, 2021) followed the same trend. With these results, observing the same trend in all studies, the results were grouped in Table I.3. Similarities can be found between the soluble and total fractions indicating some correlation between these. The ordered elements from highest to lowest concentration follow the same order in both fractions (Table I.2 and I.3). Additionally, the variability of the results of soluble concentration is high for each element (RSD > 68%).

Only in 7 digestates the available information allows to determine the relationship between the trace element concentration in the soluble and the total fraction. These digestates come from animal and vegetal waste. No significant differences in mean and standard deviation were observed when evaluating each different type of digestate, so Table I.4 is constructed considering all the results in a single data set. The ratio of trace element present in the soluble fraction was not similar for all elements, the soluble/total ratio is distributed between 0.20 and 0.68 indicating that the solubilization depends on the trace element. The trace elements found with the highest solubilization were Ni, Cd, and As, while the trace elements that had the lowest fraction in the liquid phase were Cu, Cr, and Zn. All trace elements, with the exception of Ni, showed high variability in the liquid/total ratio (RSD > 58%). Ni poorly forms precipitates in anaerobic environments (Takeno, 2005), thus less variation in its soluble fraction are expected.

Table I.3: Soluble fraction (mg kg^{-1} TS) of principal elements studied in diverse digestates

Element	mean	std	RSD (%)	min	median	max	n
Fe	3180						1
Zn	774	1148	148	2	155	2741	5
Cu	409	520	127	0	68	1137	5
Pb	64	108	168	0.9	15	225	4
Cr	56	38	68	0	69	86	4
Ni	36	30	84	1.4	35	74	4
As	9	15	169	0.2	0.3	26	3
Cd	3	5	186	0.1	0.2	10	4

Data for creation of this table extracted from: (Fernández-Rodríguez et al., 2021; Legros et al., 2017; Micolucci et al., 2016; Zhu et al., 2014)

Table I.4: Ratio between soluble/total fraction concentration of trace elements in diverse digestates

Element	mean	std	RSD (%)	min	median	max	n
Ni	0.68	0.06	9	0.62	0.66	0.76	5
Cd	0.47	0.30	65	0.11	0.50	0.79	5
As	0.39	0.36	93	0.05	0.30	0.83	6
Pb	0.34	0.36	105	0.05	0.20	0.87	5
Cu	0.27	0.23	87	0.04	0.13	0.53	7
Cr	0.26						1
Zn	0.20	0.12	58	0.06	0.16	0.43	7

Data for creation of this table extracted from: (Fernández-Rodríguez et al., 2021; Jin & Chang, 2011; Zheng et al., 2021)

I.2.3. Sequential extractions

Sequential extractions are analytical procedures based on the use of a series of selective reagents to solubilise successively the metal fractions retained in different mineralogical fractions (Gleyzes et al., 2002). In this way, in each extracted fraction, species with different availabilities are found. These techniques are important to understand the environmental behavior and bioavailability of trace elements. Initially, it was applied in soils and sediments, but recent applications has included digestates. Sequential extraction brings information about the distribution and potential mobility of trace elements (Jaradat & Momani, 1999) and it is useful for predicting the toxicity risk in the soil if the digestate is used as fertilizer (Czekała et al., 2022). Similarly, in many anaerobic digesters, the composition of the digestate is almost the same as the composition within the digester, so the information obtained by sequential extraction would help understand the behavior of trace elements within the digester. In digestates, mainly two sequential extraction methods has been used: Tessier and the modified European Community Bureau of Reference (BCR) procedure.

I.2.3.1. Principle

The Tessier procedure consists in 4 sequential solid-liquid extraction stages, where an extractant solution is added to the sample. After separation of the supernatant and residual phases by centrifugation, the supernatant phase is analyzed to quantify the trace elements corresponding to the extracted fraction. In contrast, the residual phase is used for the next extraction stage. Tessier procedure distinguishes 5 fractions, for each one a principal extractant is used. The first fraction corresponding to "exchangeable", as the extracting phase, MgCl is used at pH 7. The second is called "bound to carbonates", for which NaOAc adjusted to pH 5.0 with acetic acid (HOAc) is used. The third is "Bound to Fe-Mn Oxides", where NH_2OH-HCl in HOAc is used. The fourth fraction is "Bound to organic matter", two extractions with H_2O_2 are performed by adjusting the pH to 2 with HNO_3. Finally, for the fifth fraction called "residual", the residue is digested with a strong acid according to the procedure of determining the total metal fraction (Tessier et al., 1979).

The Tessier procedure has been modified several times, changing the extractants and/or extraction conditions to suit different samples, obtain a simpler procedure or save time. The BCR procedure was obtained in this way, and was accepted by the Standards, Measurements, and Testing Program of the European Commission (Dąbrowska, 2012; Ure et al., 1993). The BCR procedure consists of 3 sequential extraction steps, where 4 fractions are obtained. The first, "exchangeable" fraction contains metal associated with water, exchangeable cations and carbonates, and is considered mobile and easily bioavailable to organism; this fraction is obtained by extraction with acetic acid. The second, "reducible" fraction group oxides and hydroxides of Fe and Mn, compounds of this fraction are thermodynamically unstable under anoxic conditions, the reducible fraction is obtained by extraction with NH_2OH-HCl adjusting to pH 2 with HNO_3. The third "oxidizable" fraction includes metals bound to organic matter and sulfides, this fraction is obtained by a treatment with H_2O_2 and an extraction with NH_2OAc, adjusted to pH 2. Finally, the fourth "residual" fraction that considers metals that do not belong to no previous fraction which metals are associated with stable minerals, it is obtained by mineralization with concentrated acid of the residue from the previous stage (Hanay et al., 2008; Solís et al., 2002; Zdeb et al., 2020; Zheng et al., 2021).

I.2.3.2. Applicability and limitations

The Tessier and BCR procedures have been applied in digestates with satisfactory results. However, it has to be noted that different environmental conditions can change the distribution of the fractions, thus samples should avoid contact with air and should be analyzed as soon as possible the sample is obtained. The presence of oxygen could affect the mobility of metals and change the distribution within fractions, but procedures for soils and sediments do not indicate that no oxygen conditions are required (Tessier et al., 1979; Ure et al., 1993). However (Kersten & Förstner, 1987; Rapin et al., 1986) proposed the necessity of performing the sequential extraction in a glovebox under argon atmosphere for anoxic sediments. (Zehl & Einax, 2005) compared the BCR method in soil samples under no oxygen environment and exposed to air for the first 2 fractions, and found that the presence of atmospheric oxygen affected the distribution of fractions for Cd, Pb, Zn, Fe, Mn, Co and Ni.

There is no unique and clear procedure regarding the presence or absence of oxygen for studies that have applied sequential extractions in digestates. It is not usually indicated if there was any restriction on the presence of air (Cestonaro do Amaral et al., 2014; Dąbrowska, 2012; Dong et al., 2013; González-Suárez et al., 2020; Mancini et al., 2019; Marcato et al., 2009; Thanh et al., 2017; T. Wang et al., 2020), while others studies clearly indicate the extractions that were made in a glovebox with no air environment (Braga et al., 2017; Flyhammar, 1997). There are even studies where the digestate sample was dried by air and/or with temperature (Hanay et al., 2008; Ortner et al., 2014; Zdeb et al., 2020; Zhu et al., 2014) then, in these studies, the digestate sample was exposed to air during drying time.

Sequential extractions are time consuming procedures, usually each of the stages require 16 h of extraction, in addition to stages of volume reduction by heating (Sahuquillo et al., 1999). Moreover, it is easy to lose sample between each of the stages, thereby losing accuracy. In fact a 20% error in total metal mass recovery can be accepted (Van Der Veen et al., 2007).

In addition, changes in the trace element fractions distribution may occur in anaerobic samples due to the extraction steps, which would generate a problem for the quantification of trace element fractions. (Wallmann et al., 1993) found in anaerobic sediments that metals associated with acid volatile sulphides can be dissolved in extraction steps previous to oxidation extraction step, while metal sulphides of lower solubility may precipitate. As well, the extraction of reducible fractions, can oxidize the anoxic sample and overestimates oxidizable fraction compared to reducible (Osuna et al., 2004).

I.2.3.3. Application in digestates

Many studies have included sequential extraction in the digestate characterization. In the collected studies, the number of digestates that have been characterized by Tessier and BCR procedure are 16 and 15, respectively. 4 groups of digestates have been identified: derived from manure from different animals (Manure group), derived from vegetal waste (Vegetable group), derived from wastewater treatment plant (WWTP group) and derived from municipal solid waste (MSW group).

The main difference between the Tessier and BCR procedure are the first two fractions of the Tessier procedure, whose sum should be equivalent to the exchangeable fraction of the BCR procedure (Thanh et al., 2016). So, to summarize the information, the first two fractions of the Tessier methods were added and indicated as a single “exchangeable” fraction. Figure I.6 summarizes the fractionation results for the 4 groups of the trace elements with more studies.

The results show a high variability regarding the trace element and the type of digestate, actually, the RSD can be high for some fraction (for example the exchangeable fraction of Cu for manure digestate has a RSD of 156 %). This variability can be attributed to differences in substrate composition as well as changes in speciation resulting from the AD process.

Two main group of results can be observed according to the predominance of some fraction over the different groups analyzed. The first group of results are those elements that show a similar distribution for some digestate groups. In this group is found: i) Fe, where the residual fraction is one of the majority for the 3 groups of digestates in which it is quantified. ii) Cu where the fraction residual is predominant in the manure, vegetabe and WWTP digestates. iii) Pb, with a higher residual fraction in the manure, vegetabe and WWTP digestates, and iv) As, where in digestates manure and vegetable the distribution of fractions is similar, following the order reducible > residual > oxidizable > excheangeable. On the other hand, in the second group of results are those elements where there are no similarities between the predominant fraction in the different groups of digestates. This group includes Zn, Ni, Cd and Cr.

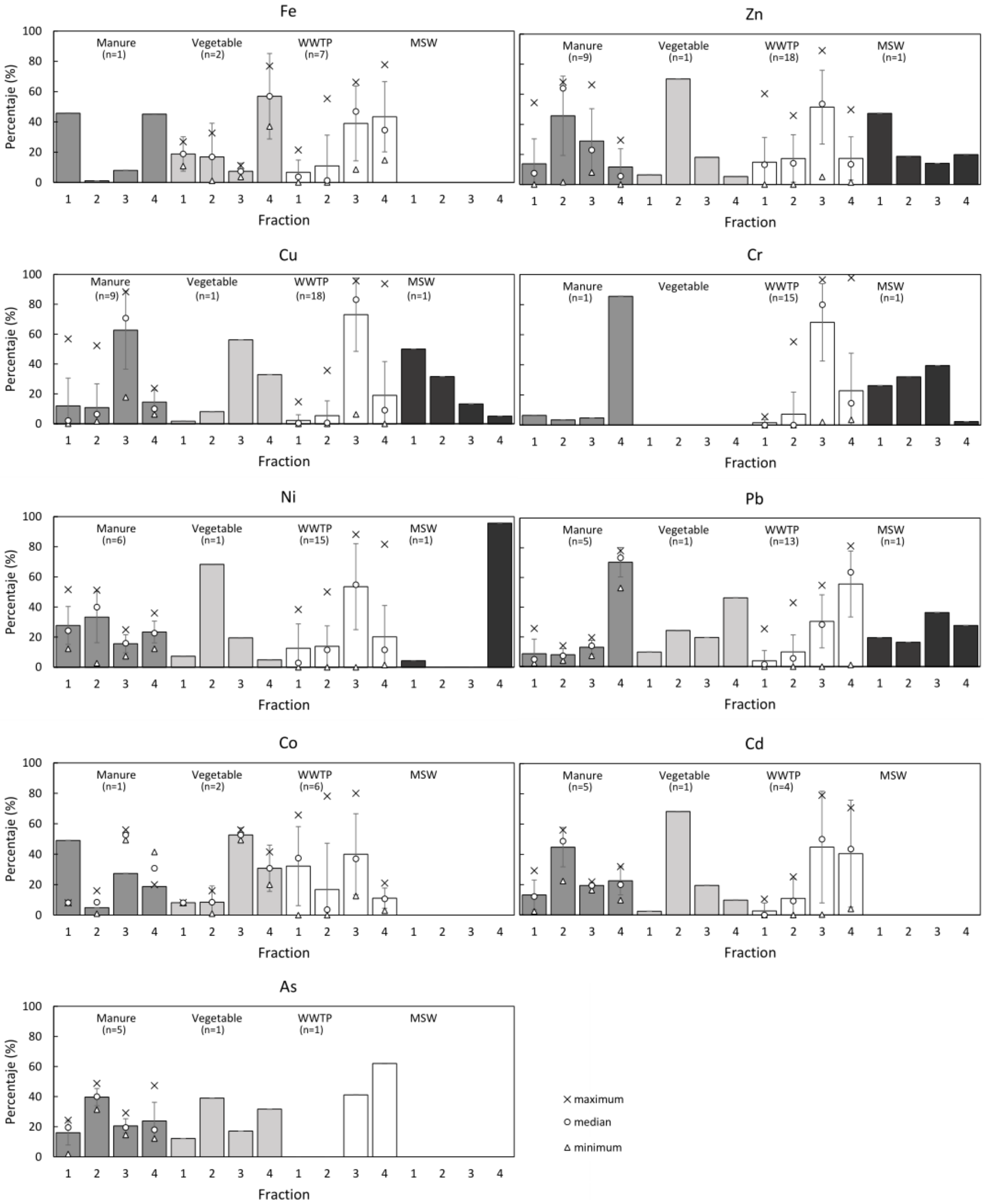


Figure I.6: Fractionation of different trace elements in 4 groups of digestates according to the origin of the waste: manure, vegetable, WWTP (Waste Water Treatment Plant) and MSW (Municipal Solid Waste). Fractions correspond to BCR sequential extraction method, 1 : exchangeable, 2 : reducible, 3 : oxidizable and 4 : residual. Data for creation of this figure extracted from: (Braga et al., 2017; Cestonaro do Amaral et al., 2014; Dąbrowska, 2012; Dacera & Babel, 2013; Dong et al., 2013; Flyhammar, 1997; González-Suárez et al., 2020; Hanay et al., 2008; Huang et al., 2018; Liu et al., 2019; Mancini et al., 2019; Marcato et al., 2009; Ortner et al., 2014; Thanh et al., 2017; Q. Wang et al., 2021; T. Wang et al., 2020; Zdeb et al., 2020; M. Zhang et al., 2016; Q. Zhang et al., 2016; Zheng et al., 2020, 2021; Zhu et al., 2014)

I.2.4. Free metal fraction

Free metal fraction consisted in the free ionic metal (M^{z+}). This fraction can be estimated on the basis of speciation models, but several information is required and the results could be inaccurate. On the other hand, it can be measured directly by the Donnan Membrane Technique (DMT) (Temminghoff et al., 2000).

I.2.4.1. Principle

DMT involves the use of a negatively charged cation exchange membrane to create an electrochemical potential gradient, allowing the selective transport of metal ions from the system/sample, called 'donor solution', to an 'acceptor solution'. Both solutions are recirculated from the original vessel to the DMT membrane (Figure I.7). The transport of metals occur until an equilibrium is reached, called 'Donnan equilibrium' (Temminghoff et al., 2000). Then the concentration of free metal accumulated in the acceptor solution is measured by a suitable instrumental analysis technique. Finally, through an equation obtained by mass balance in equilibrium, the free metal concentration in the donor solution is calculated.

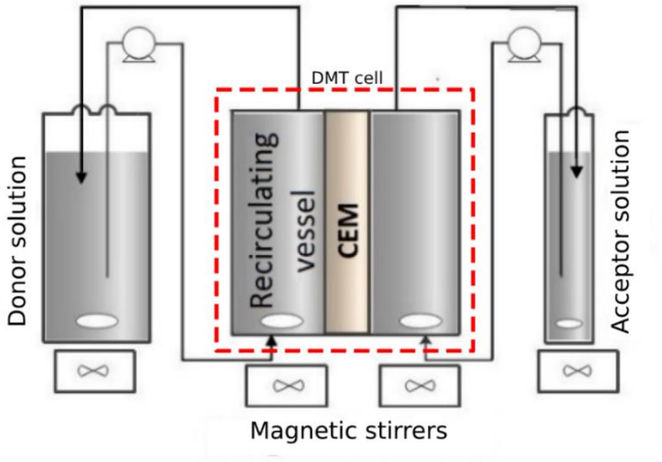


Figure I.7: General scheme for a DMT system. CEM: Cation exchange membrane. Adapted from (Asante-Sackey et al., 2021)

I.2.4.2. Applicability and limitations

DMT allows measuring the free metal fraction in solution, thus it can be applied in AD liquid systems, which usually have less than 15% total solid concentration (Yang et al., 2015). A limitation of the DMT is its relatively long equilibration time, the Donnan membrane equilibrium can be reached in 1-2 days. Moreover, the limit of detection depends upon the amount of free metal ion separated by the DMT. When the free ion concentration in the sample is below the detection limit of analysis, an ion complexing ligand with not too high affinity and at a relatively low concentration can be added to the acceptor solution to increase the accumulation in the acceptor solution (Weng et al., 2005).

I.2.4.3. Application in digestates

There is only one study that has applied free metal fraction quantification with DMT in anaerobic digestates. Bartacek et al. (2008) evaluated the free Cobalt fraction in a lab scale anaerobic reactor under 3 different Cobalt pulse dose compositions: i) $CoCl_2$, ii) $CoCl_2$ with carbonate and phosphate and iii) Cobalt complexed with EDTA ($CoEDTA^{2-}$). Half maximal inhibitory concentration (IC_{50}) in biogas production was determined for free Cobalt, obtaining 10, 14, and 15 µmol L^{-1} for the pulse dose i), ii) and iii), respectively.

I.2.5. Fractionations techniques and bioavailable fraction

The bioavailable fraction is the fraction with biological effect. It is the fraction that is the most relevant in understanding the effect of trace elements in AD. Some chemical species can be considered as biovailable, such as free ions and soluble complexes (Fairbrother et al., 2007; Takashima, 2018). Therefore, some fractionations techniques can approach the bioavailable fraction, however they are not accurate. The liquid fraction or the "excheangeable" (Tessier and BCR procedure) and part of the "bound to carbonates" fractions (Tessier procedure) could be considered bioavailable, as metal associated with water and excheangeable ions. However such fractions would not be completely restricted to bioavailable species. In fact, in AD systems, the measured bioavailability according to sequential extraction means “potential for bioavailability" (Cai et al., 2018). On the other hand, the free metal fraction is rarely a good approximation of bioavailability, since this fraction is reduced to only one species within all those that compose the bioavailable fraction.

The results of sequential extraction techniques are generaly in the order of mg L^{-1} (Hullebusch et al., 2005). The bioavailable fraction can be in the order of µg L^{-1}, therefore a technique with better sensitivity is required. An *in situ* sampling technique, diffusive gradients in thin films technique (DGT), appears as an option to approximate the bioavailable fraction.

I.3. Diffusive Gradient in Thin Films

Diffusive Gradients in Thin Films technique (DGT) was presented by Davison & Zhang (1994) as a simple technique designed to fractionate *in situ* trace elements in natural waters. Due to its versatility and simplicity, this technique has achieved extraordinary success in the scientific community and is widely used in environmental studies (Guan, 2019). Its application was quickly extended to sediments (1995), soils (1998) and wastes (2012) and the number of publications is constantly increasing. Since 2016, publications exceeded 100 a year, and by 2020 the total number of DGT publications was up to 1261, involving several areas such as agronomy, analytical chemistry, environmental, marine and soil science (Wei et al., 2022). DGT is able to sample some chemical species, which constitute the fraction called “labile fraction” and have a special interest in environmental area, due to its biological relevance. Thus, many elements and compounds have been detected and quantified by DGT (Table I.5) including potential inorganic and organic contaminants.

Table I.5: Analytes measured by DGT (Guan, 2019)

Category	Analytes
Metals and metalloids	Ag(I), Al(III), As(III), As(V), Au(III), Ba(II) , Ca(II), Co(II), Cr(III), Cr(VI), Cu(II), Fe(II), Fe(III), Ga(III), Mg(II), Mn(II), Mo(VI), Ni(II), Pb(II), Sb(III), Sb(V), Se(VI), V(V), W(VI), Zn(II)
Nutrients	F(-I),K(I), NH_4^+-N, NO_3^--N, phosphate, phosphite
Radionuclides and isotopes	Cs, Sr, Tc, U, Ra, Pu, ^{66}Zn, ^{64}Zn, ^{34}S, ^{32}S, ^{206}Pb, ^{204}Pb
Nanoparticules	ZnO, Ag
Organics	aminomethylphosphonic acid, antibiotics, bisphenols, 4-chlorophenol, diethylarsinate, estrogens, glyphosate, illicit drugs, low-molecular-weight organic phosphorus, methylmercury, monomethylarsonate, organophosphorus flame retardants, perfluoroalkyl substances, pesticides, pharmaceuticals
Others	Rare earth elements, SO_4^{2-}

I.3.1. Labile fraction and its relation with bioavailable fraction

The term lability refers to the ease with which substitution processes occur, which is relative to the kinetic stability of a particular species (Rao et al., 1986). For a metal complex, it refers to the ease with which it can dissociate or replace its ligands. The term lability has been analytically analyzed and discussed using mass transfer process (Galceran & Puy, 2015; Van Leeuwen, 2001). An experimental classification has even been proposed for different degrees of lability in trace elements, from very labile to inert (Figura & McDuffie, 1980). DGT measures the labile fraction, in practice for trace elements, this fraction is mainly constituted of free ions, inorganic complexes, and weak and low-weight organic complexes (Takashima, 2018).

On the other hand, the bioavailable fraction, in a chemistry perspective, denotes the species freely available (unbound or unsequestered), that can be uptake across a physiological membrane (D. X. Guan, 2019). Labile metal fraction contain free ions and easily dissociable complexes that may be more susceptible to interact with organisms (Fairbrother et al., 2007). Labile fraction has shown to be more accurate to relate with the bioavailable fraction than other fractions. (Bradac et al., 2009) studied the accumulation of Cd in periphyton (a complex mixture of algae, cyanobacteria, heterotrophic microbes, and detritus) in freshwater. No correlation between the intracellular Cd accumulated and the free Cd fraction was observed, while the labile fraction measured with DGT showed a good correlation with the Cd accumulated (Figure I.8). Similarly, DGT-labile concentrations has shown strong correlations with bioaccumulation of several trace elements in aquatic organisms (Eismann et al., 2020).

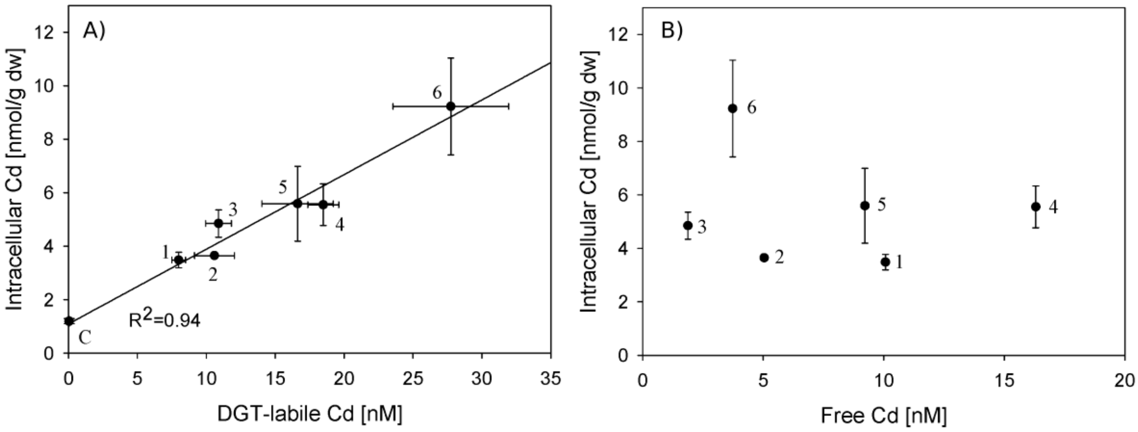


Figure I.8: Intracellular Cd concentrations in periphyton as a function of A) DGT-labile and B) free Cd concentrations in water after 6 h exposure. Numbers 1 to 6 and C represent different Co and nitriloacetic acid exposition conditions (Bradac et al., 2009)

I.3.2. Fundamentals and components

A typical DGT device consist in a piston-type device with a plastic piston holder and a cap, where 3 layers are held: a binding gel layer, an diffusive gel layer and a filter membrane (Figure I.9). The internal diameter of the cap is 18 - 20 mm. Both, the diffusive and the binding gel are usually polyacrylamide hydrogels where water is the main component (Scally et al., 2003). Diffusive gel controls the transport of the species to the binding gel, restricting the transport to diffusion only. Binding gels typically contain solid resins or powders (which are selective for the analytes or group of analytes of interest) incorporated into the polyacrylamide hydrogel. Nowadays, there are more than 40 different types of binding layers designed for a wide range of analytes (D. X. Guan, 2019). Considering trace elements, Chelex binding gel is usually used for cations (Galceran et al., 2021; Marrugo-Madrid et al., 2021), Zr-oxyde binding gel for oxyanions (D.-X. Guan et al., 2015), and for specific metalloid species, thiol binding gels have been used (Bennett et al., 2010; Österlund et al., 2010; Sahin, 2003). The filter membrane has two functions: to protect the gels inside the DGT from possible contamination from particles present in the solution, and to contain the gels to prevent damage or loss of these during the DGT deployment time. The most common membrane filter materials are : cellulose nitrate, cellulose acetate, polyvinylidene and hydrophilic polyethersulfone (Li et al., 2019).

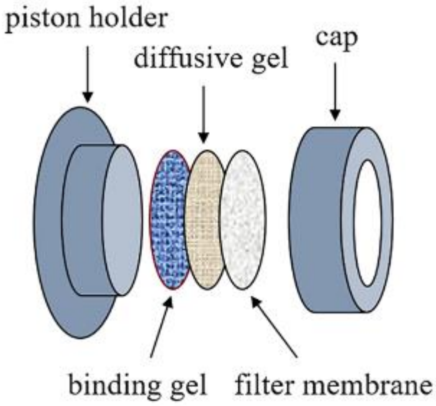


Figure I.9: Structural diagram of a piston-type DGT device (Wei et al., 2022)

The main fundamentals of the DGT technique are described by two processes: the diffusion of analytes through a diffusion layer and the binding of the analytes of interest in a binding gel. The procedure, in a simple description, is as follows: a DGT device is put in contact with the solution and the solutes are progressively accumulated in the binding gel by the continuous diffusion through the diffusive layer. The exposition time of the DGT to the solution is controlled and measured. The concentration of analytes in the sample solution is calculated from the mass of analytes accumulated in the binding gel, as determined by elution and chemical analysis (Davison & Zhang, 1994).

I.3.3. DGT Theory

The labile fraction, including free ions, labile complexes which can dissociate, , diffuse through the diffusive gel and is then rapidly and irreversibly sorbed by the binding gel. A steady state within the DGT device is reached within a few minutes to hours (Puy et al., 2012; Tusseau-Vuillemin et al., 2003), where a diffusive gradient is established (Figure I.10).

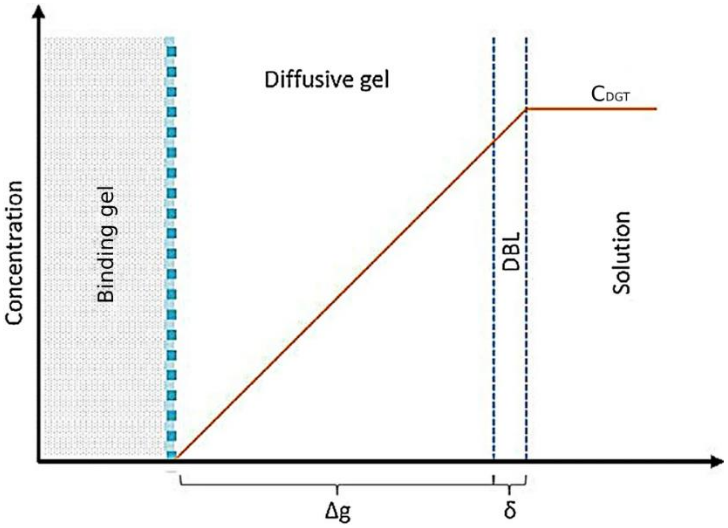


Figure I.10: Diffusive gradient of the labile species in steady state in a DGT. Δg is the thickness of the diffusive gel + filter membrane, δ is the tickness of the diffusive bounday layer (DBL), a zone where the species are transported by molecular diffusion, and C_{DGT} is the concentration of labile species in solution. Adapted from (Marrugo-Madrid et al., 2021)

The diffusion boundary layer (DBL) appears in Figure I.10. It is a zone between the filter membrane and the bulk solution where the transport of solutes is solely controlled by diffusion. However, the thickness of the DBL is assumed to be negligible compared to the thickness of the diffusive gel in well mixed systems (Davison & Zhang, 1994).

Based on Fick's first law of diffusion, and considering a well-mixed system ($\delta \approx 0$), the diffusion flux density at steady state can be described according to equation I.1 (Davison & Zhang, 1994).

$$F = -D\frac{\partial C}{\partial x} = D\frac{(C_{DGT} - C^*)}{\Delta g} \qquad (I.1)$$

Where, F is the flux density of the solute across the DGT, D is the diffusion coefficient of the solute in the diffusive gel, $\partial C/\partial x$ is the concentration gradient, C_{DGT} and C^* are the concentration of the labile species in the bulk solution and at the interface of the binding gel and the diffusive gel, respectively, Δg is the thickness of the diffusive gel and filter membrane. $C^* = 0$ because all labile metal arriving at the binding gel is assumed to be irreversibly sorbed, therefore, equation $(I.1)$ can be reduced to equation $(I.2)$:

$$F = D\frac{C_{DGT}}{\Delta g} \quad (I.2)$$

On the other hand, by definition, F can be expressed as equation (I.3):

$$F = \frac{m}{At} \quad (I.3)$$

Where m is the mass of the analyte diffused by a unit of area (A) and time (t). By combining equations $(I.2)$ and $(I.3)$, equation $(I.4)$ for determination of C_{DGT} is obtained.

$$C_{DGT} = \frac{m\Delta g}{DAt} \quad (I.4)$$

The mass m that has crossed the diffusive gel is the mass that is retained in the binding gel, therefore, the binding gel is eluted and the total mass of solute accumulated in the binding gel (m) is obtained by equation $(I.5)$ (H. Zhang & Davison, 1995):

$$m = \frac{C_e V_e}{f_e} \quad (I.5)$$

Where C_e is the metal concentration in elute, V_e is the elution volume and f_e the elution factor of the solute from the binding gel.

I.3.4. Validation of DGT theory

When DGT technique is applied in a new matrix, or when a new material for diffusive or binding gel is proposed, the performance of the DGT measurements has to be validated. There are two ways to validate the DGT theory (Davison, 2016; Ding et al., 2018) :

i) Deploy the DGT in a solution with a known concentration of the labile targeted analyte, usually the solution is prepared with a single labile species of the targeted analyte. Then, the C_{DGT} calculated is compared to the initial total analyte concentration in the bulk solution (C_b) with the parameter $U\%$ (equation $I.6$). The $U\%$ value should be within 90–110%.

$$U\% = \frac{C_{DGT}}{C_b} 100\% \quad (I.6)$$

ii) Obtaining a linear relationship between the accumulated mass in the binding gel and the deployment time. If C_{DGT} is constant over time, a linear trend can be detected between the accumulated mass (m) and the time (t). Equation I.4 can be written as equation $I.7$:

$$m = \left(\frac{C_{DGT} D A}{\Delta g}\right) t \quad (I.7)$$

The accumulated mass in the binding gel over time follows the trend shown in Figure I.11. A short transient time (minutes to hours) is observed, where the solutes start the diffusion through the diffusive gel (Puy et al., 2012; Tusseau-Vuillemin et al., 2003). The accumulated mass during the transient period must be negligible compared to the total accumulated mass. A second period corresponds to the steady state, where the flux is constant and a linear accumulation of the solute over time is observed. At longer times, the flux decreases and the accumulation rate is lower compared to the steady state. In this period, the accumulation is thermodynamically limited, due to the decreased number of free spaces in the binding gel. Finally, a saturation/equilibrium is reached, where the accumulation reaches a plateau. At this point there are no solute concentration gradients, due to all the binding sites of the binding gel are occupied, corresponding to the saturation of the DGT device. In summary, the linear trend between the accumulated mass over time is just valid during the steady time period, and this period also corresponds to the deployment time where the equation $I.4$ is valid.

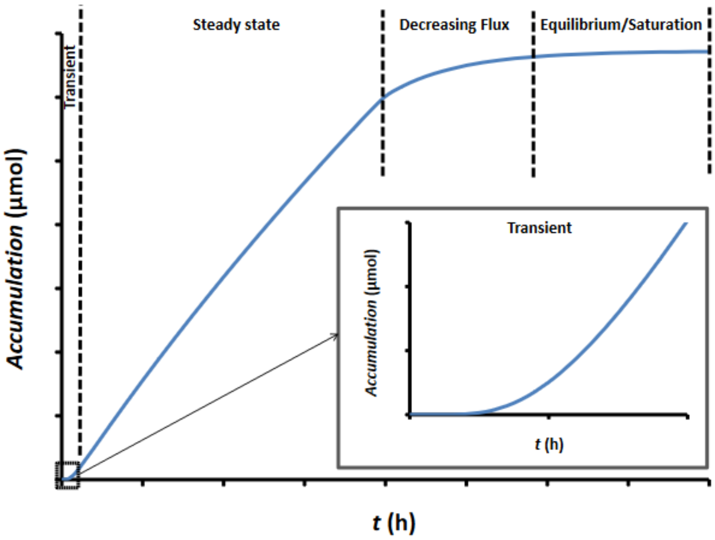


Figure I.11: Scheme of analyte accumulation over time in a DGT device. Adapted from (Jiménez-Piedrahita et al., 2017)

I.3.5. Studies of DGT in anaerobic digestion systems

The application of DGT to determine the labile concentration of trace element in AD is recent, the first publication was in 2017. Nowadays there are just 5 publications about this topic.

These studies have focused on 3 main topics: evaluation of the theory of the DGT technique, effect of various variables on measurements and determination of the labile fraction. The DGT deployment time in a WWTP digestate was optimized by Laera et al. (2019a) through the analysis of mass accumulation over time (equation 7). It was recommended to deploy DGT for more than 4 h, and 24 h was a good compromise to allow quantification of most of the targeted elements. Similarly, the DGT deployment time was evaluated in a mix of manure, energy crops and food waste digestate by Ilic et al. (2024), setting the time to 23 h.

The effect of the digestate matrix has been evaluated by Laera et al. (2019a), using diffusive gels pre-loaded with digestate. This scenario provoked an underestimation of 10–30% in the DGT labile concentrations. On the other hand, the effect on the labile fraction due to air exposure of the digestate for 12 weeks was evaluated by Laera et al. (2019b) using Chelex and Zr DGT to target 14 trace elements. It was detected that aeration promoted dissolution of the quantified elements, but this did not lead to increased labile fraction, except for some elements under forced aeration conditions.

DGT has been used to quantify the labile trace element fraction in digestate samples from a Biomethane Potential test spiked with Cd (Bourven et al., 2017). It was observed that AD inhibition was initially provoked by the labile and inert soluble fraction of Cd. The DGT deployment time was arbitrarily set to 24 h, although the results of the DGT measurements were satisfactory, and it was indicated that this time could be optimized. In another study, Takashima (2018) described the total, soluble and DGT-labile fraction in an anaerobic CSTR (Continuous Stirred Tank Reactor) in which the join effect of a thermal pretreatment (170 °C for 1 h) on the sewage sludge used as fed and subsequent doses of Co and Ni was evaluated. The thermal pretreatment did not show any significant effect on the DGT analysis in the digestates for these two trace elements. Finally, Ilic et al. (2024) evaluated the DGT technique to detect Fe, Co, Ni and Mn in 7 different digestates from waste water treatment plant and agricultural waste. Additionally, two types of diffusive gels were evaluated (restricted and open gels) to assess the occurrence of organic complexes. Half of the samples showed statistically significant differences and it was highlighted that the value of diffusion coefficient can be source of systematic errors that affect the conclusions.

All these studies show the potential of DGT for trace element determination in anaerobic digestates. DGT has many advantages compared to other fractionations techniques, it is a simple and easy to use technique. As it is an *in situ* technique, it does not require sample treatment which prevent changes in trace elements speciation.

There are common points that should be considered in the DGT deployment in digestates. Exposure of the sample with oxygen from the air should be avoided to prevent any change in speciation. Generally, during sampling the containers are fully filled to minimize air exposure until analysis (Ilic et al., 2024; Laera et al., 2019a, 2019b) and the DGT deployment is performed in an anaerobic atmosphere, as a closed beaker (Laera et al., 2019a). Takashima (2018) does not indicate the procedure of the sample to prevent exposure to air, and although (Ilic et al., 2024) indicates the procedure during sampling avoid air exposition, preventive procedure during deployment of DGTs are not indicated. The second point to consider is deployment time, which should be evaluated and optimized for each trace element and digestate sample, due to the matrix variability between different digestate samples. Takashima (2018) does not justify the selection of 24 h for DGT deployment time.

The digestates studied come from different AD systems, however it is known that there is a great variability in the physico-chemical characteristics between the digestates, even within the same group. Therefore the conclusions from these studies are useful in understanding the applicability of DGT in this type of matrix, but cannot be taken as general conclusions and more studies are needed to assess the feasibility of DGT in anaerobic systems: digestates and other AD systems, as inside AD reactors.

I.4. Outline of the thesis

The effect of trace elements on the AD has been studied extensively, generally evaluated in biogas production. However, there are other response variables that are affected by the presence of trace elements, such as the production of certain metabolic products. On the other hand, the conclusions obtained in these studies regarding the recommended or optimal values for biogas production are uncertain, being one of the main ones a lack of precision given that the indicated concentration does not correspond to the bioavailable fraction. DGT technique, which quantifies the labile fraction that may be related to the bioavailable fraction, has appeared as an option and has been applied in anaerobic systems with promising results. However more studies are needed to reliably apply DGT in AD systems.
This is why this thesis studies the global impact (i.e., in various response variables) of a trace element in an anaerobic reactor under continuous dose of trace element, which is characterized in different fractions. Similarly, DGT theory technique is evaluated in anaerobic digestates, considering the need to maintain anaerobic conditions during deployment. Additionally, the application of DGT *in situ* to an AD reactor is explored

I.5. References

Afting, C., Hochheimer, A., & Thauer, R. K. (1998). Function of H2-forming methylenetetrahydromethanopterin dehydrogenase from methanobacterium thermoautotrophicum in coenzyme F420 reduction with H2. *Archives of Microbiology*, *169*(3), 206–210. https://doi.org/10.1007/s002030050562

Appels, L., Baeyens, J., Degrève, J., & Dewil, R. (2008). Principles and potential of the anaerobic digestion of waste-activated sludge. *Progress in Energy and Combustion Science*, *34*(6), 755–781. https://doi.org/10.1016/j.pecs.2008.06.002

Asante-Sackey, D., Rathilal, S., Kweinor Tetteh, E., Ezugbe, E. O., & Pillay, L. V. (2021). Donnan Membrane Process for the Selective Recovery and Removal of Target Metal Ions—A Mini Review. *Membranes*, *11*(5), 358. https://doi.org/10.3390/membranes11050358

Bajpai, P. (2017). Process Parameters Affecting Anaerobic Digestion. In P. Bajpai (Ed.), *Anaerobic Technology in Pulp and Paper Industry* (pp. 13–27). Springer Singapore. https://doi.org/10.1007/978-981-10-4130-3_3

Bartacek, J., Fermoso, F. G., Baldó-Urrutia, A. M., Van Hullebusch, E. D., & Lens, P. N. L. (2008). Cobalt toxicity in anaerobic granular sludge: Influence of chemical speciation.

Journal of Industrial Microbiology & Biotechnology, *35*(11), 1465–1474. https://doi.org/10.1007/s10295-008-0448-0

Bennett, W. W., Teasdale, P. R., Panther, J. G., Welsh, D. T., & Jolley, D. F. (2010). New Diffusive Gradients in a Thin Film Technique for Measuring Inorganic Arsenic and Selenium(IV) Using a Titanium Dioxide Based Adsorbent. *Analytical Chemistry*, *82*(17), 7401–7407. https://doi.org/10.1021/ac101543p

Bourven, I., Casellas, M., Buzier, R., Lesieur, J., Lenain, J.-F., Faix, A., Bressolier, P., Maftah, C., & Guibaud, G. (2017). Potential of DGT in a new fractionation approach for studying trace metal element impact on anaerobic digestion: The example of cadmium. *International Biodeterioration & Biodegradation*, *119*, 188–195. https://doi.org/10.1016/j.ibiod.2016.11.007

Bradac, P., Behra, R., & Sigg, L. (2009). Accumulation of Cadmium in Periphyton under Various Freshwater Speciation Conditions. *Environmental Science & Technology*, *43*(19), 7291–7296. https://doi.org/10.1021/es9013536

Braga, A. F. M., Zaiat, M., Silva, G. H. R., & Fermoso, F. G. (2017). Metal fractionation in sludge from sewage UASB treatment. *Journal of Environmental Management*, *193*, 98–107. https://doi.org/10.1016/j.jenvman.2017.01.070

Cai, Y., Wang, J., Zhao, Y., Zhao, X., Zheng, Z., Wen, B., Cui, Z., & Wang, X. (2018). A new perspective of using sequential extraction: To predict the deficiency of trace elements during anaerobic digestion. *Water Research*, *140*, 335–343. https://doi.org/10.1016/j.watres.2018.04.047

Cecchi, F., & Cavinato, C. (2015). Anaerobic digestion of bio-waste: A mini-review focusing on territorial and environmental aspects. *Waste Management & Research: The Journal for a Sustainable Circular Economy*, *33*(5), 429–438. https://doi.org/10.1177/0734242X14568610

Cestonaro do Amaral, A., Kunz, A., Radis Steinmetz, R. L., & Justi, K. C. (2014). Zinc and copper distribution in swine wastewater treated by anaerobic digestion. *Journal of Environmental Management*, *141*, 132–137. https://doi.org/10.1016/j.jenvman.2014.03.021

Chen, Y., & Cheng, J. J. (2005). Anaerobic Processes in Waste Treatment. *Water Environment Research*, *77*(6), 1347–1388. https://doi.org/10.2175/106143005X54407

Chen, Y., Cheng, J. J., & Creamer, K. S. (2008). Inhibition of anaerobic digestion process: A review. *Bioresource Technology*, *99*(10), 4044–4064. https://doi.org/10.1016/j.biortech.2007.01.057

Czekała, W., Jasiński, T., Grzelak, M., Witaszek, K., & Dach, J. (2022). Biogas Plant Operation: Digestate as the Valuable Product. *Energies*, *15*(21), 8275. https://doi.org/10.3390/en15218275

Dąbrowska, L. (2012). Speciation of heavy metals in sewage sludge after mesophilic and thermophilic anaerobic digestion. *Chemical Papers*, *66*(6), 598–606. https://doi.org/10.2478/s11696-011-0128-9

Dacera, D. D., & Babel, S. (2013). Effect of Drying Process on Heavy Metals Fractionation of Contaminated Sludge for Land Application. *Soil and Sediment Contamination*, *22*(4), 457–468. https://doi.org/10.1080/15320383.2013.733443

Davison, W. (Ed.). (2016). *Diffusive gradients in thin-films for environmental measurements*. Cambridge University Press.

Davison, W., & Zhang, H. (1994). In situ speciation measurements of trace components in natural waters using thin-film gels. *Nature*, *367*(6463), 546–548. https://doi.org/10.1038/367546a0

Ding, Y., Yang, J., & Cai, J. (2018). Preparation of guanidinylated carboxymethyl chitosan and its application in the diffusive gradients in thin films (DGT) technique for measuring labile trace metals in water. *International Journal of Environmental Analytical Chemistry*, *98*(14), 1275–1291. https://doi.org/10.1080/03067319.2018.1545901

Dong, B., Liu, X., Dai, L., & Dai, X. (2013). Changes of heavy metal speciation during high-solid anaerobic digestion of sewage sludge. *Bioresource Technology*, *131*, 152–158. https://doi.org/10.1016/j.biortech.2012.12.112

Du Laing, G. (2010). Analysis and Fractionation of Trace Elements in Soils. In P. S. Hooda (Ed.), *Trace Elements in Soils* (1st ed., pp. 53–80). Wiley. https://doi.org/10.1002/9781444319477.ch4

Eismann, C. E., Menegário, A. A., Gemeiner, H., & Williams, P. N. (2020). Predicting Trace Metal Exposure in Aquatic Ecosystems: Evaluating DGT as a Biomonitoring Tool. *Exposure and Health*, *12*(1), 19–31. https://doi.org/10.1007/s12403-018-0280-3

Ermler, U., Grabarse, W., Shima, S., Goubeaud, M., & Thauer, R. K. (1997). Crystal Structure of Methyl-Coenzyme M Reductase: The Key Enzyme of Biological Methane Formation. *Science*, *278*(5342), 1457–1462. https://doi.org/10.1126/science.278.5342.1457

Esposito, G., Frunzo, L., Giordano, A., Liotta, F., Panico, A., & Pirozzi, F. (2012). Anaerobic co-digestion of organic wastes. *Reviews in Environmental Science and Bio/Technology*, *11*(4), 325–341. https://doi.org/10.1007/s11157-012-9277-8

European Biogas Association. (2022). *EBA Statistical Report 2022*. European Biogas Association. https://www.europeanbiogas.eu/SR-2022/EBA/

Facchin, V., Cavinato, C., Fatone, F., Pavan, P., Cecchi, F., & Bolzonella, D. (2013). Effect of trace element supplementation on the mesophilic anaerobic digestion of foodwaste in batch trials: The influence of inoculum origin. *Biochemical Engineering Journal*, *70*, 71–77. https://doi.org/10.1016/j.bej.2012.10.004

Fairbrother, A., Wenstel, R., Sappington, K., & Wood, W. (2007). Framework for Metals Risk Assessment. *Ecotoxicology and Environmental Safety*, *68*(2), 145–227. https://doi.org/10.1016/j.ecoenv.2007.03.015

Feng, X. M., Karlsson, A., Svensson, B. H., & Bertilsson, S. (2010). Impact of trace element addition on biogas production from food industrial waste - linking process to microbial communities: Biogas production from food industrial waste. *FEMS Microbiology Ecology*, *74*(1), 226–240. https://doi.org/10.1111/j.1574-6941.2010.00932.x

Fermoso, F. G., Bartacek, J., Chung, L. C., & Lens, P. (2008). Supplementation of cobalt to UASB reactors by pulse dosing: CoCl2 versus CoEDTA2− pulses. *Biochemical Engineering Journal*, *42*(2), 111–119. https://doi.org/10.1016/j.bej.2008.06.005

Fermoso, F. G., Van Hullebusch, E., Collins, G., Roussel, J., Mucha, A. P., & Esposito, G. (Eds.). (2019a). Mathematical modelling of trace element dynamics in anaerobic digestion environments. In *Trace Elements in Anaerobic Biotechnologies* (pp. 101–152). IWA Publishing. https://doi.org/10.2166/9781789060225_0101

Fermoso, F. G., Van Hullebusch, E., Collins, G., Roussel, J., Mucha, A. P., & Esposito, G. (Eds.). (2019b). Trace element enzymes in reactions essential for anaerobic digestion. In *Trace Elements in Anaerobic Biotechnologies* (pp. 51–72). IWA Publishing. https://doi.org/10.2166/9781789060225_0051

Fermoso, F. G., Van Hullebusch, E. D., Guibaud, G., Collins, G., Svensson, B. H., Carliell-Marquet, C., Vink, J. P. M., Esposito, G., & Frunzo, L. (2015). Fate of Trace Metals in Anaerobic Digestion. In G. M. Guebitz, A. Bauer, G. Bochmann, A. Gronauer, & S. Weiss (Eds.), *Biogas Science and Technology* (Vol. 151, pp. 171–195). Springer International Publishing. https://doi.org/10.1007/978-3-319-21993-6_7

Fernández-Rodríguez, M. J., Mancilla-Leytón, J. M., Jiménez-Rodríguez, A., Borja, R., & Rincón, B. (2021). Reuse of the digestate obtained from the biomethanization of olive mill solid waste (OMSW) as soil amendment or fertilizer for the cultivation of forage grass (Lolium rigidum var. Wimmera). *Science of the Total Environment*, *792*, 148465. https://doi.org/10.1016/j.scitotenv.2021.148465

Figura, Paul., & McDuffie, Bruce. (1980). Determination of labilities of soluble trace metal species in aqueous environmental samples by anodic stripping voltammetry and Chelex column and batch methods. *Analytical Chemistry*, *52*(9), 1433–1439. https://doi.org/10.1021/ac50059a015

Filgueiras, A. V., Lavilla, I., & Bendicho, C. (2002). Chemical sequential extraction for metal partitioning in environmental solid samples. *Journal of Environmental Monitoring*, *4*(6), 823–857. https://doi.org/10.1039/b207574c

Flyhammar, P. (1997). Estimation of heavy metal transformations in municipal solid waste. *Science of the Total Environment*, *198*(2), 123–133. https://doi.org/10.1016/S0048-9697(97)05439-9

Fox, J. A., Livingston, D. J., Orme-Johnson, W. H., & Walsh, C. T. (1987). 8-Hydroxy-5-deazaflavin-reducing hydrogenase from Methanobacterium thermoautotrophicum: 1. Purification and characterization. *Biochemistry*, *26*(14), 4219–4227. https://doi.org/10.1021/bi00388a007

Galceran, J., Gao, Y., Puy, J., Leermakers, M., Rey-Castro, C., Zhou, C., & Baeyens, W. (2021). Speciation of Inorganic Compounds in Aquatic Systems Using Diffusive Gradients in Thin-Films: A Review. *Frontiers in Chemistry*, *9*, 624511. https://doi.org/10.3389/fchem.2021.624511

Galceran, J., & Puy, J. (2015). Interpretation of diffusion gradients in thin films (DGT) measurements: A systematic approach. *Environmental Chemistry*, *12*(2), 112–122. https://doi.org/10.1071/EN14068

Gärtner, P., Ecker, A., Fischer, R., Linder, D., Fuchs, G., & Thauer, R. K. (1993). Purification and properties of N^5-methyltetrahydromethanopterin: Coenzyme M methyltransferase from *Methanobacterium thermoautotrophicum*. *European Journal of Biochemistry*, *213*(1), 537–545. https://doi.org/10.1111/j.1432-1033.1993.tb17792.x

Garuti, M., Langone, M., Fabbri, C., & Piccinini, S. (2018). Methodological approach for trace elements supplementation in anaerobic digestion: Experience from full-scale agricultural biogas plants. *Journal of Environmental Management*, *223*, 348–357. https://doi.org/10.1016/j.jenvman.2018.06.015

Glass, J. B., & Orphan, V. J. (2012). Trace metal requirements for microbial enzymes involved in the production and consumption of methane and nitrous oxide. *Frontiers in Microbiology*, *3*(FEB). https://doi.org/10.3389/fmicb.2012.00061

Glass, J. B., Yu, H., Steele, J. A., Dawson, K. S., Sun, S., Chourey, K., Pan, C., Hettich, R. L., & Orphan, V. J. (2014). Geochemical, metagenomic and metaproteomic insights into trace metal utilization by methane-oxidizing microbial consortia in sulphidic marine sediments. *Environmental Microbiology*, *16*(6), 1592–1611. https://doi.org/10.1111/1462-2920.12314

Gleyzes, C., Tellier, S., & Astruc, M. (2002). Fractionation studies of trace elements in contaminated soils and sediments: A review of sequential extraction procedures. *TrAC Trends in Analytical Chemistry*, *21*(6–7), 451–467. https://doi.org/10.1016/S0165-9936(02)00603-9

González-Suárez, A., Pereda-Reyes, I., Oliva-Merencio, D., & Montalvo-Martínez, S. J. (2020). Speciation of iron, nickel and cobalt in the anaerobic biodegradation of rice straw. *Revista Facultad de Ingeniería Universidad de Antioquia*, *101*, 55–63. https://doi.org/10.17533/udea.redin.20200366

Guan, D. X. (2019). Diffusive gradients in thin-films (DGT): An effective and simple tool for assessing contaminant bioavailability in waters, soils and sediments. In *Encyclopedia of Environmental Health* (pp. 111–124). Elsevier. https://doi.org/10.1016/B978-0-12-409548-9.11403-4

Guan, D.-X., Williams, P. N., Luo, J., Zheng, J.-L., Xu, H.-C., Cai, C., & Ma, L. Q. (2015). Novel Precipitated Zirconia-Based DGT Technique for High-Resolution Imaging of Oxyanions in Waters and Sediments. *Environmental Science & Technology*, *49*(6), 3653–3661. https://doi.org/10.1021/es505424m

Hagemeier, C. H., Křer, M., Thauer, R. K., Warkentin, E., & Ermler, U. (2006). Insight into the mechanism of biological methanol activation based on the crystal structure of the methanol-cobalamin methyltransferase complex. *Proceedings of the National Academy of Sciences*, *103*(50), 18917–18922. https://doi.org/10.1073/pnas.0603650103

Hamdi, M. (1991). The hydrodynamics of the environmental effects on the kinetics of methane formation in anaerobic sludge digestion. External mass transfer study. *Environmental Technology*, *12*(10), 907–913. https://doi.org/10.1080/09593339109385085

Hanay, Ö., Hasar, H., Kocer, N. N., & Aslan, S. (2008). Evaluation for agricultural usage with speciation of heavy metals in a municipal sewage sludge. *Bulletin of Environmental Contamination and Toxicology*, *81*(1), 42–46. https://doi.org/10.1007/s00128-008-9451-4

Holleman, A. F., Wiberg, E., Wiberg, N., Eagleson, M., Brewer, W., & Aylett, B. J. (Eds.). (2001). *Holleman-Wiberg inorganic chemistry*. Academic.

Huang, R., Zhang, B., Saad, E. M., Ingall, E. D., & Tang, Y. (2018). Speciation evolution of zinc and copper during pyrolysis and hydrothermal carbonization treatments of sewage sludges. *Water Research*, *132*, 260–269. https://doi.org/10.1016/j.watres.2018.01.009

Hullebusch, E., Utomo, S., Zandvoort, M., & Llens, P. (2005). Comparison of three sequential extraction procedures to describe metal fractionation in anaerobic granular sludges. *Talanta*, *65*(2), 549–558. https://doi.org/10.1016/j.talanta.2004.07.024

Ilic, A., Kouba, V., De Vrieze, J., Du Laing, G., & Bartacek, J. (2024). Diffusive gradients in thin films (DGT) as a robust and reliable technique to measure bioavailable metals in anaerobic digestates. *Environmental Technology & Innovation*, *33*, 103526. https://doi.org/10.1016/j.eti.2024.103526

Jaradat, Q. M., & Momani, K. A. (1999). Contamination of Roadside Soil, Plants, and Air With Heavy Metals in Jordan, A Comparative Study. *Turkish Journal of Chemistry*, *23*(2), 209–220.

Jiménez-Piedrahita, M., Altier, A., Cecilia, J., Puy, J., Galceran, J., Rey-Castro, C., Zhang, H., & Davison, W. (2017). Extending the Use of Diffusive Gradients in Thin Films (DGT) to Solutions Where Competition, Saturation, and Kinetic Effects Are Not Negligible. *Analytical Chemistry*, *89*(12), 6567–6574. https://doi.org/10.1021/acs.analchem.7b00704

Jin, H., & Chang, Z. (2011). Distribution of heavy metal contents and chemical fractions in anaerobically digested manure slurry. *Applied Biochemistry and Biotechnology*, *164*(3), 268–282. https://doi.org/10.1007/s12010-010-9133-7

Karlsson, A., Einarsson, P., Schnürer, A., Sundberg, C., Ejlertsson, J., & Svensson, B. H. (2012). Impact of trace element addition on degradation efficiency of volatile fatty acids, oleic acid and phenyl acetate and on microbial populations in a biogas digester. *Journal of Bioscience and Bioengineering*, *114*(4), 446–452. https://doi.org/10.1016/j.jbiosc.2012.05.010

Kersten, M., & Förstner, U. (1987). Effect of sample pretreatment on the reliability of solid speciation data of heavy metals—Implications sesfor the study of early diagenetic processes. *Marine Chemistry*, *22*(2–4), 299–312. https://doi.org/10.1016/0304-4203(87)90016-8

Laera, A., Buzier, R., Guibaud, G., Esposito, G., & van Hullebusch, E. D. (2019a). Assessment of the DGT technique in digestate to fraction twelve trace elements. *Talanta*, *192*(August 2018), 204–211. https://doi.org/10.1016/j.talanta.2018.09.033

Laera, A., Buzier, R., Guibaud, G., Esposito, G., & van Hullebusch, E. D. (2019b). Distribution trend of trace elements in digestate exposed to air: Laboratory-scale investigations using DGT-based fractionation. *Journal of Environmental Management*, *238*(November 2018), 159–165. https://doi.org/10.1016/j.jenvman.2019.02.120

Legros, S., Levard, C., Marcato-Romain, C. E., Guiresse, M., & Doelsch, E. (2017). Anaerobic Digestion Alters Copper and Zinc Speciation. *Environmental Science and Technology*, *51*(18), 10326–10334. https://doi.org/10.1021/acs.est.7b01662

Lehninger, A. L., Nelson, D. L., & Cox, M. M. (Eds.). (2003). *Lehninger principles of biochemistry* (3. ed., 7., printing). Worth Publ.

Li, C., Ding, S., Yang, L., Wang, Y., Ren, M., Chen, M., Fan, X., & Lichtfouse, E. (2019). Diffusive gradients in thin films: Devices, materials and applications. In *Environmental Chemistry Letters* (Vol. 17, Issue 2). Springer International Publishing. https://doi.org/10.1007/s10311-018-00839-9

Lindorfer, H., Ramhold, D., & Frauz, B. (2012). Nutrient and trace element supply in anaerobic digestion plants and effect of trace element application. *Water Science and Technology*, *66*(9), 1923–1929. https://doi.org/10.2166/wst.2012.399

Liu, C., Tong, Q., Li, Y., Wang, N., Liu, B., & Zhang, X. (2019). Biogas production and metal passivation analysis during anaerobic digestion of pig manure: Effects of a magnetic Fe3O4FA composite supplement. *RSC Advances*, *9*(8), 4488–4498. https://doi.org/10.1039/c8ra09451a

Mancini, G., Papirio, S., Lens, P. N. L., & Esposito, G. (2019). A preliminary study of the effect of bioavailable Fe and CO on the anaerobic digestion of rice straw. *Energies*, *12*(4), 1–11. https://doi.org/10.3390/en12040577

Marcato, C. E., Pinelli, E., Cecchi, M., Winterton, P., & Guiresse, M. (2009). Bioavailability of Cu and Zn in raw and anaerobically digested pig slurry. *Ecotoxicology and Environmental Safety*, *72*(5), 1538–1544. https://doi.org/10.1016/j.ecoenv.2008.12.010

Marrugo-Madrid, S., Turull, M., Zhang, H., & Díez, S. (2021). Diffusive gradients in thin films for the measurement of labile metal species in water and soils: A review. *Environmental Chemistry Letters*, *19*(5), 3761–3788. https://doi.org/10.1007/s10311-021-01246-3

Matheri, A. N., Ntuli, F., & Ngila, J. C. (2021). Sludge to energy recovery dosed with selected trace metals additives in anaerobic digestion processes. *Biomass and Bioenergy*, *144*, 105869. https://doi.org/10.1016/j.biombioe.2020.105869

Metcalf & Eddy (Ed.). (2014). *Wastewater engineering: Treatment and resource recovery* (5. ed., internat. student ed). McGraw-Hill.

Micolucci, F., Gottardo, M., Cavinato, C., Pavan, P., & Bolzonella, D. (2016). Mesophilic and thermophilic anaerobic digestion of the liquid fraction of pressed biowaste for high energy yields recovery. *Waste Management*, *48*, 227–235. https://doi.org/10.1016/j.wasman.2015.09.031

Oleszkiewicz, J. A., & Sharma, V. K. (1990). Stimulation and inhibition of anaerobic processes by heavy metals—A review. *Biological Wastes*, *31*(1), 45–67. https://doi.org/10.1016/0269-7483(90)90043-R

Ortner, M., Rachbauer, L., Somitsch, W., & Fuchs, W. (2014). Can bioavailability of trace nutrients be measured in anaerobic digestion? *Applied Energy*, *126*(2014), 190–198. https://doi.org/10.1016/j.apenergy.2014.03.070

Österlund, H., Chlot, S., Faarinen, M., Widerlund, A., Rodushkin, I., Ingri, J., & Baxter, D. C. (2010). Simultaneous measurements of As, Mo, Sb, V and W using a ferrihydrite diffusive gradients in thin films (DGT) device. *Analytica Chimica Acta*, *682*(1–2), 59–65. https://doi.org/10.1016/j.aca.2010.09.049

Osuna, M. B., Van Hullebusch, E. D., Zandvoort, M. H., Iza, J., & Lens, P. N. L. (2004). Effect of Cobalt Sorption on Metal Fractionation in Anaerobic Granular Sludge. *Journal of Environmental Quality*, *33*(4), 1256–1270. https://doi.org/10.2134/jeq2004.1256

Özen Daş, İ. T., Özmıhçı, S., & Büyükkamacı, N. (2024). Environmental impact analysis of different wastes to biohydrogen, biogas and biohytane processes. *International Journal of Hydrogen Energy*, *56*, 1446–1463. https://doi.org/10.1016/j.ijhydene.2023.12.184

Paudel, S. R., Banjara, S. P., Choi, O. K., Park, K. Y., Kim, Y. M., & Lee, J. W. (2017). Pretreatment of agricultural biomass for anaerobic digestion: Current state and challenges. *Bioresource Technology*, *245*, 1194–1205. https://doi.org/10.1016/j.biortech.2017.08.182

Phan, K.-H., Nguyen, A.-T., Le, S.-L., Ngo, T.-M., Cao, T. N.-D., Nguyen, T.-T., Le, L.-T., Mai, T.-P., & Bui, X.-T. (2023). Thermophilic anaerobic digestion and emerging methods for organic waste treatment: A review. *Bioresource Technology Reports*, *22*, 101402. https://doi.org/10.1016/j.biteb.2023.101402

Puy, J., Uribe, R., Mongin, S., Galceran, J., Cecília, J., Levy, J., Zhang, H., & Davison, W. (2012). Lability Criteria in Diffusive Gradients in Thin Films. *The Journal of Physical Chemistry A*, *116*(25), 6564–6573. https://doi.org/10.1021/jp212629z

Rao, A. L. J., Singh, M., & Sehgal, S. (1986). Determination of Stability Constants of Metal Complexes by Polarographic Methods—A Review. *Reviews in Analytical Chemistry*, *8*(4). https://doi.org/10.1515/REVAC.1986.8.4.283

Rapin, Francois., Tessier, Andre., Campbell, P. G. C., & Carignan, Richard. (1986). Potential artifacts in the determination of metal partitioning in sediments by a sequential extraction procedure. *Environmental Science & Technology*, *20*(8), 836–840. https://doi.org/10.1021/es00150a014

Sahin, F. (2003). Selective pre-concentration of selenite from aqueous samples using mercapto-silica. *Talanta*, *60*(5), 1003–1009. https://doi.org/10.1016/S0039-9140(03)00154-1

Sahuquillo, A., López-Sánchez, J. F., Rubio, R., Rauret, G., Thomas, R. P., Davidson, C. M., & Ure, A. M. (1999). Use of a certified reference material for extractable trace metals to assess sources of uncertainty in the BCR three-stage sequential extraction procedure. *Analytica Chimica Acta*, *382*(3), 317–327. https://doi.org/10.1016/S0003-2670(98)00754-5

Sánchez, C. (2009). Lignocellulosic residues: Biodegradation and bioconversion by fungi. *Biotechnology Advances*, *27*(2), 185–194. https://doi.org/10.1016/j.biotechadv.2008.11.001

Scally, S., Davison, W., & Zhang, H. (2003). In Situ Measurements of Dissociation Kinetics and Labilities of Metal Complexes in Solution Using DGT. *Environmental Science & Technology*, *37*(7), 1379–1384. https://doi.org/10.1021/es0202006

Schattauer, A., Abdoun, E., Weiland, P., Plöchl, M., & Heiermann, M. (2011). Abundance of trace elements in demonstration biogas plants. *Biosystems Engineering*, *108*(1), 57–65. https://doi.org/10.1016/j.biosystemseng.2010.10.010

Schauer, N. L., & Ferry, J. G. (1986). Composition of the coenzyme F420-dependent formate dehydrogenase from Methanobacterium formicicum. *Journal of Bacteriology*, *165*(2), 405–411. https://doi.org/10.1128/jb.165.2.405-411.1986

Seruga, P. (2021). The municipal solid waste management system with anaerobic digestion. *Energies*, *14*(8). https://doi.org/10.3390/en14082067

Solís, G. J., Alonso, E., & Riesco, P. (2002). Distribution of metal extractable fractions during anaerobis suldge treatment in southerrn Spain WWTPS. *Water, Air, and Soil Pollution*, *140*, 139–156. https://doi.org/10.1023/A:1020128605211

Takashima, M. (2018). Effects of Thermal Pretreatment and Trace Metal Supplementation on High-Rate Thermophilic Anaerobic Digestion of Municipal Sludge. *Journal of Environmental Engineering*, *144*(3), 04018009. https://doi.org/10.1061/(ASCE)EE.1943-7870.0001340

Takeno, N. (2005). Atlas of Eh-pH diagrams Intercomparison of thermodynamic databases. *National Institute of Advanced Industrial Science and Technology Tokyo*, *419*, 285.

Temminghoff, E. J. M., Plette, A. C. C., Van Eck, R., & Van Riemsdijk, W. H. (2000). Determination of the chemical speciation of trace metals in aqueous systems by the Wageningen Donnan Membrane Technique. *Analytica Chimica Acta*, *417*(2), 149–157. https://doi.org/10.1016/S0003-2670(00)00935-1

Templeton, D. M., Ariese, F., Cornelis, R., Danielsson, L.-G., Muntau, H., Van Leeuwen, H. P., & Lobinski, R. (2000). Guidelines for terms related to chemical speciation and fractionation of elements. Definitions, structural aspects, and methodological approaches (IUPAC Recommendations 2000). *Pure and Applied Chemistry*, *72*(8), 1453–1470. https://doi.org/10.1351/pac200072081453

Tersteegen, A., & Hedderich, R. (1999). *Methanobacterium thermoautotrophicum* encodes two multisubunit membrane-bound [NiFe] hydrogenases: Transcription of the operons and sequence analysis of the deduced proteins. *European Journal of Biochemistry*, *264*(3), 930–943. https://doi.org/10.1046/j.1432-1327.1999.00692.x

Tessier, A., Campbell, P. G. C., & Bisson, M. (1979). Sequential Extraction Procedure for the Speciation of Particulate Trace Metals. *Analytical Chemistry*, *51*(7), 844–851. https://doi.org/10.1021/ac50043a017

Thanh, P. M., Ketheesan, B., Yan, Z., & Stuckey, D. (2016). Trace metal speciation and bioavailability in anaerobic digestion: A review. *Biotechnology Advances*, *34*(2), 122–136. https://doi.org/10.1016/j.biotechadv.2015.12.006

Thanh, P. M., Ketheesan, B., Zhou, Y., & Stuckey, D. C. (2017). Effect of operating conditions on speciation and bioavailability of trace metals in submerged anaerobic membrane bioreactors. *Bioresource Technology*, *243*, 810–819. https://doi.org/10.1016/j.biortech.2017.07.040

Thauer, R. K. (1998). Biochemistry of methanogenesis: A tribute to Marjory Stephenson:1998 Marjory Stephenson Prize Lecture. *Microbiology*, *144*(9), 2377–2406. https://doi.org/10.1099/00221287-144-9-2377

Thauer, R. K. (2011). Anaerobic oxidation of methane with sulfate: On the reversibility of the reactions that are catalyzed by enzymes also involved in methanogenesis from CO2. *Current Opinion in Microbiology*, *14*(3), 292–299. https://doi.org/10.1016/j.mib.2011.03.003

Tusseau-Vuillemin, M.-H., Gilbin, R., & Taillefert, M. (2003). A Dynamic Numerical Model To Characterize Labile Metal Complexes Collected with Diffusion Gradient in Thin Films Devices. *Environmental Science & Technology*, *37*, 1645–1652.

Ure, A. M., Quevauviller, Ph., Muntau, H., & Griepink, B. (1993). Speciation of Heavy Metals in Soils and Sediments. An Account of the Improvement and Harmonization of Extraction Techniques Undertaken Under the Auspices of the BCR of the Commission of the

European Communities. *International Journal of Environmental Analytical Chemistry*, *51*(1–4), 135–151. https://doi.org/10.1080/03067319308027619

Van Der Veen, A., Fermoso, F. G., & Lens, P. N. L. (2007). Bonding Form Analysis of Metals and Sulfur Fractionation in Methanol-Grown Anaerobic Granular Sludge. *Engineering in Life Sciences*, *7*(5), 480–489. https://doi.org/10.1002/elsc.200720208

Van Leeuwen, H. P. (2001). Revisited: The Conception of Lability of Metal Complexes. *Electroanalysis*, *13*(10), 826–830. https://doi.org/10.1002/1521-4109(200106)13:10<826::AID-ELAN826>3.0.CO;2-J

Vintiloiu, A., Lemmer, A., Oechsner, H., & Jungbluth, T. (2012). Mineral substances and macronutrients in the anaerobic conversion of biomass: An impact evaluation. *Engineering in Life Sciences*, *12*(3), 287–294. https://doi.org/10.1002/elsc.201100159

Vorholt, J. A., Vaupel, M., & Thauer, R. K. (1996). A Polyferredoxin with Eight [4Fe–4S] Clusters as a Subunit of Molybdenum Formylmethanofuran Dehydrogenase from *Methanosarcina barkeri*. *European Journal of Biochemistry*, *236*(1), 309–317. https://doi.org/10.1111/j.1432-1033.1996.t01-1-00309.x

Wallmann, K., Kersten, M., Gruber, J., & Förstner, U. (1993). Artifacts in the Determination of Trace Metal Binding Forms in Anoxic Sediments by Sequential Extraction. *International Journal of Environmental Analytical Chemistry*, *51*(1–4), 187–200. https://doi.org/10.1080/03067319308027624

Wang, Q., Zhang, C., Jung, H., Liu, P., Patel, D., Pavlostathis, S. G., & Tang, Y. (2021). Transformation and Mobility of Cu, Zn, and Cr in Sewage Sludge during Anaerobic Digestion with Pre-or Interstage Hydrothermal Treatment. *Environmental Science and Technology*, *55*(3), 1615–1625. https://doi.org/10.1021/acs.est.0c05164

Wang, T., Tang, Z., Guo, Y., Zhang, X., Yang, Q., Xu, B., & Wang, H. (2020). Comparison of Heavy Metal Speciation of Sludge During Mesophilic and Thermophilic Anaerobic Digestion. *Waste and Biomass Valorization*, *11*(6), 2651–2660. https://doi.org/10.1007/s12649-018-00575-9

Wei, T.-J., Guan, D.-X., Li, X.-Y., Hao, Y.-L., Teng, H. H., Yang, J.-F., Xu, Y.-Y., & Li, G. (2022). Analysis of studies on environmental measurements using diffusive gradients in thin-films (DGT) from 1994 to 2020. *Journal of Soils and Sediments*, *22*(4), 1069–1079. https://doi.org/10.1007/s11368-022-03168-1

Weinrich, S., & Nelles, M. (2021). *Basics of anaerobic digestion biochemical conversion and process modelling*. Deutsches Biomasseforschungszentrum gemeinnützige GmbH.

Weng, Van Riemsdijk, W. H., & Temminghoff, E. J. M. (2005). Kinetic Aspects of Donnan Membrane Technique for Measuring Free Trace Cation Concentration. *Analytical Chemistry*, *77*(9), 2852–2861. https://doi.org/10.1021/ac0485435

Yang, L., Xu, F., Ge, X., & Li, Y. (2015). Challenges and strategies for solid-state anaerobic digestion of lignocellulosic biomass. *Renewable and Sustainable Energy Reviews*, *44*, 824–834. https://doi.org/10.1016/j.rser.2015.01.002

Zdeb, M., Pawłowska, M., & Pacan, J. (2020). The influence of anaerobic digestion on selected heavy metals fractionation in sewage sludge. *Journal of Ecological Engineering*, *21*(3), 27–35. https://doi.org/10.12911/22998993/118302

Zehl, K., & Einax, J. (2005). Influence of Atmospheric Oxygen on Heavy Metal Mobility in Sediment and Soil. *Journal of Soils and Sediments*, *5*(3), 164–170. https://doi.org/10.1065/jss2005.01.132

Zerkle, A. L. (2005). Biogeochemical signatures through time as inferred from whole microbial genomes. *American Journal of Science*, *305*(6–8), 467–502. https://doi.org/10.2475/ajs.305.6-8.467

Zhang, F., Li, Y., Yang, M., & Li, W. (2012). Content of heavy metals in animal feeds and manures from farms of different scales in Northeast China. *International Journal of Environmental Research and Public Health*, *9*(8), 2658–2668. https://doi.org/10.3390/ijerph9082658

Zhang, H., & Davison, W. (1995). Performance Characteristics of Diffusion Gradients in Thin Films for the in Situ Measurement of Trace Metals in Aqueous Solution. *Analytical Chemistry*, *67*(19), 3391–3400. https://doi.org/10.1021/ac00115a005

Zhang, M., Yang, C., Jing, Y., & Li, J. (2016). Effect of energy grass on methane production and heavy metal fractionation during anaerobic digestion of sewage sludge. *Waste Management*, *58*, 316–323. https://doi.org/10.1016/j.wasman.2016.09.040

Zhang, Q., Zhang, L., Sang, W., Li, M., & Cheng, W. (2016). Chemical speciation of heavy metals in excess sludge treatment by thermal hydrolysis and anaerobic digestion process. *Desalination and Water Treatment*, *57*(27), 12770–12776. https://doi.org/10.1080/19443994.2015.1055518

Zhang, X., Fan, Y., Hao, T., Chen, R., Zhang, T., Hu, Y., Li, D., Pan, Y., Li, Y.-Y., & Kong, Z. (2024). Insights into current bio-processes and future perspectives of carbon-neutral treatment of industrial organic wastewater: A critical review. *Environmental Research*, *241*, 117630. https://doi.org/10.1016/j.envres.2023.117630

Zheng, X., Wu, K., Sun, P., Zhouyang, S., Wang, Y., Wang, H., Zheng, Y., & Li, Q. (2021). Effects of substrate types on the transformation of heavy metal speciation and bioavailability in an anaerobic digestion system. *Journal of Environmental Sciences (China)*, *101*, 361–372. https://doi.org/10.1016/j.jes.2020.08.032

Zheng, X., Wu, K., Sun, P., Zhouyang, S., Wang, Y., Wang, H., Zheng, Y., Li, Q., Geng, H., Xu, Y., Zheng, L., Gong, H., Dai, L., Dai, X., Zheng, X., Liu, Y., Huang, J., Du, Z., Zhouyang, S., … Shen, X. (2020). The influence of variables on the bioavailability of heavy metals during the anaerobic digestion of swine manure. *Ecotoxicology and Environmental Safety*, *195*(December 2019), 110457. https://doi.org/10.1016/j.ecoenv.2020.110457

Zhu, N. min, Qiang-Li, Guo, X. jing, Hui-Zhang, & Yu-Deng. (2014). Sequential extraction of anaerobic digestate sludge for the determination of partitioning of heavy metals. *Ecotoxicology and Environmental Safety*, *102*(1), 18–24. https://doi.org/10.1016/j.ecoenv.2013.12.033

Zitomer, D. H., Johnson, C. C., & Speece, R. E. (2008). Metal Stimulation and Municipal Digester Thermophilic/Mesophilic Activity. *Journal of Environmental Engineering*, *134*(1), 42–47. https://doi.org/10.1061/(ASCE)0733-9372(2008)134:1(42)

Chapter II. Effect of Cobalt continuous dose in UASB, focus on metal profile and Extracellular Polymeric Substances

II.1. Introduction

Upflow anaerobic sludge blanket reactor (UASB) is a highly efficient technology for the treatment of liquid waste through anaerobic digestion, allowing operations with high organic load rates and low hydraulic residence times. The main characteristic of the UASB is biomass granulation, which entails an easy separation of liquid and solid phases in the same reactor (Latif et al., 2011). The feed enters by the bottom of the reactor and the upflow velocity causes the fluidization of the sludge blanket, allowing the agitation of the granules. This configuration gives to the UASB a hydrodynamic behavior close to a completely stirred tank reactor or extended tanks in series (Cisneros et al., 2021; López & Borzacconi, 2010). However, studies have found that the sludge may present concentration profiles. For example, it has been observed a decrease along the height in the concentration of solids (total, volatile and suspended volatile) (Elmitwalli & Otterpohl, 2007; Vadlani & Ramachandran, 2008), and number and size of granules (Ozgun et al., 2019). Similarly, a decrease in polysaccharides and protein external polymeric substances (EPS) concentration along the height in the sludge has been detected (Ozgun et al., 2019) as well as changes in the relative abundance of microorganisms (Antwi et al., 2017; Ozgun et al., 2019). However, there is a lack of knowledge on metal concentration profiles in granular sludge.

An important element in correct UASB operation is the stability of the granules of biomass. Operational problems are very likely to occur if the granules break or are not large enough to settle in the liquid and solid phase separation area of the reactor. Typical problems are reactor biomass washing out, outflow obstruction or sludge bed fluidization problems, which would lead to reduced reactor performance or even failure. The granulation of the sludge can be enhance through flocculants, such as Ca^{+2} and Fe^{+2} (Yu et al., 2001), and sporadically through flocculants secreted by microorganisms, such as extracellular polymeric substances (EPS). EPS are composed of a complex mix of biopolymers such as polysaccharides, proteins, nucleic acids, lipids, humic substances, and uronic acids (Pal & Paul, 2008). These substances can bind to the surface of the EPS cell and form a matrix that allows the flocculation of biomass. Therefore, they are key elements in the granulation processes and maintenance of the physical integrity and stability of the granule (Huang et al., 2022; Liu et al., 2010; Peng et al., 2021). Additionally, EPS covering the peripheral surface of a cell can provide a way of protection against the infiltration of toxic metals (Muthu et al., 2017). Metals have been reported to have a different binding capacity with EPS. Being trapped in the organic matrix of EPS (Huang et al., 2022), their bioavailability can significantly change in anaerobic digestion systems. EPS in UASB has been previously studied. (Ozgun et al., 2019) characterized the polysaccharides and protein EPS concentration at the end of the operational period along different heights in a UASB. On the other hand, the interrelation of Cobalt (Co) in EPS production in anaerobic system through Biomethane Potential assay has been evaluated by Hasani Zadeh et al. (2022). However, the evolution of EPS in a UASB along the height under stress with different concentrations of trace metals has not been studied yet.

Trace metals are necessary for the good performance of the anaerobic digestion process (Fermoso et al., 2019b). In particular, Co is essential for methanogens organisms, being part of the production and activation of various enzymes, coenzymes, and vitamin B12 (Li et al., 2023; Matheri et al., 2021), which are implied in methane generation. Thus a correct Co concentration can improve biogas production. Co supplementation in low concentration has shown an increase in biogas yield (Garuti et al., 2018; Li et al., 2023; Reungsang et al., 2016). However, an inhibitory effect has been observed with high concentrations (Chen et al., 2016; Matheri et al., 2021; Paulo et al., 2017). Recommended concentrations of Co in anaerobic systems have been collected by Garuti et al. (2018) and Matheri et al. (2021) and the values fluctuate between 0.6 µg L^{-1} and 10 mg L^{-1}. In these reviews it is explained that the differences could be attributable to several factors, such as inoculum origin, feedstock composition, chemical speciation, and bioavailability of Co. Indeed, the dosed metal usually forms inert species that are not bioavailable, such as sulfide and carbonates, so that the fraction available to microorganisms is less than the total dose.

There are only a few Co dose studies conducted at UASB. The relationship between UASB response variables regarding pulse dose of Co in different forms, such as chloride and complexed with EDTA has been evaluated by Fermoso et al. (2008, 2010), and in more detail, the free, dissolved, and total Co fractions has been quantified by Bartacek et al. (2008). For continuous dosing in UASB, Co concentration has been quantified in effluent and sludge, but Co is dosed in a mix of trace metals, so the effect in the reactor response cannot be attributed exclusively to Co (Pornmai et al., 2023; Reungsang et al., 2016; Subramanyam & Mishra, 2013). Co deprivation in UASB reactors has been evaluated previously (Zandvoort et al., 2002, 2006), showing a negative impact on biogas production, with a consequent improvement when Co was added to the reactor subsequently. Studies focused exclusively on the effect on reactor performance due to different continuous doses of Co in UASB reactor operation, as well as their characterization in different fractions along the sludge blanket have not yet been reported. The present study investigates the effect of the different continuous doses of Co on the performance of a UASB reactor, delivering a comprehensive and interrelated view between the metal dose, different fractions of Co (soluble and total) found in the reactor, the evolution of Co along the sludge blanket and reactor operation, with particular emphasis on EPS changes.

II.2. Materials and methods

II.2.1. Inoculum and feeding solution

The anaerobic granule inoculum was obtained from a UASB reactor of a beer factory wastewater treatment plant in Seville, Spain. The main chemical characterization of the inoculum was: pH = 7.43 ± 0.05, Total alkalinity = 3700 ± 300 mg $CaCO_3$ L^{-1}, Total Solids = 58.0 ± 3.1 g kg^{-1}, Volatile Solids = 39.3 ± 2.2 g kg^{-1}, and Total COD = 108.1 ± 2.2 g O_2 kg^{-1}.

The reactor was fed with a synthetic solution. This solution was stored separately in 2 vessels to avoid possible precipitation reactions between the different mineral compounds. The solutions were pumped and mixed in equal proportions before the reactor entrance. The first bag contained glucose and micronutrients, and the second one sodium bicarbonate and macronutrients. The alkalinity was set at 0.80 gCOD $gCaCO_3^{-1}$ and the concentration of micro and macronutrients was set according to (Brunetti et al., 1983; Fermoso et al., 2008; Lin & Lin, 1997). Thus, the concentration of the influent was: glucose: 2.85 g L^{-1}, $NaHCO_3$: 2.5 g L^{-1}, macronutrients (in mg L^{-1}): NH_4Cl: 165, KH_2PO_4: 33, $MgSO_4$: 59, $CaCl_2$: 5.9, micronutrients (in mg L^{-1}): $FeCl_2 \cdot 4H_2O$: 450, $CoCl_2 \cdot 6H_2O$: 450, $MnCl_2 \cdot 4H_2O$: 112.5, $NiCl_2$: 100, $AlCl_3 \cdot 6H_2O$: 20.25, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$: 11.25, $ZnCl_2$: 11.25, and $CuCl_2 \cdot 2H_2O$: 8.55.

II.2.2. Start-up and reactor operation

A lab scale glass UASB anaerobic reactor of 5 L with a stable operation was used for this experiment. Initially, the reactor was inoculated with 40% of the volume with granular sludge. Temperature was maintained at 35 ± 2 °C with a heating system. The UASB reactors were operated at a hydraulic retention time (HRT) of 24 h and an organic loading rate (OLR) of 3 g COD L^{-1} d^{-1}, with no recirculation. The biogas was collected in a gas bag, which was emptied periodically.

A stable operation was reached after 108 d of start-up, where the sludge bed remained in the same position inside the UASB, and no washing out of the sludge was observed. Additionally, COD removal showed a variation < 5% between consecutive measurements. Then, after this start-up period, the metal dosage experiment was performed.

II.2.3. Metal dosage in UASB reactor

Continuous doses of Co were evaluated, 6 concentrations were selected: 0.1, 10, 50, 100, 500, and 1000 µM of Co, the first value corresponded to the Co concentration in the base. These concentrations were equivalent to a dose of 0.1, 10, 50, 100, 500, and 1000 µM of Co $L_{reactor}^{-1}$ d^{-1}, respectively. For each concentration, Co was added to the feed until the selected concentration was reached. These concentrations were dosed in consecutive cycles. Each cycle consisted of 2 stages, a first stage where Co was dosed for at least 14 d, until a pseudo-steady state in biogas productivity and COD remotion was observed, and a second stage of cleaning of the reactor, where the reactor was fed with Co on dose base (0.1 µM) for at least 3 d to remove the remains of Co that could be present in the liquid phase in order to not interfere with the next stage. After this period, the next cycle started. In detail, the dosing time of each Co concentration were: for 0.1 µM, all the period of start-up, i.e., operation days 0 – 175,; 10 µM, 17 d, i.e., operation days 175 – 192; 50 µM, 14 d, i.e., operation days 217 – 231; 100 µM, 16 d, i.e., operation days 236 – 252; 500 µM, 18 d, i.e., operation days 265 – 283; 1000 µM, 14 d, i.e., operation days 292 – 306.

Volume and composition of biogas, pH, ratio volatile fatty acids – alkalinity were monitored daily. COD removal was monitored 3 times per week. At the end of the steady-state, samples were taken in 3 points of the reactor, separated by 10 cm, from the bottom of the reactor these points corresponded to bottom sludge (0 cm), top sludge (10 cm), and liquid zone (20 cm). These samples were used for metal concentration and EPS analysis.

II.2.4. Residence time distribution test

The hydrodynamic behavior of the UASB was evaluated through a residence time distribution test (RTD). A UASB reactor in parallel with the same set-up, i.e., same dimensions, pipping, HRT, OLR, flow and composition of base feed, percentage of sludge fed, etc. was assembled only for the purpose of this test. NaCl was used as a tracer. A calibration curve was performed that related the concentration of NaCl in the feed to the electrical conductivity by electrode (HACH, sensION+ MM150). 300 mL of NaCl 5 M in one pulse were added at the reactor feed inlet and the electrical conductivity was monitored at the reactor outlet during 72 h (3 HRT). The results were analyzed with the Miller dispersion index (MDI) (Metcalf & Eddy, 2014).

II.2.5. Analytical methods

Total and volatile solids and COD were measured according to standard methods (APHA, 2017). pH was measured with electrode (HACH, sensION+ MM150). Total alkalinity and ratio volatile fatty acid – alkalinity were measured by sequential titration with H_2SO_4 0.01 M at pH 5.75 and 4.3 (Ripley & Boyle, 1986). The biogas collected in bags was quantified by a volumetric meter (Ritter, TG1/5), and the composition of biogas (CH_4, CO_2, NH_3, and H_2S) with a gas analyzer (Geotech, Biogas 5000). The biogas volume is indicated in normal conditions (0 °C, 1 atm).

EPS were measured by extraction with the method performed by Hasani Zadeh et al. (2022) with the difference that the surrounding water was carefully and manually removed by decanting to avoid breaking the granules. 2 g of granules of sludge (wet mass), were used for the analysis. The main components in EPS, carbohydrates and proteins, were measured by Anthrone method and bicinchoninic acid (BCA) assay, respectively. The samples were measured in a spectrophotometer (Biorad iMark Microplate reader, Hercules, CA, USA) at 630 nm and 620 nm for carbohydrates and proteins, respectively.

II.2.6. Metal analysis

Initial sludge and samples from the 3 different points at the steady-state were analyzed in different fractions. Solid fraction was obtained through decantation of the granules, and manual separation of the liquid phase. Liquid soluble fraction was obtained from the liquid phase separated from the granules in decantation process, after centrifugation at 4000 rpm x 30 min (orto alresa, digtor22) and filtration at 0.22 µm (nylon filter, RephiQuik).

The samples, for both for liquid and solid fractions, were digested with 10 mL of HNO_3 69% (trace analysis quality, PlasmaPURE) in a microwave oven (CEM, MARS 5) at three stages program: 100°C for 3 min, 170 °C for 10 min, and 195°C for 7 min, with temperature ramp of 10 min between the stages for reaching the temperature. 0.15 g of granules (wet mass) for the solid fraction, and 3 g of the liquid fraction were used. Finally, the digested solutions were filtrated by 0.22 µm (nylon filter, RephiQuik). For all the fractions, if it was necessary, HNO_3 concentration was adjusted to $\geq 3\%$ adding HNO_3 69%, then these were analyzed by ICP-MS (Agilent 7800). Internal standard ^{103}Rh was added and blanks (solution HNO_3 3%, with ultrapure water) were analyzed every 20 samples. The recovery of the Internal standard was in the range 80 - 120%, with a relative standard deviation below 10%.

II.3. Results and Discussion

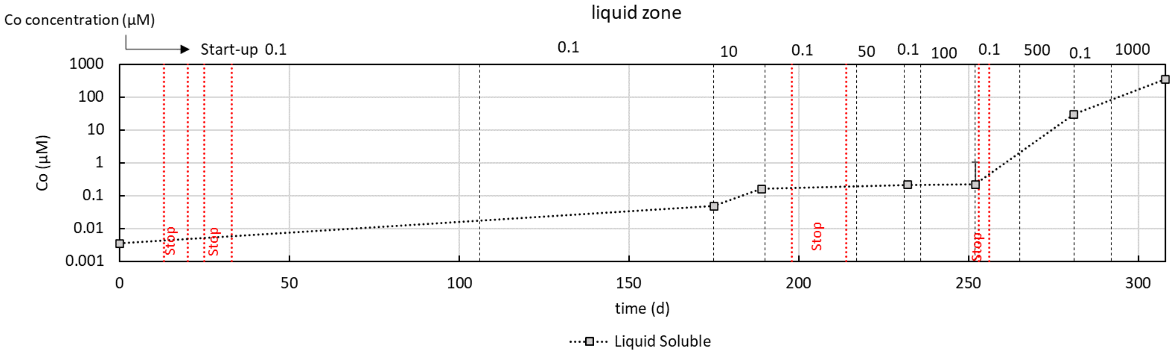
This section is divided into three parts, each focused on a factor evaluated in this study. The first part refers to the characterization of the different fractions of Co in the UASB, their evolution over time, accumulation, and profile along the reactor. The second part is focused on the UASB performance response variables: pH, alkalinity, biogas and COD removal. The time series, as well as the relationship between these responses and the different fractions of Co measured are presented. Finally, the third part refers to the analysis of EPS in the UASB. The time series, profile along the sludge bed, and relationship between EPS and the fractions of Co measured are presented.

II.3.1. Different Cobalt fractions in UASB

II.3.1.1. Time serie of Cobalt concentration

The Cobalt concentrations over time in the 3 sample points inside the reactor are presented in Figure II.1. During the 175 d of start-up, a continuous dose of 0.1 µM of Co was added to the UASB, in this period, an increase of Co concentration was observed for all the fractions. In the liquid zone, the Co concentration was found between 20 and 30 times higher than the concentration at time 0, meanwhile, in granules the accumulation of around 100 and 145 times the initial concentration of Co in the sludge was observed in the bottom and top sludge, respectively. These observations can be explained due to accumulation of Co in the reactor. During the next 5 Co continuous dose cycle, i.e., 10, 50, 100, 500, and 1000 µM of Co, an increase of Co concentration was detected in solid fraction in both sampling points in the sludge zone in the reactor over time, showing an accumulation of Co inside the reactor. At the end of the experiment, the concentration in the granules was 30 ± 7 and 26 ± 18 mM of Co for the top and bottom sludge, respectively; around 7200 times the initial Co concentration. On the other hand, the liquid soluble concentration remained relatively constant during Co continuous dose cycle of 10, 50, and 100 µM of Co. The higher increases in liquid soluble concentration were observed for the last two dose concentrations in the 3 sampling points, with an increase of 134 ± 6 and 11 ± 2 times in Co liquid soluble fraction detected from 100 to 500 and 500 to 1000 µM of Co continuous dose, respectively.

A)



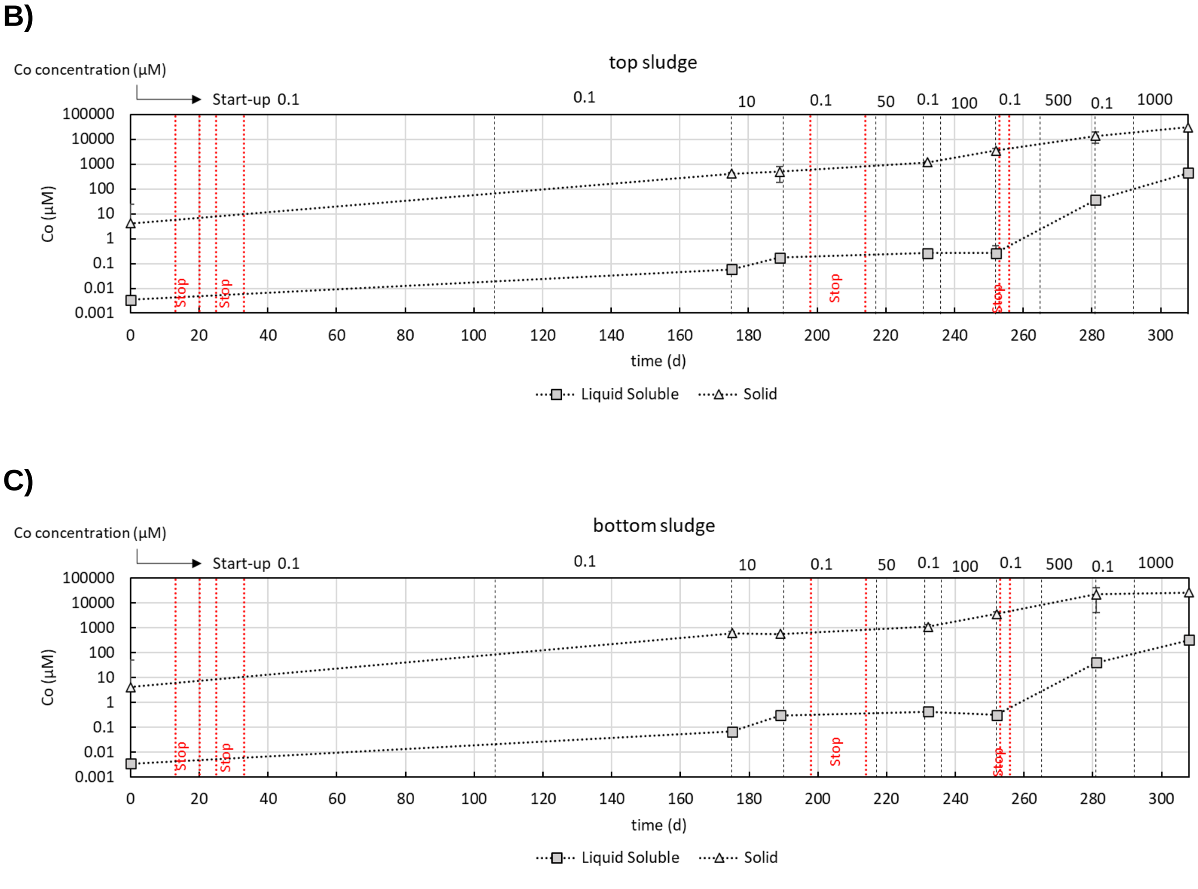


Figure II.1: Liquids and solid Co concentration in the 3 points sampled in reactor UASB. The different concentrations of Co dose are indicated with the dotted vertical lines, and the period between red dotted lines are the time when the reactor operation was stopped. A) Liquid zone, B) Top Sludge, C) Bottom sludge

II.3.1.2. Accumulation of Cobalt in UASB

The comparison between the Co concentration in feed and the Co concentration measured in the pseudo-steady state is shown in Figure II.2. Additionally, the total Co added in terms of $\mu mol_{Co}\ L_{sludge}^{-1}$ is included. This comparison allows to determine if there were accumulation of Co in the UASB. The Cobalt content added during each Co dose was 80.6, 850, 3500, 8000, 45000, and 70000 µmol of Co, for the cycles of dose 0.1, 10, 50, 100, 500 and 1000 µM of Co, respectively. At the end of the operation of 306 d, 127.4 mmol of Co were added to the UASB. The concentration in the liquid zone should be technically the same as the effluent, since in this zone no biological reactions happen, just the physical separation between liquid, granules and biogas.

The Co liquid soluble concentration for all the Co doses was lower than the Co concentration in the feeding solution, indicating that the Co added had to be accumulated in the UASB. The total added Co concentration was higher than the Co concentration in the sludge for the 2 sampling points measured in the sludge. The estimated retention of Co in the sludge for the 0.1 and 10 µM Co dose were the 80 ± 20 % and 28 ± 3 % of the total Co added, respectively.

Meanwhile for the next doses the estimated retention was around 11 – 18 % of the total Co added, indicating a decrease in the Co retained by the sludge.

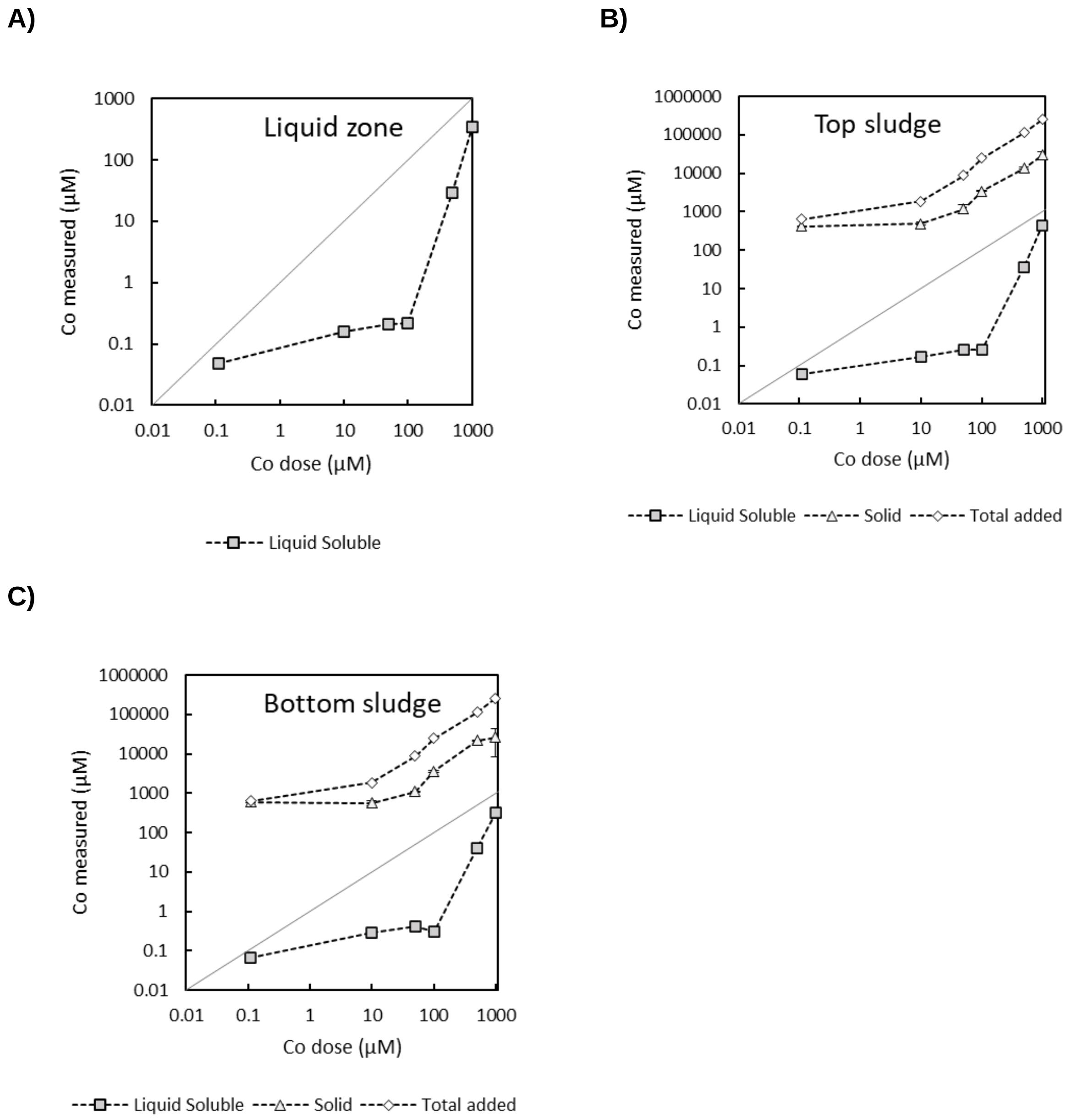


Figure II.2: Different Co concentrations measured compare to Co concentration in continuous dosage. A) liquid zone, B) top sludge, and C) bottom sludge. Diagonal gray line: Co measured = Co dose

II.3.1.3. Cobalt profile along the UASB

The hydrodynamic of the UASB was evaluated with a residence time distribution test and quantified with the Morril dispersion index (MDI), which is the ratio between the percentile 90 and 10 of the accumulate tracer recovery. A MDI of 1 indicates the hydrodynamic behavior of an ideal plug flow reactor, and a MDI of 22 corresponds to an ideal completely stirred tank reactor (CSTR). The UASB of this study had a MDI value of 16.7, indicating a CSTR hydrodynamic behavior of the liquid phase. However, despite presenting a hydrodynamic similar to a mixing tank, similarly observed in previous studies (Cisneros et al., 2021; Vian et al., 2020), the sludge bed can present a profile in various parameters, such as the metal concentration because the granules were slightly fluidized, but not mixed, generating in the sludge bed a structure similar to a fixed bed. The bottom of the UASB is the first contact zone between the feed and the sludge bed, therefore this area is more stressed by the presence of Co. In this way, Co may have interacted with microorganisms and other substances present, which causes Co to accumulate in the sludge, such as precipitation when reacting with the present sulfide. This decreases the content of Co that advances in the sludge bed, decreasing the concentration of Co to which the upper areas would be exposed. Similar profiles have been observed for solid concentration in UASB, where the concentration of solid and volatile solids decreased with the height (Elmitwalli & Otterpohl, 2007; Vadlani & Ramachandran, 2008), likewise, greater number and size of granules have been found at the bottom of the reactor (Ozgun et al., 2019). Therefore, the concentration of liquid soluble, and solid Co concentration was evaluated at the 3 sampling points of the UASB: bottom sludge, top sludge and liquid phase, for the 6 continuous Co dose.

The previous described profile was observed just for liquid soluble concentrations ≤ 500 µM of Co (Figure III.3.A), and in solid concentration for Co dose concentrations of 0.1 and 500 µM (Figure III.3.B). For Co continuous doses 10, 50, and 100 µM, in solid concentration, no differences were detected along the sludge bed (Figure III.3.B), possibly a decrease in Co concentration along the height of the sludge did happen at the beginning of the dose cycle, but due to the dose time, a chemical equilibrium between the Co in the liquid phase and in the granules was achieved from the bottom of the UASB which did not cause the decrease of Co amount for the upper areas. In this way, after the 14 -17 d of continuous dose, a chemical equilibrium would have been reached in all the sludge bed. Probably, due to the low dose of Co for the first dosage, 0.1 µM (which is 100 times less than the next continuous dose, 10 µM), Co was constantly accumulated at the bottom of the sludge and the chemical equilibrium was not reached within. A difference in the profile was observed in the Co continuous dose of 500 µM, where a greater accumulation was detected in the bottom of the sludge, possibly given by the strong decrease in H_2S for this dose (Figure 2.D). The H_2S dissociate in sulfide, which reacts with Co generating a precipitate, CoS, which accumulates in the granules (Paulo et al., 2017). Regarding the 1000 µM of Co continuous dose, probably a chemical equilibrium was again reached between the Co in the different phases along the reactor, since all the H_2S and NH_3 that could affect the precipitation and accumulation of Co had been consumed to values close to 0 in the previous continuous dose (500 µM of Co).

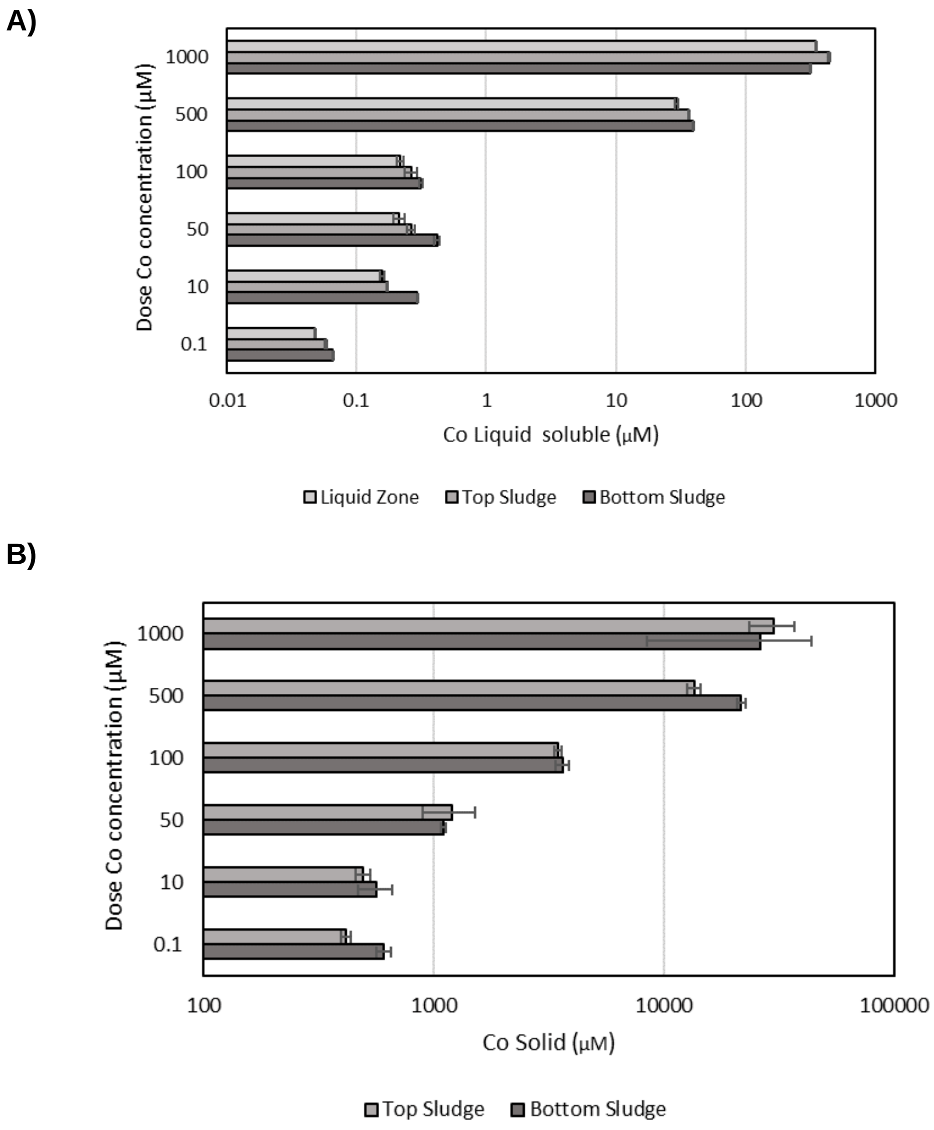


Figure II.3: Different Co concentrations along the UASB for the different Co continuous dose. A) Liquid soluble concentration and B) Solid concentration

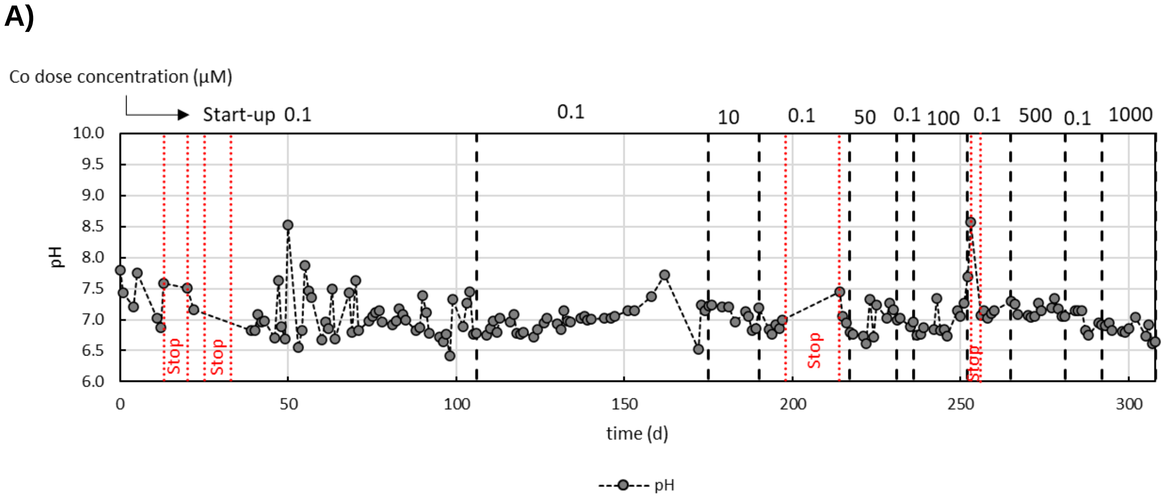
II.3.2. Relation between UASB performance and the different Co continuous dose

II.3.2.1. Time serie of response variables in UASB performance

Figure II.4 shows the reactor performance during the operation time. The period of start-up was 175 d, where stability of sludge and biogas was reached. At the end of this time, samples were taken for metal and EPS analysis, then the continuous cycle doses started. pH was stable during all operation time, in the range 6.5 – 7.5 (Figure II.4.A). Bicarbonate alkalinity was set in the feed, due to this the values of total alkalinity were relatively constant during all the experiment, with a value of 1480 ± 120 mg $CaCO_3$ L^{-1} (Figure II.4.B). Ratio of volatile fatty acids – alkalinity was mostly in the range 0.2 - 0.35, which is an indicator of stable operation in the reactor, exceptionally some days this value was out of range, but the reactor returned to normal values without making any changes (Figure II.4.B).

Biogas productivity at the end of 0.1 µM Co continuous dose was 520 ± 35 NmL g_{COD}^{-1} d^{-1} and no significant difference in the biogas productivity was observed for 10 µM of Co dose. An increase of 19 % in biogas productivity respect to 10 µM of Co dose was observed at the steady state with 50 µM of Co dose, reaching a productivity of 620 ± 30 NmL g_{COD}^{-1} d^{-1}. For the next doses a consecutive decrease in biogas productivity was detected with values of 560 ± 10, 490 ± 20 and 406 ± 55 NmL g_{COD}^{-1} d^{-1} for the dose 100, 500 and 1000 µM of Co, respectively (Figure II.4.C). COD removal was higher than 90% during all operation time, except for the last dose, where the value decreased until 79 ± 4 %, which is related to biogas decrease for the last Co dose (Figure II.4.C).

Regarding the quality of biogas, methane concentration remained virtually constant, with values between 50 – 56 % (Figure II.4.C). NH_3 concentration in biogas during start-up reached values close to 1000 ppm (days 74 – 81, and 132 – 139), however at the end of the start-up period the concentration was 610 ± 120 ppm, no significant change was observed for 10 µM of Co dose (640 ± 60 ppm). A first decrease was detected with dose 50 and 100 µM of Co dose, with NH_3 concentrations of 350 ± 30 and 315 ± 20 ppm, respectively; and a second decrease reaching values close to 0 were observed for 500 and 1000 µM of Co dose, 16 ± 3 and 13 ± 2 ppm, respectively. The NH_3 concentration remained relatively constant during the cleaning period, i.e. dose of 0.1 µM of Co between Co doses higher than 0.1 µM, except during the period between 500 – 1000 µM Co dose, where a concentration higher than 300 ppm was observed (Figure II.4.D). H_2S concentration observed at the end of start-up period was 1790 ± 430 ppm, and no significant changes were observed for 50 and 100 µM Co dose. A decrease in concentration and variability was detected for the next concentrations, a small decrease was observed for 100 µM Co dose (1620 ± 70 ppm), then for 500 µM Co dose, a very low concentration was reached (80 ± 10 ppm), and finally for 1000 µM Co dose, an increase to 200 ± 70 ppm was observed. During the periods of cleaning between Co doses, the H_2S concentration reached values close to 2000 ppm (Figure II.4.D).



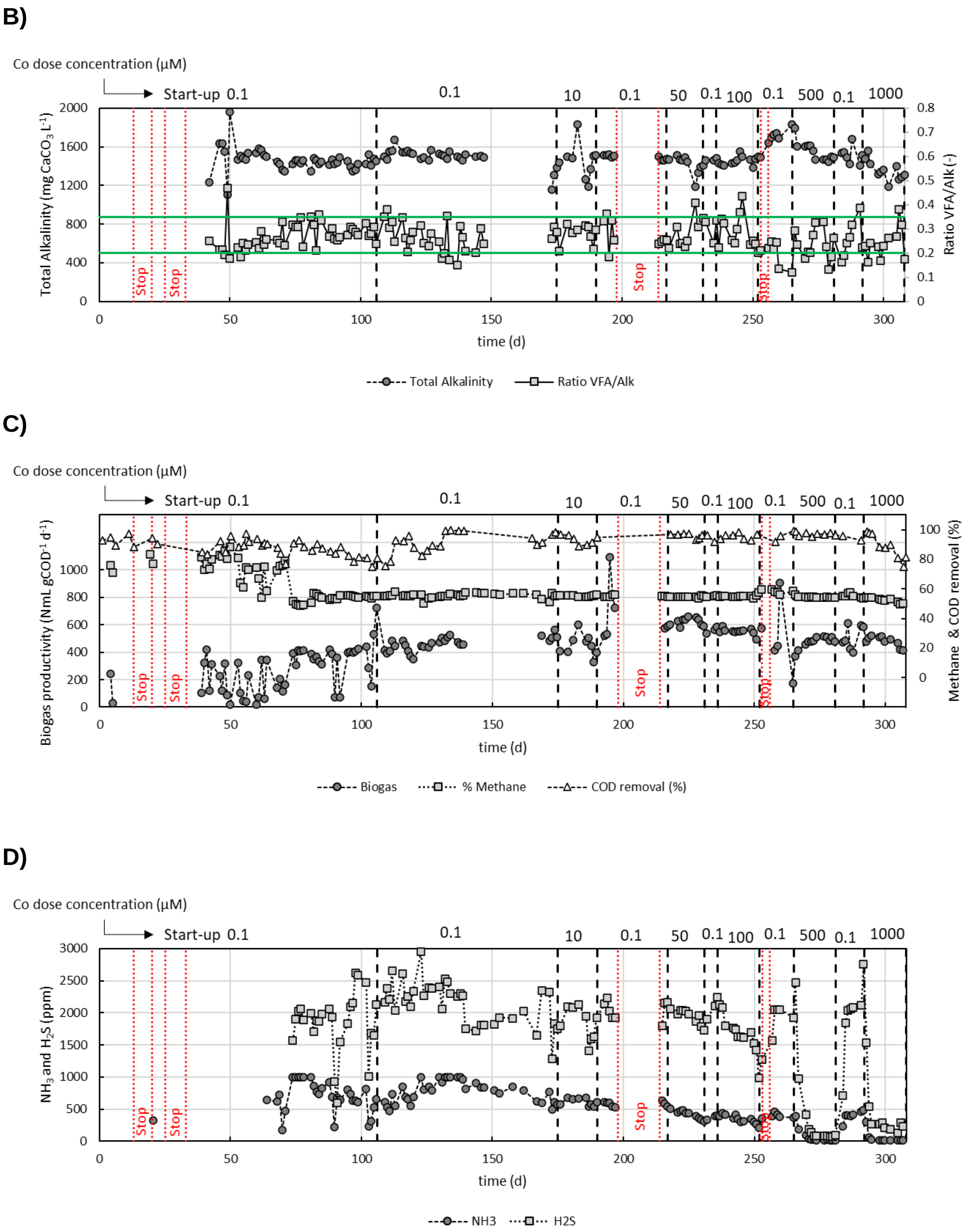


Figure II.4: Reactor performance time line. A) pH, B) Total alkalinity and ratio VFA/Alkalinity, C) Biogas productivity, methane concentration, and COD removal, and D) NH_3 and H_2S concentration in biogas. The different concentration of Co dose are indicated with the dotted vertical lines, and the period between red dotted lines are the time where the reactor was stopped. In B) graph, green horizontal lines show the normal operation range for ratio VFA/Alkalinity

II.3.2.2. Relation between Co fraction and UASB response: biogas productivity, COD removal, H_2S and NH_3

The Co added to the UASB was in the form of Co chloride ($CoCl_2$), which means that it was added in the form of free Co(II). Within the UASB, Co(II) could interact with the microorganisms and substances present modifying the chemical speciation of the Co added. At the end of each of the continuous dosing cycles of Co the concentration of liquid soluble Co was measured, and the solid concentration in the granules. The response variables analyzed correspond to the response in UASB performance where changes were observed with the different continuous doses of Co: biogas productivity, COD removal, and H_2S and NH_3 concentration in biogas.

II.3.2.2.1. Biogas productivity and COD removal

Most of the Co dosed was accumulated in the sludge (Section 3.1.2) where the concentration of Co in the sludge was more than at least 100 times the total liquid concentration. Although the concentration of Co in the sludge at the end of the first continuous dose cycle of Co was 600 µM of Co, a value that has been reported as toxic to AD microorganisms by Garuti et al. (2018) and Matheri et al. (2021), inhibition in biogas production was only observed after the concentration of Co in the sludge was greater than 3600 µM of Co (Figure II.5.A). This may be because the main Co species present in the sludge could be present in inert species, which are non-toxic and non-bioavailable to microorganisms, such as sulfide precipitates (Paulo et al., 2017), likewise as Co trapped in EPS structure, that protect microorganisms from toxic metals (Muthu et al., 2017). In section II.3.2.2.2 and II.3.3.3 are discussed the formation of CoS precipitate and EPS in the UASB, respectively. Thus, the Co that could affect microorganisms were those bioavailable species that were mainly in the liquid phase.

The Co soluble liquid fraction were found under the reported optimal dose values of dissolved Co for methanogenic organisms, which is 2 µM of Co (Fermoso et al., 2019a). No inhibition in biogas productivity was observed in the first 2 doses, even an increase in biogas productivity was observed in the third Co continuous dose (50 µM of Co) where the soluble liquid concentration was 0.42 ± 0.02 µM. When the Co soluble liquid concentration was higher than 2 µM, which was observed in the last 2 doses, inhibition in biogas productivity was observed. The Co soluble liquid concentration was 39.3 ± 0.1 and 316 ± 2 µM of Co for the continuous dose cycles of Co 500 and 1000 µM, respectively. It should be noted that inhibition in biogas productivity for the same Co concentration value was only observed when this value was associated with the soluble liquid fraction. At the end of the period of operation (day 306 of operation), the concentration of Co in the soluble liquid fraction was 316 ± 2 µM of Co, value close to the Co value in the sludge at the end of the first dose cycle (0.1 µM of Co, day 175 of operation), with values of Co solid concentration of 417 ± 20 and 607 ± 44 µM of Co for top and bottom sludge positions respectively, and inhibition in biogas productivity was only observed at the end of the operating period. Thus, it was possible to determine that the inhibition in biogas productivity was due to the Co present in the soluble liquid fraction.

Regarding organic matter, COD removal is usually related to biogas productivity, however, in this study the same trend was not observed between COD removal and Co measured in the UASB as the trend between biogas productivity and Co measured in the UASB (Figure II.5.B). COD removal was higher than 90% in all dose cycles, except in the last one where COD removal decreased to 79 ± 4%. Various processes may affect the removal of organic matter and could not impact in changes in biogas productivity, such as anabolic processes of microorganisms or granulation processes such as granule growth or granule breakage.

II.3.2.2.2. Minority compounds in the biogas: H_2S and NH_3

The presence of H_2S and NH_3 in biogas were influenced by the concentration of Co in the UASB. The H_2S generated by sulfate reducing bacteria and dissolved in the liquid medium reacts into ion sulfide (S^-), which precipitates with Co generating CoS, which accumulates in the granules (Ortner et al., 2014; Paulo et al., 2017). A correlation could be seen between the Co and H_2S present in biogas (Figure II.5.C), for the first 3 Co continuous doses the Co solid concentration in the sludge remained relatively constant, between 500 and 1000 µM of Co, as well as, the H_2S concentration in biogas, with a value around 1800 ppm. When the Co solid concentration in the sludge increased to 3000 µM of Co (at the end of Co continuous dose of 100 µM of Co) a reduction in the H_2S concentration was observed, with a 12% decrease in the H_2S concentration compared to the previous continuous dose cycle. For the next dose (500 µM of Co), the Co solid concentration in the sludge increased 6 times, which caused a decrease in the concentration of H_2S to a value of 76 ± 12 ppm, indicating the possibility that almost all of the H_2S was transformed into sulfide and was precipitated along with Co. The slight increase for the last dose cycle may have been due to early failure in the reactor where biogas production decreased, indicating problems with methanogenic microorganisms, which could temporarily promote the activity of sulfate reducing bacteria.

In this study, the source of nitrogen is NH_4Cl. The ammonium ion (NH_4^+) can be used by the microorganisms to synthesize aminoacids and also can spontaneously be deprotonated to NH_3 in the liquid medium, however, the deprotonation equilibrium reaction is not favorable in the pH range that the UASB has operated (pKa $NH_4^+= 9.24$). Thus, the main process that produced NH_3 was the fermentation of aminoacids (Sikora et al., 2018). A correlation was also observed between NH_3 in biogas and Co concentration, which can be seen for all quantified fractions (Figure II.5.D), with higher Co concentration the NH_3 concentration in biogas was lower. The reduction in NH_3 concentration in biogas is possibly due to the capture of ammonia in the liquid phase since Co(II) reacts with free NH_3 in solution forming a complex $Co(NH_3)_6^{2+}$ soluble in aqueous solution (Ma et al., 2013). This complex may be present in the liquid fraction or could be captured by the granules. When the Co concentration reached the values of 30 µM of Co in the liquid fraction or 20000 µM of Co in the sludge, the NH_3 content was reduced to 17 ± 3 ppm, indicating that at this point almost all NH_3 had already been captured by the presence of Co in the UASB.

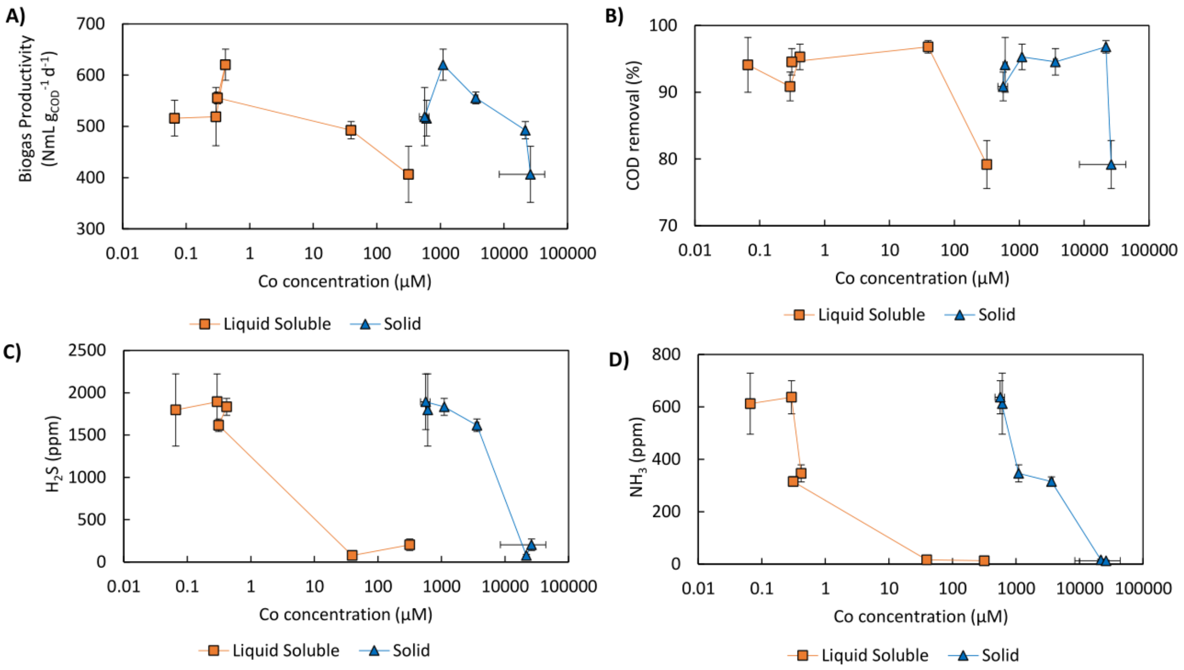


Figure II.5: Correlation between response variables that showed changes due to Co addition through continuous dose and different Co concentration at the bottom sludge . A) Biogas productivity, B) COD removal, C) H_2S in biogas, and D) NH_3 in biogas

II.3.3. Relation between External Polymeric Substances and the different Co continuous dose

II.3.3.1. Time series of EPS in UASB performance

EPS-proteins and EPS-carbohydrates in granules extract, selected as the main components in EPS, were measured at the end of each metal dose when the pseudo-steady state was reached. EPS-Proteins in the two different sampling points, bottom and top sludge, are presented in Figure II.6.A. At the bottom, no significant changes in EPS-protein concentration was observed from 0.1 to 50 µM of Co dose. Then, a decrease for 100 and 500 µM of Co dose was observed, reducing the concentration in 60 % at the end of 500 µM of Co dose compared to EPS-protein concentration at 10 µM of Co dose. Finally, an increase to 1.1 ± 0.1 $g_{albumin}$ kg^{-1} was observed at the end of the last dose, 1000 µM of Co. In the top of the sludge, the EPS-protein concentration started to decrease from 0.1 µM of Co dose to 500 µM of Co dose, from 1.08 ± 0.1 to 0.23 ± 0.08 $g_{albumin}$ kg^{-1}. Then, as well as the bottom, an increase was observed, resulting a similar value as the initial EPS-protein concentration with 0.1 µM of Co dose. On the other hand, EPS-carbohydrates showed different trend related to sludge position (Figure II.6.B). In the bottom, a non-significant increase in EPS-carbohydrates concentration was observed between 0.1 to 10 µM of Co dose, with an EPS-carbohydrates concentration of 0.41 ± 0.01 $g_{glucose}$ kg^{-1} for 10 µM of Co dose. Then a reduction of 65 % in EPS-carbohydrates concentration was observed from 10 to 100 µM of Co dose, and a consistent increase in EPS-carbohydrate concentration was observed from 100 to 1000 µM of Co dose from 0.14 ± 0.05 to 0.23 ± 0.04 $g_{glucose}$ kg^{-1}. Whereas, the top of the sludge maintained a constant EPS-carbohydrates concentration, between 0.18 – 0.26 $g_{glucose}$ kg^{-1}.

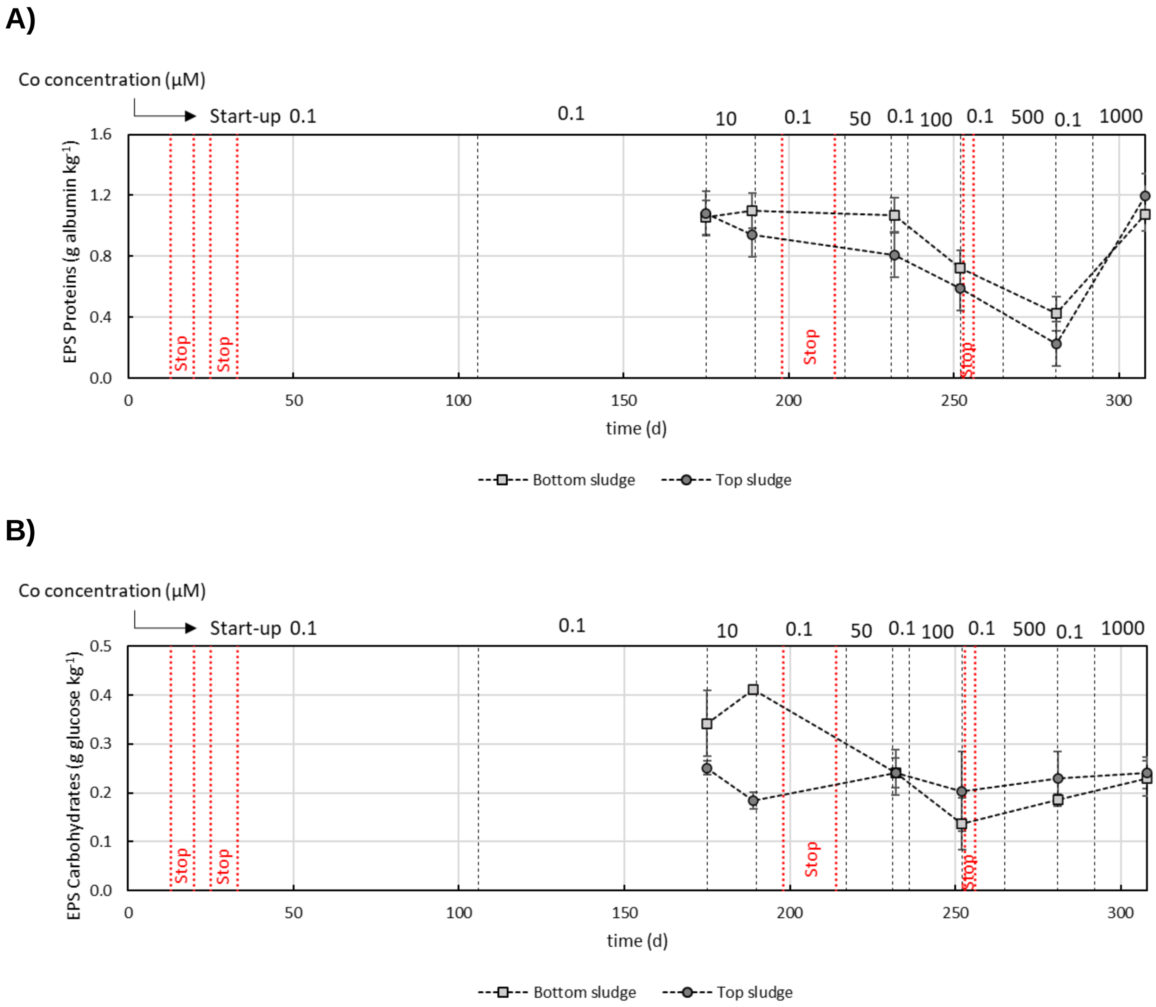


Figure II.6: EPS proteins and carbohydrates evolution over time for different continuous Co dose A) EPS-Proteins, and B) EPS-Carbohydrates. The different concentration of Co dose are indicated with the dotted vertical lines, and the period between red dotted lines are the time where the reactor was stopped

II.3.3.2. Profile of EPS along the sludge bed for the different Co dose

EPS proteins and carbohydrates were measured in the 2 sampling points, at the top and bottom of the sludge bed at the end of each Co continuous dose. The profile of EPS proteins and carbohydrates is shown in Figure II.7. Both EPS proteins and EPS carbohydrates showed no significant differences in EPS concentration along the sludge bed for all Co continuous dose evaluated, except for EPS carbohydrates at Co continuous doses of 0.1 and 10 µM, where in the bottom sludge the content of EPS carbohydrates was higher than the top sludge (Figure II.7.A and B). One of the function of EPS is protection of the microorganisms against toxic metals, thus it should be expected that Co solid concentration profile conditioned EPS profile along the sludge bed. The results of this study were consistent with this previous idea, due to no differences in Co solid concentration along the sludge bed were observed except Co continuous dose of 0.1 µM (Figure II.3.C). EPS profile in a UASB has been evaluated previously by Ozgun et al. (2019), however this study evaluated the profile in no metal stress conditions. Co dose of 0.10 µM in this current study could be considered under no Co stress, In this case, only the EPS carbohydrates decrease profile along the sludge bed was similar for both studies.

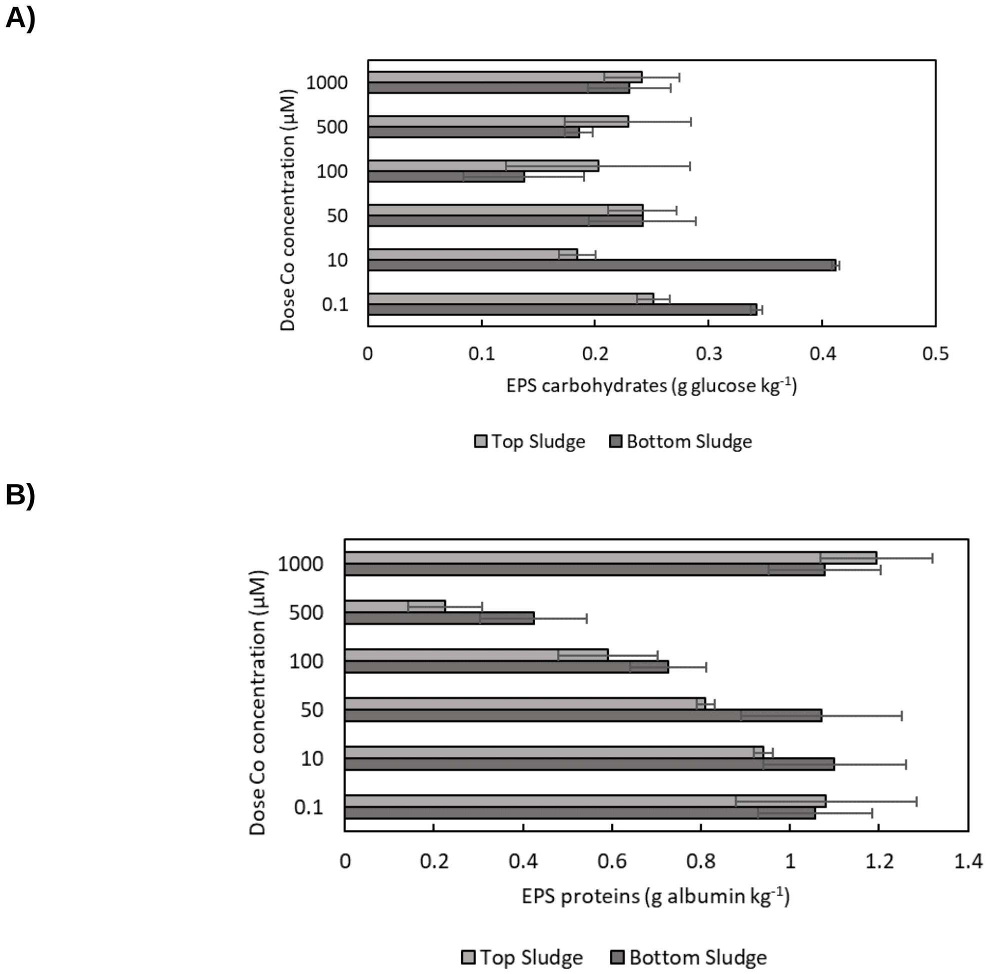


Figure II.7: Profile of EPS in sludge bed at the end of each Co continuous dose cycle. A) EPS carbohydrates, B) EPS proteins

II.3.3.3. Effect of different Co continuous doses in EPS Proteins and Carbohydrates

The concentration of EPS proteins and carbohydrates were measured at the top and bottom sludge positions at the end of each continuous dose cycle of Co. These values have been correlated with the different concentrations of Co measured in these same positions at the end of each cycle of continuous dose of Co (Figure II.8).

II.3.3.3.1. EPS proteins

In both sampling points the trend between EPS proteins concentration and Co concentration are essentially the same for Co liquid soluble and solid concentration. A trend of decreased EPS protein concentration with the increase in Co concentration was observed for all the continuous dose cycles if the last point for the top sludge position is omitted (Figure II.8.A and C). The decrease in EPS proteins due to the presence of Co may be because the Co has affected the microbial activity of production of EPS proteins (Ding et al., 2018). Even, it has even been reported that under metal stress circumstances activity of protease enzyme is increased, which causes protein degradation (Palma et al., 2002). The highest decrease in EPS proteins was observed for Co continuous dose of 100 and 500 µM, same Co dose cycles where a decrease in biogas productivity started to be detected (Figure II.5.A), supporting that the UASB was in a stress situation. The trend of decrease of EPS proteins obtained in this study coincides with the results obtained by Hasani Zadeh et al., (2022), where a decrease in the EPS protein concentration due to the increase of Co was observed in Biomethane Potentials assays.

For the last dose, an increase in the concentration of EPS proteins was detected, reaching values similar to the initial ones (Figure II.8. A and C). It is proposed that this increase occurred by a quantification of internal EPS proteins released due to granule breakage. At the end of the continuous dose of Co of 500 µM, where Co concentration in the sludge was between 10 and 30 mM of Co, the EPS proteins concentration had been reduced by 70 and 60 % over the initial concentration, for the top and bottom sludge, respectively (Figure II.8.A and C). As the EPS are elements that maintain the integrity of the granule, their reduction can favor the breaking of the granule and with it the release of the internal components of the granule, among them the EPS internal proteins. The method used for measuring EPS did not destroy the granule, therefore it quantified the EPS proteins that could be extracted from the granule surface; when the granule breaks, more EPS are available to be extracted and quantified. Yan-jun et al. (2006) observed a sudden increase in EPS in aerobic granular sludge and attributed it to the release of EPS when granules broke. This sudden increase was not observed by (Hasani Zadeh et al., 2022) because the sludge used in that study was not granular.

II.3.3.3.2. EPS carbohydrates

Differences in EPS carbohydrates concentration evolution was observed along the sludge bed, whereas in top sludge a relative value of 0.22 ± 0.04 g glucose kg^{-1} was observed (Figure II.8.B) in bottom sludge a consisted decrease in the EPS carbohydrate concentration with the consecutive doses and an increase during the last 2 doses was observed (Figure II.8.D). The trend at the bottom of the sludge with the EPS carbohydrates and Co solid concentration was similar to EPS proteins (Figure II.8. A and C) and can be explained by the same reasons, just the increase in EPS carbohydrates did not reach the initial values of concentration, possibly due to the consumption of the EPS carbohydrates by the microorganisms. The increase in EPS proteins could be detected since they were not consumed by microorganisms because carbohydrates are degraded faster and easier than proteins in anaerobic digestion (Yang et al., 2015). The effect of Co in the production of EPS carbohydrates in Biomethane potential assays has been studied by Hasani Zadeh et al., (2022) showing no changes in the concentration of EPS carbohydrates for different doses of Co, which would be consistent with the results observed in the top sludge.

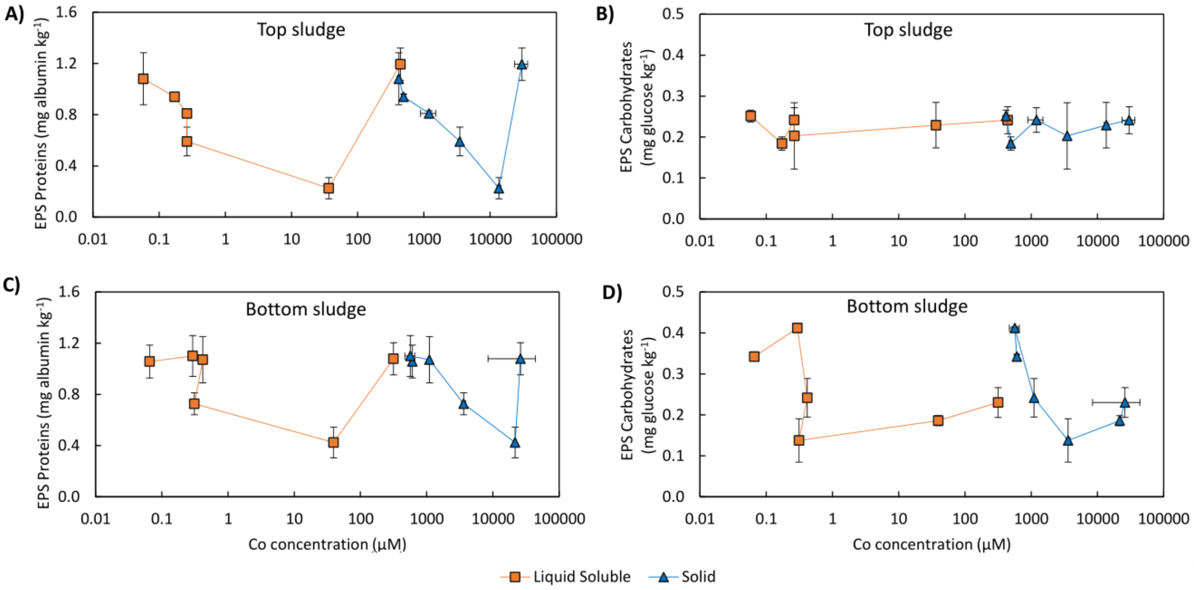


Figure II.8: EPS carbohydrates y proteins concentration in granules in 2 sample points in sludge bed related to different Co concentrations: liquid soluble and solid (granules). A) EPS proteins in top sludge, B) EPS Carbohydrates in top sludge, C) EPS Proteins in bottom sludge, and D) EPS Carbohydrates in bottom sludge

II.4. Conclusions

The effect of Co continuous dose in a UASB reactor was evaluated. Co was characterized through the liquid soluble and solid concentrations and was measured along the reactor. Various UASB response were considered and related to Co. Part of the Co added was accumulated in granules within the UASB, but this did not reflect substantial changes in the stability of the UASB. Only decreased biogas productivity and decreased H_2S and NH_3 concentrations in biogas were observed for the higher Co doses. The inhibitory effect was attributed to the Co present in the liquid soluble fraction since the solid fraction could have been composed mainly of non-bioavailable species.

A profile where the concentration decreased along the height of the UASB was observed only for some doses of Co, mainly for lower ones. These profiles were observed for Co liquid soluble and solid concentration and EPS carbohydrates concentration. The concentration of EPS proteins and carbohydrates were affected by the presence of Co. A general tendency to decrease was observed during operation time, except for the end of the operation where an increase was observed. This increase is attributed to the release of EPS from the inside of the granules due to the breaking of these by the previous decrease of the EPS.

II.5. Acknowledgments

This project has received funding from the European Union’s Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 861088. The authors thanks Victor Ramos for ICP-MS analysis.

II.6. References

Antwi, P., Li, J., Opoku Boadi, P., Meng, J., Shi, E., Xue, C., Zhang, Y., & Ayivi, F. (2017). Functional bacterial and archaeal diversity revealed by 16S rRNA gene pyrosequencing during potato starch processing wastewater treatment in an UASB. *Bioresource Technology*, *235*, 348–357. https://doi.org/10.1016/j.biortech.2017.03.141

APHA. (2017). *Standard Methods for the Examination of Water and Wastewater (23rd ed.). Washington DC: American Public Health Association*.

Bartacek, J., Fermoso, F. G., Baldó-Urrutia, A. M., Van Hullebusch, E. D., & Lens, P. N. L. (2008). Cobalt toxicity in anaerobic granular sludge: Influence of chemical speciation. *Journal of Industrial Microbiology & Biotechnology*, *35*(11), 1465–1474. https://doi.org/10.1007/s10295-008-0448-0

Brunetti, A., Boari, G., R Passino, & Rozzi, A. (1983). *Physico-chemical factors affecting start-up in UASB digesters*. 317–334. PASCAL.

Chen, J. L., Steele, T. W. J., & Stuckey, D. C. (2016). Stimulation and Inhibition of Anaerobic Digestion by Nickel and Cobalt: A Rapid Assessment Using the Resazurin Reduction Assay. *Environmental Science & Technology*, *50*(20), 11154–11163. https://doi.org/10.1021/acs.est.6b03522

Cisneros, J. F., Pelaez-Samaniego, M. R., Pinos, V., Nopens, I., & Alvarado, A. (2021). Development of an Automated Tracer Testing System for UASB Laboratory-Scale Reactors. *Water*, *13*(13), 1821. https://doi.org/10.3390/w13131821

Ding, P., Song, W., Yang, Z., & Jian, J. (2018). Influence of Zn(II) stress-induction on component variation and sorption performance of extracellular polymeric substances (EPS) from Bacillus vallismortis. *Bioprocess and Biosystems Engineering*, *41*(6), 781–791. https://doi.org/10.1007/s00449-018-1911-6

Elmitwalli, T. A., & Otterpohl, R. (2007). Anaerobic biodegradability and treatment of grey water in upflow anaerobic sludge blanket (UASB) reactor. *Water Research*, *41*(6), 1379–1387. https://doi.org/10.1016/j.watres.2006.12.016

Fermoso, F. G., Bartacek, J., Chung, L. C., & Lens, P. (2008). Supplementation of cobalt to UASB reactors by pulse dosing: CoCl2 versus CoEDTA2− pulses. *Biochemical Engineering Journal*, *42*(2), 111–119. https://doi.org/10.1016/j.bej.2008.06.005

Fermoso, F. G., Bartacek, J., Manzano, R., Van Leeuwen, H. P., & Lens, P. N. L. (2010). Dosing of anaerobic granular sludge bioreactors with cobalt: Impact of cobalt retention on methanogenic activity. *Bioresource Technology*, *101*(24), 9429–9437. https://doi.org/10.1016/j.biortech.2010.07.053

Fermoso, F. G., Van Hullebusch, E., Collins, G., Roussel, J., Mucha, A. P., & Esposito, G. (Eds.). (2019a). Mathematical modelling of trace element dynamics in anaerobic digestion environments. In *Trace Elements in Anaerobic Biotechnologies* (pp. 101–152). IWA Publishing. https://doi.org/10.2166/9781789060225_0101

Fermoso, F. G., Van Hullebusch, E., Collins, G., Roussel, J., Mucha, A. P., & Esposito, G. (Eds.). (2019b). Trace element enzymes in reactions essential for anaerobic digestion. In *Trace Elements in Anaerobic Biotechnologies* (pp. 51–72). IWA Publishing. https://doi.org/10.2166/9781789060225_0051

Garuti, M., Langone, M., Fabbri, C., & Piccinini, S. (2018). Methodological approach for trace elements supplementation in anaerobic digestion: Experience from full-scale agricultural biogas plants. *Journal of Environmental Management*, *223*, 348–357. https://doi.org/10.1016/j.jenvman.2018.06.015

Hasani Zadeh, P., Serrano, A., Collins, G., & Fermoso, F. G. (2022). Interrelating EPS, soluble microbial products and metal solubility in a methanogenic consortium stressed by nickel and cobalt. *Ecotoxicology and Environmental Safety*, *238*. https://doi.org/10.1016/j.ecoenv.2022.113579

Huang, L., Jin, Y., Zhou, D., Liu, L., Huang, S., Zhao, Y., & Chen, Y. (2022). A Review of the Role of Extracellular Polymeric Substances (EPS) in Wastewater Treatment Systems. *International Journal of Environmental Research and Public Health*, *19*(19), 12191. https://doi.org/10.3390/ijerph191912191

Latif, M. A., Ghufran, R., Wahid, Z. A., & Ahmad, A. (2011). Integrated application of upflow anaerobic sludge blanket reactor for the treatment of wastewaters. *Water Research*, *45*(16), 4683–4699. https://doi.org/10.1016/j.watres.2011.05.049

Li, J., Chen, Q., Fan, Y., Wang, F., & Meng, J. (2023). Improved methane production of two-phase anaerobic digestion by cobalt: Efficiency and mechanism. *Bioresource Technology*, *381*, 129123. https://doi.org/10.1016/j.biortech.2023.129123

Lin, C. Y., & Lin, M. L. (1997). Effect of heavy metals on the upflow anaerobic sludge blanket process. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering*, *32*(7), 1933–1944. https://doi.org/10.1080/10934529709376656

Liu, X.-M., Sheng, G.-P., Luo, H.-W., Zhang, F., Yuan, S.-J., Xu, J., Zeng, R. J., Wu, J.-G., & Yu, H.-Q. (2010). Contribution of Extracellular Polymeric Substances (EPS) to the Sludge Aggregation. *Environmental Science & Technology*, *44*(11), 4355–4360. https://doi.org/10.1021/es9016766

López, I., & Borzacconi, L. (2010). UASB reactor hydrodynamics: Residence time distribution and proposed modelling tools. *Environmental Technology*, *31*(6), 591–600. https://doi.org/10.1080/09593331003646638

Ma, S., Song, H., Chen, G., Wang, Y., & Zang, B. (2013). Cobalt(II) as an additive inhibiting ammonia escape in carbon capture using ammonia solution. *Greenhouse Gases: Science and Technology*, *3*(5), 392–396. https://doi.org/10.1002/ghg.1364

Matheri, A. N., Ntuli, F., & Ngila, J. C. (2021). Sludge to energy recovery dosed with selected trace metals additives in anaerobic digestion processes. *Biomass and Bioenergy*, *144*, 105869. https://doi.org/10.1016/j.biombioe.2020.105869

Metcalf & Eddy (Ed.). (2014). *Wastewater engineering: Treatment and resource recovery* (5. ed., internat. student ed). McGraw-Hill.

Muthu, M., Wu, H.-F., Gopal, J., Sivanesan, I., & Chun, S. (2017). Exploiting Microbial Polysaccharides for Biosorption of Trace Elements in Aqueous Environments—Scope for Expansion via Nanomaterial Intervention. *Polymers*, *9*(12), 721. https://doi.org/10.3390/polym9120721

Ortner, M., Rachbauer, L., Somitsch, W., & Fuchs, W. (2014). Can bioavailability of trace nutrients be measured in anaerobic digestion? *Applied Energy*, *126*(2014), 190–198. https://doi.org/10.1016/j.apenergy.2014.03.070

Ozgun, H., Ersahin, M. E., Zhou, Z., Tao, Y., Spanjers, H., & Van Lier, J. B. (2019). Comparative evaluation of the sludge characteristics along the height of upflow anaerobic sludge blanket coupled ultrafiltration systems. *Biomass and Bioenergy*, *125*, 114–122. https://doi.org/10.1016/j.biombioe.2019.04.001

Pal, A., & Paul, A. K. (2008). Microbial extracellular polymeric substances: Central elements in heavy metal bioremediation. *Indian Journal of Microbiology*, *48*(1), 49–64. https://doi.org/10.1007/s12088-008-0006-5

Palma, J. M., Sandalio, L. M., Javier Corpas, F., Romero-Puertas, M. C., McCarthy, I., & Del Río, L. A. (2002). Plant proteases, protein degradation, and oxidative stress: Role of peroxisomes. *Plant Physiology and Biochemistry*, *40*(6–8), 521–530. https://doi.org/10.1016/S0981-9428(02)01404-3

Paulo, L. M., Ramiro-Garcia, J., Van Mourik, S., Stams, A. J. M., & Sousa, D. Z. (2017). Effect of Nickel and Cobalt on Methanogenic Enrichment Cultures and Role of Biogenic Sulfide in Metal Toxicity Attenuation. *Frontiers in Microbiology*, *8*, 1341. https://doi.org/10.3389/fmicb.2017.01341

Peng, H., Guo, J., Li, H., Song, Y., Lu, C., Han, Y., & Hou, Y. (2021). Granulation and response of anaerobic granular sludge to allicin stress while treating allicin-containing wastewater. *Biochemical Engineering Journal*, *169*, 107971. https://doi.org/10.1016/j.bej.2021.107971

Pornmai, K., Itsadanont, S., Lertpattanapong, M., Seneesrisakul, K., Jiraprasertwong, A., Leethochawalit, M., Sekiguchi, H., & Chavadej, S. (2023). Enhancement of methanogenic activity by micronutrient control: Micronutrient availability in relation to sulfur transport. *Journal of Environmental Sciences*, *127*, 738–752. https://doi.org/10.1016/j.jes.2022.06.044

Reungsang, A., Sittijunda, S., & Sreela-or, C. (2016). Methane production from acidic effluent discharged after the hydrogen fermentation of sugarcane juice using batch fermentation and UASB reactor. *Renewable Energy*, *86*, 1224–1231. https://doi.org/10.1016/j.renene.2015.09.051

Ripley, L. E., & Boyle, W. C. (1986). Improved Alkalimetric Monitoring for Anaerobic Digestion of High-Strength Wastes. In *Converse Source: Journal (Water Pollution Control Federation)* (Vol. 58, Issue 5, pp. 406–411).

Sikora, A., Detman, A., Mielecki, D., Chojnacka, A., & Błaszczyk, M. (2018). Searching for Metabolic Pathways of Anaerobic Digestion: A Useful List of the Key Enzymes. In J. R. Banu (Ed.), *Anaerobic Digestion*. IntechOpen. https://doi.org/10.5772/intechopen.81256

Subramanyam, R., & Mishra, I. M. (2013). Characteristics of methanogenic granules grown on glucose in an upflow anaerobic sludge blanket reactor. *Biosystems Engineering*, *114*(2), 113–123. https://doi.org/10.1016/j.biosystemseng.2012.11.010

Vadlani, P. V., & Ramachandran, K. B. (2008). Evaluation of UASB reactor performance during start-up operation using synthetic mixed-acid waste. *Bioresource Technology*.

Vian, J., Vigueras-Carmona, S. E., Velasco-Perez, A., Sánchez-Sánchez, K. B., & Puebla, H. (2020). Hydrodynamics of a modified up-flow anaerobic sludge blanket reactor treating organic fraction of municipal solids waste. *International Journal of Chemical Reactor Engineering*, *18*(7). https://doi.org/10.1515/ijcre-2020-0024

Yang, G., Zhang, P., Zhang, G., Wang, Y., & Yang, A. (2015). Degradation properties of protein and carbohydrate during sludge anaerobic digestion. *Bioresource Technology*, *192*, 126–130. https://doi.org/10.1016/j.biortech.2015.05.076

Yan-jun, L., Xiufen, L., Zhao-zhe, H., & He, L. (2006). Production of Extracellular Polymeric Substances from aerobic granular sludge and its distribution. *Environmental Chemistry*. https://api.semanticscholar.org/CorpusID:100994542

Yu, H., Tay, J., & Fang, H. (2001). The roles of calcium in sludge granulation during UASB reactor start-up. *Water Research*, *35*(4), 1052–1060. https://doi.org/10.1016/S0043-1354(00)00345-6

Zandvoort, M. H., Geerts, R., Lettinga, G., & Lens, P. N. L. (2002). Effect of Long-Term Cobalt Deprivation on Methanol Degradation in a Methanogenic Granular Sludge Bioreactor. *Biotechnology Progress*, *18*(6), 1233–1239. https://doi.org/10.1021/bp020078e

Zandvoort, M. H., Van Hullebusch, E. D., Golubnic, S., Gieteling, J., & Lens, P. N. (2006). Induction of cobalt limitation in methanol-fed UASB reactors. *Journal of Chemical Technology & Biotechnology*, *81*(9), 1486–1495. https://doi.org/10.1002/jctb.1559

Chapter III. Trace elements fractionation in anaerobic digestates: recommendation for using Diffusive Gradients in Thin films passive samplers

A slightly modified version of this chapter has been accepted for publication in Journal of Environmental Management (2024). https://doi.org/10.1016/j.jenvman.2024.121223

III.1. Introduction

Trace elements (TE) are necessary for anaerobic digestion (AD) since many are essential in the metabolic pathways of microorganisms. However, depending on their concentration, they can also have inhibitory or toxic effects (Nakhate et al., 2023). The digestate, a by-product of AD, can be used as fertilizer. But the presence of trace elements could be an impediment to its use due to a potential contamination in soil and crops (O'Connor et al., 2022). Studies on element toxicity in AD usually quantify the total concentration or, at best, the soluble fraction of metal in AD systems (Mudhoo & Kumar, 2013). However, a given element is found under various chemical species in AD and many of them are not bioaccessible to microorganisms, such as sulfites and phosphates precipitates (Ortner et al., 2014) or complexes. Therefore, analytical tools allowing the monitoring of the bioavailable fraction is necessary for a better management of AD process and their by-products.

Some analytical techniques have been tested for the estimation of the bioavailable fraction in digestates. For example, the soluble and exchangeable fraction in the *ex-situ* BCR and Tessier sequential extraction method (Wang et al., 2020; Zheng et al., 2021) or the free fraction estimated with the DMT technique (Bartacek et al., 2008) has been used as a proxy for the bioavailable fraction. But these techniques have shown a lack of sensitivity in AD systems, with a sensitivity in the order of mg/kg for BCR and Tessier method (Hullebusch et al., 2005), and µg kg^{-1} for DMT (Bartacek et al., 2008). Additionally, selectivity problem has been detected in sequential extraction methods, with overestimation of some fractions in anaerobic system (Hullebusch et al., 2005) or quantification problems of soluble metals in soils (Vodyanitskii, 2006).

Alternatively, an *in situ* sampling technique, diffusive gradients in thin films technique (DGT) has been developed for more than 25 years (Davison & Zhang, 1994) and recently applied in anaerobic systems with promising results (Bourven et al., 2017; Laera et al., 2019a, 2019b; Takashima, 2018). Target elements diffuse through a diffusive gel and are accumulated in a binding gel (Zhang & Davison, 1995). Only some chemical species are therefore able to be sampled by DGT, which are mainly constituted of free ions, inorganic complexes, and weak and low-weight organic complexes (Takashima, 2018). Modelisation of the mass transfer within the sampler allows converting the accumulated mass into the concentration of all the species present in the exposure medium: the so called "labile fraction". A rigorous and detailed explanation of the labile fraction concentration can be found in Galceran & Puy (2015). The chemical species sampled by DGT are considered as among the most biologically relevant and therefore the labile fraction can be related to the bioavailable fraction (Eismann et al., 2020). When applied in anaerobic systems, the DGT technique has been shown to increase the sensitivity for trace elements monitoring compared to conventional methods that require pre-treatment of the sample, as centrifugation and acid digestion for dissolved element (Laera et al., 2019a). Bourven et al. (2017) observed a consistent evolution of AD inhibition by cadmium with its DGT-labile fraction. Considering these advantages, the DGT technique has

been applied to study the effect of Co and Ni supplementation in AD (Takashima, 2018) or the behavior of trace elements during digestate aeration (Laera et al., 2019b).
However, these first works also pointed out that DGT deployment in anaerobic digestates is not straightforward and the methodology must be carefully adapted to the targeted system (Laera et al., 2019a). Currently, the number of studies of DGT in digestates is limited and more data is necessary to reliably apply this technique in the complex AD matrix. Indeed, this matrix is heterogeneous, multi-elemental and highly organic. Thus, it can alter the functioning of the sampler through, for example, an alteration of the species diffusion or their binding in the device. To explore the validity of the DGT technique in anaerobic system, this study explores the use of DGT in different anaerobic digestates. To deepen the work of Laera et al. (2019a) performed on digestated from municipal sludges, additional types of digestates and binding gels are evaluated. In addition to the two common binding gels based on chelex resin and zirconium oxide, thiol based binding gels are used to simultaneously investigate cations, anions and reduced metalloid species in two digestates, one derived from distillery waste and the other from cow's manure. Moreover, air exposure during DGT deployment is considered to determine whether maintaining strict anaerobic conditions is mandatory. The results obtained are discussed to provide recommendations for a relevant use of the DGT technique in anerobic systems.

III.2. Materials and Methods

III.2.1. Digestate sampling and characterization

Two different digestates from anaerobic digestion process were used in this study, one from the digestion of a spirit distillery waste and the other from the digestion of cow's manure. They were sampled in November 2021 and March 2022 respectively. The digestates were collected in fully filled 10 L high density polyethylene bottles to avoid air exposure. These bottles were stored at 4°C until running the experiment, within 2 days after sampling.

The digestates were characterized for the total solids, volatile solids, suspended solids and volatile suspended solids according to the French standard AFNOR NF T90-105. In addition, total and dissolved chemical oxygen demand (COD), alkalinity and pH were measured in triplicates according to standard methods (APHA, 2017). To determine the total TE content, 5 g of sample were submitted to acid digestion in triplicate with 6 mL of 69% HNO_3 (ACS ISO reagent, Carlo Erba) and 3 mL of H_2O_2 30% (ACS reagent, Merck). The digestion was performed at 180°C for 60 min, using a microwave oven (Multiwave GO, Anton Paar GmbH). To determine the dissolved TE content, the digestates were centrifuged (5000 rpm for 20 min at 10°C). 2 mL of each supernatant was immediately sampled and digested as described above. Blank of digestion was also performed to correct the value of the samples. Digested samples were analyzed for trace elements using the procedure described in the analytical procedure section.

III.2.2. DGT preparation and elution

In this study Chelex- DGT (Ch-DGT), Zirconia-DGT (Zr-DGT) and Thiol-DGT were used for, respectively, cationic metals (Al, Cd, Co, Cr, Cu, Mn, Fe, Ni, Pb, and Zn), oxyanions (As, Mo, Sb, Se, and W) and reduced metalloid species (As(III), Sb(III) and Se(IV)). All DGTs consisted in a piston type plastic holder with three layers from bottom to top: binding gel, diffusive gel and filter membrane. Plastic holders, Chelex and Thiol binding gels were purchased from DGT Research (Lancaster, UK). Zr binding gels were prepared according to Devillers et al. (2016). Standard polyacrylamide diffusive gels (15% acrylamide and 0.3% agarose-derived cross linker, 0.80mm thick) were prepared according to Zhang et al. (1998). Polycarbonates filter membranes Nuclepore® were used (0.2 µm pore size, 0.02mm thickness, Whatman, UK). Prior to their deployment, the DGTs intended to be used in anoxic conditions (see next section) were preconditioned by removing the dissolved oxygen by immersion in a nitrogen bubbling 0.01 M NaCl solution for 4 h.

After deployment, the DGT were rinsed with ultrapure water (UPW; MilliQ, >18.2 MΩ·cm) and disassembled to recover the binding gel. Chelex binding gels were eluted in 2 mL of HNO_3 1 M for 24 h (Laera et al., 2019a), Zr binding gels in 2 mL of 0.005 M NaOH with 0.5 M H_2O_2 for 4 h (Laera et al., 2019a) and Thiol binding gels in 2 mL of 1 M HNO_3 with 0.01 M KIO_3 for 24 h (Sahin et al., 2003). All reagents were purchased from Merck and were ACS reagent grade. The metal concentrations in these eluates were determined by inductively coupled plasma mass spectrometry (ICP-MS) as described in the analytical procedure section. The accumulated mass in binding gel is determined according to equation $(III.1)$ (Zhang & Davison, 1995).

$$m = \frac{C_e V_e}{f_e} \quad (III.1)$$

Where $C_e$is the metal concentration in eluted, V_e is the elution volume (2 mL in this study) and f_e the elution factor (values in Section III.7 Annex. Table S1).

III.2.3. DGT deployment

Two distinct series of DGT deployments were performed. The first one, aiming at verifying DGT reliability in anaerobic digestates, was performed under anaerobic conditions. For this purpose, the experiments were run in a nitrogen-conditioned glovebox. Oxygen level was monitored and kept below 0.2 mg L^{-1}. The second series of deployment, aiming at testing if air exposure is conceivable for more convenient DGT deployments, was performed on a bench with open beakers allowing air contact.

For a given digestate study, 15 DGT of each type (except Thiol-DGT for the study of the distillery waste digestate) were deployed in 2 polypropylene beakers containing each 10 L of digestate agitated with a plastic propeller (between 70 and 100 rpm to ensure the formation of a vortex depending on digestate viscosity). All Zr-DGT were deployed in a single beaker, whereas Ch and Thiol-DGT were deployed in the same beaker. DGT of each type were deployed in triplicate for 8, 12, 24, 42 and 72 h. All DGT were initially simultaneously deployed (time 0). In addition, to verify the labile metal content stability over time, two additional triplicate of 24h Chelex DGT were deployed between the 24th and 48th hour, and between the 48th and 72th hour. In parallel, 2 blanks DGT of each type were kept at 4 °C in a sealed bag with a humid environment during the total DGT deployment time.

pH (Radiometer analytical pH C3001-9), redox potential (metteler toledo LE510 ORP), dissolved oxygen level (HACH LDO 101 HQ30d) and temperature (glass thermometer, Julabo) were monitored each hour from 8:00 to 18:00. At the end of the experiment, digestate samples were characterized for total and dissolved metal content as described in section III.2.1.

III.2.4. Trace element analysis

Trace element content was analyzed by ICP-MS (Agilent 7700×) using ^{115}In as internal standard (standard solution, Carlo Erba). All the samples were diluted with ultrapure water if required and finally adjusted to 2% HNO_3 (with 69% HNO_3, Merck, ACS reagent grade). In the analysis process, blank samples consisting of ultrapure water adjusted to 2% HNO_3 were included after every 10 samples. Quality controls at 10 and 20 µg L^{-1} were added to check the performance of the analysis. The global recovery was 102 ± 21% for all elements among all analyses.

III.3. Results and Discussion

III.3.1. Digestate samples characterization

The characterization of digestates samples is summarized in Section III.7 Appendix III.Table S2 and S3. For both digestates, Eh and pH values were found in normal operating ranges: neutral pH and Eh around -300 mV (Vongvichiankul et al., 2017). The digestates presented different compositions. The manure digestate had a 4 times higher content of solids as well as a 27 times higher dissolved organic matter content compared to the distillery digestate. The alkalinity of the manure digestate was 10 times higher than the distillery digestate one. This value would imply a higher presence of carbonates and bicarbonates, which could eventually react with the trace elements generating precipitates and inorganic complexes (Callander & Barford, 1983). During the DGT experiments, Eh, pH and dissolved oxygen concentrations were monitored (Section III.7 Appendix III Figures S1 and S2). An increase in pH ($\approx$ 1 unit) was observed in all conditions. It may have been due to the microbial residual activity by consumption of fatty acids (Molaey et al., 2021). Eh remains stable for experiments run under anaerobic conditions with undetectable O_2 concentration during all the time. Under anaerobic conditions, Eh raised gradually during the experiment. Finally, oxygen levels remained stable in manure digestate, but, in distillery digestate, it raises up to 2 mg L^{-1} between the 8th and 48th hours, then decreased to the initial values. The decrease in oxygen concentration could possibly results from its consumption by anaerobic aerobic facultative bacteria.

Regarding the elements characterization (Section III.7 Appendix III.Table S3), P, Cu, Fe, Al, Zn and Mn were found in higher concentrations in both digestates. The Cu content in the distillery digestate was 4 times higher than the manure, probably partly due to the distillation process, which is carried out in copper distillers. The P content was at least 2 times higher than any other element in both digestates. The soluble fraction represented less than 7 % of the total for trace elements in the manure digestate, while for the distillery digestate, it represented between 13 and 20 %, for Mo, Sb, and Cd and less than 6 % for the other elements.

III.3.2. Time accumulation of trace elements in anaerobic digestates

According to DGT theory, the mass transfer within the sampler reaches a steady state within a few minutes to hours (Tusseau-Vuillemin et al., 2003). From then, as long as the concentration in the digestate remains constant, the accumulated mass should increase linearly over time until the binding layer starts to be saturated. Plots of the accumulated mass *versus* exposure time are given for each targeted element and each DGT configuration in supplementary material (Section III.7 Appendix III. Figures S3 – S7). Only the results from the experiments under anaerobic conditions are discussed to evaluate DGT reliability in anaerobic digestates. Typical examples of the different behaviours observed are given in Figure III.1.

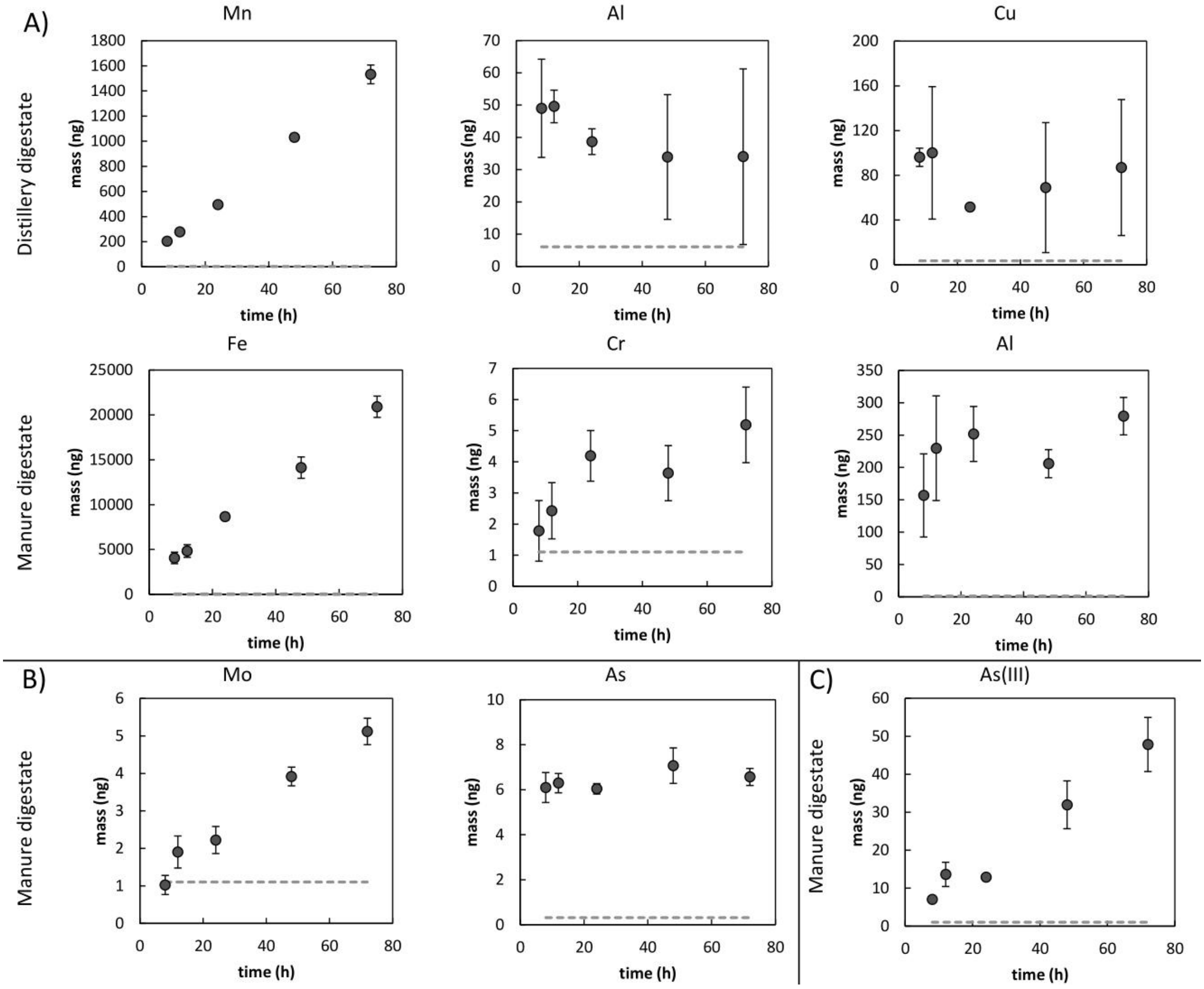


Figure III.1: Examples of some elements accumulation over time in: A) Ch-DGT samplers, B) Zr-DGT samplers and C) Thiol-DGT samplers. Dotted line: blank

For Ch-DGT deployed in the distillery waste digestate, only Mn had a linear accumulation profile over time up to about 1.5 µg. Significant masses of Al and Cu were detected, around 40 and 60 ng respectively, but without any accumulation trend over time. For the other elements, the accumulated masses were below or very close to the blanks and are consequently not discussed. When samplers were deployed in the manure digestate, Fe, Mn and Ni had a linear accumulation trend over time up to about 21 µg, 9 µg and 12 ng respectively. The accumulated masses of Cr and Co increased during the experiments but their linearity over time is questionable. For these two elements, the unclear trend could result from accumulated masses in the same order of magnitude of the blanks level. As observed for the other digestate, significant masses of Al were detected, around 200 ng, but without any accumulation trend over time.

For Zr-DGT, all trace elements in the samplers deployed in distillery waste digestate were below or very close to the blank. In the manure digestate, only Mo had a linear accumulation profile over time, up to 5 ng. As, Sb and Se were also accumulated but the masses did not increase over time. Finally, for Thiol-DGT deployed in the manure digestate, Sb(III) and Se(IV) accumulated mass were very close to the blank concentration and only As(III) shows a linear accumulation (up to 50 ng).

The linear trend observed for Ch-DGT (Fe, Mn, Mo and Ni in at least one of the two studied digestates), Zr-DGT (Mo in the manure digestate) and Thiol-DGT (As(III) in the manure digestate) indicates that the accumulation over time in the complex matrix of anaerobic digestates is consistent with the DGT theory. This is in accordance with Laera et al. (2019a), who demonstrated it for a digestate from sludges of a municipal wastewater treatment plant for all quantified elements (5 over 12 studied). However, our current study also shows that, for some elements, significant amounts can be accumulated in DGT without a linear trend over time (Al and Cu in Ch-DGT and As in Zr-DGT). This highlights that their accumulation does not comply with the usual description of mass transfer in the DGT. Several phenomena could explain these observations and are discussed hereafter.

III.3.2.1. Saturation of the samplers

The lack of linearity in accumulation over time could possibly result from a partial saturation of the binding phase. Indeed, Laera et al. (2019a) already proposed that lack of linear accumulation for As in anaerobic digestates could be explained by a competition with P for accumulation in the binding layer. To investigate this option, the accumulated amounts of each element were summed up to estimate a pseudo-total accumulated amount (Figure III.2). P was quantified and added in the calculation of the pseudo-total accumulated elements for Zr-DGT.

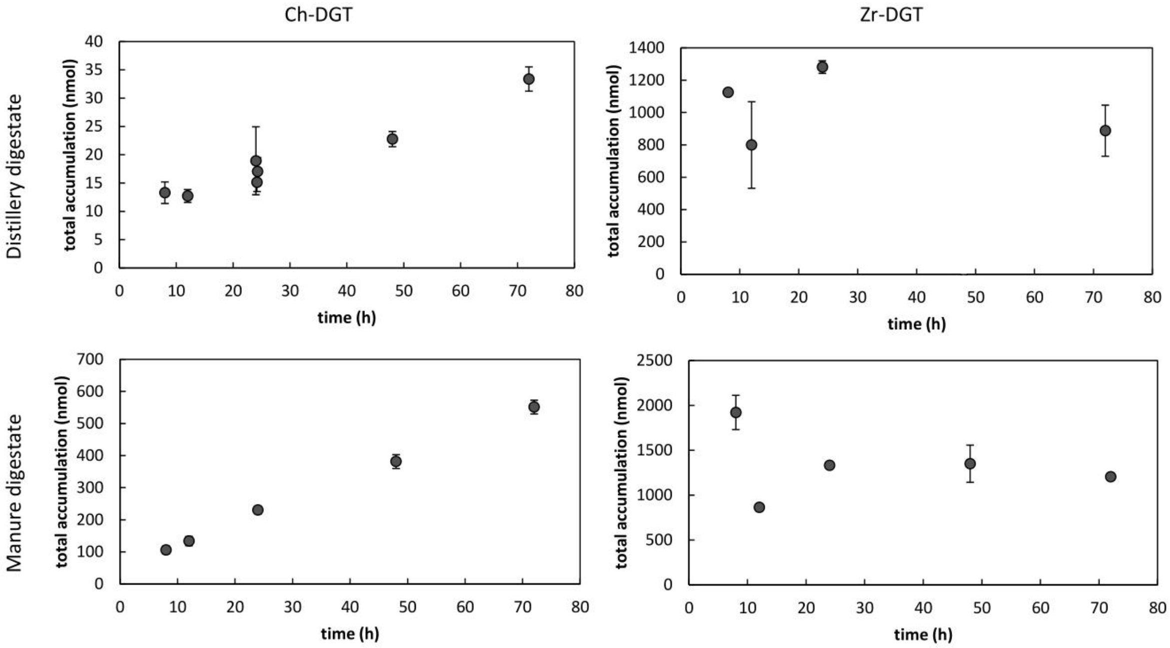


Figure III.2: Evolution of the total accumulated mass of elements (estimated by summation of all detected elements) over time

For both digestates, a linear accumulation trend could be observed for Ch-DGT up to about 550 nmol. It could thus be assumed that the effective sorption capacity of the Ch-DGT was not reached for any element. Indeed, the capacity of a standard Chelex binding gel is estimated around 15 µmol (Galceran et al., 2021). As a result, the lack of linear accumulation for Al and Cu does not result from the saturation of the binding phase and other hypothesis should be investigated.

In contrast, for Zr-DGT, the pseudo-total amount of accumulated elements does not increase over time and is nearly almost equal to the accumulation of P (*i.e.* P represent more than 99 % of the total accumulated elements). This indicates a probable saturation of the binding phase around 1 - 2 µmol for both digestates. To confirm this hypothesis, Zr binding gels capacity for P was estimated (see P accumulation capacity of Zr-DGT in supplementary material). The resulting capacity was found around 1.5 µmol, which is similar with the pseudo-total accumulated masses in both digestates (Figure III.2). This results thus supports the saturation hypothesis. As a result, the absence of linear accumulation over time for Zr-DGT likely results from a saturation of the binding phase by P.

III.3.2.2. Variation of labile concentration during experiments

Lack of linear accumulation could also result from a significant variation of the labile concentration (*i.e.* "DGT available") in the digestate during DGT exposure. This is of particular concern in digestates where significant biological activity can occur during experiments. In order to detect a potential evolution of the labile concentration, Ch-DGT were deployed for consecutive steps of 24 h (*i.e.* from the beginning to the 24th hour, from the 24th to the 48th hour and finally from the 48th to the 72th hour). All results are displayed in supplementary material (Figures S8 and S9) and only data from the experimental series run under anaerobic condition will be considered here. In most cases, no significant difference could be observed between the three 24 h consecutive steps, indicating that labile concentration did not significantly evolves during the experiment. As can be seen for Al and Cu in Figure III.3:, although slight variations could be observed, no significant evolution of labile concentration could be highlighted for both digestates. Therefore, the absence of linear accumulation observed during Ch-DGT time-series deployments for Al and Cu, cannot be explained by a significant variation of the labile concentration during the experiment.

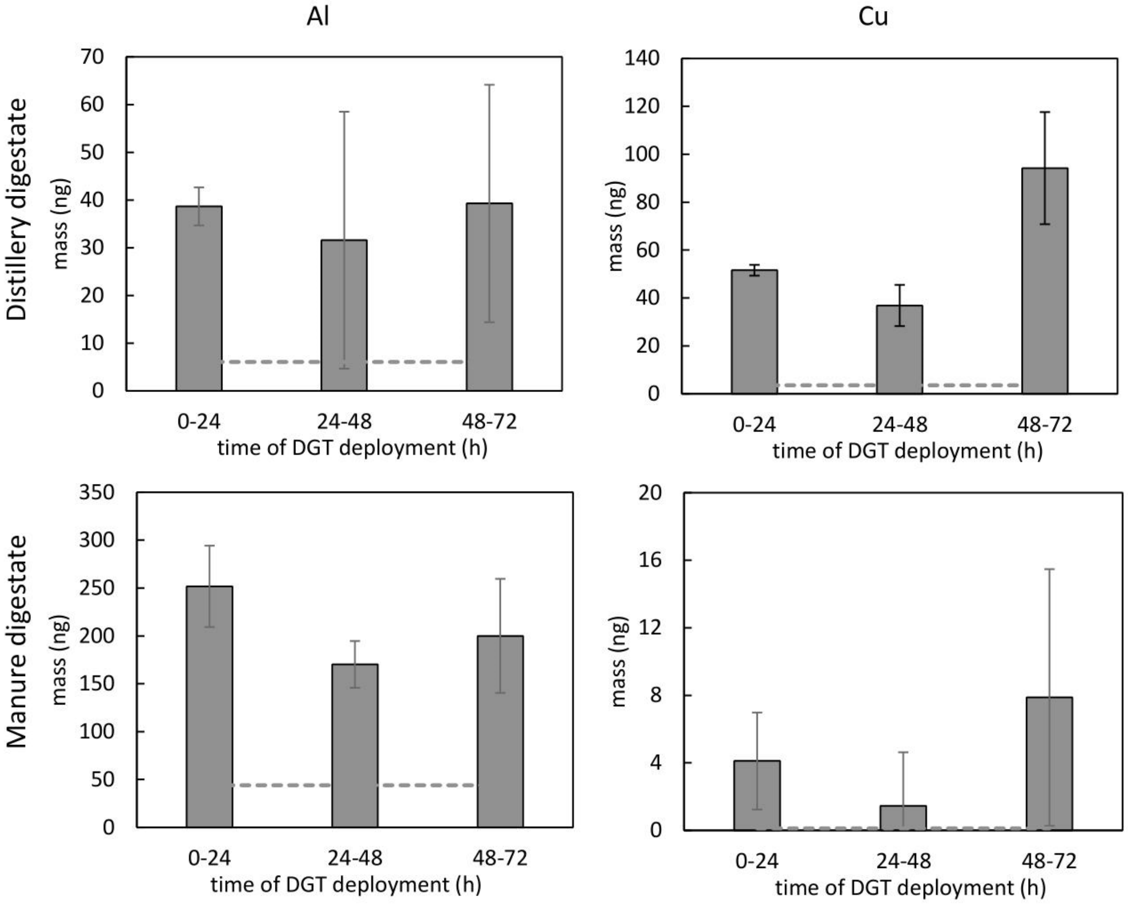


Figure III.3: Accumulated masses of Al and Cu (anaerobic conditions experiments) consecutive 24h deployments of Ch-DGT in: A) distillery waste digestate and B) manure digestate. Dotted line: blank

III.3.2.3. Alternative hypothesis

The presence of a significant amount of element in the binding layer but without an accumulation trend over time might result from the presence of significant amount of “DGT inert” species (*e.g.* strong complexes, nanoparticles that cannot be bound to the binding layer). Indeed, inert or partially-labile species of an element could be able to diffuse within the sampler and reach the binding layer. They may not react with the binding phase and a steady state between the solution and the binding layer could be established (Tusseau-Vuillemin et al., 2003). Consequently, their concentration in the gel of the binding layer would be proportional to their concentration in solution, and thus constant over time. If the concentration of such complexes is very high in the system, the amount of non-sorbed element in the binding layer could be significantly higher than the amount of sorbed element (*i.e.* labile species). In such a situation, the labile element accumulation, which evolves linearly over time, would be negligible, resulting in the observation of a constant “accumulated” mass over time. Assuming that these “DGT inert” species cannot be bound by any binding phase, similar amount of element should be detected regardless of the sorbent used in the binding layer. Table III.*1:* compares the masses of Al and Cu found in Ch-DGT and in Zr-DGT according to the deployment time by using the ratio of the mass in Ch-DGT to the mass in Zr-DGT. If the element found in the binding gel corresponds mainly to “DGT-inert” species, the ratio should be close to 1 and stable over time. The results shows that the Al and Cu masses found in Ch-DGT may be higher than in Zr-DGT. However, due to the high uncertainty on the ratios, the proposed hypothesis cannot be strictly excluded at this stage and could thus be, at least partly, responsible for the absence of accumulation over time for Al and Cu on Ch-DGT. Further work is consequently required to understand this specific behaviour.

Table III.1: Ratio between the mass found in Ch-DGT on the mass found in Zr-DGT for Al and Cu

Deployment time	Al (distillery digestate)	Cu (distillery digestate)	Al (manure digestate)
8h	3 ± 3	7 ± 2	7 ± 4
12h	3 ± 2	7 ± 5	5 ± 3
24h	5 ± 3	5 ± 6	8 ± 2
48h	2 ± 3	25 ± 43	2.3 ± 0.5
72h	0.8 ± 0.7	3 ± 4	2.8 ± 0.3

III.3.3. Practical set up for DGT deployment in anaerobic digestates

Maintaining anaerobic conditions during DGT deployments requires specific equipment (*e.g.* glove box) and makes DGT deployment more constraining. In order to simplify the procedure, deployments without strictly maintaining anaerobic conditions (*i.e.* non-degassed DGT devices deployed in samples opened to air) were also performed simultaneously with the experiments run under anaerobic conditions. The corresponding accumulated mass in the different DGT devices are given in supplementary material (Section III.7 Appendix III. Figures S2 – S7). In most cases, the absence of strictly anaerobic deployment conditions did not induce a significant change in the mass accumulation patterns. For Zr-DGT and thiol-DGT, the observations are unfortunately probably limited due to the potential saturation by P and to the low content of reduced metal(loids) in the studied digestates respectively. However, the loss of anaerobic conditions during deployment did not modify Mo accumulation in Zr-DGT and As(III) in Thiol-DGT for the manure digestate.

However, significant differences could be observed in the accumulation trend for several elements with Ch-DGT (Figure III.4*:*). Indeed, for Mn in the distillery waste digestate and for Fe, Mn and Co in the manure digestate, the accumulated masses under non-anaerobic conditions were significantly lower than the ones observed under anaerobic conditions. For Cu in the distillery digestate, a linear accumulation profile was observed under non-anaerobic conditions whereas no accumulation was observed under anaerobic conditions. It is expected that these differences result from changes in the DGT-available fractions induced by the exposure of digestates to air. This hypothesis could be verified using the consecutive 24h deployment samplers (Section III.7 Appendix III. Figures S8 and S9). For the distillery digestate, the Mn accumulation under non-anaerobic conditions were indeed lower (20-40%) than under anaerobic conditions for the three 24h steps. The same behaviour was observed for Fe, Mn and Co in the manure digestate. This indicates that air exposure promotes a decrease in the labile concentration in the digestate. Such decrease could be easily explained by the precipitation of Mn and Fe under oxide or oxyhydroxide forms when exposed to oxygen. Cobalt decrease could rather result from a coprecipitation with Mn and/or Fe. For each of these cases, the decrease observed over the 24h deployment steps is consistent with the decreasing accumulation over time observed under non-anaerobic conditions. In the case of Cu in the distillery digestate, the accumulated masses during the 24h steps under non-anaerobic conditions are strongly higher than under anaerobic conditions. This means that air introduction in the system has promoted the appearance of labile species. The oxydation of sulphide precipitates is probably the origin of such element releases. This explains, at least partly, the observation of a linear accumulation only during the time-series deployments performed under non-anaerobic conditions.

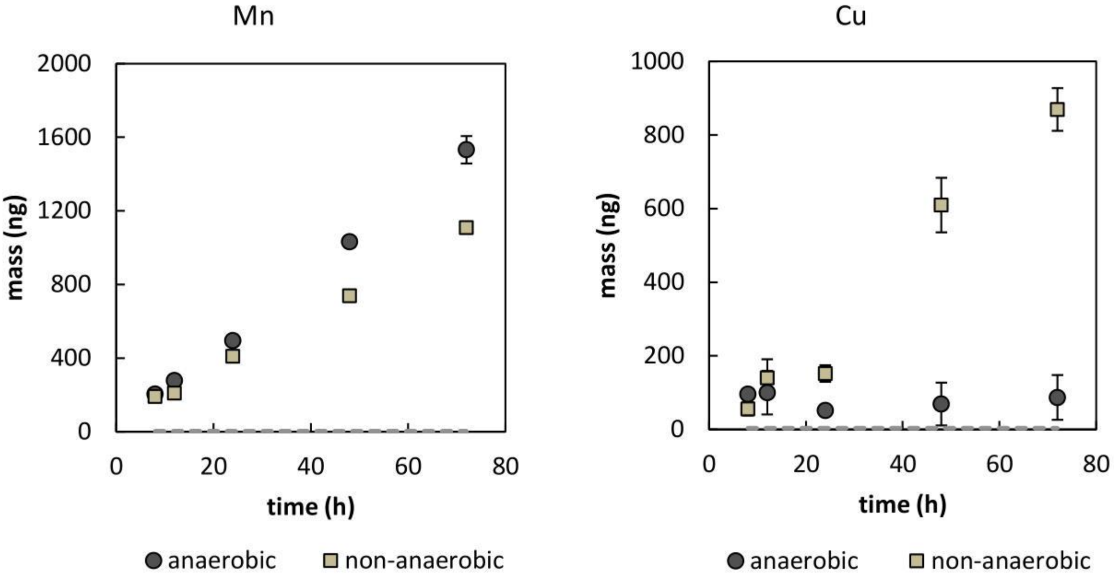


Figure III.4: Accumulated masses of Mn and Cu obtained after deployments under anaerobic and non-anaerobic conditions in the distillery digestate. Dotted line: blank

III.4. Conclusions: Recommendations for using DGT in anaerobic digestates

This study demonstrates that the DGT theory was fulfilled for most trace elements detected at a significant level, indicating that DGT can be effectively deployed in anaerobic digestates to evaluate the labile fraction of trace elements. However, precaution must be taken since several elements did not have a linear over time (Al, Cu and As in this study). Therefore, any conversion of their masses found in the samplers into a labile concentration in the digestate would lead to incorrect values. Given that it is currently not possible to anticipate these altered behaviours, we strongly recommend to systematically perform time-series deployments of DGT (*i.e.* deployment of samplers for various times) in anaerobic digestates and to consider only the elements with a linear accumulation trend. In the specific case of arsenic, the use of selective devices like thiol-DGT should be considered. Although thiol-DGT only samples labile As(III) instead of total labile As, it could still be relevant in the case of anaerobic digestates, where it is likely that As would be mostly found under its reduced form. In any case, thiol-DGT device could help detecting a potential saturation of Zr-DGT by P, when the accumulated As amount in Zr-DGT is lower than in thiol-DGT.

This study also showed that deploying DGT *ex-situ* rather than directly in the reactor for studying anaerobic digestates could be relevant in some conditions. However, erroneous results can be obtained for some elements because of air introduction (Co, Cu, Fe, and Mn in this study) and efforts must be made to keep the anaerobia during all the process in order to obtain a realistic view of the labile concentration of elements in an anaerobic digestate. Otherwise, short time and consecutive deployments of DGT (*i.e.* similar to the consecutive 24h DGT used in this study) must be performed in order to highlight any change in the labile concentration in the digestate over time that could alter element accumulation during DGT deployment. In such case, even if a linear accumulation is observed in the time series deployments, the resulting estimated labile concentration would not be representative of the real labile concentration in the anaerobic digestate.

III.5. Acknowledgements

This project has received funding from the European Union’s Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 861088. The authors thanks Patrice Fondanèche for ICP-MS analysis and Emanuelle Ducloux for digestate sampling.

III.6. References

APHA. (2017). Standard Methods for the Examination of Water and Wastewater (23rd ed.). Washington DC: American Public Health Association.

Bartacek, J., Fermoso, F. G., Baldó-Urrutia, A. M., Van Hullebusch, E. D., & Lens, P. N. L. (2008). Cobalt toxicity in anaerobic granular sludge: Influence of chemical speciation. Journal of Industrial Microbiology & Biotechnology, 35(11), 1465–1474. https://doi.org/10.1007/s10295-008-0448-0

Bourven, I., Casellas, M., Buzier, R., Lesieur, J., Lenain, J.-F., Faix, A., Bressolier, P., Maftah, C., & Guibaud, G. (2017). Potential of DGT in a new fractionation approach for studying trace metal element impact on anaerobic digestion: The example of cadmium. International Biodeterioration & Biodegradation, 119, 188–195. https://doi.org/10.1016/j.ibiod.2016.11.007

Callander, I. J., & Barford, J. P. (1983). Precipitation, chelation, and the availability of metals as nutrients in anaerobic digestion. I. Methodology. Biotechnology and Bioengineering, 25(8), 1947–1957. https://doi.org/10.1002/bit.260250805

Davison, W., & Zhang, H. (1994). In situ speciation measurements of trace components in natural waters using thin-film gels. Nature, 367(6463), 546–548. https://doi.org/10.1038/367546a0

Devillers, D., Buzier, R., Simon, S., Charriau, A., & Guibaud, G. (2016). Simultaneous measurement of Cr(III) and Cr(VI) in freshwaters with a single Diffusive Gradients in Thin Films device. Talanta, 154, 533–538. https://doi.org/10.1016/j.talanta.2016.04.009

Eismann, C. E., Menegário, A. A., Gemeiner, H., & Williams, P. N. (2020). Predicting Trace Metal Exposure in Aquatic Ecosystems: Evaluating DGT as a Biomonitoring Tool. Exposure and Health, 12(1), 19–31. https://doi.org/10.1007/s12403-018-0280-3

Galceran, J., Gao, Y., Puy, J., Leermakers, M., Rey-Castro, C., Zhou, C., & Baeyens, W. (2021). Speciation of Inorganic Compounds in Aquatic Systems Using Diffusive Gradients in Thin-Films: A Review. Frontiers in Chemistry, 9, 624511. https://doi.org/10.3389/fchem.2021.624511

Galceran, J., & Puy, J. (2015). Interpretation of diffusion gradients in thin films (DGT) measurements: A systematic approach. Environmental Chemistry, 12(2), 112–122. https://doi.org/10.1071/EN14068

Hullebusch, E., Utomo, S., Zandvoort, M., & Llens, P. (2005). Comparison of three sequential extraction procedures to describe metal fractionation in anaerobic granular sludges. Talanta, 65(2), 549–558. https://doi.org/10.1016/j.talanta.2004.07.024

Laera, A., Buzier, R., Guibaud, G., Esposito, G., & van Hullebusch, E. D. (2019a). Assessment of the DGT technique in digestate to fraction twelve trace elements. Talanta, 192(August 2018), 204–211. https://doi.org/10.1016/j.talanta.2018.09.033

Laera, A., Buzier, R., Guibaud, G., Esposito, G., & van Hullebusch, E. D. (2019b). Distribution trend of trace elements in digestate exposed to air: Laboratory-scale investigations using DGT-based fractionation. Journal of Environmental Management, 238(November 2018), 159–165. https://doi.org/10.1016/j.jenvman.2019.02.120

Molaey, R., Yesil, H., Calli, B., & Tugtas, A. E. (2021). Influence of volatile fatty acids in anaerobic bioleaching of potentially toxic metals. Journal of Environmental Management, 285. https://doi.org/10.1016/j.jenvman.2021.112118

Mudhoo, A., & Kumar, S. (2013). Effects of heavy metals as stress factors on anaerobic digestion processes and biogas production from biomass. International Journal of Environmental Science and Technology, 10(6), 1383–1398. https://doi.org/10.1007/s13762-012-0167-y

Nakhate, S. P., Gulhane, M., Singh, A. K., Purohit, H. J., Shah, M. P., & Khardenavis, A. A. (2023). Trace metals as key controlling switches regulating the efficiencies of aerobic and anaerobic bioprocesses. Biochemical Engineering Journal, 198, 108999. https://doi.org/10.1016/j.bej.2023.108999

O'Connor, J., Mickan, B. S., Siddique, K. H. M., Rinklebe, J., Kirkham, M. B., & Bolan, N. S. (2022). Physical, chemical, and microbial contaminants in food waste management for soil application: A review. Environmental Pollution, 300, 118860. https://doi.org/10.1016/j.envpol.2022.118860

Ortner, M., Rachbauer, L., Somitsch, W., & Fuchs, W. (2014). Can bioavailability of trace nutrients be measured in anaerobic digestion? Applied Energy, 126(2014), 190–198. https://doi.org/10.1016/j.apenergy.2014.03.070

Sahin, F., Volkan, M., Howard, A., & Ataman, O. (2003). Selective pre-concentration of selenite from aqueous samples using mercapto-silica. Talanta, 60(5), 1003–1009. https://doi.org/10.1016/S0039-9140(03)00154-1

Takashima, M. (2018). Effects of Thermal Pretreatment and Trace Metal Supplementation on High-Rate Thermophilic Anaerobic Digestion of Municipal Sludge. Journal of Environmental Engineering, 144(3), 04018009. https://doi.org/10.1061/(ASCE)EE.1943-7870.0001340

Tusseau-Vuillemin, M.-H., Gilbin, R., & Taillefert, M. (2003). A Dynamic Numerical Model To Characterize Labile Metal Complexes Collected with Diffusion Gradient in Thin Films Devices. Environmental Science & Technology, 37, 1645–1652.

Vodyanitskii, Yu. N. (2006). Methods of sequential extraction of heavy metals from soils: New approaches and the mineralogical control (a review). Eurasian Soil Science, 39(10), 1074–1083. https://doi.org/10.1134/S106422930610005X

Vongvichiankul, C., Deebao, J., & Khongnakorn, W. (2017). Relationship between pH, Oxidation Reduction Potential (ORP) and Biogas Production in Mesophilic Screw Anaerobic Digester. Energy Procedia, 138, 877–882. https://doi.org/10.1016/j.egypro.2017.10.113

Wang, T., Tang, Z., Guo, Y., Zhang, X., Yang, Q., Xu, B., & Wang, H. (2020). Comparison of Heavy Metal Speciation of Sludge During Mesophilic and Thermophilic Anaerobic Digestion. Waste and Biomass Valorization, 11(6), 2651–2660. https://doi.org/10.1007/s12649-018-00575-9

Zhang, H., & Davison, W. (1995). Performance Characteristics of Diffusion Gradients in Thin Films for the in Situ Measurement of Trace Metals in Aqueous Solution. Analytical Chemistry, 67(19), 3391–3400. https://doi.org/10.1021/ac00115a005

Zhang, H., Davison, W., Gadi, R., & Kobayashi, T. (1998). In situ measurement of dissolved phosphorus in natural waters using DGT. Analytica Chimica Acta, 370(1), 29–38. https://doi.org/10.1016/S0003-2670(98)00250-5

Zheng, X., Wu, K., Sun, P., Zhouyang, S., Wang, Y., Wang, H., Zheng, Y., & Li, Q. (2021). Effects of substrate types on the transformation of heavy metal speciation and bioavailability in an anaerobic digestion system. Journal of Environmental Sciences (China), 101, 361–372. https://doi.org/10.1016/j.jes.2020.08.032

III.7. Appendix III: Supplementary material

Table III.S2. Elution factors f_e used in this study

Species	**Elution factor**	**Reference**
Al	0.85	(Devillers et al., 2017)
Cd	0.85	
Co	0.85	
Cr	0.8	
Cu	0.85	
Ni	0.85	
Pb	0.85	
Zn	0.85	
As	0.7	(Laera et al., 2019)
Mo	0.86	
Sb	0.61	
Se	0.86	
W	0.7	
Fe	0.7	(Zhang & Davison, 1995)
Mn	0.82	
P	0.95	(Ding et al., 2010)

References

Devillers, D., Buzier, R., Charriau, A., & Guibaud, G. (2017). Improving elution strategies for Chelex®-DGT passive samplers. *Analytical and Bioanalytical Chemistry*, *409*(30), 7183–7189. https://doi.org/10.1007/s00216-017-0680-4

Ding, S., Xu, D., Sun, Q., Yin, H., & Zhang, C. (2010). Measurement of dissolved reactive phosphorus using the diffusive gradients in thin films technique with a high-capacity binding phase. *Environmental Science and Technology*, *44*(21), 8169–8174. https://doi.org/10.1021/es1020873

Laera, A., Buzier, R., Guibaud, G., Esposito, G., & van Hullebusch, E. D. (2019). Distribution trend of trace elements in digestate exposed to air: Laboratory-scale investigations using DGT-based fractionation. *Journal of Environmental Management*, *238*(November 2018), 159–165. https://doi.org/10.1016/j.jenvman.2019.02.120

Zhang, H., & Davison, W. (1995). Performance Characteristics of Diffusion Gradients in Thin Films for the in Situ Measurement of Trace Metals in Aqueous Solution. *Analytical Chemistry*, *67*(19), 3391–3400. https://doi.org/10.1021/ac00115a005

Table III.S3. Main initial characteristics of the studied digestates

	Distillery waste digestate	Cow manure digestate
TS (%)	2.1 ± 0.1	8.4 ± 0.3
VS (%TS)	54.3 ± 0.2	25 ± 1
COD ($gO_2\ L^{-1}$)	7.0 ± 0.6	52 ± 2
Dissolved COD ($gO_2\ L^{-1}$)	0.5 ± 0.2	14.5 ± 0.5
Alkalinity ($gCaCO_3\ L^{-1}$)	0.67 ± 0.02	6.54 ± 0.09
pH	7.9 ± 0.1	7.3 ± 0.1
Eh (mV)	-357 ± 3	-360 ± 3

Table III.S4. Total and soluble elements concentration in the studied digestates. <LD means that concentration was lower than 3 times the mean blanks concentration

Element	**Distillery waste digestate**		**Cow manure digestate**	
	Total	**Soluble**	**Total**	**Soluble**
P (mg L^{-1})	730 ± 10	14 ± 2	860 ± 80	80 ± 4
Cu (mg L^{-1})	40 ± 1	0.18 ± 0.02	10 ± 1	0.40 ± 0.01
Fe (mg L^{-1})	37 ± 1	< LD	360 ± 20	4.6 ± 0.2
Al (mg L^{-1})	33 ± 3	< LD	400 ± 30	7.8 ± 0.1
Zn (mg L^{-1})	5.7 ± 0.3	< LD	27 ± 4	1.4 ± 0.1
Mn (µg L^{-1})	4900 ± 200	47 ± 8	26000 ± 2000	1170 ± 20
Pb (µg L^{-1})	610 ± 20	< LD	770 ± 40	27 ± 3
Cr (µg L^{-1})	170 ± 30	7 ± 5	1170 ± 110	31 ± 2
Ni (µg L^{-1})	110 ± 20	< LD	570 ± 40	27 ± 1
W (µg L^{-1})	30 ± 9	< LD	150 ± 7	7 ± 1
Mo (µg L^{-1})	24 ± 16	4 ± 3	220 ± 9	14.4 ± 0.3
Co (µg L^{-1})	17 ± 2	0.4 ± 0.1	170 ± 7	10 ± 1
As (µg L^{-1})	17 ± 1	1.0 ± 0.9	280 ± 20	15 ± 7
Sb (µg L^{-1})	12 ± 3	1.7 ± 0.5	40 ± 4	1.8 ± 0.4
Se (µg L^{-1})	10 ± 2	< LD	110 ± 20	2.6 ± 0.1
Cd (µg L^{-1})	4.4 ± 0.4	0.6 ± 0.3	40 ± 2	2 ± 1

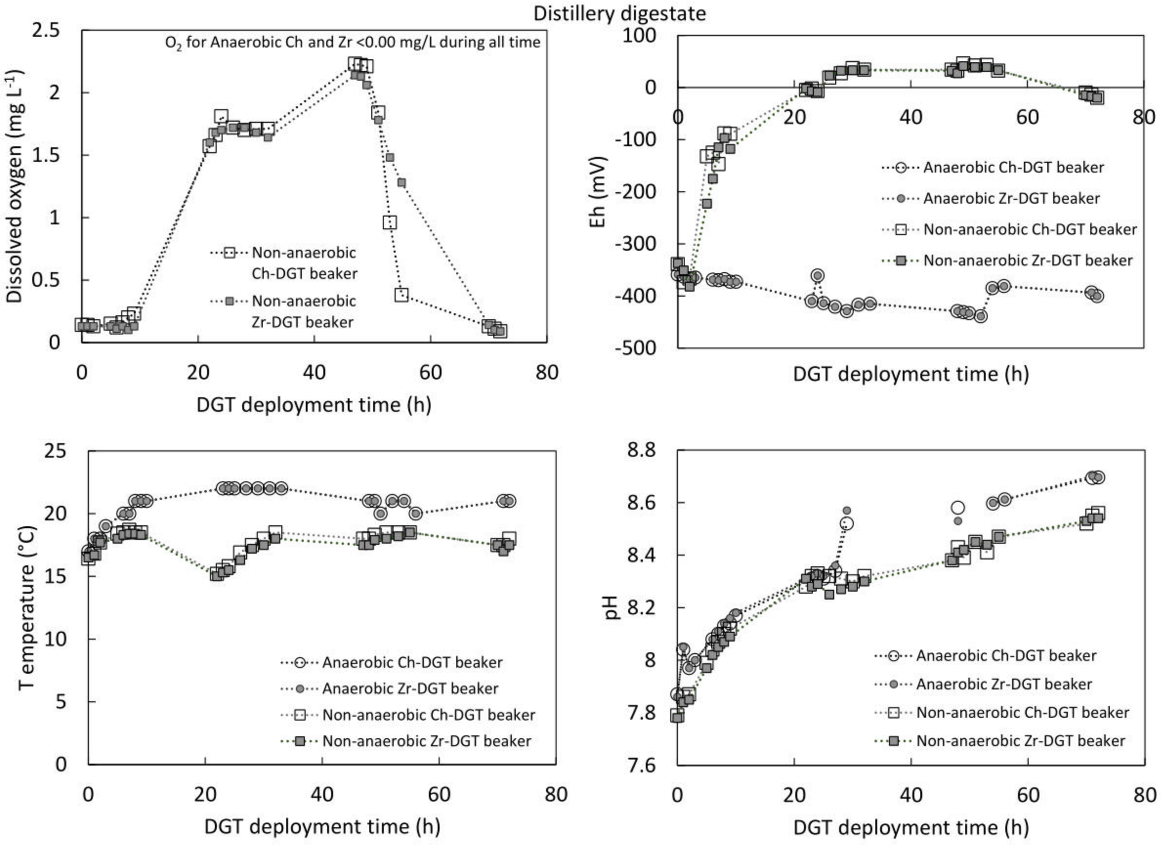


Figure II.S5. Evolution of parameters monitored during DGT experiment in distillery digestate: Dissolved oxygen, redox potential (Eh), Temperature and pH

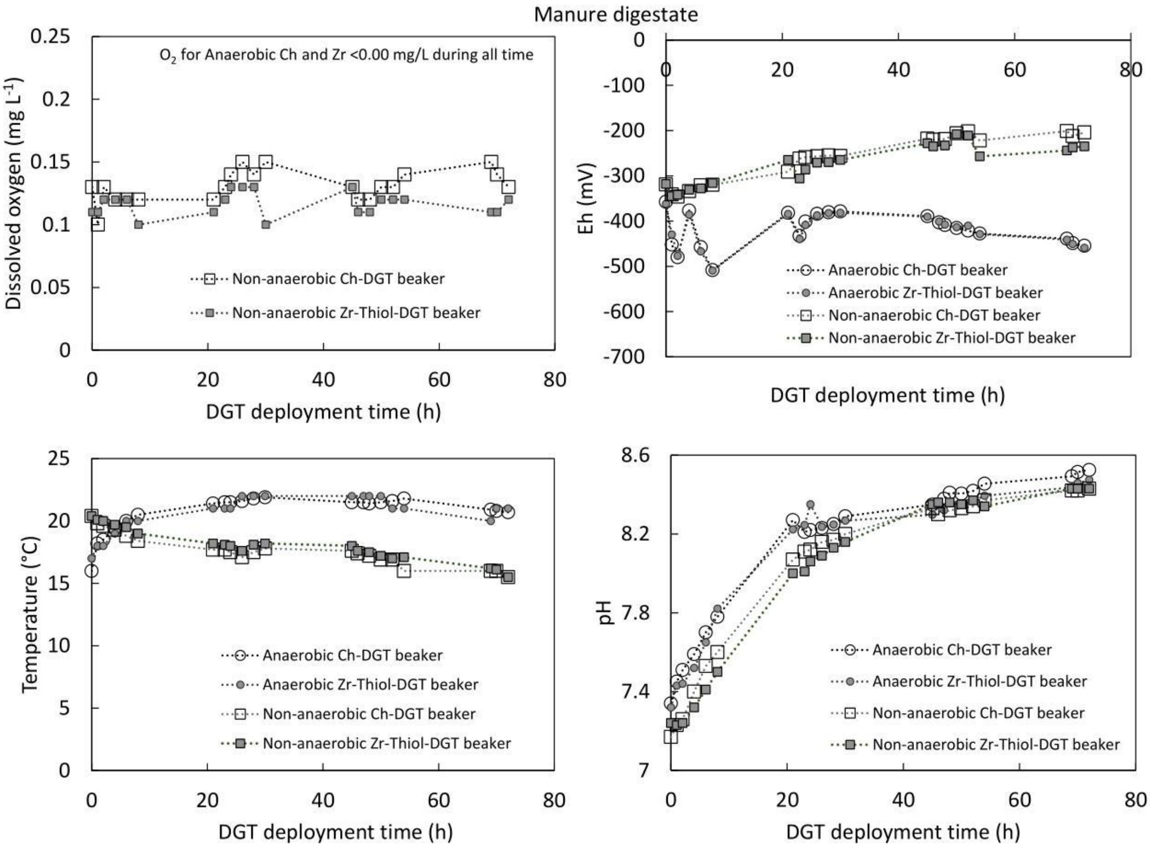


Figure III.S6. Evolution of parameters monitored during DGT experiment in manure digestate: Dissolved oxygen, redox potential (Eh), Temperature and pH

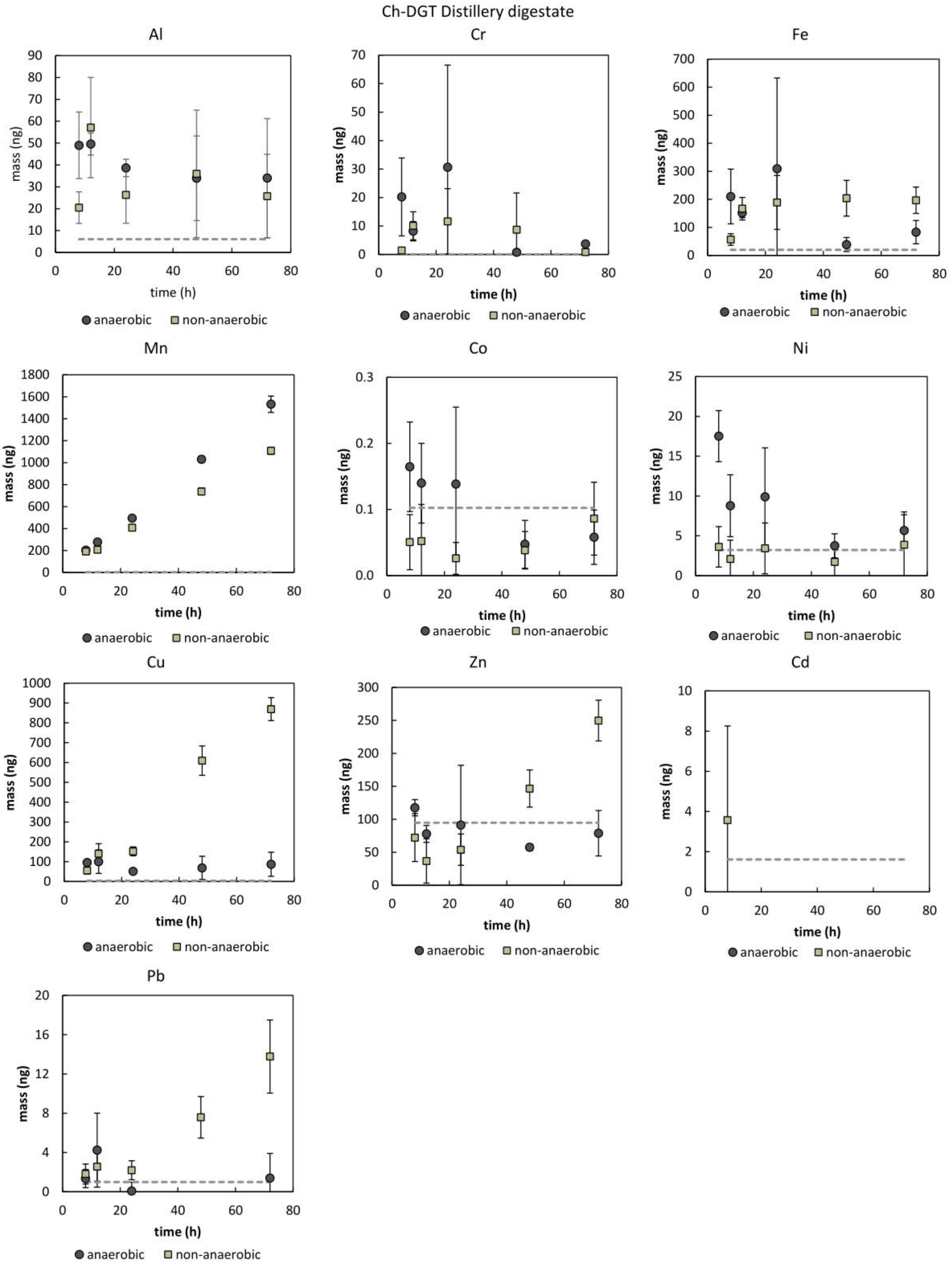


Figure III.S7. Accumulation over time of all targeted cations under anaerobic and non-anaerobic conditions in Ch-DGT samplers in distillery waste digestate. Dotted line: blank

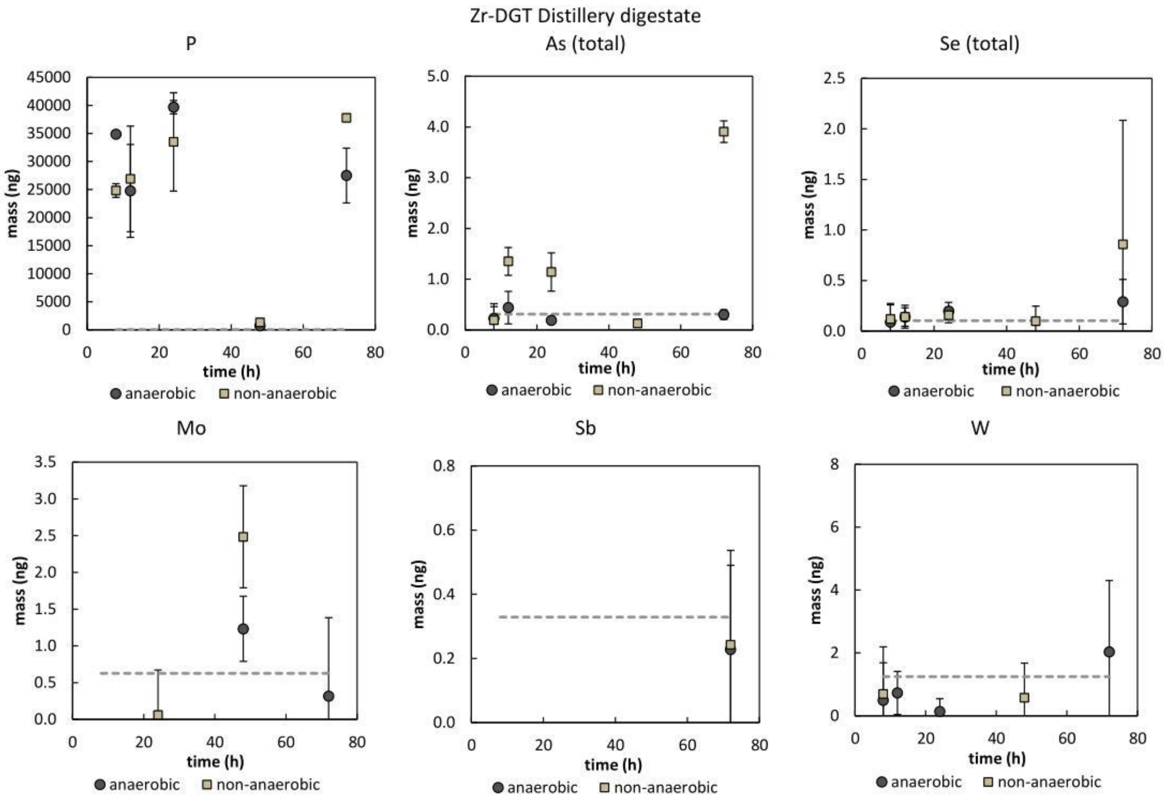


Figure III.S8. Accumulation over time of all targeted anions under anaerobic and non-anaerobic conditions in Zr-DGT samplers in distillery waste digestate. Dotted line: blank

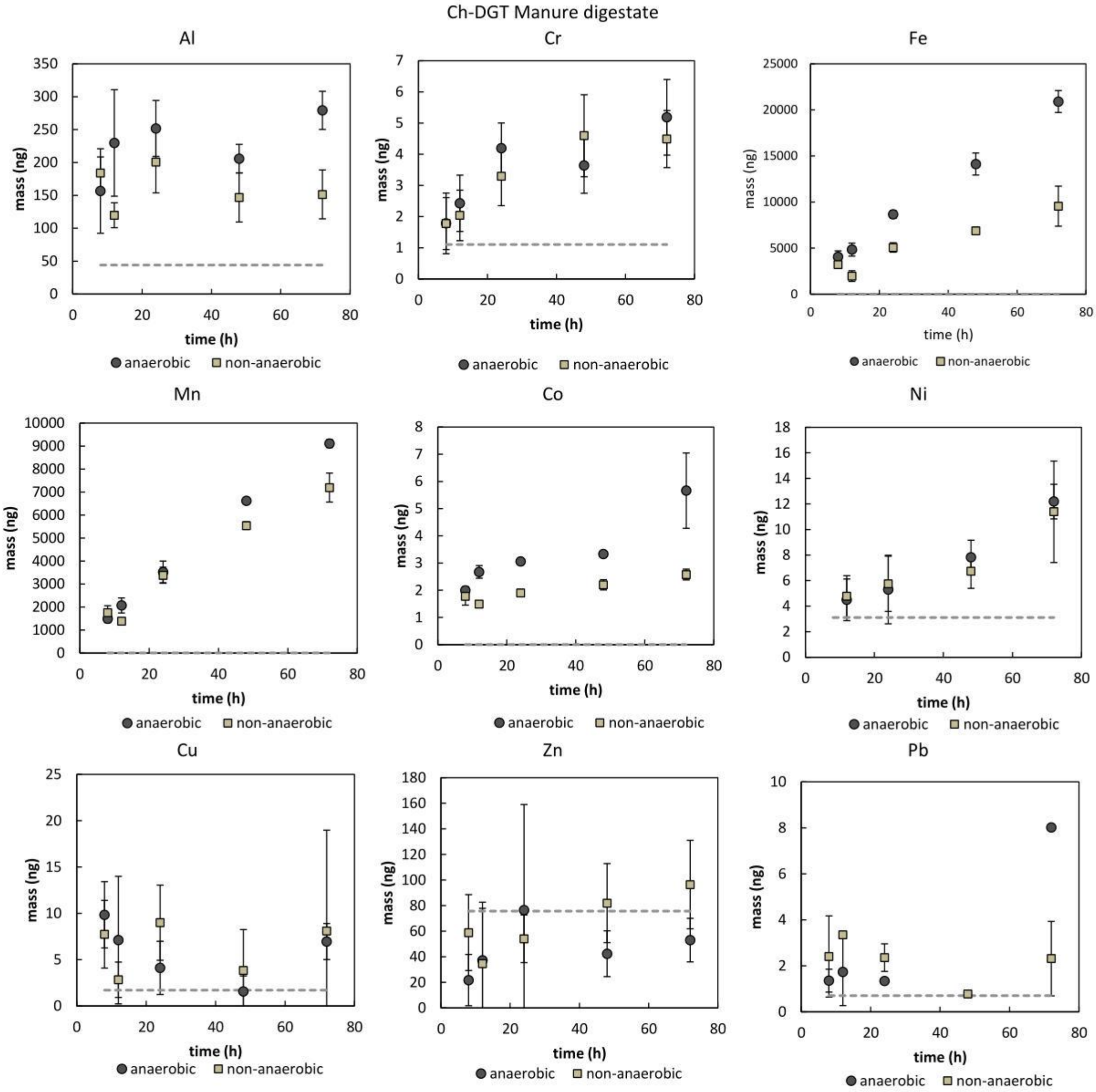


Figure III.S9. Accumulation over time of all targeted cations under anaerobic and non-anaerobic conditions in Ch-DGT samplers in manure waste digestate. Cadmium was not detected in all conditions. Dotted line: blank

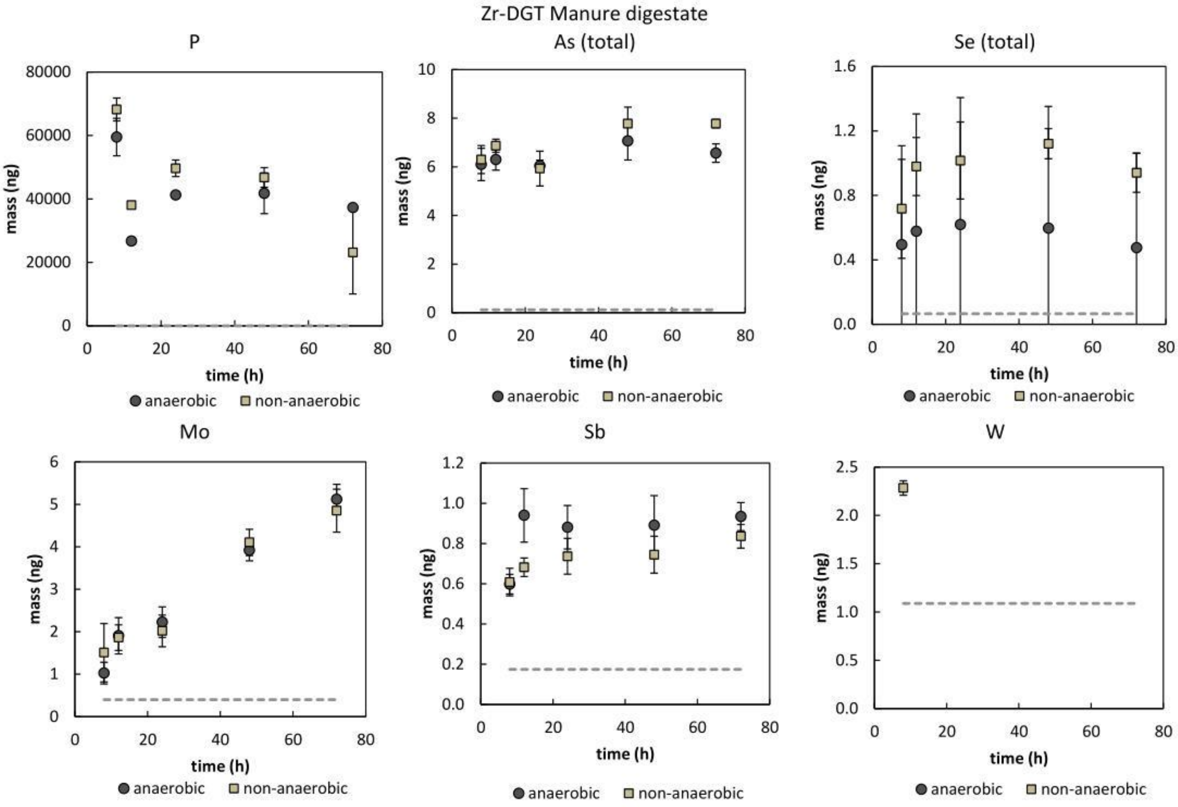


Figure III.S10. Accumulation over time of all targeted anions under anaerobic and non-anaerobic conditions in Zr-DGT samplers in manure waste digestate. Dotted line: blank

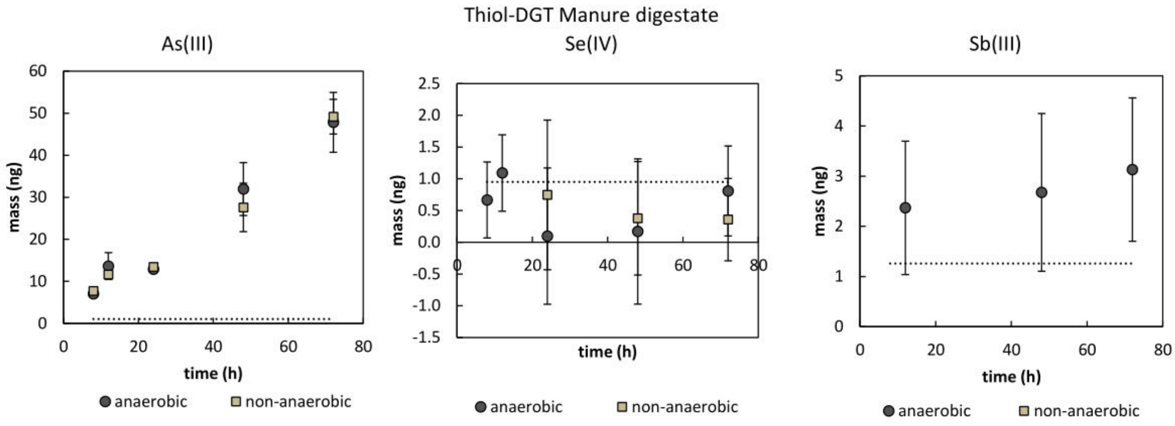


Figure III.S11. Accumulation over time of all targeted elements under anaerobic and non-anaerobic conditions in Thiol-DGT samplers in manure waste digestate. Dotted line: blank

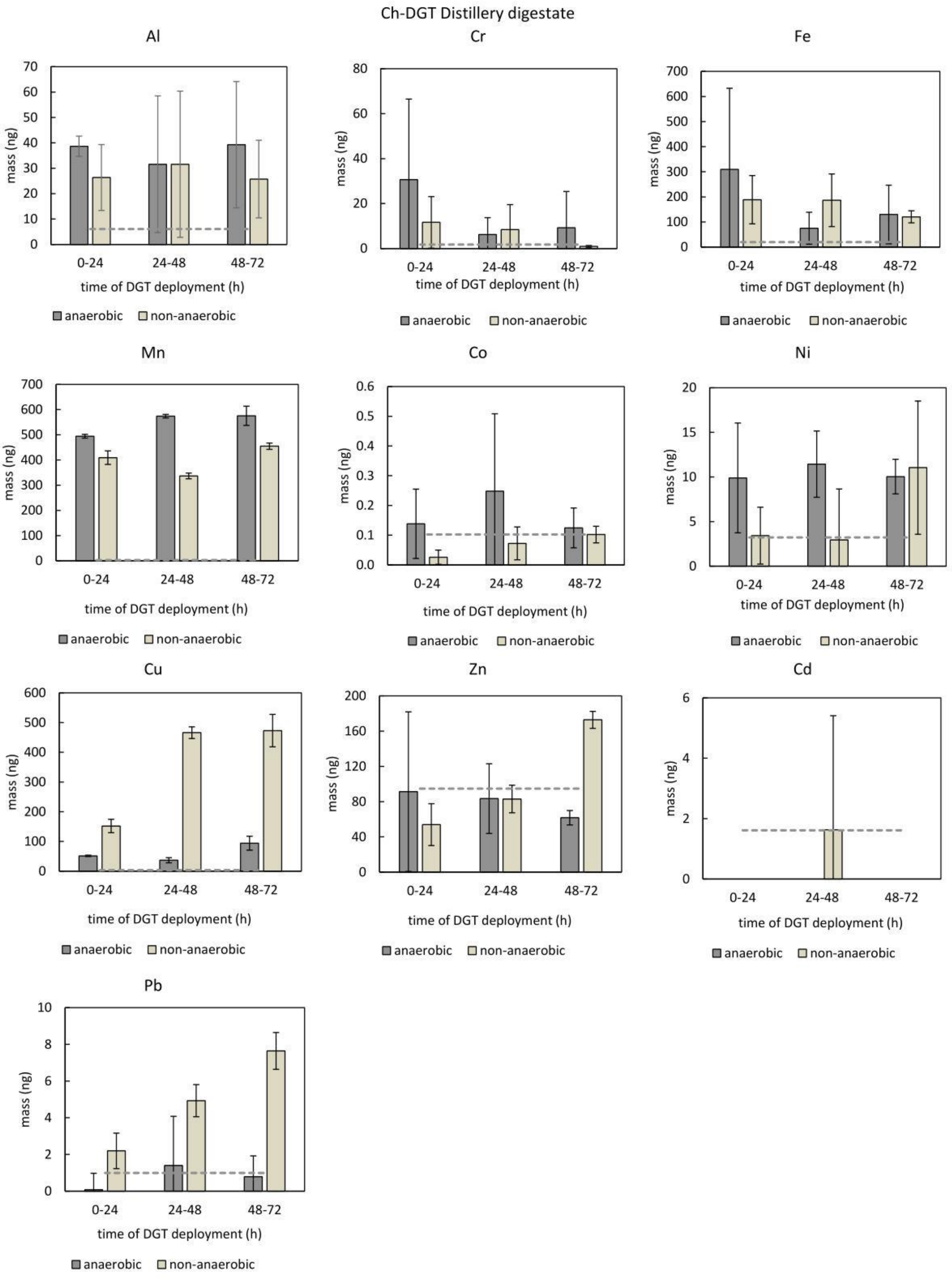


Figure III.S12. *Accumulated masses of all targeted elements under anaerobic and non-anaerobic conditions in consecutive 24h deployments of Ch-DGT in distillery waste digestate. Dotted line: blank*

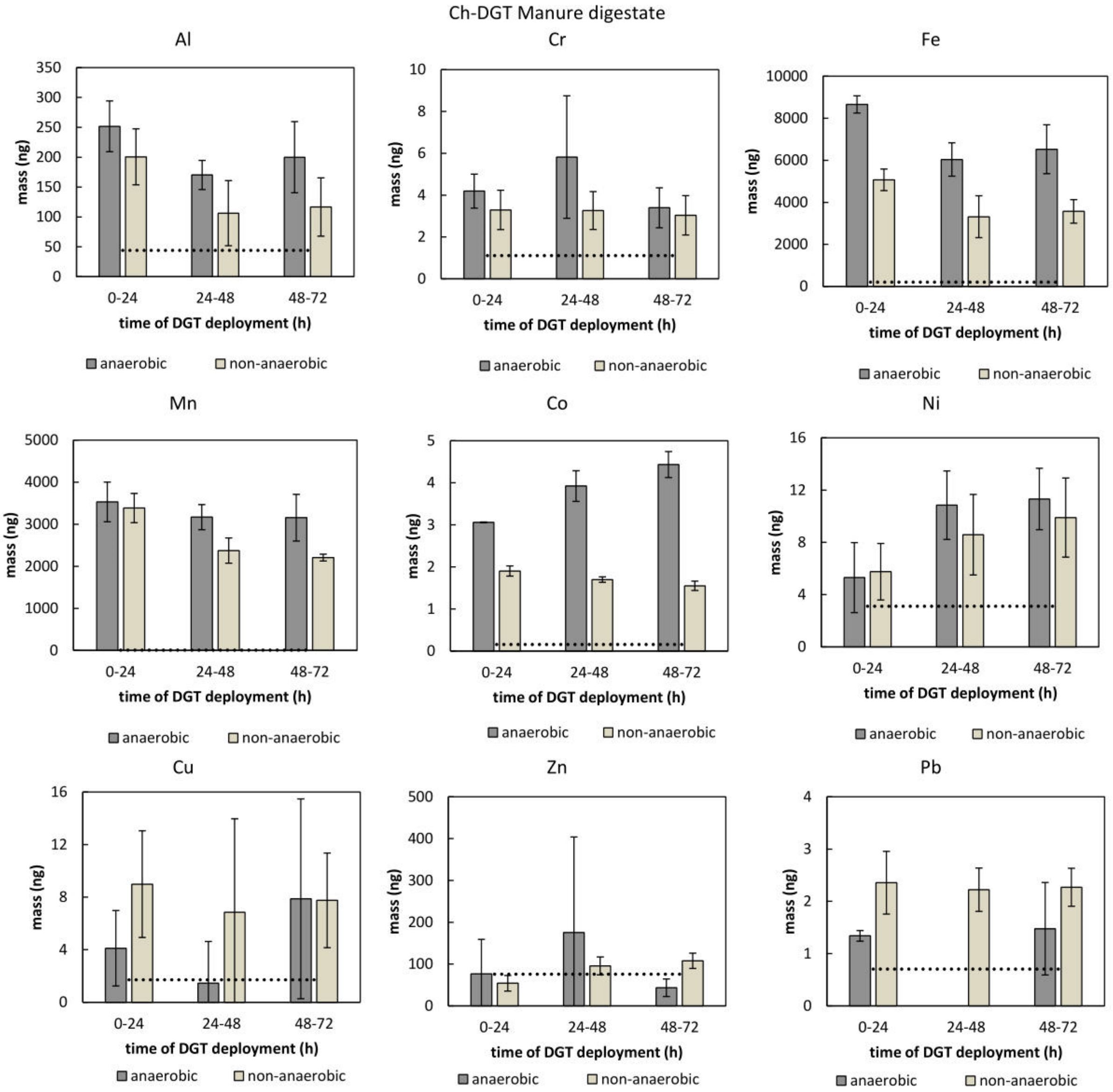


Figure III.S13. Accumulated masses of all targeted elements under anaerobic and non-anaerobic conditions in consecutive 24h deployments of Ch-DGT in manure waste digestate. Cadmium was not detected in all conditions. Dotted line: blank

P accumulation capacity of Zr-DGT

The evaluation of the accumulation capacity of Zr-binding for P was estimated by immerging directly a binding gel in 10 mL of K_2HPO_4 solution that contain from 1 to 10 µmol of P. After 17 h of stirring, the binding gels were recovered, gently rinsed with ultrapure water and eluted using 2 mL of 0.005 M NaOH with 0.5 M H_2O_2 for 4 h. The accumulated mass was then deduced by determining the eluate P concentration by ICP-MS.

Chapter IV. Use of DGT in anaerobic digestor: *in situ* trial in a UASB lab scale reactor

IV.1. Introduction

Recently, the use of Diffusive Gradient in Thin Films (DGT) has been extended to anaerobic digestion (AD) systems for the determination of the labile fraction of trace elements (Bourven et al., 2017; Ilic et al., 2024; Laera et al., 2019a, 2019b; Takashima, 2018). The results of these studies are promising, however, they were focused in anaerobic digestates, which is a by-product of the AD. The DGT deployment for a digestate is performed *ex situ* to the AD reactor, meaning that the digestate has to be sampled from the AD reactor and transported to an appropriate installation for DGT deployment. A procedure with these characteristics involves certain considerations to avoid changes in the speciation of the trace elements in the sample, mainly avoiding exposure with air. An option for avoiding possible problems in changes in speciation is to deploy the DGT device inside the AD reactor. Nevertheless, the application of DGT *in situ* to an AD reactor has not yet been studied.

The medium within the AD reactor is different from a digestate. The AD reactor is fed regularly, which means that it has more microbial activity than a digestate, and consequently biogas is constantly being generated by the AD process. These differences along with reactor construction and operation characteristics, such as turbulence within the reactor can be a problem for the physical integrity of the DGT device. Similarly, any change in the exposure area of the DGT would invalidate the measurement because this is a necessary parameter for the calculation of the labile fraction. In fact, exposure area is the parameter that generates greater uncertainty in the estimation of the labile concentration (Knutsson et al., 2014).

The validation of the DGT technique in a new system can be performed by two ways. The first is the evaluation of the relationship between the accumulated mass of the targeted element and deployment time. If the DGT device is acting according to theory, a linear trend is expected. Secondly, a recovery between 90 – 110 % of the targeted element in a solution with a known concentration is expected, knowing that all the content of this element is susceptible to be sampled by the DGT (Ding et al., 2018). The distribution of all species of an element in an anaerobic system is not known (it can be approximated, but not fully quantified) thus, the validation of the DGT technique should be done from the first option.

Cobalt (Co) is an essential trace element in AD (Fermoso et al., 2019) and it has been targeted for DGT technique validation in anaerobic digestates in previous studies (Ilic et al., 2024; Laera et al., 2019a) as well in the Chapter III of this thesis. In Laera et al. (2019a) and Ilic et al. (2024) DGT theory could be validated with the linear accumulation of Co over time. On the other hand, the results in Chapter III indicated that the reliability for Co could not be clearly established.

In this study, the upflow anaerobic sludge bed reactor (UASB) under stress with Co of the Chapter II was selected to evaluate the *in situ* use of the DGT technique. The DGT devices were deployed inside the UASB in order to evaluate them considering deployment conditions and validation of theory.

IV.2. Materials and Methods

IV.2.1. UASB operation and metal dosage

The experiment with DGT were performed during the operation of the UASB reactor used in Chapter II. The details of the UASB reactor configuration, inoculation, feed, operational parameters and Co dosage strategy can be found in Chapter II. Section II.2.2 and II.2.3.
A stable operation was reached after a period of start-up of 108 days. During the next 31 days, the DGT stability test was performed (described in Section IV.2.4). Six different doses of Co were evaluated, with concentrations of 0.1, 10, 50, 100, 500 and 1000 µM of Co. Each Co dose was applied continuously for at least 14 days, followed by a period of at least 3 d with base feed to refresh the liquid content in the UASB. The detail of the time for each Co dose is shown in Chapter II. Section II.2.3.

Three sampling points were selected in the UASB, with a separation of 10 cm, from the bottom of the UASB these points corresponded to: bottom sludge (0 cm) , top sludge (10 cm) and liquid zone (20 cm). At the end of each Co dose, DGT were deployed for validation of DGT theory using Co labile fraction (described in Section IV.2.7).

IV.2.2. DGT preparation and deployment

The labile concentration of Co in the UASB reactor was measured by Chelex DGT. Details of Chelex DGT can be found in Chapter 2 section 2.2.

3 DGTs were put in a plastic holder with a separation of 10 cm between them (see Figure 1.A). These positions corresponded to the three selected sampling points: bottom sludge, top sludge, and liquid zone. DGTs were introduced in the UASB by the solid-liquid-gas phase separation zone of the reactor, which could be opened and separated from the UASB. The process of introducing and removing DGTs from the UASB was carried out as quickly as possible to avoid excessive air entry into the UASB, which would disrupt anaerobic conditions. For each Co dose, 2 extra DGT devices corresponding to blanks were kept in a sealed bag at 4 °C throughout the entire DGT deployment.

After deployment, the DGT were rinsed with ultrapure water (MilliQ, >18.2 MΩ·cm) and disassembled to recover the binding gel. Subsequently, Chelex binding gels were eluted in 2 mL of HNO_3 1 M for 24 h (Laera et al., 2019a). The Co concentration in the eluates were determined by ICP-MS (Agilent 7800). Internal standard ^{103}Rh was added and blanks (solution HNO_3 3%, with ultrapure water) were analyzed every 20 samples. The recovery of the Internal Standard was in the range 80 - 120%, with a relative standard deviation below 10%.

IV.2.3. Evaluation of DGT deployment conditions

A preliminary test was performed at the end of the start-up period. 3 DGTs were deployed for 16 h in each of the 3 sampling zones inside the UASB (liquid zone, top and bottom sludge bed). The DGTs were put parallel to the flow direction, i.e., for this UASB in a vertical position. At the end of the deployment time, bubbles of gas were observed between the filter and diffusive gel for all the DGTs, changing the exposure area, invalidating the measurement of the device. It was decided to evaluate other configurations for DGT deployment, that could avoid bubble accumulation inside the device.

This experiment was performed during the days of operation 108 and 139, after the start-up (stabilization phase). The Co dose in the UASB reactor was 0.1 µM during this experiment. Three deployment configurations were evaluated : (1) a horizontal position with the sampling window at the opposite side of the flow to limit contact with gas, (2) a horizontal position with 3 small holes in the filter membrane to allow a release of the gas trapped between the diffusive gel and the filter membrane and finally (3) a vertical position with three small holes in the filter membrane, which allow a parallel configuration between the sampling window and the flow direction (configuration usually used in DGT deployments). The holes were made with a tip of a syringe needle. Configurations (1) and (2) were evaluated in triplicate for deployment times 4, 8, 16, 24 and 48 h. Configuration (3) was evaluated in triplicate just for a deployment time of 4 h. After deployment time, each device was visually examined and disassembled to observe any gas bubble or internal physical alteration.

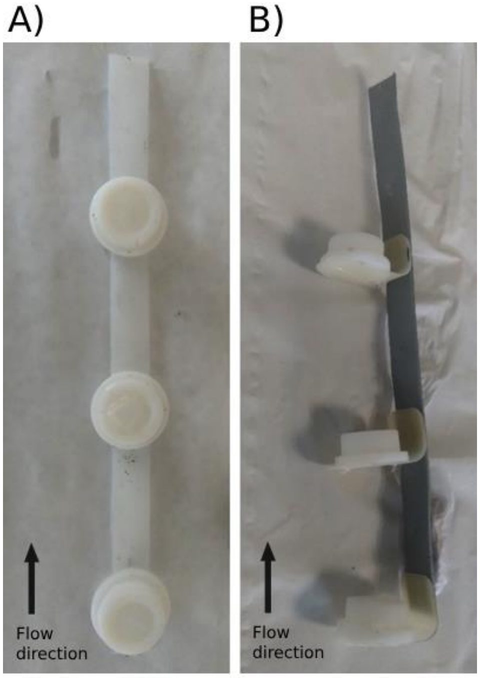


Figure IV.1: DGT configurations. A) vertical, B) horizontal

To determine the cause of alteration observed in the DGT devices, another test was performed in simplified systems (i.e. beakers). Three open beakers were used, each one was filled with a different solution, 1) feed, 2) sludge and 3) a mix of sludge, feed, and 5 g of glucose. In this last one, the glucose was added to promote biogas generation. The beakers were thermostated at 35 ± 2 °C. One DGT was deployed per beaker for 4 h in vertical position with no holes. No agitation was selected to emulate the UASB configuration. After deployment time, DGTs were visually examined for physical integrity.

IV.2.4. Validation of DGT theory in UASB

The validation of the DGT theory was evaluated by analyzing the mass accumulates in the binding gel over time. At the end of the Co continuous dose cycle of 10, 50, 100, 500, and 1000 µM, 9 DGTs were deployed in a horizontal position without holes at the bottom sludge position in triplicate, for 4, 6, and 8 h. All DGT were initially deployed at time 0.

IV.3. Results and Discussion

IV.3.1. DGT deployment conditions in UASB

The evaluation of the three deployments configurations (i) horizontal, ii) horizontal with 3 small holes in the filter membrane and iii) vertical with holes) is presented in Table IV.1. If at least 1 DGT was altered, the whole group of replicates was discarded. The only configuration where the integrity of the DGTs was not affected was the horizontal deployment without holes during 4 h. No alteration was also observed for 8 h DGT deployed in horizontal position (with and without holes) in the top and bottom sludge but bubbles were detected between the filter and diffusive gel in the DGT in the liquid zone. Moreover, for 4 h deployments with holes, some bubbles were detected (Figure IV.2.A). From 8 h for the liquid zone and 16 h in the sludge zone, serious alterations were observed in the DGT, such as a large bubble throughout the exposed area (Figure IV.2.B), the beginning of separation of the membrane filter to the plastic cap (Figure IV.2.C) or a complete separation of the filter membrane and gels, where sludge entered into the DGT (Figure IV.2.D). Therefore, the most reliable configuration seems to be DGT in horizontal position without holes deployed for 8 h or less.

Table IV.1: Stability of DGT after deployment in different configurations and deployment times in UASB

		Configuration														
		Horizontal without holes					Horizontal with holes					Vertical with holes				
	DGT deployment time	4h	8h	16 h	24 h	48h	4h	8h	16 h	24 h	48h	4h	8h	16 h	24 h	48h
Position	liquid zone	o	x	x	x	x	w	x	x	x	x	x	-	-	-	-
	top sludge	o	o	x	x	x	w	o	x	x	x	x	-	-	-	-
	bottom sludge	o	o	x	x	x	w	o	x	x	x	x	-	-	-	-

o: No problem.
w: a gas bubble was observed (size < 50% total area).
x: a gas bubble covering the area was observed, the filter was starting to be separated from the plastic cap, or the separation of the filter membrane and gels from the plastic holder.
- : Not tested

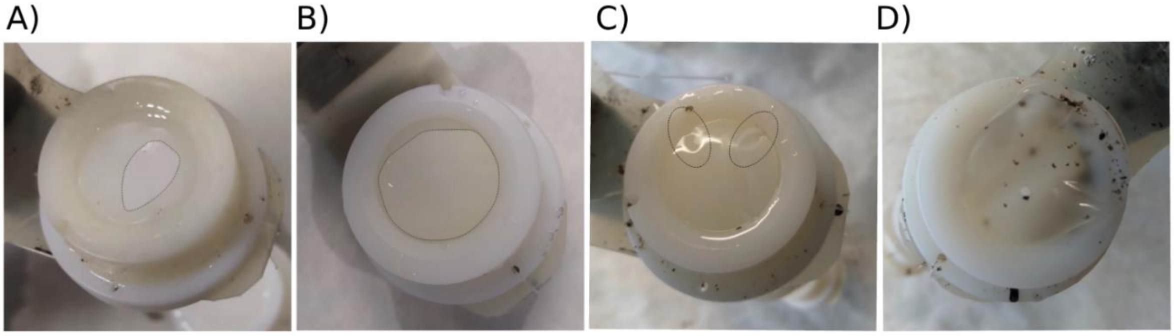


Figure IV.2: Type of damage observed during different deployment times and configurations. A) Bubble covering < 50 % of the exposure area, B) Bubble covering all the exposure area, C) Filter membrane starting to separate from the plastic cap, D) Filter membrane out of the plastic cap, with granules inside the internal space of the DGT. In A) and B) the border of the bubble are indicated with a dotted black line. In C) the fold zones of the membrane filter are indicated within the black ovals.

The DGT integrity after the stability test in beakers is displayed in (Figure IV.3). No biogas bubbles formation in the medium was observed for the beaker with liquid. Meanwhile, in the beaker where there was only sludge, some biogas bubbles were observed, but in lower number compared to the beaker containing sludge and glucose. Only the DGT deployed in the beaker of sludge and glucose showed a bubble after the deployment of 4 h (Figure IV.3.C), indicating that some production of biogas bubbles is required for these to accumulate inside the DGT. This test showed that bubbles in the DGT came from the biogas generated by the substrate degradation and not from an issue with the device itself. Moreover, the bubbles in the DGT were found when a microbial activity and therefore biogas production was present in the system at a significant level.

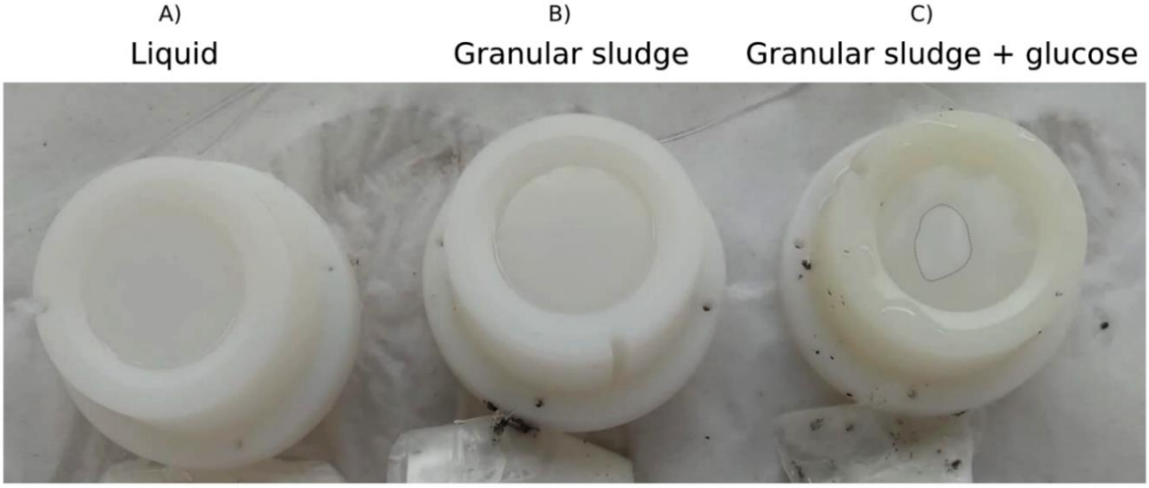


Figure IV.3: DGT after 4h of deployment in 3 conditions. The border of the bubble in granular sludge with glucose is highlighted with a black dotted line

IV.3.2. Co accumulation over time

Validation of the DGT theory in the UASB lab scale reactor was evaluated by analyzing the Co mass accumulation over time at the bottom of the reactor (it was expected to be the zone with more Co concentration in the UASB), at the end of the Co continuous dose of 10, 50, 100, 500 and 1000 µM. According to the limitations for physical stability of the device obtained in Section IV.3.1, the DGT were deployed in horizontal position without holes for times 4, 6, and 8 h.

The results of the mass accumulated in the binding gel of the DGT for all replicates are presented in Table IV.2. Despite having identified a valid interval of time for the application of DGT in the UASB (Section IV.3.1), some samples presented bubbles inside. The mass that was accumulated was still quantified to allow discussion of the buble formation impact (Table IV.2). The masses accumulated in altered DGTs differ from the valid replicas of their group. For doses ≤ 100 µM of Co, the accumulated mass was greater than in their replicates, between 1.5 and 14 times more than their replicates, whereas for the dose 1000 µM of Co, the mass accumulated in the altered DGT was less than the valid replicates, an average of 75%. This indicats that the presence of bubbles could not only lower the exposure area and thus the estimated labile concentration, but also can increase the mass that was accumulated in the binding gel. Such increase might be caused by direct penetration of solution inside the device. Additionally, 2 samples were lost by the ICP-MS due to technical problems, and 2 samples had no problems, but appear to be outliers when comparing their value with the rest of the replicates in their group (Table IV.2). The presence of bubbles in the DGT was a problem that implied that 18% of the samples had to be discarded, which together with the other 2 problems identified (possible outliers and sample losses in the ICP-MS) made 27% of the samples to be discarded. This led to undesirable situations, for example, for the accumulation of Co for the dose 10 µM of Co, only 4 points out of the 9 planned were available, which made the trend analysis less reliable.

Table IV.2: Accumulated mass (ng) in each DGT replicate of experiment Co accumulation over time

Deployment time (h)	**Replicate**	**Co dose (µM)**				
		10	**50**	**100**	**500**	**1000**
4 h	1	x	81	43	1002	46039
	2	14	29	97	4653	41218
	3	46	739	51	3294	43150
6 h	1	27	94	808	4358	46421
	2	68	198	192	6034	60940
	3	43	71	56	x	51896
8 h	1	10	100	151	6329	82069
	2	31	70	194	5819	56828
	3	29	73	300	5404	65252

code:

🟥 small bubble 🟧 big bubble 🟨 Possible outlier ☒ lost by ICP-MS

The valid results for the accumulation of Co over time are presented in Figure IV.4. The accumulated mass over time for the continuous doses of Co of 10 µM seemed to show a linear trend, however the number of points presented (4 measurements) was not sufficient to obtain a robust conclusion. Doses of 50 and 100 µM of Co did not seem to show a linear trend, and the continuous dose of 500 µM of Co would show a linear trend. In these 3 doses, the replicates were widely dispersed. The relative standard deviation (RSD) can be used to assess method precision, 10 % as sample variation threshold is suggested by Davison (2016). The RSD for the accumulated masses in the doses 10, 50 and 100 µM of Co were higher than 20 %, indicating a problem of repeatability for those measurements. The only dose at which a line trend could easily be observed is the continuous dose of 1000 µM of Co, with a RSD < 14 %. Measurement validity and repeatability problems induced that the correlation between accumulated mass and time could not be demonstrated at this stage.

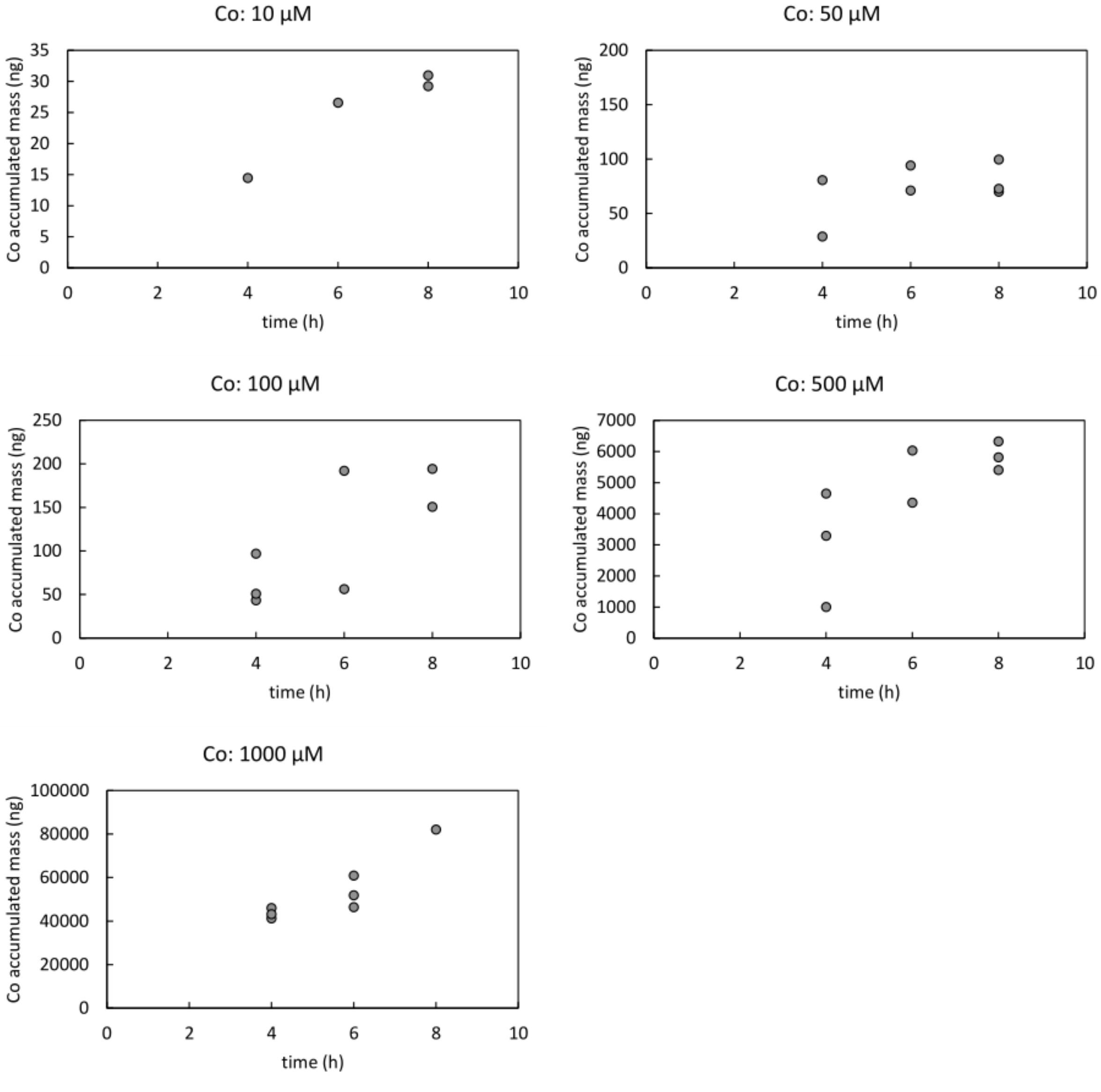


Figure IV.4: Valid results for accumulated mass of Co in DGT over time for different Co continuous doses in UASB

IV.4. Conclusions

DGT technique was deployed *in situ* in a UASB reactor stressed with 6 different Co continue doses, at 3 positions: liquid zone, top and bottom sludge. Several problems occurred during DGT deployment: the presence of bubbles between the filter membrane and the diffusive gel, rendering the device useless, and repeatability issues for valid results.

The bubbles came from biogas accumulation within the DGT and they appeared when biogas production reached a significant level. Different deployment configurations were evaluated to avoid the accumulation of the bubbles. DGTs in horizontal position (perpendicularly to flow direction and sampling window not facing it) for a maximum of 4 h for the liquid zone and 8 h in the sludge zone was selected as a valid configuration. However, bubbles were still observed in some DGTs deployed with this configuration in the subsequent experiment (accumulation of Co over time). Additionally, a time range with maximum 8 h could be considered short for a DGT deployment condition.

Accumulation of Co over time seemed to show the expected linear trend just for 1000 µM of Co continuous dose, indicating that DGT showed to be a promising technology for determine labile concentration in UASB, but its robustness over *in situ* conditions with significant biogas production still has to be demonstrated.

IV.5. Acknowledgments

This project has received funding from the European Union’s Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 861088. The authors thanks Victor Ramos for ICP-MS analysis.

IV.6. References

Bourven, I., Casellas, M., Buzier, R., Lesieur, J., Lenain, J.-F., Faix, A., Bressolier, P., Maftah, C., & Guibaud, G. (2017). Potential of DGT in a new fractionation approach for studying trace metal element impact on anaerobic digestion: The example of cadmium. *International Biodeterioration & Biodegradation*, *119*, 188–195. https://doi.org/10.1016/j.ibiod.2016.11.007

Davison, W. (Ed.). (2016). *Diffusive gradients in thin-films for environmental measurements*. Cambridge University Press.

Ding, Y., Yang, J., & Cai, J. (2018). Preparation of guanidinylated carboxymethyl chitosan and its application in the diffusive gradients in thin films (DGT) technique for measuring labile trace metals in water. *International Journal of Environmental Analytical Chemistry*, *98*(14), 1275–1291. https://doi.org/10.1080/03067319.2018.1545901

Fermoso, F. G., Van Hullebusch, E., Collins, G., Roussel, J., Mucha, A. P., & Esposito, G. (Eds.). (2019). Trace element enzymes in reactions essential for anaerobic digestion. In *Trace Elements in Anaerobic Biotechnologies* (pp. 51–72). IWA Publishing. https://doi.org/10.2166/9781789060225_0051

Ilic, A., Kouba, V., De Vrieze, J., Du Laing, G., & Bartacek, J. (2024). Diffusive gradients in thin films (DGT) as a robust and reliable technique to measure bioavailable metals in anaerobic digestates. *Environmental Technology & Innovation*, *33*, 103526. https://doi.org/10.1016/j.eti.2024.103526

Knutsson, J., Rauch, S., & Morrison, G. M. (2014). Estimation of measurement uncertainties for the DGT passive sampler used for determination of copper in water. *International Journal of Analytical Chemistry*, *2014*. https://doi.org/10.1155/2014/389125

Laera, A., Buzier, R., Guibaud, G., Esposito, G., & van Hullebusch, E. D. (2019a). Assessment of the DGT technique in digestate to fraction twelve trace elements. *Talanta*, *192*(August 2018), 204–211. https://doi.org/10.1016/j.talanta.2018.09.033

Laera, A., Buzier, R., Guibaud, G., Esposito, G., & van Hullebusch, E. D. (2019b). Distribution trend of trace elements in digestate exposed to air: Laboratory-scale investigations using DGT-based fractionation. *Journal of Environmental Management*, *238*(November 2018), 159–165. https://doi.org/10.1016/j.jenvman.2019.02.120

Takashima, M. (2018). Effects of Thermal Pretreatment and Trace Metal Supplementation on High-Rate Thermophilic Anaerobic Digestion of Municipal Sludge. *Journal of Environmental Engineering*, *144*(3), 04018009. https://doi.org/10.1061/(ASCE)EE.1943-7870.0001340

General conclusions and Recommendations for future work

The difficulty of a correct quantification of the bioavailable fraction in anaerobic digestion (AD) systems indicates the need to evaluate a more accurate technique. DGT technique is proposed as a promising solution, this technique quantifies the labile fraction, which can be related to bioavailable fraction. Although DGT has already been evaluated in anaerobic systems needs more results, as well as the evaluation of new parameters.

To assess the bioavailable fraction in anaerobic systems, DGT devices were evaluated in two different digestates (Chapter III) and an UASB reactor (Chapter IV). DGT theory was fulfilled for most trace elements detected at a significant level in the digestates. The potential saturation of Zr-DGT by P is a problem for detection of oxyanions species with Zr-DGT. However, for As, Thiol-DGT can be used for detecting As(III) instead of total As, which can be still relevant in anaerobic systems. Additionally, air introduction can provoke erroneous results.

To evaluate the effect of the continuous dose of a trace element in an AD reactor, a UASB reactor was dose with several concentrations of Co (Chapter II). The presence of Co does not presuppose problems in the stability of the operation, except for high doses where inhibition and changes in the integrity of granules are observed (due to the decrease in EPS). Most of the Co added accumulated within the granules, presumably in non-bioavailable inert forms. The inhibition was attributed to the Co in the soluble liquid fraction. For having a more precise result, as discussed in Chapter I, a fraction related to the bioavailable fraction was attempted to be quantified. DGT was evaluated in this reactor (Chapter IV), however it needs to be improved. The deployment of DGT inside the UASB reactor presented problems that invalidated the effectiveness of the DGT device in this system: device useless due to presence of biogas bubbles between the filter membrane and the diffusive gel of the DGT and low repeatability for valid devices. Therefore, the robustness of DGT applied in AD reactors is not fulfilled.

Recommendations

The results of the validation of the DGT theory in digestates contribute to state effectiveness of the DGT technique. However, this results could differ respect to other digestates. In order to state the robustness of this technique in anaerobic digestates in a general way, DGT devices should be deployed in more types of digestates. Analysis in anaerobic digestates from various sources of origin (for example, digestate obtained from lignocellulosic waste and granular digestate), with different composition in solids and organic matter content, would contribute to this objective. It must be considered the perform time-series deployments of DGT in digestates under anaerobic conditions and to consider only the trace elements with a linear accumulation trend.

The study of Co dose in a UASB could be expanded to include higher concentrations, reaching toxic levels and forcing the UASB failure. Similarly, this study was restricted to Co, but there are other trace elements that are necessary in AD, specifically in the methanogenesis, such as Ni, Fe and Zn, which could be studied in the same way.

The first step before validating the DGT technique *in situ* an AD reactor is to be able to perform a feasible deployment without any of these problems. It was possible to identify that a source of problems was the generation of biogas in the system, therefore the effect of the presence of gas in the medium on the DGT device should be evaluated. Parameters such as gas bubble production and bubble size could be starting parameters. In this way, some DGT configuration and AD reactor operation could be identified that allows a feasible DGT deployment. It can also allow long-time deployments because the 8 h obtained in this study may not be enough to justify the DGT theory with a time-series deployment. Increased exposure time allows a higher accumulation of the element of interest within DGT, which increases sensitivity since the element can be validly detected with analytical techniques. On the other hand, the problem of low repeatability must be solved as well. The evaluation of DGT device in different types of AD reactors, where the biomass is not in granular form, could indicate whether this problem is intrinsic to the UASB reactor.

List of Scientific Publications

Publications

- Caroca Sepulveda, E., Buzier, R., Simon, S., Fermoso, F., Zahedi, S., & Guibaud, G. (2024). Trace elements fractionation in anaerobic digestates: Recommendation for using diffusive gradients in thin films passive samplers. Journal of Environmental Management, 360, 121223. https://doi.org/10.1016/j.jenvman.2024.121223
- Effect of Cobalt continuous dose in UASB, focus on metal profile and Extracellular Polymeric Substances – in preparation.

Oral Presentations

- E. Caroca, R. Buzier, S. Simon, F. Fermoso, G. Guibaud. Uso de DGT (diffusive gradient in thin films) para la determinación de fracción lábil de metales en digestatos para posterior uso como fertilizante. IX Congreso Internacional de Ecología. Seville, Spain, 2023.
- E. Caroca, R. Buzier, S. Simon, F. Fermoso, S. Zahedi, G. Guibaud. Applicability of DGT (Diffusive gradient in thin film) in anaerobic digestates. IMAB23-International Congress on Metal-microbe applications for circular economy. Porto, Portugal, 2023.

Poster Presentations

- Eric Caroca, Rémy Buzier, Stephane Simon, Fernando Fermoso, Gilles Guibaud. Applicability of DGT (Diffusive gradient in thin films) in anaerobic digestate. EMBO, SIAM, Practical Course Breathless microbes: Life with no oxygen. Wageningen, The Netherlands, 2022.
- Eric Caroca, Rémy Buzier, Stephane Simon, Gilles Guibaud, Soraya Zahedi, Fernando Fermoso. Interrelation of EPS and metal content in a UASB under Cobalt continue dose. 18th IWA World Conference on Anaerobic Digestion (IWA AD18). Istanbul, Türkiye, 2024.

Caractérisation de la fraction métal labile dans des matrices complexes contenant des microbes

Les éléments traces (TE) sont nécessaires à la digestion anaérobie car plusieurs d'entre eux jouent un rôle essentiel dans les voies métaboliques des micro-organismes. Cependant, leur concentration peut également avoir des effets inhibiteurs ou toxiques. Le digestat, un sous-produit de la digestion anaérobie, peut être utilisé comme engrais, mais la présence d'TE pourrait en limiter l'utilisation en raison d'une contamination potentielle du sol et des cultures. Ces TE peuvent être présents sous différentes formes chimiques et seule la fraction biodisponible est responsable de la réponse biologique. La technique de gradients de diffusion en couches minces (DGT) est une méthode d'échantillonnage passif qui permet de quantifier la fraction labile, pouvant être reliée à la fraction biodisponible. Son application aux digestats anaérobies pourrait s'avérer pertinente mais la méthodologie doit être soigneusement adaptée.

Les principaux objectifs de cette recherche étaient d'analyser l'impact de la présence d'un TE sur le fonctionnement d'un réacteur anaérobie et d'évaluer la faisabilité de l'utilisation de la technique DGT dans le contexte de la digestion anaérobie : dans les digestats et directement à l'intérieur d'un bioréacteur.

L'effet de l'ajout continu de différentes concentrations de cobalt (Co) dans un réacteur à lit de boues anaérobies ascendant (UASB) a été étudié. Les performances de l'UASB ont été suivies à trois positions différentes le long du réacteur : boues de fond, boue de surface et zone liquide. Les concentrations en Co des fractions solides (granules), liquide soluble et substances polymériques extracellulaires (EPS) ont été mesurées. La production de biogaz a été affectée positivement pour des doses de Co < à 50 µM et négativement pour des doses > à 100 µM. Une diminution générale des EPS a été observée en raison de la dose de Co, ce qui a compromis la stabilité des granules. Aucune différence significative n'a été observée dans le profil de Co en fonction de la profondeur dans le réacteur pour les fractions solide et liquide soluble. La concentration en Co particulaire était environ 1000 fois plus élevée que les concentrations solubles, indiquant une forte accumulation de Co dans les granules. Cependant, le Co accumulé s'est avéré être sous des formes inertes.

Des dispositifs DGT de type Chelex, Zirconium et Thiol ont été déployés dans deux digestats différents pour évaluer l'applicabilité de la technique. Des déploiements de durées variables ont été réalisés en présence et en absence d'air afin de déterminer si le maintien de conditions anaérobies était nécessaire. Le profil d'accumulation linéaire théoriquement attendu n'a été observé que pour certains TE (Fe, Mn, Ni ou As(III)), selon le digestat, tandis que d'autres éléments ont été détectés sans tendance cohérente (Al, As et Cu). La fraction labile de Co, Cu, Fe et Mn a montré des variations sur une période de 72 h lorsque les déploiements ont eu lieu en présence d'air.

Des dispositifs DGT ont été déployés à l'intérieur de l'UASB supplémenté en Co pour évaluer la faisabilité de l'utilisation des DGT directement à l'intérieur d'un réacteur. Problèmes de reproductibilité et une accumulation de biogaz à l'intérieur des DGT ont été observée, ce qui a invalidé leur utilisation. Différentes configurations de déploiement ont été évaluées pour limiter ce phénomène. Un déploiement considéré valide a été obtenu pour un flux perpendiculaire pendant une durée maximale de 4 h dans la zone liquide et de 8 h dans les boues.

La DGT semble donc applicable pour évaluer la fraction labile de certains TE dans les digestats, mais nécessite une vérification minutieuse. Il est recommandé d'effectuer des déploiements de durées variables pour identifier uniquement les TE présentant une accumulation linéaire. Les conditions anaérobies doivent être maintenues pendant le déploiement. Le déploiement direct des DGT dans un UASB a induit des problèmes qui doivent encore être résolus.

Mots-clés : Métaux, échantillonnage passif, fractionnement, biodisponibilité, digestion anaérobie, réacteur ascendant à lit de boue anaérobie, substances polymériques extracellulaires

Characterization of the labile metal fraction in complex matrices containing microbes

Trace elements (TE) are necessary for anaerobic digestion (AD) since many are essential in the metabolic pathways of microorganisms. However, depending on their concentration, they can also have inhibitory or toxic effects. The digestate, a by-product of AD, can be used as fertilizer but the presence of TE could impede its use due to potential contamination in soil and crops. TE can be present under different species amongst which only the bioavailable ones are responsible for the biological response. Diffusive gradients in thin films (DGT) is a passive sampling technique that quantifies the labile fraction, which can be related to the bioavailable fraction. DGT has been studied in waterbodies, sediments, and solids systems, but its application to anaerobic digestates is not straightforward and the methodology must be carefully adapted. The main objectives of this research were to analyze the impact of the presence of a trace element, Cobalt (Co), in the operation of an anerobic reactor and to evaluate the feasibility of using the DGT technique in anaerobic systems: in digestates and also directly inside a bioreactor.

Firstly, the effect of continue addition of different concentrations of Co in an upflow anaerobic sludge bed reactor (UASB) was studied. UASB performance was monitored at three different positions along the reactor: bottom and top sludge, and liquid zone. Co concentration for solid (granules), liquid soluble, and extracellular polymeric substances (EPS) were measured in these samples. Biogas production was affected positively for Co dose lower and equal to 50 µM of Co and negatively for Co dose higher than 100 µM of Co. A general decrease in EPS was observed due to Co dose, which compromised the granular stability. No strong differences were observed in Co profile along the depth in the reactor for solid and liquid soluble fractions. The solid Co concentration was around 1000 times higher than the liquid concentrations, indicating a high accumulation of Co in the granules. However, the Co accumulated was found as inert species.

In a second stage, Chelex, Zirconia, and Thiol DGT devices were deployed in two different digestates to evaluate the applicability of the passive sampling technique. Time-series deployments were performed in presence and absence of air to determine whether maintaining anaerobic conditions is mandatory. A theoretically expected linear accumulation profile was observed for some TE only (Fe, Mn, Ni or As(III)), depending on the digestate, whereas other elements were detected without a consistent trend over time (Al, As, and Cu). The labile fraction of Co, Cu, Fe and Mn demonstrated changes over a 72 h period when deployments occurred in the presence of air.

In the third stage, DGT devices were deployed inside the UASB under Co stress to assess the feasibility of using DGT directly inside a reactor. Biogas accumulation was observed inside the DGT, which invalidated the device. Different deployment configurations were evaluated to limit the phenomenon. The valid deployment was restricted to a perpendicular flow for a maximum of 4 h in the liquid zone, and 8 h in the sludge zone. Additionally, repeatability problems were observed, which indicates the presence of unidentified problems still to be solved.

DGT thus appears applicable to evaluate the labile fraction of some TE in digestates but requires careful verification. Consequently, it is recommended to conduct time-series deployments to identify only the elements displaying a linear accumulation trend. Anaerobic conditions should be maintained during deployment to avoid significant shifts in the labile fraction over time. The direct deployment of DGT in an UASB induced some problems and limitations still to be solved.

Keywords: Metals, passive sampling, fractionation, bioavailability, anaerobic digestion, Upflow Anaerobic Sludge Bed reactor, Extracellular polymeric substances