Silesian University of Technology
Faculty of Energy and Environmental Engineering
Department of Environmental Biotechnology

The influence of chosen antibiotics on the anammox process

DOCTORAL THESIS

mgr inż. Filip Gamoń

Supervisor: dr hab. Aleksandra Ziembińska-Buczyńska, prof. PŚ
Assistant supervisor: dr inż. Grzegorz Cema

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„Powiedz mi, to zapomnę. Naucz mnie, to może zapamiętam. Zaangażuj mnie, to się nauczę.”
– Benjamin Franklin

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Contents

ACRONYMS

AMX – amoxicillin

anammox – anaerobic ammonia oxidation

AnAOB – anaerobic ammonia oxidizing bacteria

ARB – antibiotic resistant bacteria

ARGs – antibiotic resistance genes

AOB – ammonia oxidizing bacteria

CAP – chloramphenicol

CESs - contaminants of emerging concern

CIP – ciprofloxacin

CLA – clarithromycin

CMR – Continuous membrane reactor

Comammox – complete ammonia oxidation

DDD – defined daily doses

DHA – dehydrogenase activity

DOX – doxycycline

ECDC – European Centre for Disease Prevention and Control

ENR – enrofloxacin

DO – dissolved oxygen

EU – enzyme unit

EPSs – extracellular polymeric substances

ERY – erythromycin

FBR – fluidized bed reactor

FF – florfenicol

HAO – hydroxylamine oxidoreductase

HDH – hydrazine dehydrogenase

HET – heterotrophic bacteria

HZO – hydrazine oxidoreductase

HZS – hydrazine synthase

IC – inhibitory concentration

MGE – mobile genetic element

MRGs – metal resistance genes

NGS – next generation sequencing

N^4-AcSMX – N^4-acetyl sulfamethoxazole

NIR – nitrite reductase

NLR – nitrogen loading rate

NOB – nitrite oxidizing bacteria

NOR – norfloxacin

NRE – nitrogen removal efficiency

NRR – nitrogen removal rate

OTC – oxytetracycline

qPCR – quantitative polymerase chain reaction

QS – quorum sensing

PCA – principal component analysis

PN – protein

PS – polysaccharides

SAA – specific anammox activity

SBR – sequential batch reactor

SDM – sulfadimethoxine

SM – sulfamethazine

SMX – sulfamethoxazole

SS – suspended solid

TC – tetracycline

TCH – tetracycline hydrochloride

TEM – transmission electron microscopy

TIA – tiamulin

TNRR – total nitrogen removal rate

TRM – trimethoprim

UAF – upflow anaerobic biological filter

VSS – volatile suspended solid

WWTPs – wastewater treatment plants

FOREWORD

The basis of the presented dissertation is a series of three publications. The text of this dissertation uses references to articles using the numbering method presented:

PUBLICATION 1: Gamoń F., Cema G., Ziembińska-Buczyńska A., The influence of antibiotics on the anammox process - a review. Environ Sci Pollut Res 29, 8074–8090 (2022) DOI: https://doi.org/10.1007/s11356-021-17733-7 IF = 5.190, MEiN = 100 points.

PUBLICATION 2: Gamoń F., Banach-Wiśniewska A., Jaspreet Jandoo Kaur, Cema G., Ziembińska-Buczyńska A., Microbial response of the anammox process to trace antibiotic concentration. Journal of Water Process Engineering, Volume 46, 102607 (2022), DOI: https://doi.org/10.1016/j.jwpe.2022.102607 IF = 7.340, MEiN = 100 points.

PUBLICATION 3: Gamoń F., Banach-Wiśniewska A., Poprawa I, Cema G., Ziembińska-Buczyńska A. Insight into the microbial and genetic response of anammox biomass to broad range concentrations of different antibiotics: Linking performance and mechanism. Chemical Engineering Journal, 451(1), 138546 (2023). DOI https://doi.org/10.1016/j.cej.2022.138546 IF = 16.744, MEiN = 200 points.

Streszczenie

Szerokie i niekontrolowane stosowanie antybiotyków w medycynie, weterynarii i hodowli zwierząt ma bezpośrednie odzwierciedlenie w ich występowaniu w środowisku, zwłaszcza wodnym. Szacuje się, że rocznie do środowiska trafia kilka tysięcy ton antybiotyków oraz produktów ich przemiany metabolicznej. Za główne źródło antybiotyków w wodach powierzchniowych i podziemnych uznaje się oczyszczalnie ścieków (ang. *wastewater treatment plants*, WWTPs). Obecność antybiotyków w ściekach wiąże się z rozwojem bakterii opornych na antybiotyki (ang. *antibiotic resistant bacteria*, ARB) i występowaniem genów oporności na antybiotyki (ang. *antibiotic resistance gens*, ARGs), które stanowią szczególne zagrożenie dla zdrowia ludzi i zwierząt na całym świecie. Ponadto antybiotyki mają negatywny wpływ na proces biologicznego oczyszczania ścieków, takich jak na przykład nitryfikacja i denitryfikacja, powodując obniżenie aktywności metabolicznej mikroorganizmów prowadzących te procesy, czego efektem może być obniżenie efektywności oczyszczania ścieków. Jednym z takich procesów, który jest szczególnie wrażliwy na działanie antybiotyków jest anammox (ang. *anaerobic ammonia oxidation*). Anammox jest znany jako innowacyjna i bardziej ekonomiczna alternatywa dla konwencjonalnych metod biologicznego usuwania azotu ze ścieków (nitryfikacji i denitryfikacji). Jednakże bakterie prowadzące proces anammox są podatne na czynniki toksyczne występujące w ściekach (w tym antybiotyki), co może utrudniać wdrożenie procesu w skali technicznej.

Prezentowana praca doktorska obejmuje trzy publikacje opisujące interakcję pomiędzy trzema popularnymi antybiotykami (oksytetracykliną (OTC), cyprofloksacyną (CIP) i klarytromycyną (CLA)) a osadem czynnym anammox. Badania zostały poprzedzone szczegółową analizą literatury (**Publikacja 1**), która ujawniła kierunki badawcze, w których obecnie dostępna wiedza z zakresu działania antybiotyków na proces anammox była ograniczona. **Publikacja 1** ukazała w szczególności brak wystarczającej liczby badań na niskich stężeniach antybiotyków (występujących w ściekach) oraz brak badań nad antybiotykami, które powszechnie i w dużych ilościach występują w ściekach, jak cyprofloksacyna i klarytromycyna. Badania opisane w niniejszej pracy były prowadzone w sekwencyjnym reaktorze porcjowym (ang. *sequencing batch reactor*, SBR). W ramach pracy określono: (I) działanie śladowego stężenia antybiotyków (0,001 mg L^{-1}) na wydajność procesu anammox, (II) efekt wzrastających stężeń antybiotyków (0,001-100 mg L^{-1}) na proces

anammox, (III) zmian w strukturze zbiorowiska bakteryjnego anammox wywołanych działaniem antybiotyków, (IV) liczebność genów funkcyjnych (adaptatywnych) przemian azotowych w osadzie czynnym anammox, (V) efekt działania antybiotyków na właściwości mikroorganizmów i strukturę komórek bakterii anammox, (VI) mechanizmy obronne bakterii anammox przed działaniem antybiotyków w oparciu o produkcję zewnątrzkomórkowych substancji polimerowych (ang. *extracellular polymeric substances*, EPSs) i wymianę genów oporności na antybiotyki (ARGs).

Wyniki przedstawione w **Publikacji 2** wskazują, że krótkoterminowy wpływ antybiotyków w stężeniu 0,001 mg L^{-1} spowodował wzrost efektywności procesu anammox o około 7,1% podczas działania OTC, natomiast zarówno CIP, jak i CLA spowodowały spadek aktywności odpowiednio o 8,4% i 3,2%. Test długoterminowy nie wykazał istotnych zmian w efektywności procesu anammox, co może wynikać z ochronnego działania polimerów zewnątrzkomórkowych przed tak niskimi stężeniami antybiotyków. Niemniej jednak każdy z antybiotyków powodował zmiany w strukturze zbiorowiska bakteryjnego. Zaobserwowano spadek liczebności Candidatus *Brocadia*, natomiast liczebność bakterii *Nitrospira* (nitryfikator II fazy) wzrosła o 1,68% (bioreaktor z dodatkiem OTC), 4,43% (bioreaktor z dodatkiem CIP), 3,08% (bioreaktor z dodatkiem CLA). Obecność bakterii *Nitrospira* może być związana z jej zdolnością do przeprowadzania całkowitej nitryfikacji (comammox, ang. *complete ammonia oxidation*) w warunkach ograniczonego dostępu do tlenu. Zmiany w strukturze społeczności bakteryjnej odpowiadały zmianom w obfitości genów przemian azotowych, gdzie obfitość genu *hzo* zmniejszyła się, natomiast geny odpowiedzialne za nitryfikację (*amoA* i *nxrA*) miały w całym okresie trwania eksperymentu wyższą liczebność niż gen *hzo*. Śladowe stężenie antybiotyków spowodowało również rozwój antybiotykooporności. Spośród 6 wykrytych genów determinujących oporność na badane antybiotyki (*tetX*, *tetC*, *tetW* - ARGs-OTC; *mphA* - ARGs-CLA; *qnrB4*, *qnrS* - ARGs-CIP) liczebność aż 4 wzrosła (*tetW, tetC, qnrB4, qnrS*). W **Publikacji 3** odnotowano, że przy długim czasie ekspozycji antybiotyków na proces anammox jedynie stężenia antybiotyków powyżej 1 mg L^{-1} mają istotny wpływ na efektywność usuwania azotu ze ścieków. Dla badanych antybiotyków efektywność usuwania azotu zmniejszyła się o 27% (OTC), 30% (CIP) i 56% (CLA) na koniec eksperymentu, co wykazało, że CLA ma najbardziej niekorzystny wpływ na proces anammox. Analiza struktury społeczności bakterii anammox i genów przemian związków azotowych wykazała rozwój bakterii należących do typu *Planctomycetes* (w tym bakterii anammox) pod wpływem każdego z antybiotyków, podczas gdy większość tych bakterii nie była zdolna do prowadzenia procesu anammox. Podobnie jak

w badaniach przedstawionych w **Publikacji 2**, liczebność bakterii z rodzaju *Nitrospira* wzrastała wraz ze wzrostem stężenia antybiotyków. W czasie trwania eksperymentu zaobserwowano rozwój oporności na antybiotyki, gdzie liczebność poszczególnych ARGs determinujących oporność na badane antybiotyki była wyższa od poziomu na początku eksperymentu. Dodatkowo, analiza zależności pomiędzy dominującymi gatunkami a ARGs i genami funkcyjnymi wskazała znaczącą rolę Candidatus *Jettenia* w transferze oporności na CLA.

Podsumowując, w prezentowanej rozprawie udowodniono, że antybiotyki w stężeniach takich, jakie występują w ściekach (0.001 mg L^{-1}), nie wpływają na efektywność procesu anammox. Powodują natomiast zmiany w strukturze zbiorowiska bakterii osadu czynnego oraz powodują rozwój antybiotykooporności na badane antybiotyki. Co więcej, dopiero wyższe stężenia antybiotyków (powyżej kilku mg L^{-1}) powodują znaczne obniżenie efektywności procesu anammox oraz powodują zmiany w strukturze komórek bakterii anammox. Dodatkowo, wykazano, że bakterie żyjące w zbiorowisku osadu czynnego anammox są w stanie ochronić się przed działaniem antybiotyków poprzez produkcję EPSs i transfer ARGs. Udowodniono, że Candidtus *Jettenia* uczestniczy w transferze ARGs determinujących oporność CLA.

Abstract

Extensive and uncontrolled use of antibiotics in medicine, veterinary, and animal breeding are directly reflected in their occurrence in the environment (especially in the aquatic environment). A few thousand tons of antimicrobials and their transformation products are estimated to be annually worldwide introduced to the environment. Wastewater treatment plants (WWTPs) have been found as the main source of antibiotics in natural waters (surface water, groundwater, etc.). The occurrence of antibiotics in WWTPs is related to the development of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs), which pose a worldwide risk to human and animal health. In addition, antibiotics have a negative impact on the process of biological wastewater treatment, including nitrification, and denitrification, for example, causing a decrease in metabolic activity resulting in a reduction in the efficiency of wastewater treatment. One such process that is particularly vulnerable to antibiotics is anammox (anaerobic ammonia oxidation). Anammox is known as an innovative and more sustainable biological rich-nitrogen removal alternative to conventional nitrification-denitrification technology. However, the bacteria that carry out the anammox process are susceptible to toxic substances found in wastewater (including antibiotics), which can make it difficult to implement the process on a technical scale.

The presented doctoral dissertation includes three publications describing the interaction between three popular antibiotics (oxytetracycline (OTC), ciprofloxacin (CIP), and clarithromycin (CLA)) and anammox process and microorganisms which perform it. The research was preceded by a detailed literature analysis (**Publication 1**), which revealed research directions in which currently available knowledge was limited. In particular, **Publication 1** showed a lack of sufficient research on low concentrations of antibiotics (that can be found in wastewater) and a lack of research on antibiotics that are commonly found in wastewater in large quantities, such as ciprofloxacin and clarithromycin. The anammox process was carried out in a sequencing batch reactor (SBR). Experiments evaluated: (I) the effect of trace concentration (0.001 mg L^{-1}) of antibiotics on the anammox process performance, (II) the influence of successive concentration (0.001-100 mg L^{-1}) of antibiotics on the effectiveness of anammox process performance, (III) antibiotics influence on community structure of anammox bacteria in activated sludge, (IV) effect of antibiotics on the abundance of the nitrogen-cycle functional genes in the anammox community, (V) antibiotics impact on the microbial properties and anammox cell structures, (VI) investigating the

protective mechanisms of anammox bacteria against antibiotics based on extracellular polymer substances (EPSs) production and exchange of antibiotic resistance genes (ARGs).

The results presented in **Publication 2** show that the short-term effect of antibiotics at 0.001 mg L^{-1} on the anammox process caused an increase in anammox activity by about 7.1% under OTC pressure while both CIP and CLA caused a decrease in anammox activity by about 8.4% and 3.2%, respectively. The long-term test showed no significant change in the efficiency of the anammox process, which may be due to the protection of anammox bacteria from such low concentrations of antibiotics mainly by the production of EPSs. Nevertheless, each antibiotic caused changes in community structure decreasing the abundance of Candidatus *Brocadia*, while the abundance of *Nitrospira* increased by 1.68% (bioreactor with OTC addition), 4.43% (bioreactor with CIP addition), 3.08% (bioreactor with CLA addition). The presence of *Nitrospira* bacteria may be related to its ability to carry out complete nitrification (comammox) under oxygen-limited conditions. The changes in the community structure corresponded with changes in the abundance of functional genes, where the abundance of the *hzo* gene decreased, while the gene responsible for nitrification (*amoA* and *nxrA*) had a much higher abundance than the *hzo* gene during the whole experiment. Trace concentrations of antibiotics have also caused a development in antibiotic resistance. Among 6 detected genes that determined resistance to tested antibiotics (*tetX*, *tetC*, *tetW* – ARGs-OTC; *mphA* – ARGs-CLA; *qnrB4*, *qnrS* – ARGs-CIP) up to 4 obtained an increase in abundance (*tetW*, *tetC*, *qnrB4*, *qnrS*). **Publication 3** noted that long-term exposure to the antibiotic at concentrations above 1 mg L^{-1} has a significant effect on reducing the efficiency of the anammox process. Correspondingly, the nitrogen removal rate (NRR) decreased by 27% (OTC), 30% (CIP), and 56% (CLA), suspecting that CLA has the most adverse effect on the anammox process. Co-occurrence analysis of community structure and anammox functional genes revealed the development of *Planctomycetes* under each antibiotic's suppression, while probably most of these bacteria were unable to perform of anammox process. Similar to the results presented in publication 2, the abundance of *Nitrospira* increased as the concentration of antibiotics increased. During the experimental period, it was observed the development of antibiotic resistance was almost in all found ARGs determining tested antibiotics. The abundance of each ARGs was higher than its initial level. Additionally, co-occurrence analysis between dominant species, ARGs, and functional genes indicated a significant role for Candidatus *Jettenia* in the transfer of CLA resistance.

In conclusion, the presented dissertation proved that antibiotics in concentrations such as those found in wastewater do not affect the effectiveness of the anammox process. However,

they cause changes in the bacterial community structure of the activated sludge and cause the exchange of resistance genes determining the resistance towards the antibiotics being tested. Moreover, only higher concentrations of antibiotics (above few mg L^{-1}) cause significant decreases in the effectiveness of the anammox process and caused changes in anammox cell structures. Additionally, as tested, bacteria living in the anammox biomass community are able to counteract antibiotics suppression by the production of EPSs and transfer of ARGs. It was proven that Candidtus *Jettenia* participated in the transfer of ARGs determining CLA resistance.

1. Introduction

Several organic substances such as pesticides, herbicides, pharmaceuticals, personal care products, hormones, and their metabolites are uncontrolled released into the environment with WWTPs effluents because conventional biological treatment is not designed to remove these kinds of pollutants from wastewater (Krzeminski et al., 2019; Kowalska et al., 2020). Due to their unknown environmental effect, they are considered as contaminants of emerging concern (CECs). Among all CECs high attention has been paid to antibiotics, because there are extensively used in human, and animal treatment, and aquaculture as antimicrobial therapeutics (Kümmerer, 2009). The worldwide consumption of antibiotics for medical purposes has been estimated at 70 billion standard units/year, while antibiotics up-take in livestock at 63,515 tons per year (Van Boeckel et al., 2014; Van Boeckel et al., 2015). Only between 2000 - 2010, worldwide antibiotics consumption has been found to increase by 36% (Van Boeckel et al., 2014). Moreover, in 2012 the total consumption of antibiotics in the European Union only in outpatient care reached 3,400 tons and the uptake of the antimicrobial drug for this purpose is still increasing. According to the data from European Centre for Disease Prevention and Control (ECDC), in 2018 the average total consumption of antimicrobial pharmaceuticals for systemic use (including community and hospital sector) was estimated at 23.4 defined daily doses (DDD) per 1000 inhabitants per day (ECDC, 2018). Unpredictably, the global pandemic of SARS-CoV-2 contributed to an increase in antibiotics consumption (Rawson et al., 2020; Langbehn et al., 2021). Although COVID-19 is caused by a virus infection, antibiotics are very often prescribed to treat the co-infection of bacteria (Rawson et al., 2020). Nevertheless, antibiotics are often given to patients without confirmation of bacterial infection and possibly used as self-medication (Usman et al., 2020).

1.1. Antibiotics as environmental hazards

Most antibiotics are not completely metabolized by humans and animals. It is considered that about 25% - 75% of antibiotics are excreted in feces and urine (Karthikeyan et al., 2006). Then antibiotics are released into the sewer and reach WWTPs (Michael et al., 2013; Rizzo et al., 2013). Unfortunately, similar to the other CECs, antibiotics are recalcitrant to biological treatment, thus they are poorly removed from wastewater and are therefore released into the environment with WWTPs effluent. These results in their presence in aquatic environments, including streams, rivers, lakes, and the marine environment (Moldovan, 2006; Lapworth et al., 2012), and their subsequent entry into soil and groundwater. Although antibiotics are

usually detected at low concentrations (from a few ng/L to a few µg/L) in the environment they can cause sub-chronic or chronic toxicity. Potential collateral effects on aquatic ecosystems and human health through the food chain can take place when treated wastewater is reused for crop irrigation (Rand-Weaver et al., 2013; Malchi et al., 2014). Antibiotics can accumulate in plants, which are then eaten by humans and animals. Despite the risk related to the occurrence of antibiotics in the environment, there is still a lack of discharge guidelines or standards for antibiotics monitoring in wastewater and the environment as well. However, it is worth noting that the European Commission, as the first international organization, established the first and second substance watch lists (both of which include antibiotics) through EU Decision, 2015/495, and then EU Decision 2018/480 respectively, for the EU-wide water policy framework.

1.2. Risks of occurrence of antibiotics in the environment

The presence of antibiotics in the water bodies causes not only a significant risk for aquatic organisms, but the occurrence of antimicrobial drugs in the environment poses also a problem to human health. One of the major problems related to the presence of antibiotics in the environment is the development and spread of ARGs and the increasing occurrence of antibiotic-resistant bacteria (ARB), which cause risks to public and animal health (Giannakis et al., 2018a). Worldwide about 700,000 deaths are annually caused by broadly understood antimicrobial resistance (AMR), including antibiotic resistance (AR). If no proper actions are taken, the number can reach up to 10 million per year in 2050 (O'Neill, 2016). The spread of AMR does not have only an environmental aspect - many different factors affect the scale of this phenomenon. However, guided by the principles described in the "One Health European Joint Programme", environmental conditions should also be taken under consideration as they play a significant role in better understanding and preventing antibiotic resistance (Destoumieux-Garzón et al., 2018; Margalida et al., 2014; Felis et al., 2020).

AR is developed in two ways: mutations towards the target antibiotics and acquiring resistance genes (Munita & Arias, 2016). However, the main problem is the acquisition of antibiotic resistance through gene transfer. ARGs transfer can take place through vertical (by cell division) and horizontal gene transfer, including transformation (DNA uptake from death cells), transduction (bacterial DNA is moved from one bacterium to another by a phage), and conjugation (gene exchange between donor bacteria, and acceptor bacteria). ARGs are mainly located on mobile genetic elements (MGEs), such as plasmids, transposons, integrons, and

outer membrane vesicles (OMVs) enabling the transfer of ARGs between bacteria (Dell'Annunziata et al., 2021). Moreover, the biological methods used in the WWTPs are designed to promote the exponential growth of bacteria and as a result, the biological chamber is a potentially suitable place for ARGs transfer (Fiorentino et al., 2019).

1.3. Occurrence of antibiotics in wastewater

One of the main reasons for antibiotic occurrence in wastewater is indiscriminate consumption (Michael et al., 2013 Wang et al., 2020). The main route of entry into wastewater is through excretion in urine and feces, where antibiotics may be presented in active as well as metabolized forms (Jjemba, 2006). Routes for antibiotics to enter the wastewater treatment plant are shown in **Figure 1**.

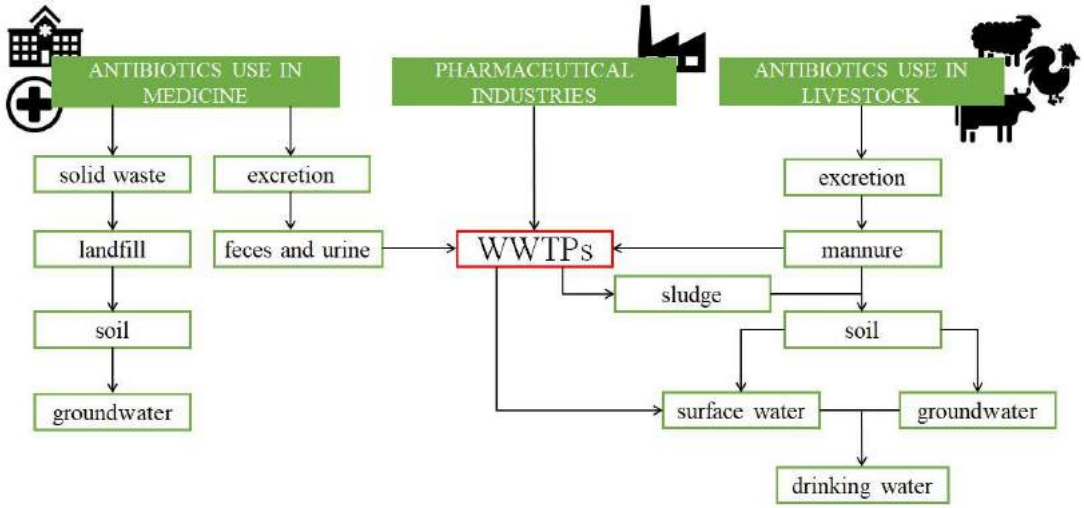

Figure 1. Routes for antibiotics to enter the wastewater treatment plant are shown (according to Langbehn et al., 2021).

Antibiotics have been detected in almost every kind of wastewater including hospital, domestic, industrial, agricultural as well as landfill leachate (Langbehn et al., 2021) The occurrence and concentration of antibiotics in wastewater depend on many factors such as demographic, and seasonal changes, geographical region, and economic data. Usually, antibiotics are found in wastewater at a concentration between ng L^{-1} up to several µg L^{-1} (Felis et al., 2020). The literature data show that ciprofloxacin (CIP), sulfamethoxazole (SMX), and trimethoprim (TRM) were the most detectable antibiotics in hospital and urban wastewater. Their concentration in raw wastewater reached a relatively high value in some studies: SMX in urban wastewater was detected at the level of 54.83 µg L^{-1} (K'oreje et al.,

2016); the concentration of CIP in hospital wastewater was found at 40.90 μg L^{-1} (Thai et al., 2018); the concentration of TRM in urban wastewater was reached 72.85 μg L^{-1}, while in hospital wastewater 30.38 μg L^{-1} (K'oreje et al., 2016; Szekeres et al., 2017). Another group of antibiotics frequently detected in surface water is macrolides. Their occurrence frequency, from 24 European countries, was equal to 58.8% (from 2792 samples) (Loos et al., 2018). Moreover, clarithromycin, belonging to the macrolide class was found at the level 1433 μg L^{-1} in the influent of municipal WWTPs (Senta et al., 2008). Wastewater from animal breeding is remarked to contain high antibiotics concentration. High concentrations of SMX and CIP were found in such kind of sewage (Leon-Aguirre et al., 2019; Zhu et al., 2020). Studies conducted on two Chinese swine wastewater treatment plants detect 22 antibiotics with a total concentration of 3.78 mg L^{-1}. Moreover, about 15 of these antibiotics are also used in medicine and are detectable in hospital and urban wastewater (Zheng et al., 2018). Generally, antibiotics are poorly degraded in the biological wastewater treatment process. Information about the degradation of SMX is different and varies between 20 – 90%. Such differences may be caused by the presence of its human metabolites N^4-acetyl sulfamethoxazole (N^4-AcSMX) which under environmental conditions can be transformed to SMX, but it is possible to transfer it once again to N^4-AcSMX by bacterial community (Göbel et al., 2007, Felis et al., 2020). Tetracyclines are considered to be antibiotics that remove well in the wastewater treatment process (up to 90%), however, the observed removal efficiency is a result of their sorption onto activated sludge but not biodegradation (Michael et al., 2013). A similar situation is observed for macrolide antibiotics, which despite the high reduction of concentration during the treatment process, they have mainly been removed from wastewater along with activated sludge, not because of degradation (Felis et al., 2020). The stability of antibiotics in the wastewater treatment plant is highly dependent on sorption capacity which involves various physicochemical properties, such as hydrophobicity, partition coefficient, and dissociation constant. Therefore, antibiotics that have a high adsorption capacity are more often presented in wastewater in relatively high concentrations (Tran et al., 2016).

1.4. Anammox process – from bacteria to technology

Anaerobic ammonia oxidation (anammox) is an anoxic and autotrophic biological process conducted by bacteria affiliated with the *Planctomycetes* phylum. Bacteria capable of carrying out the anammox process include several genera: Candidatus *Kuenenia*, Candidatus *Jettenia*, Candidatus *Brocadia*, Candidatus *Scalindua*, Candidatus *Anammoxoglobus*, and Candidatus *Anammoximicrobium*. The development of technology, mainly the molecular tool has brought

information about the discovery of a new genus of anammox bacteria tentatively named Candidatus *Loosdrechtia aerotolerans* (Yang et al., 2022). Due to the very thin murein layer, anammox bacteria were recently classified as gram-negative bacteria (Van Teeseling et al., 2015). Unlike most prokaryotes, anammox bacteria have complex cell structures (van Niftrik & Jetten, 2012). Generally, their cell structures are composed of three specific membrane systems. The outer membrane along with the murein layer builds the cell wall. The second membrane coats the cytoplasm. A major part of the anammox cell is the organelle called anammoxosome which is surrounded by the last membrane layer. This structure is a center of the metabolism of anammox bacteria (Neumann et al., 2014; Ozumchelouei et al., 2022). The anammox process allows the oxidation of ammonium nitrogen to gaseous nitrogen (N_2), using nitrite as an electron acceptor (Strous et al., 1999). Briefly, the possible anammox process mechanism is carried out as follows: ammonium and hydroxylamine are converted to hydrazine using hydrazine hydrolase, then hydrazine (N_2H_4) is oxidized dinitrogen gas, which generates electrons transferred via an electron transport chain to nitrite reducing enzyme where nitrite is reduced to hydroxylamine NH_2OH (Ni & Zhang, 2013). The scheme of the anammox biochemical pathway is presented in **Figure 2**.

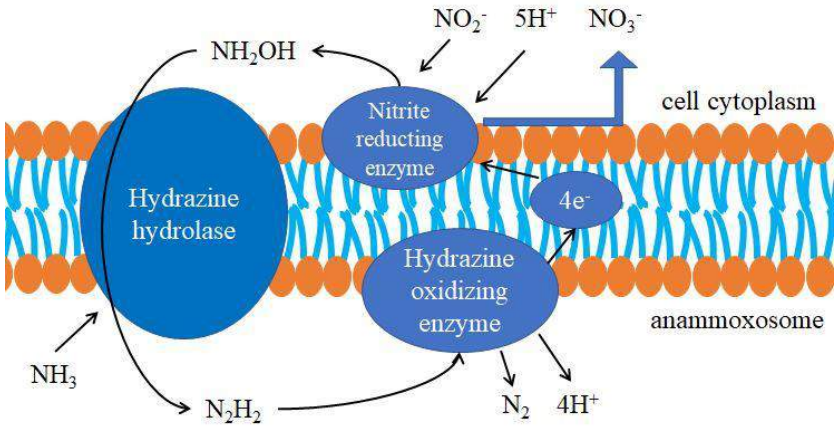

Figure 2. Biochemical pathway of the anammox process (according to Ni & Zhang, 2013; Ozumchelouei et al., 2022).

Compared to conventional processes for removing nitrogen from wastewater, the anammox process allow to reduce the aeration (necessary in nitrification) and reduce the supply of an external carbon source (as in heterotrophic denitrification process). Therefore, the implementation of the anammox process in wastewater treatment plants could contribute to reducing operating cost (Jetten et al., 2001). In addition, the anammox process makes it

possible to reduce excess sludge production while maintaining a high rate of nitrogen removal from wastewater (Jetten et al., 2001). Lower sludge production is related to the very long doubling time of anammox bacteria. The literature data describes that the doubling time of anammox bacteria is between 9 and 29 days (Jin et al., 2012; Tomaszewski et al., 2017). Although according to recent studies, it can be shortened to as much as 2.1 days (Lotti et al, 2015a; Zhang et al, 2017). Such slow growth requires a long time to implement the process into a new bioreactor and also increases the recovery time after a possible failure. In wastewater treatment systems, anammox bacteria show activity in the temperature range from 6 to 43˚C, however, the optimal conditions for conducting the anammox process are temperatures between 30 and 40˚C. Generally, temperature above or below the optimum for the anammox process may cause a decrease in the efficiency of the anammox process, but this depends on the technology used (Miksch & Sikora, 2012; Lotti et al., 2015b). Optimal pH conditions are between 6.7 and 8.3, and the process is reversibly inhibited in the presence of oxygen (Miksch & Sikora, 2012). The technologies that using anammox process is also assumed as ecologically friendly compared to the nitrification-denitrification system, as it reduces emissions of carbon dioxide and nitric oxide (IV), which are considered greenhouse gases (Ma et al., 2016).

The anammox process has found its practical application in many technical installations. In recent years, there has been a significant increase in the number of full-scale plants using the anammox process worldwide. According to the literature, the first implementation took place in 2002 (van der Star et al., 2007). By 2008, there were at least 5 installations, and according to the latest information, there were already more than 200 facilities (Jetten et al., 2009; Lackner et al., 2014; Cao et al., 2017). Since the substrates of the anammox process are ammonium nitrogen and nitrite, it must be preceded by partial nitrification, whereby about half of the ammonium nitrogen is oxidized to nitrate by ammonia oxidizing bacteria (AOB). Such a combination is the process of partial nitrification - anammox (PN/A) (Zhang et al., 2019a). About half of these plants are sequencing batch reactors (SBRs) but moving bed biofilm reactors (MBBRs) and granular sludge reactors are also encountered (Lackner et al., 2014). Among the technologies that use the anammox process, you can mention DEMON®, ANITA™ Mox, and TERRA-MOX (Christensson et al., 2013; Gonzalez-Martinez et al., 2015; Parin et al., 2020). Despite the rapid growth in the number of such treatment plants, they are mainly limited to wastewater characterized by high nitrogen loading, low organic loading, and temperatures different than optimal for anammox bacteria (van Hulle et al., 2010), as well as a large number of toxicants in wastewater, there is a possibility of anammox

process inhibition. In a group of the most harmful substances, antibiotics are recognized to be important.

1.5. Effect of antibiotics on the anammox process

Despite previous research related to the effects of various micropollutants present in wastewater on the anammox process, there is still a lack of reliable research in this field. For this reason, more and more scientists have begun to study antibiotics as substances which presence in wastewater can have a negative effect on anammox bacteria. The first studies mainly focused on the effect of antibiotics on the anammox process performance. Generally, the antibiotics at a concentration reached a few mg L^{-1} with long-term incubation (above few days) have a strong effect on the anammox process performance. A significant reduction in nitrogen removal rate (NRR) from 12.4 to 2 kg N m^{-3} d^{-1} within 26 days at 50 mg L^{-1} of OTC was presented by Yang et al. (2013). Authors demonstrated suppression of anammox bacteria by OTC due to growth inhibition and cell lysis. Shi et al. (2017) obtained stable performance of the anammox process at 1 mg L^{-1} of OTC reaching total nitrogen removal efficiency (TNRE) at 82.7%, while at 2 mg $L^{-1,}$ the TNRE reduced to 19.7%. Other studies indicated that anammox bacteria are able to acclimate to antibiotics' presence. Norfloxacin (NOR) at 0.001-50 mg L^{-1} can be somewhat tolerated by anammox bacteria (Zhang et al., 2018a). The NRR under NOR suppression fluctuated firstly decreased from 0.345 to 0.220 kg N m^{-3} d^{-1}, then recovered to 0.354 kg N m^{-3} d^{-1} at 50 mg L^{-1} of NOR. Notably, in some concentration's antibiotics may stimulate the activity of anammox bacteria. Meng et al. (2019) reported that tetracycline (TC) at 1-100 μg L^{-1} caused a slight increase in the nitrogen removal performance which contributed to elevated heme c content and multiplied the abundance of anammox bacteria.

Many studies have shown that the anammox bacterial community is susceptible to antibiotics. However, the effect of antibiotics on the bacterial community depends on many factors, including the bacteria that coexist with anammox bacteria in activated sludge. It was reported that the dominant phyla in the anammox systems are *Planctomycetes*, *Proteobacteria* while *Firmicutes* usually existed although at low abundance (Liu et al., 2012; Zhang et al 2018b; Chen et al., 2019). The high abundance of *Proteobacteria* in anammox biomass may reduce the negative effect of antibiotics because of the ability to consume some antibiotics as a carbon source (Duan et al., 2017). Moreover, nitrifying bacteria coexist in the anammox community, mainly *Nitrosomonas* (belongs to AOB) and *Nitrospira* (belongs to NOB), which

show greater resistance to antibiotics than anammox bacteria (Zhang et al., 2019e; Fu et al., 2021). An increase in the abundance of nitrifying bacteria during antibiotic suppression compensates for the nitrogen removal efficiency of the system (Fe et al., 2021). A similar relationship has been shown in **Publication 2** and **Publication 3** constituting this doctoral dissertation. Generally, the anammox bacteria can tolerate low concentrations of antibiotics, but at higher concentrations remodeling of the anammox community took place. An example of the response of the anammox bacteria community to various antibiotics is shown in **Table 1**.

Table 1. Variation of anammox bacteria abundance under various antibiotic suppression.

Antibiotic	**Concentration**	**Microorganism**	**Variates of abundance**	**Reference**
Oxytetracycline	1 mg L^{-1}	*Planctomycetes*	Decreased 26.2%	Zhang et al., (2019d)
Sulfamethoxazole	1 mg L^{-1}	*Planctomycetes*	Decreased 15.9%	Zhang et al., (2019d)
Tetracycline	100 μg L^{-1}	*Planctomycetes*	Increased 7.11%	Meng et al., 2019
Norfloxacin	1 μg L^{-1}	*Planctomycetes*	Decreased 2.44%	Zhang et al., (2019c)
Sulfadimethoxine	7 mg L^{-1}	Candidatus *Brocadia*	Decreased 2.18%	Du et al., (2018)
Norfloxacin	100 mg L^{-1}	Candidatus *Kuenenia*	Increased 4.54%	Zhang et al. (2018a)
Oxytetracycline	1 μg L^{-1}	Candidatus *Brocadia*	Decreased 4.3%	Gamoń et al. (2022b)
Ciprofloxacin	1 μg L^{-1}	Candidatus *Brocadia*	Decreased 5.1%	Gamoń et al. (2022b)
Clarithromycin	1 μg L^{-1}	Candidatus *Brocadia*	Decreased 3.5%	Gamoń et al. (2022b)

Nitrogen removal efficiency in the anammox process is directly related to enzymatic activity. Among the anammox enzymes which activity has been analyzed in the context of antibiotic action are the following: HZS – hydrazine synthesis, HAO – hydraxyloamine oxidoreductase, and HZO – hydrazine oxidase. Additionally, heme c was investigated as an important component of some enzymes playing a crucial role in the transfer and storage of electrons (Kleingardner & Bren, 2015; Ozumchelouei et al., 2022). It has been reported that sulfadimethoxine (SMD) at 1-5 mg L^{-1} did not significantly affect the abundance of *hzsA* while at a concentration of 7 mg L^{-1}, the abundance dropped from 1.1×10^6 to 3.0×10^5 copies/ng DNA. Further, at 1 mg L^{-1} of SMD, the abundance of *hzo* was markedly increased from 8.0×10^6 to 3.3×10^7 and next decreased at 7 mg L^{-1} (Du et al., 2018). Moreover, 0.1 mg L^{-1} of SMX stimulated the expression of *hdh* and *hzsA*, which could demonstrate a certain anammox bacteria's protective mechanism of increasing enzymatic activity as a result of low concentration of antibiotics (Gamoń et al., 2022b)

The ability of bacteria to resist the effects of antibiotics has drawn increasing attention from scientists. Among the main protective mechanisms of bacteria against antibiotics are the production of extracellular polymer substances (EPSs), and the exchange of antibiotic-resistance genes. Zhang et al. (2018a) reported that the secretion of EPSs increases as the concentration of NOR increased from 0.001 mg L^{-1} to 50 mg L^{-1} from 24.04 mg g^{-1} SS 198.93 mg g^{-1} SS (suspended solid), respectively. Similar results were presented by Zhang et al., (2019b) indicating that erythromycin (ERY) at concentrations of 0.001 mg L^{-1} to 50 mg L^{-1} induced a significant increase in EPSs production. Both authors pointed out that anammox bacteria begin to acclimatize to the presence of antibiotics by secreting more EPSs, mainly proteins which had a significant impact on antibiotic protection. Antibiotic resistance genes (ARGs) are a very effective mechanism of bacteria protection against antibiotics, but at the same time very dangerous to human health and life. Therefore, it is important to study this element of antibiotic protection in all wastewater treatment systems. Zhang et al., (2019b) evaluated the abundance of six different ARGs (*tetC*, *tetG*, *tetM*, *sul1*, *sul2*) in the anammox biomass under OTC, SMX suppression. The results showed that OTC caused the transfer of resistance genes targeting this antibiotic to a more significant degree than SMX. The OTC resistance genes (*tetA*, *tetB*, *tetC*, and *tetX*) were also investigated by Shi et al. (2017). This work showed that the abundance of all genes increased at an OTC concentration of 1 mg L^{-1}, while the highest abundance was achieved at a concentration of 2 mg L^{-1}. The link between the presence of ARGs and the efficiency of the anammox process has been shown by Zhang et al. 2019e. The authors compared the effects of two antibiotics (NOR and ERY) at a concentration of 0.001 mg L^{-1} on the process of anammox. In the case of NOR, no ARGs determining NOR resistance were found which corresponded to a decrease in nitrogen removal efficiency. The opposite situation was reported for ERY, where two ARGs were detected (*ermB* and *mphA*), which affected negligibly the process, highlighting the role of ERY-ARGs on anammox bacteria in the induction of protection against this antibiotic. Li et al. (2022) summarized the response of anammox bacteria to antibiotics consists of extracellular and intracellular defenses with a dependence on antibiotics concentration. At relatively low concentrations of antibiotics (different for each type of antibiotic) most of the antibiotics are retained by EPSs via adsorption, while at a concentration of the antibiotic that exceeds the sorption capacity of the EPSs, it begins to enter the cell causing the accumulation of ARGs.

In detail, the effect of antibiotics on the anammox process was described in the review article that constitutes this dissertation ‘The influence of antibiotics on the anammox process

– a review' (**Publication 1**). Furthermore, this publication showed the gaps in current knowledge which allow forming of the framework for this dissertation. In the main, it showed that many antibiotics, that concentrations in wastewater are very high and should be monitored (like ciprofloxacin and clarithromycin), are not tested for their influence on the anammox process. Additionally, the studies mainly deal with concentrations above 1 mg L^{-1}, which is practically non-existent in wastewater. Another important gap is the lack of thorough studies that describe the role of other bacteria coexisting in anammox sludge and their activities, which would help illustrate the relationship between microorganisms in the anammox community.

2. Thesis, aim, and scope of dissertation

Current literature studies show that antibiotics occurred in wastewater negatively affect biological wastewater treatment. Despite numerous studies on the effect of antibiotics on the nitrification and denitrification process, there is still not enough research on the effect of these substances on the anammox process, which is increasingly being applied in wastewater treatment plants in its side-stream. Furthermore, some antibiotics have never been tested for their effects on microorganisms residing in wastewater treatment plants.

A thesis was formulated according to which oxytetracycline, ciprofloxacin, and clarithromycin, which occur in wastewater, interact with the anammox bacteria differently than other nitrogen removal bacteria due to their specific cell structure leading to a decrease in the efficiency of nitrogen removal from wastewater and changes in the metabolism of anammox bacteria.

As a result of this thesis, the main objective of the dissertation is to evaluate the effects of oxytetracycline, ciprofloxacin, and clarithromycin on the activity of anammox bacteria and analyze the response of the anammox system to antibiotics suppression. The dissertation was achieved by realizing the following detailed goals:

- analysis of dominant species as well as functional genes of nitrogen cycle bacteria in response to antibiotics suppression,
- study the change in the relative abundance of antibiotic resistance genes targeting studied antibiotics in the anammox community,
- examination of the long-term effect of trace concentration and broad-range concentration of antibiotics on the anammox process performance,
- investigating the production of extracellular polymers by bacteria as a response to antibiotics,
- determination of the change in the structure of bacterial cells under antibiotic suppression,
- analysis of the relationship between dominant species, functional genes, and antibiotic resistance genes.

3. Research Methodology

A series of experiments were planned to achieve the aim of the dissertation. A detailed description of the materials and methodology are presented in the three publications which are part of the dissertation

PUBLICATION 1: The influence of antibiotics on the anammox process — a review

First, an extensive and in-depth literature analysis was performed on the effects of antibiotics on the anammox process. The publication has made it possible to systematize the available knowledge and to identify new potential research areas in this field. It was noted that the studies available in the literature are focused on similar types of antibiotics, omitting number of antibiotics that are important in terms of concentration and activity in the wastewater. Moreover, current studies are mainly based on concentrations of antibiotics that do not occur in the environment, especially in wastewater. Based on the literature analysis, it was decided to choose the following antibiotics for the study: oxytetracycline (OTC), ciprofloxacin (CIP), and clarithromycin (CLA). Both ciprofloxacin and clarithromycin have never been studied for their effect on the anammox process, while oxytetracycline is one of the most common TCs detected in environmental samples.

PUBLICATION 2: Microbial response of the anammox process to trace antibiotic concentration

In this research, short- and long-term exposure tests of trace concentration (0.001 mg L^{-1}) of oxytetracycline, ciprofloxacin, and clarithromycin were carried out on the anammox process. The short-term tests were performed using bath tests measuring changes in the total nitrogen concentration according to the methodology described by Tomaszewski et al. (2019a). Briefly, initial substrate concentrations were 25 mg NH_4-N L^{-1} and 30 mg NO_2-N L^{-1} and the average biomass concentration was 0.9 ± 0.2 g VSS L^{-1} (VSS, volatile suspended solid). The tests were conducted at 35˚C and a pH of 7.5. Samples from the batch test reactors were collected at 20–120 minutes intervals. The results were presented as relative efficiency (%) calculated as a percentage of specific anammox activity (SAA) to the control (without antibiotic) according to equation 1:

$$Relative\ efficiency\ (\%) = \frac{SAA(0)}{SAA} \times 100\% \qquad \text{(Eq 1.)}$$

where:
SAA(0) - specific anammox activity for control test (without antibiotic),
SAA - specific anammox activity for the test with antibiotic addition.

The long-term experiment was performed in four sequencing batch reactors with an active volume of 1 L, as presented in **Figure 3**. Three bioreactors were carried out with oxytetracycline (R-OTC), ciprofloxacin (R-CIP), and clarithromycin (R-CLA) addition, while one was a control bioreactor (without antibiotic addition).

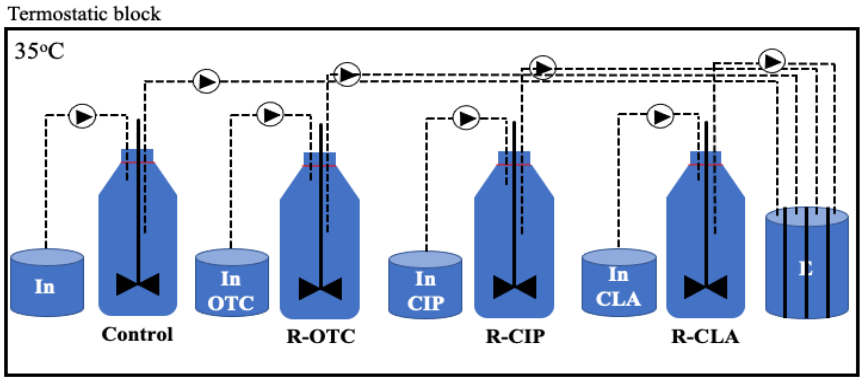

Figure 3. The scheme of the experiment based on the four SBRs. In – influent, E – effluent, control – bioreactor without antibiotic addition, R-OTC - bioreactor with OTC addition, R-CIP - bioreactor with CIP addition, R-CLA - bioreactor with CLA addition.

All bioreactors were operated in one cycle per day for 30 days with 2 days hydraulic retention time (HRT), and pH of 7.5 ± 0.7, dissolved oxygen (DO) below 0.1 mg $L^{-1,}$ and temperature at 35˚C. The reactors were fed with the mineral medium as described previously by Van der Graaf et al. (1996). The total nitrogen concentration in the medium was at the level of 230 mg L^{-1} and was regulated by adding NH_4Cl and $NaNO_2$. The nitrogen load rate (NLR) was 0.115 kg m^{-3} d^{-1}. Monitoring of reactors performance was done by measuring three forms of nitrogen (ammonium (NH_4-N), nitrite (NO_2-N), and nitrate (NO_3-N)) in the reactor influent and effluent using photometric tests (MERCK Millipore) with a spectrophotometer (MERCK Spectroquant® NOVA60). At the beginning (day 1) and at the end of the experiment (day 30) samples for EPSs and for molecular biology analysis were collected. EPSs were analyzed based on their two main components (protein (PN) and polysaccharide (PS)) according to the Flint-phenol method and Anthrone method respectively. The influence of antibiotics on nitrogen cycle functional genes (**Table 2**) and changes in the abundance of antibiotic resistance genes (**Table 3**) were investigated using quantitative polymerase chain reaction (qPCR). ARGs were selected for the study based on a literature analysis of genes that are most common and in the highest abundance occurred in wastewater treatment plants. The genotypic structure of the anammox biomass was analyzed

using next generation sequencing (NGS) based on the V3-V4 region of the 16S rRNA coding gene.

Table 2. Target genes of nitrogen cycle bacteria used in qPCR analysis.

Specificity	Target gen	Aim
Total bacteria	16S rDNA	universal bacterial marker, reference gene
Ammonia oxidizers (AOB)	*amoA*	ammonia monooxygenase
Nitrite oxidizers (NOB)	*nxrA*	nitrite oxidoreductase
Denitrifiers	*nirS* *nirK*	nitrite reductase
All known *Planctomycetes*	*hzo*	hydrazine oxidoreductase
All bacteria	*IntI1*	integrase I class

Table 3. Antibiotic resistance genes investigated in the experiment using qPCR .

Targeted antibiotic	Targeted gen	Resistance mechanism
OTC	*tetC*	Efflux pump
OTC	*tetX*	Drug modification
OTC	*tetM*	Ribosomal protection protein
OTC	*tetW*	Ribosomal protection protein
CLA	*mphA*	Drug modification
CLA	*mphB*	Drug modification
CIP	*qnrB*	DNA gyrase protection
CIP	*qnrB4*	DNA gyrase protection
CIP	*qnrS*	DNA gyrase protection

PUBLICATION 3: Insight into the microbial and genetic response of anammox biomass to broad range concentrations of different antibiotics: Linking performance and mechanism

The research was performed in the four sequencing batch reactors with a volume of 5 L. One bioreactor was a control (without antibiotic) while in three bioreactors oxytetracycline (R-OTC), ciprofloxacin (R-CIP), and clarithromycin (R-CLA) were dosed with increasing concentration (0.001 – 100 mg L^{-1}). The bioreactors were operated for 340 days. Total length of the experiment was divided into five phases (P_0 -P_4) as presented in **Table 4**.

Table 4. The operational strategy of SBRs bioreactors. Control – bioreactor with no antibiotics, R-OTC - bioreactor with OTC addition, R-CIP - bioreactor with CIP addition, R-CLA - bioreactor with CLA addition.

Stage	Time (d)	Control	R-OTC	R-CIP	R-CLA
			OTC (mg L^{-1})	CIP (mg L^{-1})	CLA (mg L^{-1})
P_0	1-48	0	0	0	0
P_1	49-111	0	0.001	0.001	0.001
P_2	112-201	0	1	1	1
P_3	202-288	0	10	10	10
P_4	289-340	0	100	100	100

Similar to **Publication 2**, the reactors were fed with a mineral medium described by Van der Graaf et al. (1996). The concentration of total nitrogen was 260 mg L^{-1} and was controlled by the addition of NH_4Cl and $NaNO_2$. The nitrogen loading rate (NLR) was 0.254 ± 0.014 kg m^{-3} d^{-1}. The reactors were performed in stable condition at a pH of 7.5 ± 0.4, a temperature of 32 ± 0.5˚C, dissolved oxygen (DO) below 0.1 mg L^{-1}, and the HRT was 1 day with a volume exchange ratio of 25%. The VSS in reactors was in the range of 1200–1248 mg VSS L^{-1}. The reactors were performed in four cycles per day. All technological parameters of used SBRs and the time of cycles were shown in **Figure 4**. Throughout the study period, the concentrations of the NH_4-N, NO_2-N, and NO_3-N in the influent and effluent were measured regularly to monitor the performance of the anammox process. Each nitrogen form was analyzed using photometric tests (MERCK Millipore) with a spectrophotometer (MERCK Spectroquant® NOVA60).

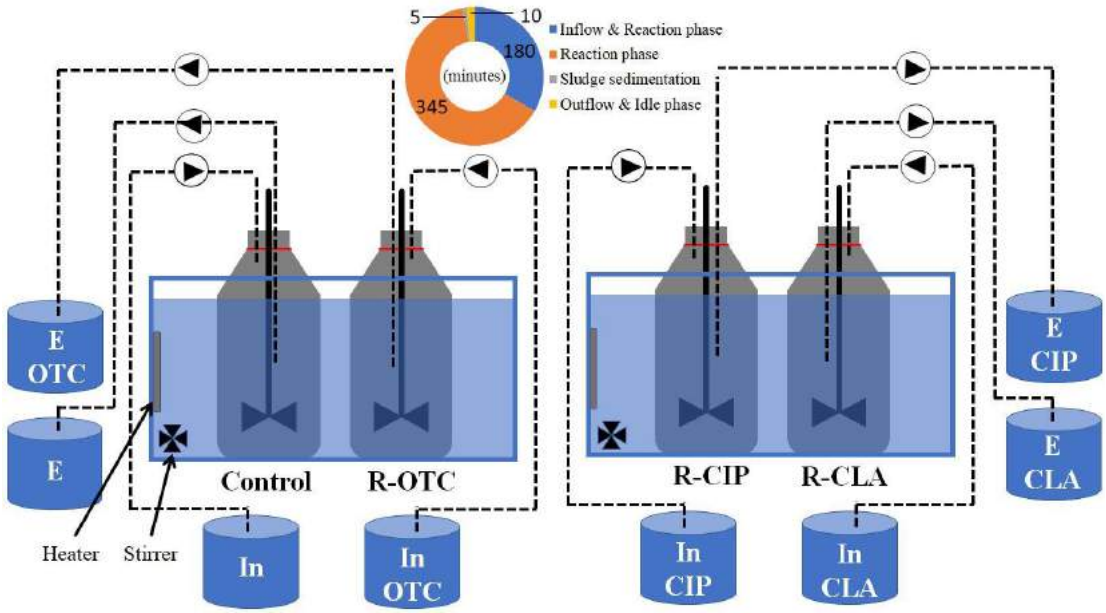

Figure 4. Experimental set-up of the four SBR bioreactors and their cycle schedule and time of cycle steps. In: influent, E: effluent, Control – bioreactor with no antibiotics, R-OTC - bioreactor with OTC addition, R-CIP - bioreactor with CIP addition, R-CLA - bioreactor with CLA addition.

At the beginning of the experiment (day 1) and at the end of each phase the samples of anammox activated sludge were collected for molecular biology analysis including metagenomic analysis, changes in functional nitrogen-cycle genes, and antibiotic resistance genes. Both functional nitrogen-cycle genes and antibiotic-resistance genes were analyzed using the qPCR method. **Publication 3** analyzed the same genes as in **Publication 2**. Metagenomic analysis was performed using NGS based on the 16S rRNA coding gene. At the end of phases P_1-P_4, the anammox activated sludge was prepared for visualization of changes in the ultrastructure of anammox bacteria using transmission electron microscopy (TEM), according to the method described by Ziembińska-Buczyńska et al. (2019), To understand the relationship between dominant bacterial species in the anammox activated sludge and functional genes as well as antibiotic resistance genes, a co-occurrence analysis was conducted using statistical data (Spearman's correlation coefficients) and then visualized using Gephi (V 0.9.1) software. Moreover, principal component analysis (PCA) was used to visualize and elucidate the impact of environmental factors on changes in dominant genera. R software (https://www.r-project.org) with the factoextra package was used for PCA preparation.

4. Results

In the present dissertation, the effects of ciprofloxacin and clarithromycin on the anammox process were evaluated for the first time. In addition, the current knowledge on the effect of oxytetracycline on the anammox process was expanded. The study focused on the effect of the tested antibiotics on the efficiency of the anammox process as well as community structure, nitrogen cycle functional genes, and mechanisms of protection against antibiotics action.

One of the main reasons for **Publication 1** was the need to systematize knowledge about the influence of antibiotics on the anammox process. A literature review showed that most studies describing the effect of antibiotics on the anammox process are conducted with very high concentrations of antibiotics, which range from a few to even several thousand mg L^{-1} (Hu et al., 2013; Yang et al., 2013; Zhang et al., 2019b). Such concentrations are much higher than those found in wastewater (few ng L^{-1} to few µg L^{-1}) (Felis et al., 2020). The literature studies presented in **Publication 1** describing the short- and long-term exposure of antibiotics to the anammox process concluded that a short exposure time is insufficient to get anammox bacteria to respond to antibiotics. These differences mainly result from characteristics of anammox bacteria, which have a low growth rate, as well as produced EPSs, which form a kind of barrier to antibiotics, protecting anammox bacteria. The literature review also addressed the effect of antibiotics on the key metabolic pathways of anammox bacteria related to nitrogen conversion as well as community structures of anammox biomass. Special attention was paid to the fact that the available literature studies mainly focus on a few selected antibiotics, omitting those which presence in wastewater is particularly dangerous, such as ciprofloxacin or clarithromycin. Both these antibiotics are included in the second European Watch List for substances that need to be monitored in the environment (EU Decision, 2018/840 of June 5, 2018).

Based on the literature review, three antibiotics were selected for the study: oxytetracycline, ciprofloxacin, and clarithromycin. In **Publication 2**, the trace (0.001 mg L^{-1}) concentration effect of antibiotics on the anammox process was analyzed. Short-term antibiotic exposure studies showed that OTC increased by about 7.1% activity of the anammox process, while both CIP and CLA caused a decrease in anammox activity by about 8.4% and 3.2%, respectively. The increase in the activity of the anammox process under OTC action was most likely triggered by a stress-induced growth in the enzymatic activity of anammox bacteria, as previously reported in the literature during low concentrations of

antibiotics stress (Zhang et al., 2019c). Long-term experiment (30 days) showed that the control reactor, R-OTC, and R-CLA had disturbed performance during all tests, however, on days 2, 9, and 17 the nitrogen removal efficiency (NRE) reached levels of 86.9%, 82.56%, and 82.52% respectively, which is close to the maximum theoretical anammox NRE (~90%). On the other hand, the R-CIP presented relatively stable performance during the experimental period with an NRE level of 62.4 ± 6.37% while the maximum NRE was presented on day 15 reaching 70%. Because in this publication the anammox process was performed for 30 days under antibiotics suppression without earlier acclimatization to the new conditions, the deviation in the NRE may be associated with biomass adaptation or bacterial community remodeling (Banach-Wiśniewska et al., 2021). This thesis confirms the results obtained for control, R-OTC, R-CLA where also deviations in NRE were observed. The role of the acclimatization period on the stability of anammox performance can be seen in **Publication 3**, where after phase P_0 also started to dose antibiotics to anammox systems at a concentration of 0.001 mg L^{-1} (phase P_1). Each bioreactor (R-OTC, R-CIP, R-CLA) maintained stable performance after antibiotics addition in 0.001 mg L^{-1} (**Publication 3, Table S3**). Nevertheless, despite the minor deviation in the anammox process performance in **Publication 2**, it can be concluded that the tested antibiotics at a concentration of 0.001 mg L^{-1} did not significantly affect the efficiency of the anammox process. Molecular analysis of functional genes showed that significant activity in the biomass at the beginning of the experiment (day 1) was presented by AOB, NOB, and HET (heterotrophic) bacteria which functional genes *amoA*, *nxrA*, *nirS*/*nirK*, respectively showed significantly higher abundance than the functional genes of anammox bacteria (*hzo*) (**Publication 2, Figure 5**). The abundance of each gene (*amoA*, *nxrA*, *nirS*/*nirK*) decreased under each antibiotic suppression, however, their level was still higher than *hzo* gene. Similarly, the abundance of the *hzo* gene decreased in each reactor with the addition of antibiotics during the running of the experiment, while in the control the *hzo* gene abundance increased. Moreover, the differences between the control and reactor with antibiotics addition were statistically significant ($p < 0.05$) suggesting that antibiotics in trace concentration have a negative effect on the activity of the anammox bacteria functional gene. The high activity of AOB and NOB functional genes has been confirmed by studies of community structure (**Publication 2, Figure 7**). The results showed a significant contribution of *Nitrospira* genus in the activated sludge community in all research bioreactors. On day 1 the abundance of *Nitrospira* was 8.1% (Control), 7,9% (R-OTC), 7,6% (R-CIP), and 8.37% (R-CLA) while at the end of the experiment it reached 9.59%, 9.6%, 12.03%, and 11.45% respectively. From the point of nitrogen removal

processes *Nitrospira* belongs to the NOB bacteria, however, it contains a full set of ammonia-converting genes, which makes it possible to carry out full nitrification in one step under anaerobic condition – named comammox (He and He, 2017). *Planctomycetes* phylum to which anammox bacteria belong represents the third most numerous group of bacteria in all bioreactors at the beginning of the test while all antibiotics resulted in a decrease in the abundance of *Planctomycetes* relative to the entire community and a drop to 4th place in the taxonomic abundance. The dominant species among anammox bacteria in the systems were Candidatus *Brocadia* and Candidatus *Jettenia*, however, the abundance of both genera decreased under antibiotics suppression, which corresponded with a decrease in *hzo* gene abundance. One of the protective mechanisms of bacteria against harmful substances (among other antibiotics) is the production of extracellular polymers (EPSs) (Zhang et al., 2019b; Zhang et al., 2020). At both, the beginning and end of the experiment, samples were taken for the determination of EPSs presence. The results obtained show that EPSs production (**Publication 2, Figure 4**) in anammox systems with the addition of antibiotics OTC, CIP, and CLA slightly increased by 0.6%, 1.2%, and 1.8%, respectively, while in control decreased by 1.4%. This result indicates an attempt to protect anammox bacteria from antibiotics through the production of EPSs. Although the total production of EPSs increased in anammox systems with the addition of CIP and CLA, the PN/PS ratio decreased by 8.0% and 10.5%, respectively. A decrease in this ratio may suggest a reduction in anammox sediment flocculation and sludge settling capacity (Meng et al., 2019; Wu et al., 2020). One of the most effective mechanism and at the same time unsafe for human health mechanisms for protecting bacteria against antibiotics is the exchange of antibiotic resistance genes. The acquisition of antibiotic resistance by bacteria through the exchange of ARGs allows bacteria to resist the antibiotics. As a result, infection by humans or animals with antibiotic-resistant bacteria can result in prolonged treatment and even death. In **Publication 2**, nine different primers (**Publication 2, Table S3**) that encoded resistance to tested antibiotics were used for the creation of an antibiotic resistance profile of the tested anammox systems. However, only six of them were detected (**Publication 2, Figure 6).** Both, the genes determining OTC (*tetW*, *tetC*) and CIP (*qnrB4*, *qnrS*) resistance showed an increase in their abundance, while the abundance of the *mphA* (CLA) gene decreased over the period of the experiment. However, the literature concludes that *mphA* gen can develop under environmental rather than laboratory conditions, due to the special resistance mechanism of regulating the synthesis of oxidoreductase (Zhang et al., 2021). The above-described studies on the production of EPSs and the abundance of antibiotic resistance genes indicate that even trace concentrations of

antibiotics result in the activation of the protective systems of anammox biomass against the effects of antibiotics.

To properly understand the effect of OTC, CIP, and CLA on the anammox process, a long-term experiment (340 days) was conducted in which the concentration of antibiotics was gradually increasing (range between 0.001 mg L^{-1} to 100 mg L^{-1}) in the influent of the bioreactors (**Publication 3**). The experiment was divided into five phases P_0-P_4 (**Publication 3, Table 1**) as presented in Table 4. The results obtained show that, as in **Publication 2**, a concentration of 0.001 mg L^{-1} of antibiotics did not cause a change in the nitrogen removal efficiency of the anammox process. Moreover, increasing the concentration of antibiotics to 1 mg L^{-1} also did not result in significant changes in the performance of the anammox process. For all antibiotics tested, NO_2-N removal efficiency in the anammox process did not drop below 99.8% in P_2. The stoichiometric ratios (NO_2-N removal/NH_4-N removal and NO_3-N production/NH_4-N removal) in this phase were also close to theoretical values (1.32 and 0.26, respectively). When the concentration of antibiotics increased to 10 mg L^{-1} significant changes in the efficiency of the anammox process were observed. For bioreactors dosed with OTC, CIP, and CLA, nitrogen removal efficiency (NRE) decreased by 19.9%, 26.57%, and 29.9%, respectively. A further reduction in the efficiency of the anammox process was observed when the concentration of antibiotics was 100 mg L^{-1}. Correspondingly, nitrogen removal decreased by 27% (R-OTC), 30% (R-CIP), and 56% (R-CLA). In Addition, the stoichiometric values of the anammox process in all bioreactors fed with antibiotics were above the theoretical values which indicate the contribution of the nitrification process to the activated sludge processes. These results correspond with the results of community structure (**Publication 3, Figure 6**) where *Nitrospira* abundance was increased with increasing antibiotic concentrations. These results are also consistent with those obtained in **Publication 2**. These studies indicate high resistance of *Nitrospira* to various antibiotics, which was also noted previously by Wang et al. (2021). *Planctomycetes* phylum, which includes anammox bacteria, also received an increase in abundance during the experiment. Despite the increase in the abundance of *Planctomycetes*, the abundance of the dominant anammox bacteria genus (Candidatus *Brocadia*) decreased during the experiment. An explanation for this situation can be found in the results of functional anammox gene (*hzo*) activity, in which abundance decreased with increasing antibiotic concentrations. This may suggest that all the antibiotics tested have a negative effect not on all *Planctomycetes* bacteria, but only those capable of conducting the anammox processes.

The co-occurrence of anammox bacteria with other nitrogen-cycle bacteria, like AOB and NOB was previously reported by Ziembińska-Buczyńska et al. (2019), Tomaszewski et al. (2019b) and Banach-Wiśniewska et al. (2020). These authors highlighted that the nitrogen removal bacteria support each other, especially in case of anammox process introduction when nitrogen removal eficacy should be maintained. These AOB/NOB support to anammox bactera was clearly visible duringf start-up of the anammox process (Ziembińska-Buczyńska et al.,2019), when oxygen is present in the bioreactor (Banach-Wiśniewska et al. 2020), or when anammox bacteria were exposed to potentially harmful substances, such as reduced graphene oxide Tomaszewski et al. (2019b). These suggest that both AOB and NOB couldsupport anammox bacteria under antibiotics suppression mitigating their effects.

In both **Publication 2** and **Publication 3**, it can be seen that *mphA* gene abundance decreases at a CLA concentration of 0.001 mg L, while in **Publication 3** when CLA concentration began to increase, the *mphA* gene abundance also increased. Generally, the abundance of almost all detected ARGs were higher than the initial level in all reactors fed with antibiotics. Moreover, their abundance is highly dependent on the concentration of antibiotics. The only gene which abundance decreased during antibiotic suppression was *tetW* (targeting OTC). Its level decreased to a value undetectable by the qPCR method. It was suspected that the occurrence of *tetW* gene in laboratory systems could be negligible, because of its occurrence mainly in raw wastewater and livestock (Li et al., 2021).

Previous studies have described antibiotic-induced changes in the internal structure of anammox cells (Zhang et al., 2015). The anammox bacteria cell inner structure analyzed by TEM (**Publication 3, Figure 3**) show that both CIP and CLA at concentrations above 10 mg L^{-1} caused lysis of anammox cells. Moreover, irregular cell shapes were observed. In the case of OTC, there were no lysed cells, while the irregular shape of anammox bacteria cells also was observed. Although OTC, CIP, and CLA do not act directly on cell wall synthesis, they can disturb the synthesis of the protein components of the cell wall. The ultrastructure image of the anammox bacteria also showed the deposition of polymeric substances around the cells. This also corresponds with the results of the EPSs production (**Publication 3, Figure 2**). The highest EPSs production was observed when the concentration of antibiotics was 100 mg L^{-1} and reached 73.7 mg g^{-1} VSS (OTC), 67.1 mg g^{-1} VSS (CIP), and 55.3 mg g^{-1} VSS (CLA). It was reported that biomass with higher specific anammox activity (SAA) tends to secrete more EPSs (Liu et al., 2020), which corresponds to the SAA in this experiment (**Publication 3, Figure S3**).

Co-occurrence analysis between functional genes, dominant species, and antibiotic resistance genes showed that nitrifying and denitrifying bacteria are mainly responsible for transmitting resistance to the antibiotics tested. There was no significant correlation between the *hzo* gene, studied ARGs, and anammox bacteria. Only in the case of the gene determining resistance to CLA (*mphA*), and Candidatus *Jettenia*. a significant correlation was found Moreover, there was a positive correlation between *mphA* and *intI1* genes ($r = 0.86$, $p < 0.05$), as well as Candidatus *Jettenia* and *intI1* gene ($r = 0.99$, $p < 0.05$). The *IntI* encode integron 1 class gene which is a part of integron recognized as a mobile genetics element (MGE) playing an important role in the transmission of antibiotic resistance genes (Gillings et al., 2017; Li et al., 2020; Li et al., 2021). Thus, the positive correlation between the resistance gene and *IntI1* may indicate that the *intI1* gene is involved in the transmission of ARGs. On the other hand, there was a negative correlation between *hzo* and *mphA* genes ($r = -0.90$, $p < 0.05$). This all may suggest that Candidatus *Jettenia* is the main anammox bacteria that exhibit resistance against CLA in opposition to other bacteria using the *hzo* pathway, as well as Candidatus *Jettenia* may participate in the transfer of the *mphA* gene.

5. Conclusion

The realization of all the stated objectives made it possible to confirm the thesis. A multifaceted picture of the interactions between bacteria and antibiotics (oxytetracycline, ciprofloxacin, and clarithromycin) was obtained, taking into account the efficiency of the anammox process, the structure of the microbial community conducting it, and the analysis of protective mechanisms against the action of antibiotics. The results of the research carried out within the framework of this dissertation allowed to conclude that:

- the tested antibiotics in concentrations similar to those found in wastewater (0.001 mg L^{-1}) do not adversely affect the conduct of the anammox process. Nevertheless, these concentrations affect the structure of the bacterial community as well as lead to the development of antibiotic resistance in the activated sludge. Only concentrations above 10 mg L^{-1} lead to a reduction in wastewater treatment efficiency, which can pose a serious problem for the quality of treated wastewater,
- in case of antibiotic suppression, anammox bacteria are supported by other nitrogen cycle bacteria present in activated sludge in order to maintain stable nitrogen removal efficacy. An increase in the abundance of *amoA* and *nxrA* genes was noted for both trace concentrations of antibiotics and concentrations reaching several mg L^{-1}. The activity of bacterial groups using these two genes (AOB and NOB) contributes to reducing the negative effect of antibiotics on the efficiency of nitrogen removal from wastewater,
- studies of the community structure of the anammox activated sludge highlight the role of *Nitrospira* (belonging to NOB), capable of conducting the comammox process under anaerobic conditions in nitrogen removal processes. This dissertation also shows that *Nitrospira* appears to be particularly resistant to the tested antibiotics and plays an important role in the transfer of ARGs.

Additional conclusions that can be drawn from the study:

- clarithromycin showed the strongest negative effect on the anammox process among of all antibiotics tested,
- relation between the abundance of the *hzo* gene and *Planctomycetes* induced that both CIP and CLA increase the abundance of *Planctomycetes* that are probably unable to conduct the anammox process,

- the abundance of Candidatus *Jettenia* and *mphA* gene (determining CLA resistance) and *intI1* gene were positively correlated indicating the role of this genus in spreading the macrolide resistance gene.
- EPSs play an important protecting role against antibiotics by creating some kind of barrier to antibiotics,
- depending on the concentration between 0.001 to 100 mg L^{-1} of OTC, CIP and CLA, different antibiotic resistance genes are induced in the anammox community.

6. Further research proposals

The presented research is in line with the rising trend of interest in antibiotics in the context of environmental risk. Therefore, on the basis of the obtained results further research directions can be proposed:

- study of the simultaneous effect of all tested antibiotics on the anammox process and anammox community,
- investigating the ability of the anammox process to recover after tested antibiotics suppression,
- examination of the by-products of tested antibiotics occurring in wastewater after treatment by biological treatment process,
- explore the role of quorum sensing in the acquisition of antibiotic resistance with special interests in the anammox bacteria role in this transfer.

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Filip Gamoń

Grzegorz Cema

Aleksandra Ziembińska-Buczyńska

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REVIEW ARTICLE

The influence of antibiotics on the anammox process — a review

Filip Gamoń[1] · Grzegorz Cema[1] · Aleksandra Ziembińska-Buczyńska[1]

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Abstract
Anaerobic ammonium oxidation (anammox) is one of the most promising processes for the treatment of ammonium-rich wastewater. It is more effective, cheaper, and more environmentally friendly than the conventional process currently in use for nitrogen removal. Unfortunately, anammox bacteria are sensitive to various substances, including heavy metals and organic matter commonly found in the wastewater treatment plants (WWTPs). Of these deleterious substances, antibiotics are recognized to be important. For decades, the increasing consumption of antibiotics has led to the increased occurrence of antibiotics in the aquatic environment, including wastewater. One of the most important issues related to antibiotic pollution is the generation and transfer of antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs). Here, we will discuss the effect of short- and long-term exposure of the anammox process to antibiotic pollutants; with a special focus on the activity of the anammox bacteria, biomass properties, community structures, the presence of antibiotic resistance genes and combined effect of antibiotics with other substances commonly found in wastewater. Further, the defense mechanisms according to which bacteria adapt against antibiotic stress are speculated upon. This review aims to facilitate a better understanding of the influence of antibiotics and other co-pollutants on the anammox process and to highlight future avenues of research to target gaps in the knowledge.

Keywords Antibiotics · Anammox · Process activity · Wastewater pollutants · Antibiotic resistance mechanisms

Introduction

The anaerobic ammonium oxidation process (anammox) has been used in the treatment of ammonium-rich wastewater with organic carbon deficiency, where ammonium is oxidized by anammox bacteria to nitrogen gas using nitrite as electron acceptor (Strous et al. 1998). Additionally, the anammox process for the treatment of wastewater provides important benefits compared to widespread nitrification and denitrification process such as the reduction of oxygen demand (by ca. 60%), the elimination for the addition of external carbon sources (which caused a decrease in operational cost (up to 60%)), low excess sludge production due to long doubling time (9–29 days), a high nitrogen removal rate, and a lower greenhouse gases emission (by ca. 90%) (Tomaszewski et al. 2017). While superior to older technologies, the anammox process is sensitive to changes in environmental and chemical parameters such as temperature, pH, and nitrogen load. Further, the anammox process is also vulnerable to a wide range of inhibiting substances, such as antibiotics, heavy metals, free ammonia, free nitrous acid, nitrite, salts, organic matter, phosphate, sulfides, and 1,4-dioxane (Tomaszewski et al. 2017, Jin et al. 2012, Lotti et al. 2012a, Ismail et al. 2021a, b). This sensitivity makes the anammox process impossible to be implemented for treatment in most types of wastewater due to the excessive number of pollutants which are found in these aqueous environments (Shi et al. 2017).

More and more scientific attention is being given to antibiotics as micropollutants in the environment. Globally, antibiotics are widely used in human and veterinary medicine, both for treating bacterial infection and for prophylaxis (Kümmerer 2009; Jin et al. 2012). High concentrations of antibiotics have been detected in a diverse range of aquatic environments including bank filtrates, wastewater, surface water and even groundwater (Kümmerer 2009, Watkinson et al. 2009; Yang et al. 2013). The most common antibiotics

Responsible Editor: Gerald Thouand

✉ Filip Gamoń
filip.gamon@polsl.pl

[1] Environmental Biotechnology Department, Silesian University of Technology, Akademicka 2A, 44-100 Gliwice, Poland

detected in these environments include amoxycillin (AMX), penicillin G, norfloxacin (NOR), enrofloxacin (ENR), ciprofloxacin (CIP), erythromycin (ERY), tetracycline (TC), oxytetracycline (OTC), and sulfamethoxazole (SMX) (Watkinson et al. 2009). The occurrence of antibiotics in the environment is a serious problem for living organisms due to the adverse effects these compounds have on their physiology. Additionally, antibiotics are designed to exert specific biological activity and, in some species, may have an immediate effect on bacterial homeostasis (Loos et al. 2013). Moreover, long-term exposure to even sub-inhibitory concentrations of antibiotics may cause chronic toxicity (Bengtsson-Palme and Larsson 2016).

Antibiotics and their metabolized products can induce unforeseen changes in an individual bacterium which can then become common in bacterial communities (Grenni et al. 2018). For example, the occurrence of antibiotics in the aquatic environment has been linked to the development of antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria (ARB) (Gonzalez Ronquillo and Angeles Hernandez 2017; Kumar et al. 2019; Agrawal et al. 2020). ARGs are located on the chromosome or embedded in mobile genetic elements. Further, their location on the mobile genome elements, such as plasmids or transposons, determines their mobility among microorganisms. Additionally, WWTPs have been identified as important reservoirs of antibiotic resistance genes, due to the high density of microorganisms and high concentration of organic substances which can facilitate the transfer of antibiotic resistance genes (Osińska et al. 2019).

Previous studies have shown that anammox activity is inhibited by antibiotics (van de Graaf et al. 1995, Fernández et al. 2009, Yang et al. 2013, Shi et al. 2017). However, it was reported that the anammox process and partial nitrification were successfully performed in the treatment of piggery wastewater which was reported to include a high concentration of antibiotics (Suto et al. 2017). Moreover, the anammox process is used for landfill leachate treatment as well as livestock and swine wastewater and pharmaceutical wastewater (Jin et al. 2012; Tang et al. 2011), where antibiotics were also found. For instance, it was presented that concentration of OTC can reach a few mg L^{-1} in livestock wastewater, because of the broad administration of these antibiotics in livestock, while concentration of antibiotics in pharmaceutical wastewater reach a few μg L^{-1} (Felis et al. 2019). Despite the application of the anammox process to the treatment of pharmaceutical wastewater (for example Jin et al. 2012 Tang et al. 2011), the authors suggested that the conventional anammox process is not suitable for this kind of wastewater because of cumulative toxicity. Moreover, antibiotics can variably affect the anammox process leading to the inhibition of nitrogen removal efficiency, altering the structure of microbial communities and stimulating the transfer of ARGs (Rodriguez-Sanchez et al. 2017; Du et al. 2018; Zhang et al. 2019b). Moreover, previous studies have reported that some antibiotics can be adsorbed or metabolized by anammox bacteria (Zhang et al. 2014). However, the exact mechanism by which antibiotics influence the anammox process remains unclear.

Here, we review the current studies and literature which interrogate the short- and long-term influence of antibiotic exposure on the anammox process. Further, we plan to link these data to bacterial activity, activated sludge granule properties, microbial community structure and the combined effect of antibiotics and other contaminants in wastewater. Additionally, we will also discuss the way of future study which will allow for more comprehensive understanding the impact of antibiotics on the anammox process. From the point of the implementation of anammox process, this review will provide the information about the possible problems that might have occurred in the anammox system in the WWTPs as a result of the presence of antibiotics in wastewater. Moreover, we would like to point out the directions in which further research on the influence of antibiotics on biological nitrogen removal processes, especial Anammox, should follow.

Effect of antibiotics on the anammox process

Short-term exposure effect of antibiotics on anammox process

Current research into the effect of antibiotics on the anammox process has primarily focused on both the short- and long-term exposure of antibiotics. However, both, short- and long-term should be specifically distinguished due to the various abilities of bacteria to adapt at these two timepoints (Du et al. 2018; Zhang et al. 2019b). Thus, a comprehensive data set describing the biological changes which may occur in anammox bacteria in response to short-term antibiotic exposure is urgently required. Table 1 reports the short-term effects of different concentrations of antibiotics on anammox process. From this table, it is clear that the most investigated antibiotic assessed during short-term antibiotic exposure on the anammox process is chloramphenicol (CAP). CAP is widely used for animal breeding (Phanwilai et al. 2020). Further, it has been shown that residual CAP can leach into the aquatic environment from the animal excrement (Díaz-Cruz et al. 2003; Phanwilai et al. 2020). Moreover, it has been demonstrated that CAP affected most bacteria in WWTPs (Dapena-Mora et al. 2007; Fernández et al. 2009; Phanwilai et al. 2020) and also the human hemopoietic system (Festing et al. 2001). Van der Graff reported that CAP, at a concentration of 200 mg L^{-1}, caused the inhibition of anammox activity by 95% (van der Graff et al. 1995). A similar finding

Table 1 Summary of short-term effect of antibiotics on AnAOB

Antibiotic	Concentration	Biomass	Activity	Exposure time	References
Chloramphenicol	200 mg L^{-1}	Granules	Decreased by 95%	First 3 days of incubation	Van der Graaf et al. (1995)
	250–1000 mg L^{-1}	Granular	Decreased by 20–80%	24 h	Fernández et al. 2009
	100–1000 µg L^{-1}	Granular	No effect	24 h	Fernández et al. 2009
	1000 mg L^{-1}	Granular, Anammox biomass enriched in bacteria belonging to the specie Candidatus *Kuenenia stuttgartiensis*	No effect	No information	Dapena-Mora et al. (2007)
	5–100 mg L^{-1}	Biofilm	Decreased by 16.2–44.7%	7 h	Phanwilai et al, (2020)
	5–100 mg L^{-1}	Suspended-growth	Decreased by 29.9–45.5%	5 h	Phanwilai et al. (2020)
	100–1000 µg L^{-1}	Biofilm	No effect	24 h	Phanwilai et al. (2020)
	100–1000 µg L^{-1}	Suspended-growth	No effect	24 h	Phanwilai et al. (2020)
Tetracycline hydrochloride	100–1000 mg L^{-1}	Granular	Decreased by 20–80%	24 h	Fernández et al. 2009
Oxytetracycline	25–100 mg L^{-1}	Granular	TNRR decreased from 74.73% to 33.45–22.15%	7 h	Noophan et al. (2012)
Doxycycline	50–100 mg L^{-1}	Granular	Decreased by 14–17%	24 h	Sguanci et al, (2017)
	100 mg L^{-1}	Granular	Decreased by 47.6%	24 h	Alvarino et al. (2014)
	50–100 mg L^{-1}	Granular	Decreased by 22%	48 h	Sguanci et al. (2017)
Tiamulin	50 mg L^{-1}	Granular	Decreased by 5%	24 h	Sguanci et al. (2017)
	50–500 mg L^{-1}	Granular	Decreased by 5–64%	48 h	Sguanci et al. (2017)
Enrofloxacin	50 mg L^{-1}	Granular	Decreased by 13%	24 h	Sguanci et al. (2017)
	100–200 mg L^{-1}	Granular	Decreased by 42–60%	48 h	Sguanci et al. (2017)
Penicilin G	0–2000 mg L^{-1}	Flocs, *Kuenenia stuttgartiensis* (free-living planktonic cells)	No effect	1 h	Hu et al. (2013)
Streptomycin	0–200 mg L^{-1}	Flocs, *Kuenenia stuttgartiensis* (free-living planktonic cells)	No effect	1 h	Hu et al. (2013)
Norfloxacin	1 µg L^{-1}*	Biofilm	Decreased by 2.2%	6 h	Zhang et al. (2019a)
Erythromycin	1 µg L^{-1}*	Biofilm	decreased by 0.56%	6 h	Zhang et al. (2019a)

TNRR, total nitrogen removal rate. * Studies using the concentration of antibiotics that occur in real wastewater

was reported by Fernández et al. (2009) and Phanwilai et al. (2020). Both studies provided evidence that CAP could significantly affect anammox activity. However, there were several experimental differences in studies mentioned. Namely, Fernández et al. (2009) used higher concentrations of CAP (250, 500, 1000 mg L^{-1}) in comparison to Phanwilai et al. (2020) (5, 10, 20, 50, 100 mg L^{-1}). Moreover, Phanwilai et al. (2020) used two types of biomass (suspended- and attached-growth), while Fernández et al. (2009) investigated only granular sludge. Additionally, Phanwilai et al. (2020) assessed the effect of CAP at an earlier exposure time (7 h), relative to Fernández et al. (2009) (24 h). In contrast to these studies, an entirely different result for 1000 mg L^{-1} CAP was obtained by Dapena-Mora et al. (2007), where no inhibitory effect was observed at 5–6 h. It is important to note, however, that Dapena-Mora et al. (2007) used flock biomass enriched in bacteria belonging to *Kuenenia stuttgartiensis*. Van de Graaf et al. (1996) indicated that the degree to which CAP can affect anammox enrichment is linked to the microbial parameters of the culture being assessed. Hu et al. (2013) elucidated the effect of penicillin G and streptomycin at concentrations ranging from 0–2000 mg L^{-1} to 0–200 mg L^{-1}, respectively. Their data indicated that a microbial biomass, with low biodiversity, is more resistant to antibiotic stress during short-term exposure. Therefore, due to the fact that anammox bacteria co-exist with other nitrogen cycle bacteria in the anammox biomass (Ziembińska-Buczyńska et al. 2019; Banach-Wiśniewska et al. 2019), it can be assumed

that the high diversity of these microorganisms will influence the activity of anammox biomass.

One of the antibiotics group most often used in medicine and animal breeding are tetracyclines (TCs). TCs inhibit the biosynthesis of proteins by preventing the binding of aminoacyl-tRNA to the 30S ribosome subunit (Yang et al. 2005). Noophan et al. (2012) investigated the effect of oxytetracycline at concentrations ranging from 23 to 100 mg L^{-1}. In that study, the authors reported a partial reduction of the anammox activity. Further, Sguanci et al. (2017) compared the effect of three antibiotics: doxycycline (DOX), tiamulin (TIA) and enrofloxacin (ENR) after 24 and 48 h. From their data, they reported that the inhibition of the anammox process was recorded after 48-h exposure to 50 mg L^{-1} of the following compounds in the order of DOX > ENR > TIA.

The major problem with the current studies is the level of antibiotics concentrations used. Most of the studies presented the results of the experiments with the antibiotics concentration that does not occur in real wastewater; therefore, it is hard to evaluate the real impact of antibiotics on the anammox process implemented in full-scale reactor. A few studies have investigated the effect of trace concentrations (< 1 mg L^{-1}) of antibiotics on the anammox bacteria. However, current evidence suggests that concentrations less than 1 mg L^{-1} do not affect the anammox bacteria (Fernández et al. 2009; Phanwilai et al. 2020). In contrast to these data, Zhang et al. (2019a) reported the effect on anammox bacteria to a low concentration of NOR and ERY (< 1 µg L^{-1}). In this study, the authors found that specific anammox activity (SAA) slightly decreased from 10.8 mg g^{-1} SS h^{-1} for both NOR and ERY to 10.56 and 10.74 mg g^{-1} SS h^{-1}, respectively. Despite the low impact of ERY and NOR on the SAA, it was further reported that dehydrogenase activity (DHA) under NOR exposure was significantly suppressed in the short-term test where it decreased from 0.49 to 0. 39 EU g^{-1} SS.

Based on short-term exposure, the half-maximal inhibitory concentration (IC_{50}) and half-maximal effective concentration (EC_{50}) value for antibiotics was designated by some authors. The IC_{50} and EC_{50} values of chosen antibiotics are shown in Table 2. However, different values are reported by the authors for the same antibiotics, especially OTC. This phenomenon may be due to the diversity of the biomass used or the test length as well as exposure time and concentration of the antibiotic (Sguanci et al. 2017). Moreover, it is worth to notice, that all presented EC_{50} and IC_{50} values (Table 2) are much higher than concentration of antibiotics that occur in real wastewater. For instance, the OTC concentration in manure samples have reached even the concentration of 250 mg L^{-1} (Zhang et al. 2014) which is also lower than IC_{50} value presented for this antibiotic. Therefore, there is no possibility to observe acute toxicity of the anammox bacteria used for real wastewater treatment.

Most of the antibiotics used show a negative dose-dependent effect on the anammox process during short-term tests. However, microbial biomass with low biodiversity was found to be more resistant to antibiotics. For antibiotics such as penicillin G and streptomycin, the long doubling time of the anammox bacteria makes it impossible to determinate its inhibition in such a short incubation time (Hu et al. 2013). Thus, to test the effect of these antibiotics, a longer experiment is required. Moreover, in most cases, antibiotic concentrations used in short-term tests were similar to those showing acute effect; therefore, it is difficult to assess short-term effect of antibiotics in practice, because such high concentrations do not occur in real wastewater.

Long-term exposure effects

Due to fact that anammox bacteria have long double times (9–29 days) as well as a slow growth rate (0.0033–0.001 d^{-1}) (Van de Graaf et al. 1996; Strous et al. 1998, 1999), the response of anammox bacteria to antibiotics may be longer. For these reasons, long-term exposure must be investigated. Table 3 reports on the long-term effect of different concentrations of antibiotics on the anammox process.

Phanwilai et al. (2020) compared the effect of CAP on granular and flock anammox biomass. In that study, the authors reported that the impact of 6 mg L^{-1} CAP on both types of biomass was similar. Further, their data indicates that the SAA decreased by 86% and 82%, respectively. However, when the concentration was increased to 1000 µg L^{-1}, a slight increase in SAA was observed. The authors concluded that a granular system is more resistant to antibiotic inhibition relative to a flock system. Therefore, anammox granules seem to be preferred for use in antibiotic-contaminated wastewater treatment. This is primarily because of the multilayered structures with two distinct regions — dispersible and stability layers. The outer layer is dispersible because the particles adhere to each other through weak interaction, i.e., extracellular polymeric substances (EPSs) ion bridging via multivalent ions and van der Waals forces. The inner region, composed of compact EPSs, is stable and the particles interact strongly through polymer entanglement (Zhang et al. 2015). However, the concentration of CAP in the effluent of anaerobic treatment process could be higher than in the effluent of WWTPs, where conventional systems (nitrification/denitrification) are used, because CAP would not bio-degrade under anaerobic processes (Michael et al. 2013).

Yang et al. (2013) indicated that the IC_{50} value of OTC is 517.5 mg L^{-1} for the anammox process, however, a concentration of only 50 mg L^{-1} can deteriorate the nitrogen removal ability of anammox bacteria within 7 days. Furthermore, 5 ± 3.5 mg L^{-1} of OTC completely inhibited the anammox process by 5 weeks (Noophan et al. 2012). An equally strong effect on anammox bacteria was caused by

Table 2 EC_{50} and IC_{50} values of antibiotics for anammox bacteria

Antibiotics	EC_{50} value	IC_{50} value	Exposure time	References
Chloramphenicol	420 mg L^{-1}	-	5 min	Fernández et al. 2009
	390 mg L^{-1}	-	15 min	Fernández et al. 2009
Tetracycline hydrochloride	94 mg L^{-1}	-	5 min	Fernández et al. 2009
	42 mg L^{-1}	-	15 min	Fernández et al. 2009
Oxytetracycline	-	517.5 mg L^{-1}	-	Yang et al. (2013)
	-	518 mg L^{-1}	-	Zhang et al. (2014)
	-	1100 mg L^{-1}	24 h	Lotti et al. (2012b)
Doxycycline	-	121 mg L^{-1}	24 h	Alvarino et al. (2014)
	-	665 mg L^{-1}	24 h	Sguanci et al. (2017)
	-	378 mg L^{-1}	48 h	Sguanci et al. (2017)
Tiamulin	-	920 mg L^{-1}	24 h	Sguanci et al. (2017)
	-	371 mg L^{-1}	48 h	Sguanci et al. (2017)
Enrofloxacin	-	157 mg L^{-1}	24 h	Sguanci et al. (2017)
	-	144 mg L^{-1}	48 h	Sguanci et al. (2017)
Sulfathiazole	-	650 mg L^{-1}	24 h	Lotti et al. (2012b)

TCH (Fernández et al. 2009). From these data, Fernández et al. (2009) suggested that the application of the anammox process to pharmaceutical wastewater treatment requires the simultaneous dual-usage of a degradation technique, which should mitigate the negative effect of these biodegradable-resistant antibiotics. Such methods may include photo-degradation and chlorination (Addamo et al. 2005; Qiang et al. 2006). It was found that anammox bacteria can adapt to the low concentration of SM and SDM (< 3 mg L^{-1}) when incubated in an up-flow anaerobic sludge blanket (UASB) reactor, because of increasing EPSs production as a defense mechanism. Further, the inhibition and accumulation of ammonium at 9 mg L^{-1} of SM and SMD were observed. However, SM in concentrations between 5 and 9 mg L^{-1} was more inhibitory than SDM due to the decrease in the use of nitrite by anammox bacteria, which led to the accumulation of nitrite (Du et al. 2018).

In contrast to the short-term effect, trace concentrations (1 μg L^{-1}) of NOR and ERY for 30 days was reported to decrease anammox activity by 30% and 1.4%, respectively (Zhang et al. 2019a). These results highlight the stronger inhibitory action of NOR relative to ERY for the suppression of the anammox bacterial. However, the action of ERY seems to be different for each type of wastewater treatment bacteria. For example, the addition of 1 mg L^{-1} of ERY slightly inhibited nitrite-oxidizing bacteria (NOB) during long-term exposure (Du et al. 2016), and dramatically decreases phosphorus removal process within 7 days (Hu et al. 2018a, b). A comparison of the action of the three broad-spectrum antibiotics: mainly amoxicillin (AMX), florfenicol (FF), sulfamethazine (SMX) was studied by Zhang et al. (2015). Long-term testing in the UASB has shown that the significant inhibition of the SAA was observed when the concentration of antibiotics was 1000, 230, 128 mg L^{-1} for AMX, SMZ, FF, respectively. Moreover, the SAA decreased by approximately 50% within the first 3 days.

Similar to the short-term exposure studies, the long-term exposure studies mainly used high concentrations of antibiotics that do not occur in real wastewater. There are only a few studies that use concentrations of antibiotics found in the aquatic environment (few μg L^{-1}) including wastewater. Zhang et al. (2019a, b) used ERY and NOR to investigate their effect on the anammox process under 1 μg L^{-1}. In both studies, no inhibitory effect of such low concentrations of antibiotics on the anammox process was demonstrated. Furthermore, the results presented in Table 3, obtained in these works, should not be taken as a real decrease in the activity of the anammox process, since there is a large scatter in the results when based on activated sludge. Therefore, such slight changes in the activity of the anammox process should not be considered as a direct effect of the antibiotics influence on the process.

Antibiotics co-occur in aquatic environments. Thus, it is necessary to study their joint effect on the anammox systems. To assess this, the individual and combined effect of OTC and SMX was investigated by Zhang et al. (2019c). Their results showed that the anammox process deteriorated when the concentration of both antibiotics reached 1 mg L^{-1}. However, the impact of separate antibiotics on anammox was higher than the combined groups.

Long-term effect of antibiotics should be connected with sludge retention time (SRT) that is used in experiment. For the anammox process, SRT should range from 30 to 60 days or even more (Chowdhury and Nakhla 2021). In the results presented in Table 3, it can be seen that, in most cases, the exposure time is lower than the optimal SRT for the anammox process mentioned above. The use of too short SRT may lead to a decrease in the efficiency of the anammox

Table 3 Summary of long-term effect of antibiotics on AnAOB

Antibiotic	Concentration	Reactor	Biomass	Effect	Operating time	References
Chloramphenicol	200 mg L^{-1}	FBR	Granules	Anammox process activity decreased by 68%	After 3 days	Van der Graaf et al. (1995)
	20 mg L^{-1}	FBR	Granules	Anammox process activity decreased by 36%	After 3 days	Van der Graaf et al. (1995)
	20 mg L^{-1}	SBR	Granules	Anammox process activity decreased by 25%	22 days	Fernández et al. 2009
	6 mg L^{-1}	SBR	Granules	SAA decreased by 86%	41 days	Phanwilai et al. (2020)
	6 mg L^{-1}	SBR	Flocs	SAA decreased by 82%	27 days	Phanwilai et al. (2020)
	1000 µg L^{-1}	SBR	Granules	SAA increased from 0.43 to 0.46 g N g^{-1} VSS d^{-1}	14 days	Phanwilai et al. (2020)
	1000 µg L^{-1}	SBR	Flocs	SAA decreased from 0.63 to 0.57 g N g^{-1} VSS d^{-1}	14 days	Phanwilai et al. (2020)
Tetracycline hydrochloride	10 mg L^{-1}	SBR	Granules	Anammox process activity decreased by 60%	37 days	Fernández et al. 2009
Oxytetracycline	50 mg L^{-1}	UASB	Granules	NRR decreased by 4.5 kg N m^{-3} d^{-1}	7 days	Yang et al. (2013)
	5 ± 3.5 mg L^{-1}	SBR	Granules	Complete inactivation of the anammox process	35 days	Noophan et al. (2012)
	1 mg L^{-1}	UASB	Granules	TNRE decreased from 88 to 62.4 ± 12.5% NRR decreased from 20 to 14.3 kg N $m^{-3}d^{-1}$	27 days	Zhang et al. (2019c)
	1–2 mg L^{-1}	UASB	Granules	TNRE decreased from 92.0 to 50.3% NRR decreased from to 3.6 ± 1.0 kg N m^{-3} d^{-1}	20 days	Shi et al. (2017)
	2 mg L^{-1}		Granules	NRR decreased to 60.0%	120 days	Zhang et al. (2018a, b)
Penicillin G	100 mg L^{-1}	FBR	Granules	Anammox process activity decreased by 36%	After 3 days	Van der Graaf et al. (1995)
	500–5000 mg L^{-1}	CMR	Flocs, Candidatus *Kuenenia stuttgartiensis* (free-living planktonic cells)	Completely inactivate of anammox process	21 days	Hu et al. (2013)
Ampicillin	800 mg L^{-1}	FBR	Granules	Anammox process activity decreased by 94%	After 3 days	Van der Graaf et al. (1995)
Streptomycin	100 mg L^{-1}	CMR	Flocs, *Kuenenia stuttgartiensis* (free-living planktonic cells)	Completely inactivate of anammox process, washout of anammox biomass and increase nitrite concentration in effluent from 0 to 5 mM	17 days	Hu et al. (2013)
Norfloxacin	1 µg L^{-1}*	Cylindrical biofilter	Biofilm	SAA decreased from 10.8 to 7.56 mg g^{-1} SS h^{-1}	30 days	Zhang et al. (2019a)

Table 3 (continued)

Antibiotic	Concentration	Reactor	Biomass	Effect	Operating time	References
Erythromycin	1 μg L^{-1}*	Cylindrical biofilter	Biofilm	SAA decreased from 10.8 to 10.65 mg g^{-1} SS h^{-1}	30 days	Zhang et al. (2019a)
Erythromycin	0.001*; 1; 10; 50 mg L^{-1}	UAF	Biomass enriched with Candidatus *Kuenenia* sp.	NRR decreased from 0.34 kg N m^{-3} d^{-1} to 0.3, 0.274, 0.2, 0.17 g N m^{-3} d^{-1}, respectively	119 days	Zhang et al. (2019b)
Sulfamethoxazole	1 mg L^{-1}	UASB	Granules	TNRE decreased from 88 to 57.3 ± 11.5% NRR decreased form 20 to 13.2 ± 2.7 kg N m^{-3} d^{-1}	27 days	Zhang et al. (2019c)
Oxytetracycline + Sulfamethoxazole	1 + 1 mg L^{-1}	UASB	Granule	TNRE decreased from 88 to 68.6 ± 10.7% NRR decreased from 20 to 15.2 ± 1.9 kg N m^{-3} d^{-1}	27 days	Zhang et al. (2019c)

CMR, Continuous membrane reactor; *FBR*, fluidized bed reactor; *NRR*, nitrogen removal rate; *SAA*, specific anammox activity, *SBR*, sequential batch reactor; *SS*, suspended solid; *TNRE*, total nitrogen removal efficiency; *UAF*, up-flow anaerobic biological filter; *UASB*, up-flow anaerobic sludge blanket digestion; *VSS*, volatile suspended solid. * Studies using the concentration of antibiotics that occur in real wastewater

process, which may be important for studies involving the effects of antibiotics (Chowdhury and Nakhla 2021).

To sum up, the differences between long- and short-term exposure are mainly due to the characteristics of anammox bacteria, which have a low growth rate. Thus, a short incubation period may be too short to characterize the effect mediated by antibiotic pollution (Hu et al. 2013). Additionally, the production of EPSs seems to be a self-protection mechanism of bacteria against antibiotics during short-term tests. Antibiotics could be adsorbed by the EPSs, reducing the short-time risk of direct contact of antibiotics and anammox bacteria (Xu et al. 2013; Zhang et al. 2015). Moreover, the inhibitory concentration of antibiotics is highly dependent on the different types of antibiotics. For example, tetracycline and sulfonamides present more significant disturbing in the anammox process than chloramphenicol or penicillin. In addition, the level of inhibition might also be attributed to the community structures, type of biological reactor/technology, and operational conditions.

Effect of antibiotics on anammox functional genes

The relative abundance of certain functional genes may be a useful tool to assess the efficiency of biological processes mediated by microorganisms (Zhi and Ji 2014). In the anammox metabolic pathways, there are three functional genes which are recognized to be the most important: nitrite reductase (*nir)* which catalyzes the conversion of nitrite into nitric oxide (Li et al. 2011), hydrazine synthase (*hzs*) which induces the transformation of nitric oxide into hydrazine, and hydrazine dehydrogenase (*hdh*) which covers hydrazine to nitrogen gas (Kartal et al. 2011).

It has been reported that SMD in concentrations ranging between 1 and 5 mg L^{-1} did not significantly affect the absolute abundance of *hzsA*. However, when the concentration reached 7 mg L^{-1}, the abundance dropped significantly from 1.1×10^6 to 3.0×10^5 copies/ng DNA. Further, at 1 mg L^{-1} of SMD, the abundance of *hzo* was markedly increased from 8.0×10^6 to 3.3×10^7 and next decreased at 7 mg L^{-1}. Similarly, SD at 1 mg L^{-1} caused a significant increase in *hzs* abundance. These data indicate that anammox bacteria are more sensitive to SMD (Du et al. 2018). A low concentration of SMX (0.1 mg L^{-1}) stimulated the expression of *nirS*, *hzsA* and *hdh* mRNA. Additionally, when the concentrations of OTC and SMX increased from 0.1 to 0.5 mg L^{-1}, the sensitivity of *nirS*, *hzsA* and *hdh* to these antibiotics was as follows: SMX > OTC > SMC + OTC. Thus, the response of *hzsA* to OTC at 1 mg L^{-1} is higher than SMX and SMX + OTX. On the other hand, *hdh* was the most sensitive to 0.1 mg L^{-1} OTC (Zhang et al. 2019c). Zhang et al. (2019c) demonstrated that within anammox bacteria, 2 mg L^{-1} of OTC was able to inhibit the *hzsA*, *hdh*, *nirS* genes at the mRNA level. The combined effect of Zn (II) and TC

on the *hzsA*, *hdh*, *nirS* expression level was studied by Fan et al. (2019). The authors found a similar trend in the relative abundance of these genes. When the concentration of Zn (II) and TC was 3.39 and 0.5 mg L^{-1}, respectively, the relative abundance was slightly increased. At higher concentrations of antibiotics (1.0 mg L^{-1}), the relative abundance of the functional genes gradually dropped below 0.1% and was significantly lower than that of the control reactor. Taken together, these lines of evidence highlight the impact of antibiotics on the expression of functional genes within the anammox process.

Antibiotic influence on the anammox microbial community

Current papers have shown that dominant phyla in the anammox system are *Planctomycetes*, *Proteobacteria* and *Firmicutes*, however, the quantity of *Firmicutes* was significantly lower than other phyla (Zhang et al. 2019b, c). However, physical and chemical parameters within these anammox systems strongly influence functional bacteria group responsible for the process. It has been reported in the literature that *Proteobacteria* include antibiotic-resistant bacteria (Shi et al. 2013). Further, Duan et al. (2017) also reported that *Proteobacteria* can use OTC as a carbon source. Similarly, *Betaproteobacteria* have a high antibiotic resistance genes exchange capability, for this reason, they are the dominant bacteria found in the OTC-rich wastewater (Liu et al. 2012). Additionally, the groups of bacteria with tetracycline resistance genes also include the *Firmicutes* phylum (Ghosh and LaPara 2007). Zhu et al. (2017) stated that *Gammaproteobacteria* have a good ability to obtain ARGs under high antibiotic concentration conditions.

Studies have reported that OTC at a concentration of 5 ± 3.5 mg L^{-1} induced significant changes in anammox community structure during a 5-week experiment. Further, it was found that Candidatus *Brocadia anammoxidans* and Candidatus *Kuenenia stuttgarteinsis* had a lower density at the 5-week timepoint as compared to the beginning of the experiment. However, higher numbers of *Nitrosomonas* sp. and *Nitrospira* sp. were observed (Noophan et al. 2012). From these data, the authors concluded that as nitrifying bacteria can survive at a concentration of 250 mg L^{-1} with only a 50% reduction in activity (Campos et al. 2001), they have a higher tolerance to OTC than anammox bacteria. Zhang et al. (2019c) found that Candidatus *Kuenenia* sp. gradually adapted to the OTC and SMX exposure with a relative abundance of over 31%. These data illustrate that the long-term adaptation to OTC and SMX may improve the tolerance to these antibiotics. However, the combined effect of these two antibiotics was found to be more severe for Candidatus *Kuenenia* sp. causing a significant decrease in their abundance which was proportional to the exposure time to the antibiotics. Nonetheless, *Candidatus Kuenenia* sp. is considered to be potentially an antibiotic-resistant bacterium (Zhang et al. 2018a, b). The combined effect of OTC and copper nanoparticles on the anammox bacterial community was shown in *Planctomycetes.* In contrast to the findings in *Proteobacteria*, the authors reported the occurrence of low antibiotic resistance after acclimatization to the potential inhibitor concentration (Cheng et al. 2020). The dominant bacteria in the anammox system, under combined TC and Zn exposure, were *Candidatus Kuenenia* sp. However, as the concentration of the inhibitors was increased, a dose-dependent decrease in relative bacterial abundance was observed. In contrast, when *Caldilinea* were exposed to an increasing concentration of the inhibitors, a significant increase in its relative abundance was observed. Together, these data suggest that *Caldilinea* is a potentially antibiotic-resistant bacteria (Fan et al. 2019).

The long-term exposure of anammox bacteria to ERY resulted in a decrease in bacterial abundance and the deterioration of nitrogen removal performance in the up-flow anaerobic biological filter (UAF) bioreactor (Zhang et al. 2019b). The most dominant genus in the anammox sludge was *Candidatus Kuenenia* sp., whose relative abundance decreased from 22.04% to 3.60% when exposed to 100 mg L^{-1} of ERY. Without antibiotic stress, the relative abundance recovered to 12.47% after 29 days, however, its abundance could not be rescued to its initial value. Similar changes were observed for another major species of anammox bacteria in the reactor, mainly *Candidatus Brocadia* sp., whose abundance decreased from the initial level of 0.45% to well below detection threshold during exposure to 100 mg L^{-1} of ERY and then increased to 0.01% during recovery phase (Zhang et al. 2019b). Zhang et al. (2019a) indicated that exposure to 1 μg L^{-1} of NOR and ERY has a slight impact on the abundance of the anammox bacteria in the system, which perhaps was due to a very low concentration of antibiotics. In agreement with the study above, the dominant bacteria were identified as *Candidatus Kuenenia* sp. Further, the analysis of the anammox bacterial community abundance showed that the relative abundance of *Candidatus Kuenenia* sp. decreases from initial level 4.31% (NOR) and 5.0% (ERY) to 1.87% and 4.99%, respectively, after 30 days of incubation with these antibiotics.

In a recent study, the inhibitory effect of SDM on anammox bacteria and the abundance of *Candidatus Brocadia* sp. were assessed. Du et al. (2018) found that exposure to SDM facilitated a decrease in relative bacterial abundance from 2.57% to 0.39%. However, the abundance of *Planctomycetes* first increased to 6.76% when exposed to 3 mg L^{-1} and then continuously decreased to 1.52% at a dose of 9 mg L^{-1}. Comparatively, the abundance of *Planctomycetes* steadily increased to 28.61% at a dose of 7 mg L^{-1} of SM and dropped rapidly to 11.42% at 9 mg L^{-1}. Together, these

data show that *Plactomycetes* and *Proteobacteria* are the main groups of bacteria present in the anammox systems. Further, these species have been shown to tolerate a low concentration of antibiotics. Moreover, *Candidatus Kuenenia* sp. belonging to the *Planctomycetes* phylum seems to be the most resistant bacteria of all anammox bacteria assessed.

Mechanism of anammox bacteria inhibition by antibiotics

The mechanisms of action of antibiotics can be divided into two basic categories: bactericidal drugs, which kill bacteria with high efficiency (> 99.9%), and bacteriostatic drugs, which inhibit bacterial growth (Kohanski et al. 2007). Antibiotics can enter through the transporters located in the cell membrane or may connect with receptors on the surface cell (Lu et al. 2021). It is understood that there are four inhibition or bactericidal mechanisms by which antibiotics act on the bacterial cell: (i) inhibition of cell wall synthesis; (ii) interaction with a cell membrane; (iii) inhibition of protein synthesis; (iv) inhibition of the transcription and replication of DNA (Zhang et al. 2019b). Further, the exact mechanism of bacteria inhibition, or killing, is dependent on the antibiotic concentration used. For example, antibiotics belonging to the TCs and CAP group interfere with the synthesis of bacterial mRNA by binding to the subunit of the ribosomes. TCs bind with the small subunit and CAP bind with the large ribosomal subunit (Nguyen et al. 2014). β-lactam antibiotics, such as penicillin inhibit the synthesis of the cell wall through the interaction with the penicillin-binding proteins and glycopeptides that associate with peptidoglycan of cell wall, inducing cell death. Antibiotics belonging to macrolide and aminoglycoside class can easily enter to cytoplasm and then produce intermediates D-glucose and L-streptose which can be further decomposed in the tricarboxylic acid cycle (Lu et al. 2021).

Anammox bacteria are characterized as slow-growth microorganisms. Furthermore, Levin-Reisman et al. (2017) indicated that slow-growing bacteria are more prone to develop antibiotic resistance than fast-growing bacteria. For instance, Liu et al. (2018) showed that slow-growing bacteria involved in the nitrification process are more likely to accept tetracycline resistance genes under trace TC exposure. It could be suspected that bacterial slow growth predisposes them to such adaptation. Zhang et al. (2019c) theorized that, due to the presence of OTC and SMX, there are four possible mechanisms of anammox bacteria that can be used to avoid the deleterious effects of antibiotic exposure: (i) produce protective layer of EPSs, (ii) aggregation of dominant microbes as a potential resistance species, (iii) regulation of the functional genes by dominant microbes as the adaptation effect to the external disturbance, (iv) increasing tolerance of anammox bacteria to SMX and OTC by the increased relative abundance of antibiotic resistance bacteria and ARGs transfer (Zhang et al. 2019c).

Analysis of the ARGs responsible for the acquired antibiotic resistance within anammox systems has highlighted the efflux pump as the most common resistance mechanism against OTC (Shi et al. 2017). Moreover, OTC stress may induce cell lysis (Yang et al. 2013). Sguanci et al. (2017) compared the efficacy of ENR and DOX on the anammox system. In their study, they showed that ENR can inhibit anammox bacteria at a faster rate than DOX. They attributed this effect to intracellular tetracycline and fluoroquinolone transport which may be facilitated by channel-forming proteins (Nikaido and Thanassi 1993). Further, channel-forming proteins genes are common in anammox bacteria (Kartal et al. 2011).

Additionally, ERY inhibited anammox bacteria by binding to the 23S rRNA molecule in the 50S ribosomal subunit, thereby inhibiting the translocation of peptidyl-tRNA and disrupting protein synthesis (Alighardashi et al. 2009; Zhang et al. 2019b). Recent research has shown that a low dose exposure of anammox bacteria to ERY (1 μg L^{-1}) can induce the expression of the two ARGs, *ermB* and *mphA*. The presence of *ermB* and *mphA* facilitates obtaining an antibiotic tolerant phenotype. Further, the authors reported that, at similar trace levels of NOR (1 μg L^{-1}), there was no protective effect induced (Zhang et al. 2019a). It is understood that *ermB* induces antibiotic tolerance by enhancing the production of polysaccharides on the cell membrane, thus reducing the antibiotics' ability to penetrate the cell (Zhu et al. 2013). Moreover, *mphA* induces chemical modifications on macrolide antibiotics thereby inducing the degradation of antibiotic or replacing the active group via the expression of synthetic oxidoreductase (Guo et al. 2015, Zhang et al. 2019a). CAP belongs to the class of antibiotics which are effective against Gram-negative bacteria. However, due to the fact that antibiotics are selectively toxic, it can also inhibit prokaryotic and eukaryotic cells alike. CAPs mode of action is achieved through the inhibition of peptide bond synthesis during protein synthesis (Dapena-Mora et al. 2007).

Theoretically, anammox bacteria should not be sensitive to antibiotics which affect the synthesis of the cell wall such as β-lactam antibiotics as many of these species have peptidoglycan-free cell walls (Cayrou et al. 2010; Hu et al. 2013; Zhang et al. 2019b). AMX is a broad-spectrum β-lactam antibiotic belonging to the aminopenicillin family which inhibits cell wall synthesis by interfering with the cross-linking of peptidoglycan (Hu et al. 2013). Sulfonamides act by inhibiting the synthesis of nucleic acids (Lotti et al. 2012b). FF is a widely used chloramphenicol-type antibiotic, which inhibits transpeptidase and disrupts the functional ability of the 50S ribosomal subunit thereby inhibiting protein synthesis (Ding et al. 2015).

An interesting phenomenon was observed during the combined effect of azithromycin, norfloxacin, sulfamethoxazole and trimethoprim on aerobic granules. Bacteria were distributed in the external and intermediate layer of the granule. Further, this external layer was compact, and bacteria were arranged perpendicularly like a protective barrier. This compact layer was likely formed by the accumulation of dead cells (Rodriguez-Sanchez et al. 2017).

The influence of antibiotics on the antibiotic resistance genes presented in anammox system

Antibiotic resistance is recognized as a global threat to human and animal health, with many bacterial species, including anammox bacteria, having developed some form of resistance toward antibiotics (Felis et al. 2019). One of the main risks for the presence of antibiotics in wastewater is the spread of ARGs (Kumar et al. 2019; Felis et al. 2019). Specifically, the presence of antibiotics in wastewater has been identified as an important factor in the selective adaption of resistance genes in many bacterial species (Zhang et al. 2019b; Shi et al. 2017; Li et al. 2016). Moreover, it is suggested that slow-growing bacteria, as anammox, could contribute to the enrichment of antibiotic resistance genes due to development of antibiotic tolerance (Fu et al. 2020; Langbehn et al. 2021). It is claimed that increasing abundance of ARGs can contribute to proper nitrogen removal, as the bacterial community gains resistance to the exposed antibiotic (Langbehn et al. 2021), which seems to be advantage to nitrogen removal. Moreover, it has been proven that many bacteria involved in nitrogen removal from wastewater or occurring in denitrifying systems, such as *Nitrospira*, *Ignavibacterium*, and *Comamonas* play an important role as ARG-carrying bacteria. The role of anammox bacteria as hotspot of antibiotics resistance genes was investigated by Fan et al. (2021). The authors presented positive correlation between *Candidatus Kuenenia* and *sul1* (gene encoding resistance for sulfonamides). On the other hand, network analysis has shown that *Candidatus Kuenenia* was predicted to be a host of *sul1* and e*rmF* under ETS (erythromycin) + SMZ stress, while under single antibiotics stress both genes were independent, which suggest that potential host of ARGs may depended on different antibiotics stress. Zhang et al. (2020) found strong interaction between *intI1* and *ereA* in anammox system under spiramycin (SPM) exposure. In fact, strong correlation between *intI1* and *ereA* has been previously reported (Thungapathra et al. 2002; Liu et al. 2014) indicating that anammox bacteria belonging to *Planctomycetes* can be the host of ARGs in nitrogen removal systems.

Zhang et al. (2019c) investigated the presence and changes in the absolute abundance of ARGs such as *tetX*, *tetC*, *tetG*, *tetM*, *sul1*, *sul2* in the anammox biomass conducted in UASB reactor under OTC, SMX and OTC + SMX antibiotic pressure. These genes can be divided into three groups according to the different resistance mechanisms: efflux pump (*tetC*, *tetG*), ribosome protection (*tetM*) and enzymatic inactivation (*tetX, sul1*, *sul2*). In their study, the highest abundance was presented by *tetX* which was more than one order of magnitude greater than that of *tetG*, *tetM*, *intI1*, *sul1*, *sul2*. Additionally, *tetC* was identified to have the lowest abundance (Zhang et al. 2019d). Similarly, Zhang et al. (2019d) reported that, under OTC drug pressure, the highest absolute abundance of ARGs were *tetX* and the lowest for *tetC*. Further, the total number of ARGs increased after the acclimation of anammox sludge to the long-term exposure of OTC. Typical tetracycline resistance genes: *tetA*, *tetB*, *tetC* and *tetX* were also detected by Shi et al. (2017). In that study, the relative abundance of *tetA*, *tetB*, *tetC* increased after the addition of OTC (1 mg L^{-1}) and the highest peak was reached on day 52, where the concentration of OTC was 2 mg L^{-1}. Overall, the relative abundance of the ARGs assessed in descending order was as follows: *tetA* > *tetX* > *tetC* > *tetB*. After 1 month of exposure to NOR and ERY (1 μg L^{-1}), two resistance genes (*ermB*, *mphA*) were detected at a low abundance, both of which target ERY (Zhang et al. 2019a). The relative expression of *ermB* increased from 2.08×10^{-4} to 3.53×10^{-4}%. Additionally, *mphA* increased from 4.48×10^{-5} to 5.00×10^{-4}%. It is worth noting that during this experiment the presence of NOR caused a significant decrease in nitrogen removal efficiency. Further, ERY exposure slightly impacted the anammox process, highlighting the role of ERY-ARGs on anammox bacteria in the induction of protection against this antibiotic.

Recovery of anammox process after antibiotics inhibition

Depending on the type of the substance and concentration of the antibiotic tested, the negative effect of antibiotics on anammox performance can be either reversible or irreversible (Gonzalez-Martinez et al. 2018). Fernández et al. (2009) reported that, after incubation with 20 mg L^{-1} of CAP, the anammox process can recover up to 59% of its initial level after 2 months. However, exposure to tetracycline hydrochloride (100–1000 mg L^{-1}) induced a complete deactivation of the biomass. In this instance, the recovery of the anammox process would require a fresh inoculation of anammox bacteria. Other authors have suggested that the long-term acclimatization of anammox bacteria to the conditions in the reactor, in which the process is conducted, is useful for the recovery of stable anammox performance (Zhang et al. 2015; Rodriguez-Sanchez et al. 2017).

Two strategies of the recovery of anammox performance after OTC shock were assessed by Yang et al. (2013): (i) addition of low concentration of antibiotics within a long

recovery period, (ii) no OTC addition combined with sludge addition. Despite the use of strategy (i) comprising of low concentration exposure of OTC (6 mg L^{-1}) and long recovery period (36 days), it would be difficult to recover the anammox process after high level of OTC used (50 mg L^{-1}). A more useful strategy to facilitate the recovery if the anammox performance would be the addition of fresh anammox sludge. The increase in volatile suspended solid (VSS) by approximately 3.3 g resulted in an increase in SAA from a 0.8 mg TN g^{-1} VSS^{-1} to 13.7 mg TN g^{-1} VSS^{-1}. The addition of sludge is a powerful method to recover the nitrogen removal capability which is lost due to the action of contaminants. This strategy was also confirmed by Tang et al. (2011) and Yang and Jin (2012). Zhang et al. (2019d) investigated two possible mechanisms of the anammox recovery after OTC exposure: addition of fresh sludge without discharge from this reactor throughout the experiment and addition of fresh anammox sludge with discharge the same volume of sludge. Obtained results have shown that exchange of biomass was superior to addition biomass in recovering the anammox performance after OTC exposure. Moreover, they reported that recovery mechanism performance aided with BA is connected with the combined contribution of functional gene regulation, the presence of efflux pumping ARGs and the self-defense role of EPSs (Zhang et al. 2019d). Yao et al. (2018) suggested that the addition of anammox sludge can stimulate the recovery process as a result of quorum sensing (QS). However, this hypothesis requires further validatory research. Taken together, bio-augmentation, through the addition of sludge, can be a useful method to mitigate the inhibitory effect of OTC, due to gradually acclimation of the microbial communities (Jin et al. 2014). Moreover, Jin et al. (2014) suggested that there are two major parameters influencing the proper recovery process: bio-augmentation time (BAT) and bio-augmentation dosage (BAD).

The negative effect of antibiotics on the anammox process can be also mitigated by adsorption and biodegradation of antibiotics by anammox bacteria (Alvarino et al. 2015). Zhang et al. (2014) suggested that recovery was mainly attributed to biodegradation of residual OTC under anaerobic conditions. However, there is lack of information about possible biodegradation mechanism. Degradation of sulfadiazine; sulfamethazine, and sulfamethoxazole by nitrogen cycle bacteria was speculated by Langbehn et al. (2021). The suggested degradation pathway was deamination catalyzed by deaminase, hydroxylation by ammonia monooxygenase, and nitration by hy-droxylamine dehydrogenase. Therefore, several enzymes are able to catalyze antibiotic degradation during the biological nitrogen removal. However, it depends on the antibiotic molecular properties and cannot be unified to all antibiotics (Langbehn et al. 2021). In case of OTC, no sorption and volatilization would be expected (Carucci et al. 2006; Sponza and Çelebi 2012). On the other hand, some studies have reported good sorption capacity of tetracyclines antibiotics onto activated sludge flocs surface (Felis et al. 2019), which caused the accumulation of this substance in sludge. Moreover, trace concentration of such antibiotics as SM, sulfamerazine and, sulfadiazine can be adsorbed by hydrophobic regions of EPSs, making anammox process easy to recover, because these antibiotics don't affect the bacteria (Hou et al. 2016). However, this led to accumulation of these antibiotics in sludge. Unfortunately, there is a lack of knowledge describing the impact of antibiotics accumulation on the anammox process and process recovery. Zhang et al. (2013) concluded that the changes in the pH value, stoichiometric ratios, SAA and properties of the anammox granules could be useful indicators for the degree of anammox recovery. Moreover, the recovery velocity, recovery time and degree of recovery are important indices for evaluating the recoverability of the anammox performance within the system (Cai et al. 2009; Zhang et al. 2014).

Influence of antibiotics on the anammox biomass properties

Production of EPSs

EPSs play an important role in anammox sludge aggregation. Their physicochemical properties such as surface charge, settling properties, dewatering properties, flocculation and adsorption ability and location outside the cell make them an important part for maintaining microbial aggregate's structure and function (Sheng et al. 2010; Zhang et al. 2014). Moreover, EPSs can affect wastewater treatment capacity and are produced in response to stressors such as the presence of antibiotics (Zhang et al. 2014, 2018a, 2019c). The main components of EPSs are proteins and polysaccharides which can influence the bacterial surface charge, hydrophobicity and sorption capacity. These proteins can provide adsorption abilities due to the hydrophobic region which include functional groups such as carboxyl, amine and hydroxyl group (Sheng et al. 2010). Therefore, EPSs can adsorb tetracycline antibiotics and sulfamethazine, sulfamerazine and sulfadiazine (Hou et al. 2016; Zhang et al. 2019c).

Zhang et al. (2018b) reported that increasing the concentration of NOR from 0.001 to 100 mg L^{-1} facilitated a drastic decrease in detectable protein from 74.06 mg g^{-1} SS to 24.04 mg g^{-1} SS at 0.001 mg NOR L^{-1} which gradually increased to 198.93 mg g^{-1} SS in 50 mg NOR L^{-1}. However, at 100 mg L^{-1} NOR, the decrease of detectable proteins was observed again. Like the changes in protein abundance, the dynamics of detectable polysaccharides elicited a similar trend in response to antibiotics pressure. These studies show that as the anammox bacteria begin to acclimatize to the presence of NOR, the anammox biofilm secreted more EPSs, mainly proteins which had a significant impact on antibiotic

protection (Zhang et al. 2018b). However, at 100 mg L^{-1} of NOR, the concentration of antibiotic exceeded the resistance capacity of the anammox biofilm, and the process was inhibited, as measured by a decrease in EPSs. These results agree with the finding of Zhang et al. (2019b) who indicated that at a concentration of 0.001–50 mg L^{-1}, ERY induced a significant increase in EPSs production. However, at a concentration of 100 mg L^{-1}, the secretion of EPSs was reduced. Zhang et al. (2019c) observed that, during a gradual increase in OTC and SMX (0.1–1 mg L^{-1}) concentration, the EPSs content initially decreased, then increased at 0.5 mg L^{-1} and then decreased further with 1 mg L^{-1}. It is worth noting that the final EPSs content was higher than the initial measurement (Zhang et al. 2018b, 2019a, b, c, d). Du et al. (2018) observed that SDM and SM above 5 mg L^{-1} caused a decrease in EPSs secretion, however, at concentrations lower than 3 mg L^{-1} the opposite effect was observed. Taken together, these data show that EPSs serves as a protective barrier to delay the penetration of hazardous agents into the cell, however, EPS-defense can be ablated by antibiotic overload (Zhang et al. 2015, 2016).

Heme c synthesis

Heme is an important part of the anammox key enzymes such as hydrazine synthesis (*hzs*), hydroxylamine oxidoreductase (*hao*) and hydrazine oxidase (*hzo*) which are involved in energy metabolic pathways (Jetten et al. 2009; Zhang et al. 2014). Moreover, the content of heme c may relate directly to anammox activity (Chen et al., 2013; Sabine Marie et al. 2015). Zhang et al. (2014) investigated influence of OTC (518 mg L^{-1}) on the anammox process under shock exposure while monitoring the changes of heme c in pre-, during- and post-shock period. The content of heme c before the shock period was 0.17 ± 0.001 μmol g^{-1} VSS. During the shock period, the level of heme c was reduced to 0.14 ± 0.001 μmol g^{-1} VSS. Additionally, after 105 h of recovery time, the heme c content was 0.12 ± 0.001 μmol g^{-1} VSS. A similar trend was observed during a higher OTC concentration (1731 mg L^{-1}) shock treatment. Changes in heme c coincided with a decrease in the anammox activity, but the recovery of heme c was delayed. Other studies have indicated that concentrations of OTC and SMX ranging between 0.1 and 1 mg L^{-1} elicited a decrease in heme c content in the anammox granules assessed. However, a mixture of these antibiotics caused fluctuations in the heme c content. For instance, the addition of 0.5 mg L^{-1} OTC + SMX increased the heme c content by 8.78%, meanwhile the addition of 1 mg L^{-1} OTC + SMX decreased the heme c content by 70.1%. These results differ in resistance properties to anammox bacteria by separated and mixed antibiotics (Zhang et al. 2019c). An increase in heme c content was also observed by Meng et al. (2019). The authors found that under a low concentration of TC (1, 10 and 100 μg L^{-1}) heme c content was increased, whereas exposure to 1000 μg L^{-1} caused a decrease in heme c content. Further, the authors found that FF rapidly reduced the heme c content by 20% from within 3 days, however, when the addition of FF is stopped, heme c returned to baseline (Zhang et al. 2015). It was reported that under OTC exposure, the color of anammox granules were fading (Yang et al. 2013). The red color of anammox sludge is attributed to the presence of heme c (Shi et al. 2017). Thus, the loss of red color is frequently regarded as an indicator of anammox activity (Tang et al. 2011; Yang et al. 2013).

Conclusion and prospects

Anammox is one of the most promising processes for nitrogen-rich wastewater removal, thus it is important to understand the impact of potential inhibitors present in the wastewater which may affect this process. There is no doubt that antibiotic pollution is a serious threat to the anammox process. Previous studies have shown that they can decrease anammox process activity, change community structures and alter the properties of the anammox biomass. Further, the effect of antibiotics depends on numerous factors such as antibiotic type, concentration or presence of coexisting inhibitors/pollutants (heavy metals, sulfides, nanoparticles). Knowledge of the impact of antibiotic and other co-pollutants on the anammox process is still unclear. Within this context, further research should focus on:

- Extending knowledge on the different antibiotics present in wastewater which may impact the anammox process. Further, the varying cellular structure and metabolism of anammox bacteria relative to other wastewater treatment bacteria, implies that the effect of antibiotics may be different and should be assessed further. However, there are a lot of antibiotics that occur in high concentrations in the aquatic environment. One such example is ciprofloxacin which was found in 6453 ng L^{-1} in raw wastewater. It is likely that, at these concentrations, this antibiotic may constitute a serious risk to activated sludge microorganisms. Moreover, as discussed in this review, some antibiotics such as β-lactams, have been thought to have no effect on anammox processes. However, their negative effect on the anammox system has been experimentally confirmed. Therefore, it is worthwhile to study a broad spectrum of antibiotics on the anammox process to create a holistic understanding of these compounds on this system.
- Extending knowledge about the combined effect of antibiotics and other pollutants which may co-occur in wastewater. As was reported above, antibiotics always occur

simultaneously with other contaminants in the aquatic environment, especially in wastewater. Besides the synergistic or antagonistic effect of combined contaminants on the anammox process effectiveness, these contaminants can also affect the spread of ARGs. The presence of antibiotics and heavy metals may stimulate the development of ARGs and heavy metals resistance gene expression, which are located in one of the mobile elements, such as plasmids or transposons. Moreover, a positive correlation between heavy metals stress and the abundance of ARGs was reported (Xu et al. 2017).

- Extending knowledge on the fate of antibiotics in the anammox systems. Antibiotics may undergo various changes in biological systems, i.e., biodegradation, biotransformation and sorption. Sorption is an important aspect in the field of ciprofloxacin (CIP) research. Previous studies have shown that 80% can be adsorbed into sludge (Golet et al. 2003, Michael et al. 2013, Felis et al. 2019). A similar effect was described for TC (Felis et al. 2019). Biodegradation and biotransformation may result in the accumulation of metabolites or incomplete transformation products. Further, the partial metabolites of antibiotics can be more dangerous than antibiotics themselves. For example, amoxicillin-S-oxide is a metabolite of amoxicillin and contain β-lactam ring, which may lead to the development of resistance in bacterial cells (Elizalde-Velázquez et al. 2016). Understanding the fate of antibiotics and their metabolites will be key to establishing efficient anammox systems in the future.
- The effect of antibiotics on combined processes. To improve the efficiency of nitrogen removal from wastewater, the anammox process is often combined with partial nitrification or partial denitrification. Few studies have investigated the effects of antibiotics on coupled processes, where their response to antibiotics may vary. This is a topic on which research should be focused on future studies.
- Previous studies focused mainly on synthetic antibiotic-contaminated wastewater treatment in the anammox process under laboratory condition. Therefore, there is a need to evaluate effect of real antibiotic-contaminated wastewater in the pilot-scale or full-scale systems to allow practical application of the anammox process.
- Studies should be conducted to determine the concentration of antibiotics that occur in wastewater treated by the anammox process. There are many studies in which the anammox process is used to treat wastewater containing antibiotics, but the concentration of antibiotics in the wastewater is not tested. Therefore, it is not possible to evaluate the concentration effect of antibiotics on the anammox process, because the concentration is unknown. This will be essential for both, future process studies and the establishment of efficient anammox systems in the future.

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Microbial response of the anammox process to trace antibiotic concentration

Filip Gamoń

Anna Banach-Wiśniewska

Jaspreet Jandoo Kaur

Grzegorz Cema

Aleksandra Ziembińska-Buczyńska

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Microbial response of the anammox process to trace antibiotic concentration

Filip Gamoń [a,*], Anna Banach-Wiśniewska [a,b], Jaspreet Jandoo Kaur [a], Grzegorz Cema [a], Aleksandra Ziembińska-Buczyńska [a]

[a] Environmental Biotechnology Department, Silesian University of Technology, Akademicka 2A, 44-100 Gliwice, Poland
[b] Regional Center for Water and Wastewater Management Co., Tychy, Poland

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ABSTRACT

This study investigated the inhibitory effect of three antibiotics: oxytetracycline (OTC), ciprofloxacin (CIP), and clarithromycin (CLA) on the anammox process conducted in four sequencing batch reactors (SBRs). The concentration of each antibiotic was 0.001 mg L^{-1}, similar to concentrations observed in municipal wastewater treatment plants. The specific anammox activity (SAA) of the anammox process was measured using a batch test, the microbial community structure was analyzed using high-throughput sequencing, and the variation of functional genes and antibiotic resistance genes (ARGs) abundance were measured by qPCR. These results indicated that none of the antibiotics significantly impacted nitrogen removal rate (NRR) in the anammox process and specific anammox activity (SAA). Analysis of the functional gene abundance variation showed significant inhibition of *hzo* by antibiotics while in control and increased from 3.88×10^{-6} to 1.26×10^{-5}. The relative abundance of almost all detected ARGs increased (*tetC* and *tetW* targeted OTC, *qnrB4* and *qnrS* targeted CIP). Only two ARGs (*tetX* targeted OTC and *mphA* targeted CLA) decreased (from 1.62×10^{-2} and 7.45×10^{-3} to 3.74×10^{-4} and 9.3×10^{-5}, respectively). Metataxonomic analysis showed a decrease in the relative abundance of anammox bacteria genera (Candidatus *Brocadia*, Candidatus *Jettenia*) in each tested reactor, and a significant increase of *Nitrospira*, regarded as a comammox microorganism. The purpose of this study was to evaluate the effect of trace concentration of three commonly detected antibiotics (OTC, CIP, CLA) in wastewater on the anammox process. Furthermore, CIP and CLA have never been investigated relative to the anammox process.

1. Introduction

Environmental contamination with antibiotics has been an important issue for decades, especially for wastewater treatment. Antibiotics exist in many types of wastewaters including municipal wastewaters [1], landfill leachates, livestock [2], hospital and pharmaceutical industry wastewaters [3,4]. The concentration of selected antibiotics in various wastewater types ranged from a few ng L^{-1} to a few μg L^{-1} [5]. The concentrations of ciprofloxacin (CIP) and norfloxacin (NOR) in hospital wastewaters reached 26 μg L^{-1} and 37 μg L^{-1} [3], respectively, and 0.82–147 ng L^{-1} and 11.1–964 ng L^{-1} in municipal wastewaters [6]. However, the concentration of CIP in wastewater may reach even 6.453 μg L^{-1} [5]. Moreover, the concentrations of sulfonamides and macrolides were measured as 22.07 and 85.1 μg kg^{-1} dry weight, respectively, in sewage sludge from wastewater treatment plants (WWTPs). The occurrence of antibiotics in wastewater systems may negatively impact microorganisms with short-term effects [7]. Moreover, long-term exposure of antibiotics towards microorganisms, even at sub-inhibitory concentrations, can cause chronic toxicity [8]. With that in mind, the presence of antibiotics in wastewater has become a challenge for biological treatment systems. Another problem connected with the release of antibiotics into wastewater relates to the development of antibiotic resistance genes (ARGs) and antibiotic resistant bacteria (ARB) [5]. Wastewater provides a fertile environment for ARGs transfer and hosting ARGs into the environment; therefore, the problem with ARGs requires attention [9].

Anaerobic ammonium oxidation (anammox) became one of the more promising options for ammonia-rich wastewater treatment because of its high nitrogen removal efficiency combined with significant cost savings for aeration, dosing external organic carbon, and production of less sludge [10]. Anammox bacteria convert ammonium into nitrogen gas using nitrite as electron acceptors under anaerobic conditions [11].

* Corresponding author.
E-mail address: filip.gamon@polsl.pl (F. Gamoń).

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However, anammox bacteria are sensitive to various substances present in wastewater and may inhibit the anammox process. Therefore, the sensitivities of anammox bacteria to different wastewater contaminants have restricted implementing anammox treatments in WWTPs, and anammox inhibition has been previously reported. In a short-term test, Fernandez et al. [12] showed that 250 mg L^{-1} of chloramphenicol reduced anammox activity by 20% within 24 h. In a long-term test, oxytetracycline (OTC) at 2 mg L^{-1} decreased nitrogen removal efficiency by 60% for 120 days [13]. However, the effects of antibiotics on anammox bacteria abated when the microorganisms produce extracellular polymers (EPSs) that have a protective function [14]. Occurrence of antibiotics in wastewater is related to the development of antibiotic resistance genes (ARGs) and antibiotic resistant bacteria (ARB), which become risk for human healthcare system [5] Moreover, the presence of antibiotic resistance-inducing genes in the bacterial genome allows bacteria to protect themselves against negative antibiotic actions. Despite the fact, that there are a few studied investigated development of ARGs in the anammox system during antibiotic stress [23,27], the knowledge about the transfer of ARGs between bacteria in the wastewater treatment system is still poor. Therefore, clarifying the behavior of ARGs in anammox system is essential.

The main objective of this study was to investigate the effect of three antibiotics (oxytetracycline (OTC), ciprofloxacin (CIP), and clarithromycin (CLA)) on the effectiveness of the implemented anammox process in a sequencing batch reactor (SBR) and a microbiological community structure, and formation of antibiotic resistance in an anammox system under environmentally relevant antibiotic concentrations (~0.001 mg L^{-1}). OTC, CIP, and CLA were selected because they are widely used in medicine, animal breeding and as a growth promoter for animals. A previous study reported that OTC strongly suppressed anammox behavior, however, its effect was tested at concentrations above 1 mg L^{-1}, significantly higher than concentrations observed in municipal wastewaters [13,15]. From previous data, CIP (0.2–2.0 mg L^{-1}) reduced nitrogen removal efficiency during nitrification-denitrification by 10.6–15.3% [16]. However, there is a lack of information regarding CIP and CLA on the anammox process. Furthermore, to account for the reaction of anammox bacteria to antibiotic stresses, short-term and long-term experiments were conducted (a batch test and in an SBR, respectively).

2. Materials and methods

2.1. Experimental set-up and analytical methods

Experiments were conducted in four 1 L SBRs and placed in a thermostatic chamber at 35 °C, as shown in Fig. 1. Each reactor was operated on 1 cycle per day for 30 day with 2 days of hydraulic retention time (HRT) and replacement rate 50% and DO concentration was below 0.1 mg L^{-1}. The first reactor (control) was carried out without adding antibiotics, while 0.001 mg L^{-1} of OTC, CIP, and CLA were added to the other three reactors (R-OTC, R-CIP, and R-CLA, respectively). Each reactor was inoculated with an equal amount of biomass and volatile suspended solids (VSS) (1.2 ± 0.6 g VSS L^{-1}). Suspended anammox biomass used in this experiment was taken from a laboratory-scale sequencing batch reactor (SBR; a volume of 20 L), previously inoculated with anammox sludge from a full-scale deamonification SBR in Germany. The anammox process was performed as described previously by Ziembińska-Buczyńska et al. [17]. Antibiotics were fed to the SBRs along with a mineral medium based on van de Graaf et al. [18] that had been adapted to anammox bacteria requirements. The concentration of ammonium and nitrite was regulated by adding NH_4Cl and $NaNO_2$, respectively. The total nitrogen concentration was 230 mg L^{-1}, while other elements were dosed at constant concentrations: 0.336 g L^{-1} ($CaCO_3$), 0.048 g L^{-1} ($KHCO_3$), 0.041 g L^{-1} (KH_2PO_4), 0.228 g L^{-1} ($MgSO_4 \cdot 7\ H_2O$), 0.007 g L^{-1} ($FeSO_4 \cdot 7\ H_2O$), 0.004 g L^{-1} (EDTA). Table 1 lists the operational conditions of each SBR. Regular measurements of ammonium, nitrite, and nitrate nitrogen in SBR effluent were conducted using photometric tests (MERCK Millipore) with a photometer (MERCK Spectroquant® NOVA60). Solution pH levels were monitored using a pH meter (WTW pH 330i). DO concentrations were measured using an ELMETRON Conductivity/Oxygen Meter CCO-505 equipped with an ELMETRON COG-1 oxygen sensor. VSS concentrations were measured according to the standard method [61].

2.2. Batch tests

Short-term experiments were performed as batch tests according to

Table 1
Operational conditions of the SBRs used. R-OTC - reactor with OTC addition, R-CIP - reactor with CIP addition, R-CLA - reactor with CLA addition.

Reactor	N-NH_4 (mg L^{-1})	N-NO_2 (mg L^{-1})	HRT (d)	pH	T (°C)	OTC (mg L^{-1})	CIP (mg L^{-1})	CLA (mg L^{-1})
Control	100	130	2	7.5 ± 0.6	35	0	0	0
R-OTC	100	130	2	7.5 ± 0.3	35	0.001	0	0
R-CIP	100	130	2	7.5 ± 0.5	35	0	0.001	0
R-CLA	100	130	2	7.5 ± 0.7	35	0	0	0.001

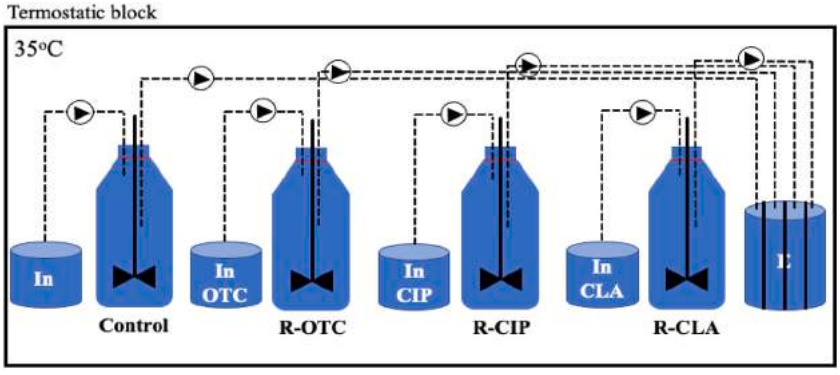

Fig. 1. The scheme of SBRs used in experiment. In – influent, E – effluent, control – reactor without antibiotic addition, R-OTC - reactor with OTC addition, R-CIP - reactor with CIP addition, R-CLA - reactor with CLA addition.

the method described by Tomaszewski et al. [19]. For the batch tests, biomass samples in a medium containing antibiotic at concentrations of 0 (control) and 0.001 mg L^{-1} (for OTC, CIP, and CLA) were prepared in 125 mL anaerobic batch reactors. The tests were stirred at 250 rpm, initial substrate concentrations were 25 mg N-NH_4 L^{-1} and 30 mg N-NO_2 L^{-1} (as NH_4Cl and $NaNO_2$, respectively), and the average biomass concentration was 0.9 ± 0.2 g VSS L^{-1}. The tests were conducted at 35 °C and a pH of 7.5, adjusted using 10% HCl or 10% NaOH. Samples from the batch test reactors were collected at 20–120 min intervals for N-NH_4 and N-NO_2 concentration measurements, while N-NO_3 was calculated based on stoichiometry.

2.3. EPSs extraction and determination

The biomass samples were centrifuged at 8000 rpm for 15 min to extract EPSs, followed by supernatant removal. Phosphate-buffered saline 1× (pH = 7) was added to the precipitate for sonication (3 min, 40 kHz). The mixture was heated in a water bath at 80 °C for 3 min and centrifuged again at 8000 rpm for 15 min. The supernatant was collected for EPS analysis, while the remaining precipitate was used to measure VSS levels. The supernatant was analyzed for EPSs components, including protein (PN) and polysaccharide (PS). The amounts of PN and PS were measured using the Flint-phenol method at 500 nm and the anthrone-sulfuric acid method at 625 nm, respectively.

2.4. qPCR analysis

Quantitative PCR (qPCR) detected quantity changes of functional genes of nitrogen cycle bacteria and variation of antibiotic resistance genes (ARGs). Initial (1 day) and long-term (30 days) sludge samples were collected and stored at −45 °C for genetic material isolation. Total bacterial DNA was extracted using a GeneMATRIX Soil DNA Purification Kit (EurX®, Poland) according to the manufacturer's instructions. The subsequent detection and amounts of five functional genes (*nirS*, *nirK*, *amoA*, *nxrA*, *hzo*) and nine ARGs (*tetX*, *tetM*, *tetC*, *tetW*, *mphA*, *mphB*, *qnrB*, *qnrB4*, *qnrS*) were determined using a QuantStudio™5, Real-Time PCR System, 96-well, 0.2 mL (TermoFisher Scientific, USA) according to the manufacturer's instructions with PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific, USA). Sequences of the primers used for functional genes and ARG analyses are presented in Tables S1 and S1, respectively.

Relative genes abundance (q) was calculated according to the equation below:

$$q = 2^{\Delta C_t}$$

where $\Delta C_t = C_{tref} - C_{tgen}$, Ct_{ref} is C_t of the reference genes (16S rRNA genes), while C_{tgen} is C_t for the analyzed gene [20].

2.5. Next-generation sequencing (NGS)

High throughput sequencing was conducted according to the method described by Banach-Wiśniewska et al. [21]. Amplification of the V3–V4 region of the 16S rRNA gene was performed with following primers: S-D-Bact-0341-b-S-17 (5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 3′) and S-D-Bact-0785-a-A-2: (5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC3′) [22] and NEBNext® High-Fidelity 2× PCR Master Mix, according to the manufacturer's instructions (Bio Labs Inc., USA). Samples were dual indexed using a Nextera® XT Index Kit. A MiSeq sequencer was used for sequencing reactions by applying paired-end technology, 2 × 250 nt with a MiSeq Reagent Kit V2 (Illumina, USA), according to the manufacturer's protocols. Results were analyzed automatically using a MiSeq Reporter (MSR) v 2.4 software (Illumina, USA) and uploaded to the MetaGenome Rapid Annotation Subsystems Technology (MG-RAST) server as FASTQ files. During the classification step, a ClassifyReads algorithm provided a species-level classification.

2.6. Statistical analysis

A significance level of α = 0.05 was assumed for all statistical analyses. Data normality was tested using the Shapiro–Wilk test, and Leven's test examined variance equalities. Based on preliminary statistical results, the Kruskal-Wallis test examined the significance of the differences between the relative gene abundance change in the presence of antibiotics. Dunn's test was conducted as a *post-hoc* pairwise multiple comparison test to discern which pairs had significant differences. Dunn's test was further adjusted by the Holm method. In addition, the Shannon and Simpson biodiversity index were calculated to examine microbial biodiversity in the collected samples. Spearman rank correlation was calculated between SAAs obtained in each reactor. All calculations were conducted using MS Excel 2013 and STATISTICA 13.1 Software.

3. Results and discussion

3.1. Short-term effects of OTC, CIP, and CLA on the microbial activity

Short-term test results of the anammox process conducted using 0.001 mg L^{-1} of OTC, CIP, and CLA are shown in Fig. 2. Relative activity of the anammox process was presented as a percentage of SAA relative to the control (without antibiotics addition). Those results showed that OTC caused an increase of 7.1% in relative anammox activity while CIP and CLA caused slight decreases in anammox activity (8.4% and 3.2%, respectively). Reports covering the effects of CIP and CLA on the anammox process are scarce, however, one study did investigate the effect of antibiotics in the same class. Erythromycin (ERY) and norfloxacin (NOR) belong to macrolide and fluoroquinolone classes, respectively, and were investigated by Zhang et al. [23]. During short-term experiments (4 h), they observed decreases in anammox activity of 1.7% and 2.2% under ERY and NOR suppression (0.001 mg L^{-1}), respectively. Similarly, this study reports that CIP, which belongs to the fluoroquinolone class, showed higher suppression of anammox than CLA (a macrolide). Sguanci et al. [24] reported that doxycycline (DOX), which belongs to the same class as OTC, had no impact on the anammox process in short-term (24 h) experiments at 5 and 10 mg L^{-1}. Noophan et al. [25] reported that OTC decreased total nitrogen removal efficiency from 74.73% to 33.48–22.15% at concentrations between 25 and 100 mg L^{-1}, within 7 h. Moreover, the half inhibitory concentration (IC_{50}) for OTC was calculated as 517.5 mg L^{-1} in the batch test [26]. However, this inhibition concentration was several mg L^{-1}, whereas in this study the trace concentration amount was much smaller (0.001 mg L^{-1}). Therefore, OTC inhibited the anammox process at concentrations above

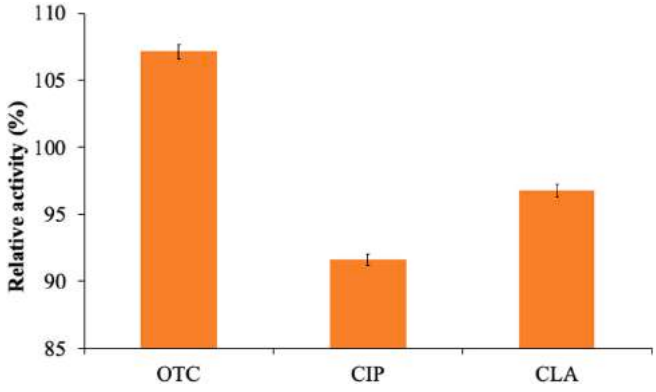

Fig. 2. Relative activity of the anammox process during short-term exposure of oxytetracycline (OTC), ciprofloxacin (CIP), clarithromycin (CLA). Results are presented as a percentage of SAA to the control (without antibiotic addition).

several mg L^{-1}. The OTC results suggested that suppression by this antibiotic at trace levels could activate some resistance mechanism, which induced anammox stimulation. Zhang et al. [27] reported that very low concentrations of sulfamethoxazole (SMX, e.g., 0.1 mg L^{-1}) stimulated up-regulation of anammox functional genes (*hdh*, *hzsA*); that suggests that under OTC suppression, an increase in anammox genes can be observed as a self-protective mechanism that increases anammox activity. Moreover, OTC adsorbs onto the EPSs that protect against direct effects of the antibiotic on bacterial cells [27]. This mechanism may play an important role in the short-term protection of anammox bacteria.

3.2. Long-term effects of OTC, CIP, and CLA on anammox performance

The nitrogen removal performance of the anammox systems suppressed by three antibiotics (OTC, CIP, CLA) in 0.001 mg L^{-1} is shown in Fig. 3a (nitrogen removal efficiency, NRE) and Fig. 3b (specific anammox activity, SAA). All reactors were subjected to a similar nitrogen loading rate (NLR) with 0.115 kg. The stabilities in the control reactor, R-OTC, and R-CLA were disturbed while the R-CLA reactor operated relatively stable with NRE and SAA reaching 62.4 ± 6.37% and 0.058 ± 0.06 kg N kg^{-1} VSS d^{-1}, respectively. At days 2, 9, and 17 in the control reactor, R-OTC, and R-CLA, the maximum theoretical anammox NRE (~90%) was reached (86.9%, 82.56%, and 82.52%, respectively), while the NRE in R-CIP did not exceed 70% (15th operating day). The deviation in the nitrogen removal efficiency may be associated with biomass adaptation or microbial community remodeling [21]. Anammox system start-ups are characterized by endogenous denitrification, which may reflect an increase in the nitrogen removal efficiency at the beginning of the experiment. Denitrification, especially endogenous in anammox systems, is characteristic of this process [17]. The denitrification contribution is also analogous to the molecular analysis of functional genes. The *nirS* and *nirK* genes in all reactors showed relatively high abundance during inoculation (day 1) and maintained those values after antibiotic suppression (day 30, described in Section 3.3). Comparing SAA and NRR from the control reactor with reactors containing OTC, CIP, and CLA, there was no significant correlation ($p > 0.05$) between the reactors (Table S3). Therefore, implementing the anammox process had a more significant effect on anammox biomass and further adaptation to new conditions than the presence of antibiotics at 0.001 mg L^{-1}.

3.3. EPSs content

Microorganisms produce EPSs in response to stress in the presence of antibiotics [27,28,29] and affect biomass characteristics [30]. Proteins and polysaccharides are viewed as important among all EPSs components. Proteins (PN) and polysaccharides (PS) are hydrophobic and hydrophilic components within EPSs, respectively. Lowering the PN/PS ratio decreases the hydrophilicity and might reduce the flocculation of activated sludge [31]. Meng et al. [32] suggested the PN/PS ratio decrease could lead to sludge settling capacity reductions. According to Fig. 4, the PN/PS ratio increased in the control and R-OTC by 7.9% and 3.35%, respectively, but decreased in R-CIP and R-CLA by 8.0% and 10.5%, respectively. However, the total EPSs production (PN + PS) slightly decreased in the control by 1.4%, while in SBRs with added OTC, CIP, and CLA increased by 0.6%, 1.2%, and 1.8%, respectively. It showed that initial exposure of streptomycin (belonging to macrolide

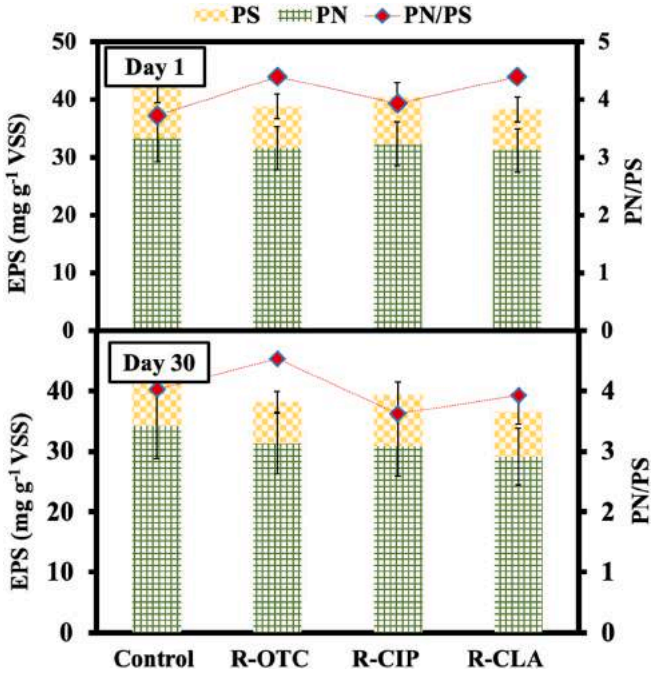

Fig. 4. Changes in the EPS of anammox biomass suppressed by 0.001 mg L^{-1} of OTC, CIP and CLA during the first and last day of long-term experiment.

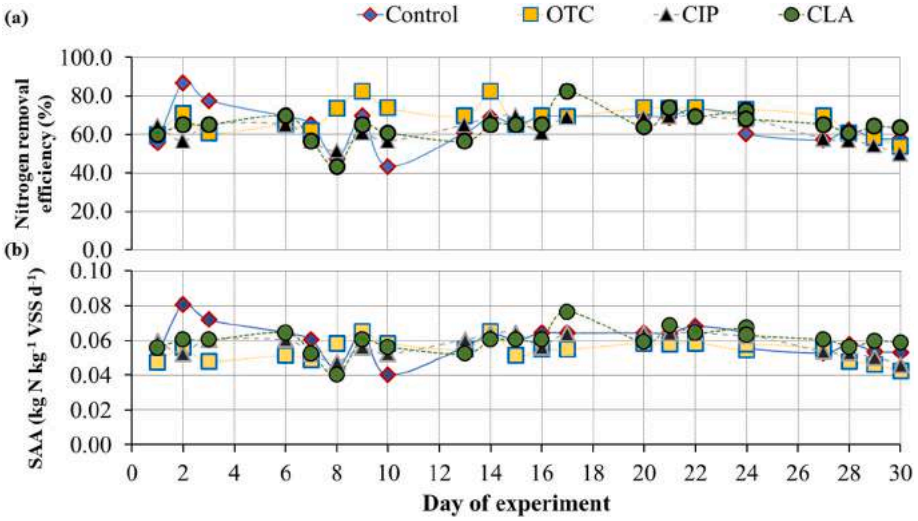

Fig. 3. Long-term operation of four SBRs. (a) The nitrogen removal efficiency (NRE), (b) The specific anammox activity (SAA).

class) stimulated EPSs production in the sludge [33]. Similar effects were described by Zhang et al. [27], where EPS levels increased from 11.9 mg g^{-1} VSS to 317.3 mg g^{-1} VSS at treatment levels of 0.5 mg L^{-1} OTC. Thus, the production of EPSs was viewed as an important self-protection mechanism against antibiotics. Moreover, EPSs are the first barrier for a bacterial cell that directly contacts and interacts with antibiotics in activated sludge [29]. The EPSs component responsible for antibiotic sorption is PN, it provides an adsorption binding site via functional groups such as: amine, carboxyl, hydroxyl group, and hydrophobic regions [30]. As previously reported by Zhang et al. [27], the presence of 0.5 mg L^{-1} OTC increased the adsorption sites and was related to the PN/PS ratio. Stronger hydrophobicity and greater adsorption site availability contributed to the higher PN/PS ratio [27,30] and was observed in the current study for R-OTC. Thus, sorption onto the PN fraction of EPSs may be an important part of the protective mechanism against antibiotics, especially OTC.

3.4. Variation of functional genes abundance

The relative abundance of functional genes responsible for nitrogen transformation: *amoA*, *nxrA*, *nirK*, *nirS*, and *hzo* genes and integron 1 class (*intI1*) were determined via qPCR using bacterial DNA as a template. All qPCR reactions were conducted at least in triplicate with efficiencies close to 1.0. The results are presented as the ratio of the specific reference gene to 16S rRNA gene (relative abundance gene). The significance of the difference between relative genes abundance changes in the presence of these antibiotics was revealed by statistical methods (Table 2). As shown in Fig. 5, changes in *amoA* and *nxrA* genes showed similar trends during the trials. On day 1, the abundance of *amoA* and *nxrA* in almost all reactors fed with antibiotics was higher compared to the control reactor (*amoA* in the R-OTC reactor was the exception). After the experiments concluded, the abundance of both *amoA* and *nxrA* genes in each reactor decreased. Moreover, the abundances of *amoA* were higher than *nxrA* in the operating SBRs reactor, indicating a significant role of ammonium oxidizing bacteria (AOB) in the anammox biomass. The occurrence of AOB in anammox systems closely related to anammox bacteria has been previously confirmed [17,34]. The presence of AOB in anammox biomass is explained in two ways: AOB protects anammox bacteria against oxygen and produces nitrite or AOB performs some anaerobic metabolism [17,35,36]. However, the relatively high abundance of the *amoA* gene, commonly attributed to AOB in wastewater treatment, is also present in the *Nitrospira* genus, which undergoes complete nitrification in one step, called comammox, under anaerobic conditions [37]. Moreover, *Nitrospira* contains a full set of ammonium converting gene encoding enzymes: ammonium monooxygenase (AMO), hydroxylamine dehydrogenase (HAO), and nitrite oxidoreductase (NXR) [37]. Therefore, an abundance of *amoA* and *nxrA* genes result in the occurrence of *Nitrospira* in these systems and confirmed in metataxonomic analysis (Section 3.3), where the relative abundance of this genus in each reactor increased.

Levels of *hzo* genes were the lowest of all investigated genes. This was caused by inoculation to SBRs and anammox adaptation. As shown in Fig. 5, the abundance of the *hzo* gene in the reactors fed with antibiotics decreased during the experimental period (the abundance increased in the control reactor). The difference in the change of *hzo* gene abundance in the control reactors and reactors with antibiotics addition was statistically significant ($p < 0.05$) (Table 3). These results suggest the presence of (0.001 mg L^{-1}) OTC, CIP, and CLA may suppress anammox bacteria, which showed low enzymatic activity during the adaptation period. Zhang et al. [27] compared the effects of OTC and SMX from 0 to 1 mg L^{-1} on anammox functional genes (*hzsA*, *hdh*), and reported that both genes were more sensitive on SMX than OTC from 0.1–0.5 mg L^{-1}. However, they also proved that *hdh* was sensitive to a low OTC dose (0.1 mg L^{-1}) rather than a higher dose. This suggested that trace concentrations of OTC (0.001 mg L^{-1}) may have an adverse effect on the *hzo* gene. Other studies showed that macrolide spiramycin at 1–5 mg L^{-1} did not lead to significant changes in the abundance of *hzsA*, *hdh*, and metabolic processes of anammox bacteria were not disturbed by long-term exposure [38].

Table 2
Statistical analysis of the significance of the differences between changes in the relative bacterial abundance in the presence of antibiotics (results with $p < 0.05$ were considered statistically significant).

	nxrA	*amoA*	*nirK*	*nirS*	*hzo*	*intI1*
Kruskal-Wallis chi-squared statistic	6.728	2.853	3.121	5.08	11.399	8.834
Degrees of freedom	3	3	3	3	3	3
p-value	0.081	0.415	0.373	0.16	0.01	0.032

The abundances of *nirK* and *nirS* were relatively similar (order of magnitude) on both the first and last day of the experiment in all reactors tested. High abundances of both *nirK* and *nirS* in anammox systems may be due to using a carbon source derived from the decomposition of dead biomass by denitrifiers in a process called endogenous denitrification. As shown in Fig. 5, *nirS* present a higher abundance than *nirK*. This result suggested that denitrifiers in the biomass preferred the *nirS* pathway over *nirK*. Similar results in anammox systems have been previously reported by Banach-Wiśniewska et al. [39]. The prevalence of the *nirS* gene abundance over *nirK* may be attributed to fact that *nirK* is Cu (Copper)-dependent, while *nirS* is cytochrome cd_1-containing [40]. Therefore, bacteria utilizing the *nirK* pathway require Cu, limited in the laboratory-scale anammox system. From results obtained for both *nirS* and *nirK*, the antibiotics used had a negligible impact on denitrification genes.

Integron – integrase 1 class gene (*intI1*) has been proposed as a marker for anthropogenic ARG pollution, mainly due to significant linkage with ARGs and rapid response to diverse environmental stresses [41]. Moreover, a positive correlation was found in the relationship between *intI1* and abundance of ARGs in aquatic environments [42,43], which accentuates the need to study *intI1* genes relative to ARGs. In the present study, the control and reactors fed with antibiotics maintained constant levels ($1.4 \times 10^{-4} \pm 2.7 \times 10^{-5}$), except for R-OTC, which reached 9.7×10^{-4} towards the end of the experimental period. A significant correlation ($p < 0.05$) between the abundance of *intI1* and genes targeting tetracycline resistance (*tetC*, *tetX*) was found in the anammox system suppressed by OTC at 2 mg L^{-1} [15]. Therefore, there may be some linkage between *intI1* and *tet* genes. On the other hand, *intI1* integrated and expressed more than 100 types of ARGs by genes cassette [44], though no *tet* genes were detected in any gene cassette by *intI1* [45]. However, previous studies revealed that *intI1* coexists with *tetC* in conjugative plasmid, which made them easier to transfer [46,47].

3.5. Variation of ARGs

Nine primers were used to detect ARGs in these experiments: four primers encoding ARGs for OTC (*tetX*, *tetC*, *tetM*, *tetW*), three primers for CIP resistance (*qnrB*, *qnrB4*, *qnrS*), and two primers for CLA resistance (*mphA*, *mphB*). Moreover, all primers were used to detect ARGs in the control reactor. As shown in Table S4, only six ARGs were detected, three of which were targeted to OTC (*tetX*, *tetC*, *tetW*), two targeted to CIP (*qnrB4*, *qnrS*), and one targeted CLA (*mphA*). As shown in Fig. 6, the number of each detected gene was expressed as a ratio of the functional genes to the 16S rRNA gene (relative genes abundance). Those results show that the relative abundance of each ARG targeted OTC increased after long-term antibiotic feeding, while the control reactor saw a decrease (excluding *tetW*, which slightly increased). Each OTC-ARGs induced a different resistance mechanism: efflux pump (*tetC*), ribosomal protection protein (*tetW*), and enzymatic modification (*tetX*); however, the dominant resistance mechanism was efflux pump and enzymatic modification. Furthermore, the highest increase was observed in *tetC* during long-term testing. Zhang et al. [38] obtained the

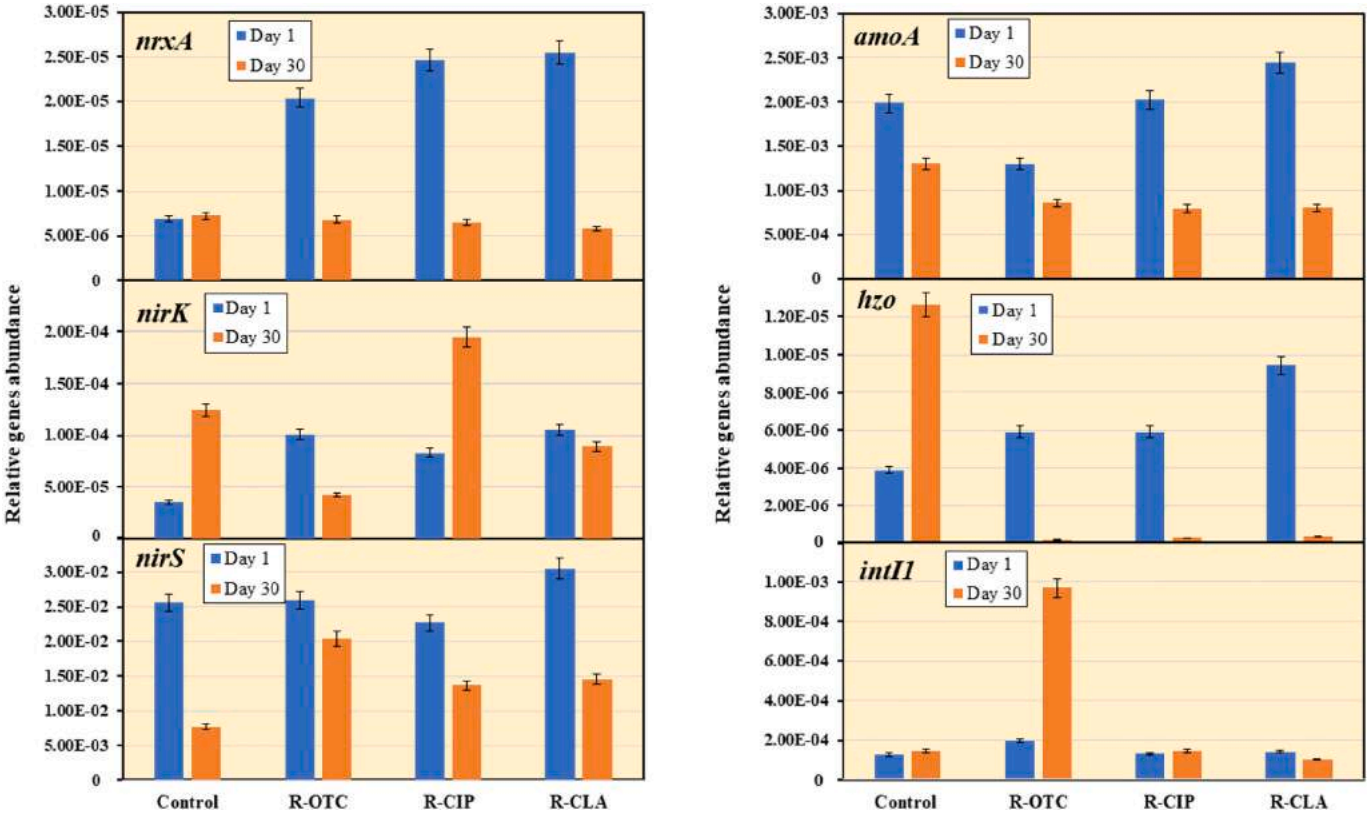

Fig. 5. Variation of the relative abundance of *nxrA*, *amoA*, *nirK*, *nirS*, *hzo*, and *intI1* in anammox biomass in four tested SBRs.

Table 3
Results of post – hoc Dunn test, further adjusted by the Holm – Bonferroni method (results with $p < 0.05$ were considered statistically significant).

	hzo				*intI1*			
	Control	R-OTC	R-CIP	R-CLA	Control	R-OTC	R-CIP	R-CLA
Control	–	0.003	0.004	0.006	–	0.053	0.896	0.863
R-OTC	0.003	–	0.912	0.834	0.053	–	0.049	0.041
R-CIP	0.004	0.912	–	0.705	0.896	0.049	–	0.712
R-CLA	0.006	0.834	0.705	–	0.863	0.041	0.712	–

overall absolute abundance of *tetX*, which was 2–3 orders of magnitude higher than the other OTC-ARGs (*tetC*, *tetG*, and *tetM*). It is worth noting they used a higher concentration of OTC (>0.1 mg L^{-1}) than in this study. Similar effects were reported by Zhang et al. [15] and Shi et al. [48], where the abundance of *tetX* far exceeded that of *tetC* under OTC pressure at 1.0, 2.0 mg L^{-1}. Therefore, anammox bacteria use different protective mechanisms depending on the environmental concentration of the antibiotic. The relatively low abundance of *tetW* genes may stem from the anammox process conducted in the laboratory condition using a mineral medium. *TetW* is recognized as a typical marker for tetracycline ARGs and is unique to WWTPs and livestock [43]. Therefore, its presence in a laboratory system can be marginal.

The relative abundances of both *qnrB4* and *qnrS* increased slightly in the reactor fed with CIP from 4.4×10^{-5} to 7.69×10^{-5} and from 7.4×10^{-8} to 6.7×10^{-7}, respectively, while the abundance in the control reactor decreased from 1.16×10^{-4} to 3.95×10^{-6} (*qnrB4*) and from 3.78×10^{-7} to 3.25×10^{-8} (*qnrS*), which indicated that CIP feeding induced *qnrB4* and *qnrS*. Both *qnrB4* and *qnrS* are protective genes and protect DNA gyrase from inhibition by quinolones. *QnrS* has become increasingly prevalent in anthropogenically influenced environments [49,50]. The presence of *qnrS* genes in the laboratory system fed with a mineral medium was validated by Ślipko et al. [51], where the relative abundance of *qnrS* fluctuated over time, spanning from below 10^{-5} copies/16S rRNA gene to above 10^{-2} copies/16S rRNA gene between 0.0001 and 0.1 mg L^{-1} of CIP. However, these results were obtained for a conventional nitrification-denitrification system in which the microorganisms have a higher growth rate than anammox bacteria [52]. Therefore, the transfer of ARGs can be faster. On the other hand, the relative abundance of *mphA* decreased in the reactor fed with CLA (from 7.45×10^{-4} to 9.0×10^{-5}) and decreased in the control reactor from 8.9×10^{-4} to 2.2×10^{-5}. Zhang et al. [38] suggested that *mphA* might be more susceptible to macrolide antibiotics in environmental samples due to a resistance mechanism. The presence of the m*phA* gene in the bacteria genome induced ability of bacteria chemical modification of antibiotics caused the degradation or replacement of the active group by synthesis of oxidoreductase [53]. Therefore, a reduction in the relative abundance of this gene was observed.

Zhang et al. [23], concluded that it was not easy to induce ARGs by NOR in the anammox system. Although NOR at 0.001 mg L^{-1} suppressed the anammox system, they did not detect that genes targeted NOR resistance. On the other hand, this study showed that CIP at 0.001 mg L^{-1} induced development of genes targeted for quinolones resistance. Therefore, these results suggest that CIP more readily induces NOR resistance to quinolones.

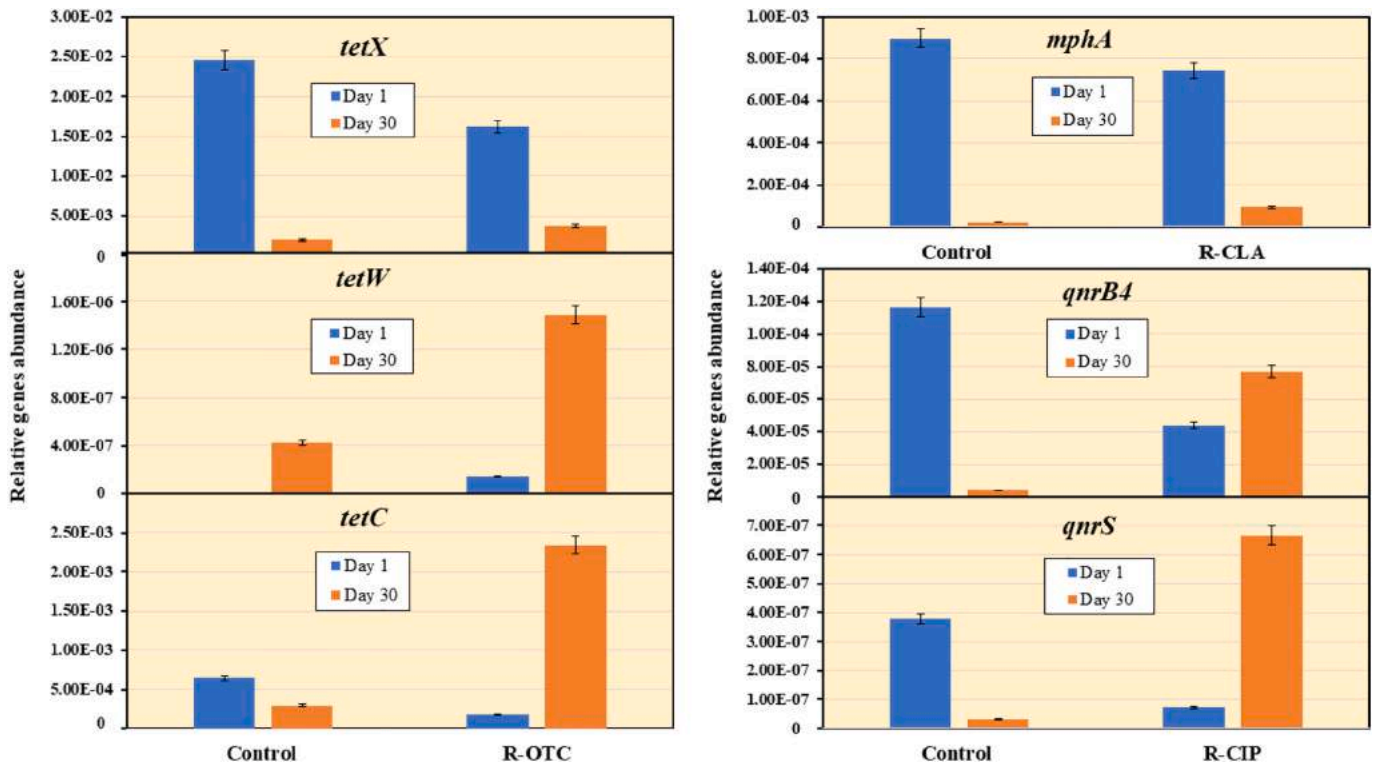

Fig. 6. Relative abundance of different ARGs (*tetX*, *tetW*, *tetC* targeted OTC, *mphA* targeted CLA, *qnrB4*, *qnrS* targeted CIP) in the four SBRs at the beginning and at the end of the anammox process performance.

3.6. Microbial community changes

A 16S rDNA-based high throughput sequencing technique determined the microbial community composition changes during long-term exposure of OTC, CIP, and CLA. The sequence numbers (OTU) of the eight samples were 69,027 (Control_1), 76,350 (Control_30), 48,991 (R-OTC_1), 70,008 (R-OTC_30), 69,311 (R-CIP_1), 83,064 (R-CIP_30), 48,004 (R-CLA_1), and 75,487 (R-CLA_30). The OTU was defined as the

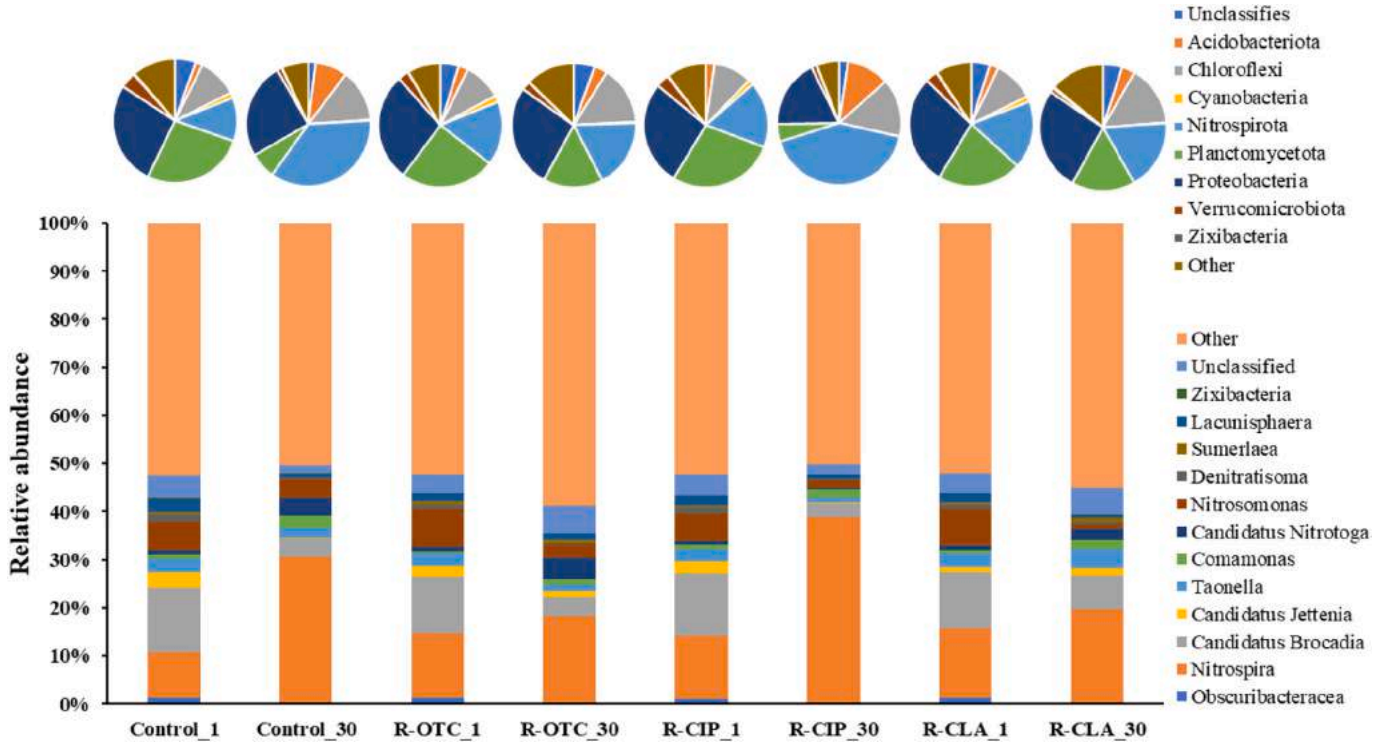

Fig. 7. Microbial community structure of anammox sludge at the phylum (pie chart) and the genus level (bar chart) in the four reactors at the beginning and at the end of the experiment.

sequence with the similarity >95%. The same OUT values co-existed in the four samples (day 1) and four samples (day 30) were 37 and 42, respectively as shown in Fig. S1. These results indicated a microbial community change occurred under antibiotic suppression. Based on OTUs values, the Shannon and Simpson biodiversity indexes were calculated (Fig. S2). For each reactor, both indexes slightly decrease at the end of the experiment. *Planctomycetes*, *Proteobacteria*, and *Bateroidetes* were the dominant phyla in this study on day 1 (Fig. 7). Similar dominant communities were observed by Cheng et al. [54] under OTC and Cu nanoparticle stresses. However, after day 30, as the main phyla functioned *Proteobacteria*, *Bacteroidetes*, and *Nitrospira*, such a high abundance of *Proteobacteria* was similar to conventional nitrification-denitrification systems in which this phylum dominates [55]. However, the occurrence of *Proteobacteria* in this study can traced to the presence of death cells of bacteria in anammox biomass as a result of the implementation period. On the other hand, it was reported that *Proteobacteria* readily utilizes antibiotics like OTC as a carbon source [56]; therefore, antibiotic addition may improve the conditions for this phylum. On day 1, *Plantomycetes* accounted for 21.28% (control), 19.56% (R-OTC), 21.27% (R-CIP), and 17.42% (R-CLA). In the end, significant changes were observed where relative abundances dropped to 4.75% (Control), 9.84% (R-OTC), 3.59% (R-CIP), and 12.00% (R-CLA). During the experiment, the third phylum transformed from *Platomycetes* to *Nitrospira* reached 25.27% (control), 11.33% (R-OTC), 33.13% (R-CIP), and 13.31% (R-CLA), up from 9.01%, 13.05%, 13.16%, and 14.02%, respectively. Previous studies reported that *Nitrospira* is relatively abundant in anammox systems [17,52,57]. *Chloroflexi* was quite abundant in these systems and the abundances of these bacteria increased from 8.1% (control), 7.9% (R-OTC), 7.6% (R-CIP), and 8.37% (R-CLA) to 9.59%, 9.6%, 12.03%, and 11.45%. *Chloroflexi* exists in technical- and lab-scale anammox reactors and utilize EPSs produced by anammox bacteria in anaerobic systems [54]

The taxonomic results at the genus level are shown in Fig. 7 (bar chart). The major genera of anammox bacteria in each reactor were Candidatus *Brocadia* and Candidatus *Jettenia*, whose abundances dropped during the trial period. For Candidatus *Brocadia*, it dropped from 7.0% (Control), 5.6% (R-OTC), 5.9% (R-CIP), and 5.7% (R-CLA), to 1.1%, 1.3%, 0.8%, and 2.2%, respectively, while for Candidatus *Jettenia*, it dropped from 5.6 (Control), 2.3% (R-OTC), 2.6% (R-CIP), and 1.0% (R-CLA) to 0.2%, 0.6%, 0.2%, and 0.9%. Because the highest drop in anammox bacteria was observed in the control reactor, it suggested these changes were caused by remodeling of the anammox biomass community after inoculation. It is worth noting that previous studies reported that Candidatus *Brocadia* adapts to certain antibiotics like sulfamethazine at concentrations below 7 mg L^{-1} [58]. This also suggested that some adaptation mechanism may have taken place towards CLA, which had the lowest decrease of all reactors in Candidatus *Brocadia* abundance (by 3.5%). Despite the decrease in anammox bacteria abundance in the control, this was the only reactor in which the *hzo* gene abundance (Fig. 5) increased. This may be due to unclassified *Planctomycetes*, with an abundance of 11.29% in the control reactor, which may exhibit enzymatic activity. The role of unclassified *Planctomycetes* in anammox systems was also pointed out by Tomaszewski et al. [52]. As shown in Fig. S3, the dominant genera were clustered into two main groups, where both Candidatus *Brocadia* and *Candidatus Jettenia* demonstrated a small distance between nodes showing similarity between the data sets obtained for each one.

Significant abundance increases were observed for *Nitrospira* (*Nitrospirae* phylum), which belongs to NOB and converts nitrite into nitrate. However, in 2015, it was reported that *Nitrospira* performed both stages of nitrification [59]. This suggested that *Nitrospira* helps anammox bacteria with nitrogen removal under anaerobic conditions. Similar results were reported by Tomaszewski et al. [52], where *Nitrospira* supported anammox bacteria under reduced graphene oxide (RGO) suppression. Moreover, increasing the abundance of this genus in the system during trials could lead to a suspicion that *Nitrospira* was more resistant to antibiotic suppression than anammox bacteria. In contrast, Zhang et al. [60] reported that *Nitrospira* was present in concentrations much lower than anammox bacteria under ERY suppression. *Nitrosomonas* and *Denitratisoma*, representing AOB and denitrifiers, respectively, were detected with lower abundance than primary anammox bacteria genera in the reactors studied. Moreover, both *Nitrosomonas* and *Denitratisoma* were inhibited throughout the experiment. Zhang et al. [60] documented that the presence of these genera had an impact on anammox performance deviation under ERY. Furthermore, for NOR at 0.001 mg L^{-1}, an increase was observed (0.57%), while ERY (0.001 mg L^{-1}) caused slight variation [23].

4. Conclusions

Short- and long-term effects on anammox performance, including production of EPSs, variation of functional genes, and ARG abundances as well as community structure were analyzed. Nitrogen removal performance showed that antibiotics (0.001 mg L^{-1}) had no significant impact on the anammox process. Each antibiotic decreased the biodiversity of anammox biomass. All antibiotics decreased the abundance of the *hzo* gene. Both OTC and CIP increased ARGs (*tetC*, and *tetW* for OTC; *qnrB4* and *qnrS* for CIP) in the anammox system, while ARGs targeted CLA (*mphA*) decreased during anammox testing. The antibiotics at trace concentrations had negative impacts on anammox at the gene level and had negligible effects on nitrogen removal mainly due to co-existing with other nitrogen-cycle bacteria in the biomass. Furthermore, the antibiotics induced development of anammox bacteria resistance via production of EPS and increased ARGs abundance. Further studies involving the effects of OTC, CIP and CLA on the anammox process should focus on elucidating the mechanism of resistance gene transfer in the anammox biomass and investigating all the protective mechanisms of anammox bacteria, including regulation of gene expression and production of EPSs.

Declaration of competing interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jwpe.2022.102607.

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Supplementary materials for

Microbial response of the anammox process to trace antibiotic concentration

Gamoń Filip[1*], Banach-Wiśniewska Anna[1,2], Kaur Jaspreet Jandoo[1], Cema Grzegorz[1], Ziembińska-Buczyńska Aleksandra[1]

[1]*Environmental Biotechnology Department, Silesian University of Technology, Akademicka 2A, 44-100 Gliwice, Poland*
[2]*Regional Center for Water and Wastewater Management Co., Tychy, Poland*

Corresponding author e-mail: filip.gamon@polsl.pl
Corresponding author phone: +48 237 29 15

Table S1. Primers used for qPCR analysis of nitrogen cycle bacteria functional genes.

Specificity	Target gen	Primers	Sequence 5'-3'
Total bacteria	16S rRNA	1055F	ATGGCTGTCGTCAGCT
		1392R	ACGGGCGGTGTGTAC
Ammonia oxidizers	*amoA*	amoA-1-F	GGGGTTTCTACTGGTGGT
		amoA-2R	CCCCTCKGSAAAGCCTTCTTC
Nitrite oxidizers	*nxrA*	nxrA-RT-F	GTGGTCATGCGCGTTGAGCA
		nxrA-RT-R	TCGGGAGCGCCATCATCCAT
Denitrifiers	*nirS*	nirS 1f	TACCACCCSGARCCGCGCGT
		nirS 3r	GCCGCCGTCRTGVAGGAA
	nirK	nirK876	ATYGGCGGVCAYGGCGA
		nirK1040	GCCTCGATCAGRTTRTGGTT
All known Planctomycetes	*hzo*	hzoCl1f1 1f	TGYAAGACYTGYCAYTGG
		hzoCl1r2	ACTCCAGATRTGCTGACC
All bacteria	*IntI1*	*int*I1_F	GGCTTCGTGATGCCTGCTT
		*int*I1_R	CATTCCTGGCCGTGGTTCT

Table S2. Primers used for ARGs analysis in anammox biomass.

Targeted antibiotic	Targeted gen	Primers	Sequence 5'-3'	Resistance mechanism
OTC	*tetC*	tetCf	GCGGGATATCGTCCATTCCG	Efflux pump
		tetCr	GCGTAGAGGATCCACAGGACG	
OTC	*tetX*	tetXf	GAAAGAGACAACGACCGAGAG	Drug modification
		tetXr	ACACCCATTGGTAAGGCTAAG	
OTC	*tetM*	tetMf	ACAGAAAGCTTATTATATAAC	Ribosomal protection protein
		tetMr	TGGCGTGTCTATGATGTTCAC	
OTC	*tetW*	tetW_F	GAGAGCCTGCTATATGCCAGC	Ribosomal protection protein
		tetW_R	GGGCGTATCCACAATGTTAAC	
CLA	*mphA*	mphAf	CTGACGCGCTCCGTGTT	Drug modification
		mphAr	GGTGGTGCATGGCGATCT	
CLA	*mphB*	mphBf	ATTAAACAAGTAATCGAGATAGC	Drug modification
		mphAr	TTTGCCATCTGCTCATATTCC	
CIP	*qnrB*	qnrBf	GGCACTGAATTTATCGGC	DNA gyrase protection
		qnrBr	TCCGAATTGGTCAGATCG	
CIP	*qnrB4*	qnrB4f	AGTTGTGATCTCTCCATGGC	DNA gyrase protection
		qnrB4r	CGGATATCTAAATCGCCCAG	

CIP	*qnrS*	qnrS_F qnrS_R	ACGACATTCGTCAACTGGAA TTAATTGGCACCCTGTAGGC	DNA gyrase protection

Table S3. Spearman range correlation value (p>0.05) between SAA in the operating reactors during long-term experiment period.

Reactor	Control	R-CLA	R-CIP	R-OTC
Control	1			
R-CLA	0.51	1		
R-CIP	0.43	0.60	1	
R-OTC	0.13	0.10	0.38	1

Table S4. Detection of studied ARGs in the experiment period. '+' – detected, '-' – not detected, NI – not investigated.

	Reactor	*tetX*	*tetM*	*tetC*	*tetW*	*mphA*	*mphB*	*qnrB*	*qnrB4*	*qnrS*
Day 1	Control	+	-	+	-	+	-	-	+	+
	R-OTC	+	-	+	+	NI	NI	NI	NI	NI
	R-CIP	Ni	NI	NI	NI	NI	NI	-	+	+
	R-CLA	NI	NI	NI	NI	+	-	NI	NI	NI
Day 30	Control	+	-	+	+	+	-	-	+	+
	R-OTC	+	-	+	+	NI	NI	NI	NI	NI
	R-CIP	NI	NI	NI	NI	NI	NI	-	+	+
	R-CLA	NI	NI	NI	NI	+	-	NI	NI	NI

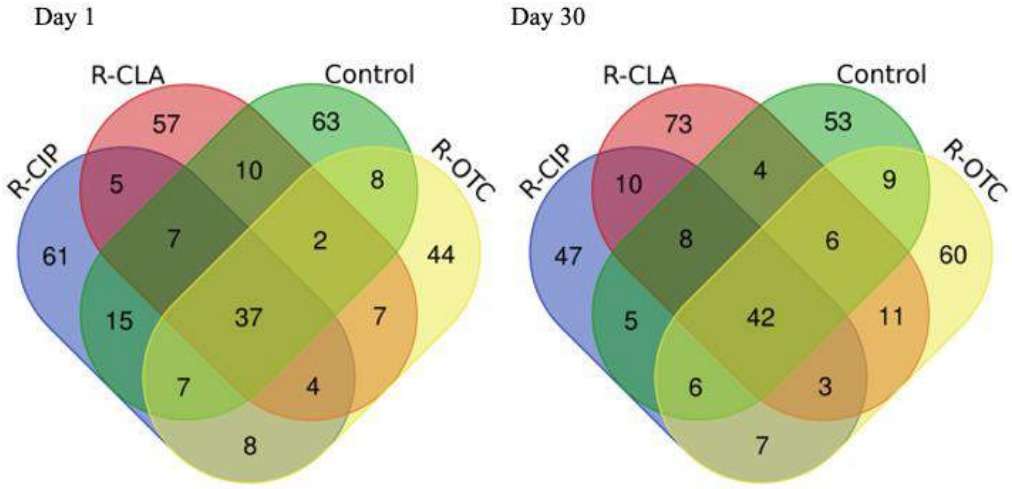

Fig. S1. Venn diagram showing the number of shared and unique OTUs among tested reactors at the beginning and end of the experiment.

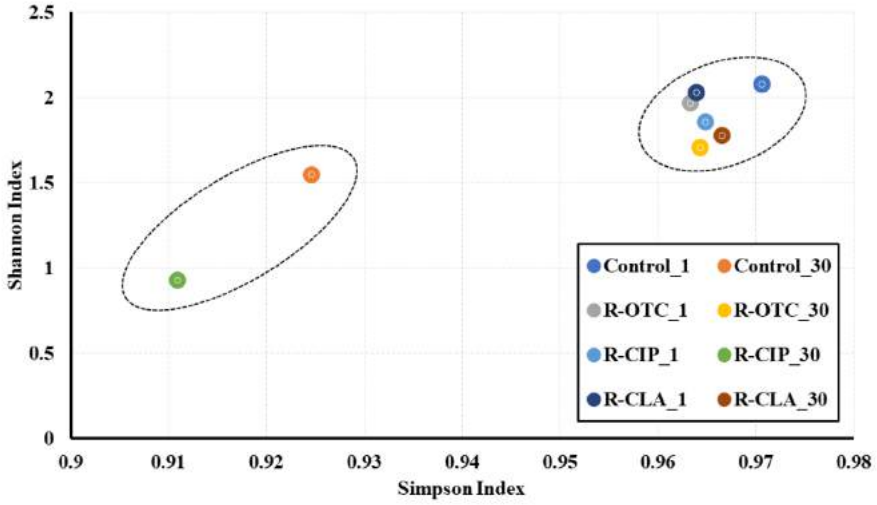

Fig. S2. Relation between Simpson and Shannon biodiversity indexes in biomass and at the beginning and at the end of experiment.

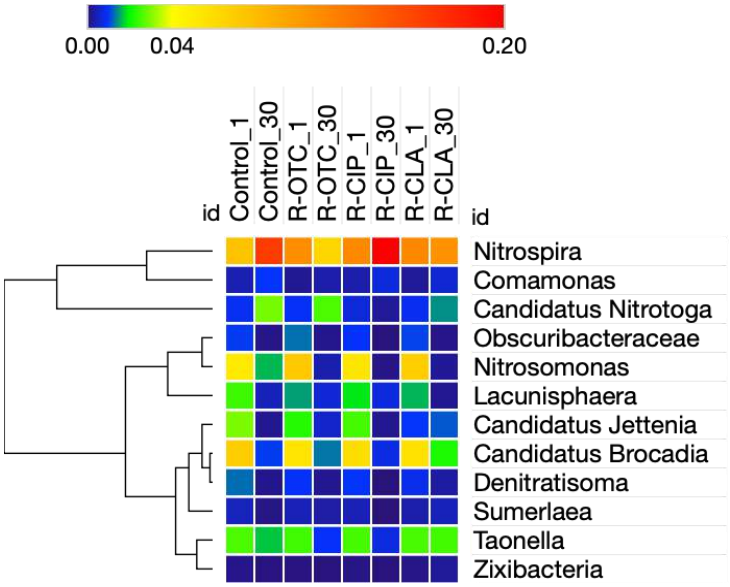

Fig. S3. Heatmap of the most abundant genera identified in four SBRs at the beginning and at the end of the long-term experiment with hierarchical clustering.

Insight into the microbial and genetic response of anammox biomass to broad range concentrations of different antibiotics: Linking performance and mechanism

Filip Gamoń

Anna Banach-Wiśniewska

Izabela Poprawa

Grzegorz Cema

Aleksandra Ziembińska-Buczyńska

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Insight into the microbial and genetic response of anammox biomass to broad range concentrations of different antibiotics: Linking performance and mechanism

Filip Gamoń [a,*], Anna Banach-Wiśniewska [b], Izabela Poprawa [c], Grzegorz Cema [a], Aleksandra Ziembińska-Buczyńska [a]

[a] Department of Environmental Biotechnology, Silesian University of Technology, Akademicka 2A, 44-100 Gliwice, Poland
[b] Regional Center for Water and Wastewater Management Co., Tychy, Poland
[c] Institute of Biology, Biotechnology and Environmental Protection, Faculty of Natural Sciences, University of Silesia in Katowice, Bankowa 9, 40-007 Katowice, Poland

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ABSTRACT

Antibiotics have become emerging pollutants occurring in wastewater, influencing the activity of microorganisms responsible for wastewater treatment. Moreover, the potential application of the anammox process in the treatment of antibiotic-containing wastewater has paid much attention. A common antibiotic, OTC (oxytetracycline), CIP (ciprofloxacin), and CLA (clarithromycin) are recognized to be monitored in wastewater due to their relatively high concentration and hazardous impact on the environment. However, their effect on the anammox process remains unknown. Therefore, this paper presents the study concerning the long-term effects of a successive concentration of three antibiotics (OTC, CIP, CLA) on the anammox process, with special emphasis on treatment efficiency, resistance mechanism, bacteria cell morphology, and activated sludge community structure. It is worth noting that the influence of a successive concentration of CIP and CLA has been studied for the first time. Results revealed that anammox community could adapt to CIP, OTC, and CLA at low concentrations (<1 mg L^{-1}), while the high concentration of antibiotics (100 mg L^{-1}) reduced the nitrogen removal rate (NRR) by 27 % (OTC), 30 % (CIP), and 56 % (CLA). Community structure analysis showed that the abundance of *Planctomycetes* increased with the increase of CIP and CLA concentration while decreasing under OTC stress. On contrary, other nitrogen-cycle bacteria (e.g., *Nitrospira*) contributed to the nitrogen removal, especially during antibiotic suppression. The abundance of corresponding ARGs (OTC resistance gens: *tetX, tetC, tetW*, CIP resistance gens: *qnrB4*, *qnrS*, CLA resistance gens: *mphA*) generally increased under antibiotic suppression. In addition, co-occurrence analysis showed that anammox bacteria might participate in the transfer of macrolide resistance genes. The findings of this study are essential in understanding the mechanisms of three antibiotics commonly occurring in wastewater during the anammox process. Moreover, the results have implications for using the anammox process for antibiotic-containing wastewater treatment and provide the operational guidance for stable conducting of the anammox process under antibiotics suppression.

1. Introduction

The discovery of antibiotics is regarded as one of the most important milestone achievements of the last century, which revolutionized human and veterinary medicine [1]. Currently, antibiotics play a crucial role in medicines, as well as their use at globally in farming to improve meat production [2,3]. Among all antibiotic classes, tetracycline, quinolones, and macrolides antibiotics are widely used in livestock and healthcare [1,2]. β-lactam antibiotics dominate in terms of global production and usage of antimicrobial pharmaceuticals, followed by tetracycline antibiotics, while in China tetracycline antibiotics dominate the market [4,5]. It is estimated that the consumption of fluoroquinolones is 7 % of total antibiotic consumption [6]. With the increasing consumption of antibiotics, there is a problem with the deposition of antibiotics in the environment. It is considered that 25 % – 75 % of environmentally occurring antibiotics are excreted in feces and urine [7]. These antibiotics are released into wastewater reaching wastewater treatment plants (WWTPs) and further, surface and groundwater [8,9]. The average

* Corresponding author.
E-mail address: filip.gamon@polsl.pl (F. Gamoń).

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concentration of antibiotics in the environment is at the level of few ng L^{-1} to a few μg L^{-1} [1]. Moreover, it was reported that in China approximately 53,800 tons of antibiotics were released directly into the environment annually [10]. Due to the risk of the occurrence of antibiotics in the environment, The European Commission established the first (Decision EU, 2015/495) and second (Decision EU 2018/480) watch list of substances for the Union-wide water policy framework. According to the first watch, it was stated that clarithromycin (CLA) is one of the most frequently occurring macrolide antibiotics in surface water samples in 58.8 % of 2792 samples [11]. Moreover, both these lists include ciprofloxacin (CIP) belonging to fluoroquinolone antibiotic family due to its increasing detection in the environment. For instance, CIP was detected in the effluent of 90 WWTPs located in the European Union (Loos et al., 2013). One of the more commonly detected antibiotics in wastewater is oxytetracycline (OTC). Despite the fact, that OTC is not included in the EU watchlist, this antibiotic is becoming increasingly interesting due to its potential for increased environmental risks – it is poorly degradable in WWTPs and the transformation products can be harmful to aquatic organisms. Moreover, similar to other micropollutants presented in the wastewater (i.e., heavy metals) [85,86], OTC has very good sorption properties on activated sludge. The occurrence of antibiotics in wastewater systems could suppress the activity and growth of microorganisms and become a challenge to the biological treatment performance [12]. Antibiotics also promote the development of antibiotic-resistant bacteria (ARB) and the spread of antibiotic-resistant genes (ARGs) causing threats to human and animal health and cannot be ignored [1]. Based on the above, depending on the type of wastewater or the area in which it is produced, it contains different concentrations and types of antibiotics. Therefore, there is a need to explore the effect of multiple antibiotics on the wastewater treatment processes to adjust the operating modes to types of antibiotic-containing wastewater.

Anaerobic ammonium oxidation (anammox) is an efficient and low-cost high nitrogen-loaded wastewater treatment technology that has been extensively studied [13–15]. Nevertheless, there are limits to the wider operation and application of the anammox process that are related to the low growth rate of anammox bacteria and their sensitivity to various chemical substances, including antibiotics [16]. As widespread pollutants, the effect of antibiotics on the anammox process have been investigated. Bi et al. [17] showed that the anammox process had a stable performance at concentrations of less than 10 mg L^{-1} sulfadiazine (SDZ), while 1 mg L^{-1} of chlortetracycline (CTC) caused an unrecoverable inhibitory impact on nitrogen removal performance. Zhang et al. [18] reported that anammox bacteria could resist erythromycin (ERY) below 1 mg L^{-1}, while 10 mg L^{-1} of ERY highly inhibited the bioactivity of anammox bacteria. Moreover, the combined effect of antibiotics was also investigated. For instance, simultaneous suppression of oxytetracycline (OTC) and sulfamethoxazole (SMX) at 1 mg L^{-1} inhibit the anammox process performance Zhang et al. [19]. Gamoń et al. [27] previously presented that the anammox process is stable operated under trace concentration (0.001 mg L^{-1}) of OTC, CIP, and CLA pointing out that even such low concentration of antibiotics influences the community structure and development of ARGs targeting these antibiotics. However, in this study, the anammox process was exposed to the antibiotics without acclimatization of biomass to a new condition and there was used only one concentration of antibiotics. As shown in previous studies, exposing the anammox process to a successive concentration of antibiotics may stabilize the anammox process performance even under higher antibiotic concentrations [32,72]. Therefore, it is necessary to study the effect of increasing concentrations of these antibiotics on anammox process. Such studies will be an important aspect in the implementation of the anammox process at the technological scale, where increasing concentrations of antibiotics have been observed.

An important aspect of antibiotic action on anammox bacteria is the effect on their community structure. Zhang et al. [20] reported that trace concentration (1 μg/L) of norfloxacin (NOR) decreased the relative abundance of Candidatus *Kuenenia* from 4.31 % to 1.87 % after 30 days of incubation. A previous study indicated that sulfadimethoxine (SDM) and sulfamethazine (SM) at 3 mg L^{-1} and 7 mg L^{-1}, respectively could slightly increase the abundance of *Planctomycetes* phylum [21]. Nevertheless, further increasing of these antibiotics concentration to 9 mg L^{-1} led to a decrease in *Planctomycetes* abundance by 5.24 % (SDM) and 17.1 % (SM). Therefore, the effect of antibiotics on the microbial community of anammox biomass is still hard to explain and requires further research.

In this study, the long-term effects of three antibiotics (oxytetracycline (OTC), ciprofloxacin (CIP), and clarithromycin (CLA) at a broad range concentration (0.001–100 mg L^{-1}) on the anammox process were evaluated. OTC, CIP, and CLA were selected because they exist in actual wastewater and reported hazardous impacts on the environment. However, few studies have investigated the effect of OTC on the anammox process, while CIP and CLA have been investigated only once at trace concentration (0.001 mg L^{-1}) [27]. Therefore, studies of the effects of increasing concentrations of CIP and CLA on the anammox process are a novelty in this study and it will bring important knowledge from the point of its practical usage due to the fact that in real wastewater antibiotics concentration usually fluctuates, The focuses of this study are: (i) determining the impact of OTC, CIP and, CLA on the anammox process performance; (ii) revealing the effect of antibiotics on anammox biomass community structure; (iii) exploring the influence of antibiotics on extracellular polymers and morphology of the anammox bacterial cell; (iv) illustrating the changes in nitrogen-cycle functional genes and antibiotic resistance genes, as well as the correlation between these genes and dominant genera with their network analysis. Compared to previous work investigating the effects of antibiotics on the anammox process, this research has been extended to study the ultrastructure of anammox bacteria by transmission electron microscopy (TEM), which allows for assessing antibiotic-induced changes in the structure of anammox bacteria cells. In addition, five functional genes responsible for enzymes production, which are involved in nitrogen removal from wastewater (*amoA* determining first step nitrification, *nxrA* determining second step nitrification, *nirS/nirK* determining denitrification, *hzo* determining anammox) were analyzed bringing detailed information on the relationships between microorganisms in the anammox-dominatedbiomass and assess their activity. Moreover, this paper also presents research over the EPSs presence in the analyzed activated sludge. Due to the fact that EPs as the first barrier against antibiotics the protective features of these compounds need to be verified. The outcomes of this study extend the knowledge about the effect of antibiotics on the anammox process and provide information about the role of anammox bacteria in the spreading of ARGs in the biological treatment system. Moreover, the results bring a theoretical guide for the implementation of the anammox process in practice.

2. Materials and methods

2.1. Seeding sludge

The inoculated anammox sludge was collected from a sequencing batch reactor (SBR) with an active volume 20 L that had been stably performing for more than six years at the laboratory scale. The anammox process was operated under optimal conditions for anammox bacteria requirements (32.0 °C, pH = 7.8), and the nitrogen removal efficiency (NRE) was approximately 90 % [22]. Candidatus *Brocadia* was the dominant anammox genera in this system.

2.2. Construction and operation of the bioreactor

Four SBRs (sequencing batch reactors) named Control, R-OTC, R-CIP, and R-CLA with an effective volume of 5 L were inoculated with seeding sludge (5 L each). R-OTC, R-CIP, and R-CLA were used as experimental bioreactors and fed with an antibiotic-containing medium (OTC, CIP, and CLA, respectively), while the Control was operated with

an antibiotic-free medium. In natural conditions, antibiotic concentrations in the wastewater gradually increase with continuous exposure to antibiotics, such as in case of hospital wastewater [31]. Because the hospital wastewater is discharged to a municipal WWTPs where also the concentration of antibiotics constantly increases. Therefore, accompanied by the synthetic medium, the tested reactors were fed with successively increasing concentrations of antibiotics. The operating period of the reactors was divided into five specific phases as shown in Table 1. The antibiotics were constantly dosed to the SBRs with a synthetic medium to maintain a relatively stable concentration in the system. The reactors were fed with a mineral medium that has been adapted from van de Graaf et al. [23]. The total nitrogen concentration was 260 mg L^{-1} and was regulated by NH_4Cl and $NaNO_2$ addition. The other components of mineral medium were 0.048 g L^{-1} ($KHCO_3$), 0.041 g L^{-1} (KH_2PO_4), 0.228 g L^{-1} ($MgSO_4{\cdot}7\ H_2O$), 0.007 g L^{-1} ($FeSO_4{\cdot}7\ H_2O$), 0.004 g L^{-1} (EDTA). The synthetic wastewater was prepared on tap water which include 318 mg $CaCO_3$ L^{-1}; therefore, no additional calcium ion source was added. The medium pH was regulated to level of 7.0. During the experiment, reactors were operated at a pH of 7.5 ± 0.4, temperature 32 ± 0.5 °C, dissolved oxygen (DO) below 0.1 mg L^{-1}, the hydraulic retention time (HRT) was 1 day with a volume exchange ratio of 25 %. The volatile suspended solid (VSS) in reactors was in range 1200–1248 mg VSS L^{-1} and changed through the experiment period as shown in Table S3. Each bioreactor was operated for four-cycles per day. The scheme and technical parameters of the anammox systems used in this study and cycle parameters are shown in Fig. S1.

2.3. Collection and characterization of samples

The influent and effluent of each bioreactor was sampled for NH_4-N, NO_2-N, and NO_3-N analysis using photometric tests (MERCK Millipore) with a spectrophotometer (MERCK Spectroquant® NOVA60). pH levels were monitored using a pH meter (WTW pH 330i). DO concentrations were measured using an ELMETRON Conductivity/Oxygen Meter CCO-505 equipped with an ELMETRON COG-1 oxygen probe. VSS concentrations were measured according to the standard method [24].

At days 1, 48, 111, 201, 288 and 340, as well as from the inoculum (IN), sludge samples were taken from the bioreactors for genetic analysis of factors including ARGs, functional genes, and community structure changes, and extracellular polymeric substances (EPSs) production. At days 111, 201, 288 and 340, sludge samples were collected for microscopic analysis. EPSs were extracted by the 'heating' method according to the method described previously [25], and determination of polysaccharides (PS) and proteins (PN) was performed using the modified Lowry and Anthrone methods, respectively.

2.4. Genetic material extraction and qPCR performance

Anammox bacteria always co-exist with other nitrogen-cycle bacteria such as nitrifiers and denitrifies in the anammox systems. Therefore, to clarify their role in nitrogen removal, enzymatic activity should be studied based on molecular biology studies. Moreover, the abundance of selected genes determining OTC, CIP and CLA resistance also were evaluated via molecular tools. GeneMATRIX Soil DNA Purification Kits (EurX®, Poland) were used for total genomic DNA extraction from collected sludge samples. The quantity of isolated DNA was verified using a Qubit® Fluorometer (Invitrogen, USA) and was stored at −45 °C. The functional nitrogen-cycle genes (*nirS, nirK, amoA, nxrA, hzo),* integrase first-class (*intI1*) and antibiotic resistance gens for OTC (*tetX*, *tetC*, *tetW*), for CIP (*qnrB4*, *qnrS*) and for CLA (*mphA*) were determined using quantitative PCR (qPCR) via a QuantStudio™5, Real-Time PCR System, 96-well, 0.2 mL (TermoFisher Scientific, USA) with PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific, USA) according to the manufacturer's instructions. The qPCR technology is widely used in quantitative gene analysis due tothe its reliable and repeatable results. The corresponding primer pairs are listed in Tables S1 and S2 were used. Results were presented as a relative abundance of a reference gene (16S rRNA) according to the method described by Livak and Schmittgen [26].

Table 1
The operational strategy in this study.

Stage	Time (d)	Control	R-OTC OTC (mg/L)	R-CIP CIP (mg/L)	R-CLA CLA (mg/L)
P_0	1–48	0	0	0	0
P_1	49–111	0	0.001	0.001	0.001
P_2	112–201	0	1	1	1
P_3	202–288	0	10	10	10
P_4	289–340	0	100	100	100

2.5. Community structure analysis

To properly understand the changes that occur in anammox biomass during antibiotic-induced stress, it is necessary to analyze the community structure of this biomass. The changes in the microbial community structure were analyzed by high-throughput sequencing according to the methodology described by Gamoń et al. [27]. Briefly, the V3-V4 region of the 16S rDNA coding gene was amplified using S-D-Bact-0341-*b*-S-17 and S-D-Bact-0786-a-A-21 primers [28] and NEBNext® High-Fidelity 2X PCR Master Mix, according to the manufacturer's instructions (Bio Labs Inc., USA). Sequencing reactions using paired-end technology 2 × 250 nt with a MiSeq Reagent Kit V2 (Illumina, USA) were performed by a MiSeq sequencer, then automatically analyzed using the MiSeq Reporter (MSR) v 2.4 software (Illumina, USA). Obtained data were uploaded to the MetaGenome Rapid Annotation Subsystems Technology (MG-RAST) and a ClassifyReads algorithm was used for a species-level classification step. Changes in community diversity were evaluated with Shannon and Simpson indices.

2.6. Microscopic analysis

Antibiotics may affect the structure of the anammox cell, therefore, changes in the ultrastructure of anammox cells under antibiotic suppression were evaluated using transmission electron microscopy (TEM). The samples of anammox biomass were fixed with 2.5 % glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.4) and stored at 4 °C, then washed in a phosphate buffer (3 × 15 min) and postfixed with 1 % OsO_4 in a 0.1 M phosphate buffer at room temperature (30 min). After fixation and washing in phosphate buffer (3 × 10 min), the samples were dehydrated in a graded series of ethanol (30, 50, 70, 90, 95, and 4 × 100 %, each for 5 min.), and ethanol: acetone (1:1) for 5 min, and in acetone (2 × 5 min.). Then, the samples were embedded in epoxy resin (Epoxy Embedding Medium Kit, Sigma). The material was cut into ultrathin sections (50 nm) using a Leica EM UC7 ultramicrotome, mounted on formvar coated copper grids (mesh 100), and stained with uranyl acetate and lead citrate according to the methodology described by Reynolds [29]. The microscopic observations were performed using a transmission electron microscope (Hitachi H500) at 75 kV.

2.7. Statistical analysis

A network analysis was performed to analyze the interaction between selected genera in the tested reactors, ARGs, and nitrogen-cycle functional genes. The networking relations were constructed based on Spearman's correlation coefficients between the analyzed data. The significant level was considered 5 %, as the commonly used in statistical analysis. The visualization of statistical results was achieved using Gephi (V 0.9.1) software. R software (https://www.r-project.org) with the factoextra package was used for principal component analysis (PCA) and the corresponding visualization.

3. Results and discussion

3.1. Performance of the anammox process exposed in OTC, CIP, CLA

The nitrogen removal performance of four SBRs (Control, R-OTC, R-CIP, R-CLA) is shown in Fig. 1a–d. The operating period of these reactors was divided into five phases (Table 1). The control reactor exhibited relatively stable operation for 340 days with an average nitrogen removal rate (NRR) of 0.195 ± 0.018 kg N m^{-3} d^{-1} and an average NO_2^--N concentration in the effluent of 0.4 ± 0.5 mg L^{-1} (Fig. 1a). Before adding antibiotics, SBRs were stabilized for 48 days (P_0). After this time 0.001 mg L^{-1} of OTC, CIP, and CLA were added to the R-OTC, R-CIP, and R-CLA bioreactors, respectively, which had no significant impact on the anammox process performance. Similar results were obtained previously, where the anammox process maintained stable performance for 30 days at trace concentration (0.001 mg L^{-1}) of OTC, CIP, and CLA [27]. Zhang et al. [20] compared the action of ERY and NOR at 0.001 mg L^{-1} on the anammox performance, indicating that ERY (macrolide class) had no impact on anammox while NOR (fluoroquinolones class) reduced the specific anammox activity (SAA) from 10.8 to 7.56 mg g^{-1} SS h^{-1} (reduction by 30 %). Resistance genes targeting ERY played a crucial role in the negligible effect of ERY on the anammox process. Two genes that targeted ERY (*mphA* and *ermB*) were found in the reactor fed with ERY, while no ARG induced NOR was detected in the system [20].

In phase 2, the addition of 1.0 mg L^{-1} of antibiotics caused a deterioration of nitrogen removal rate (NRR) by 12.21 % (R-OTC), 13.5 % (R-CIP), and 9.1 % (R-CLA) from the initial level. Moreover, the concentration of NO_2-N had not exceeded 0.7 mg L^{-1} in the effluent of each reactor, with maintained stable NO_2 removal of 99.8 % during P_2. Thus, these results indicated that the concentration of 1 mg L^{-1} of tested antibiotics had negligible effect on the anammox process performance deterioration. Previous studies have determined that 0.5 mg L^{-1} of spiramycin (macrolide class) could be considered the threshold concentration for the anammox process performance during long-term antibiotic suppression [30]. Nevertheless, Jing-Wu et al. [31] presented that anammox bacteria could adapt to the concentrations of spiramycin below 1 mg L^{-1}, mainly due to defense mechanisms such as the production of EPSs being employed. Moreover, the anammox process could be resistant to erythromycin (macrolide) in concentrations below 1 mg L^{-1} [30]. The stoichiometric molar ratios R_S (NO_3-N production/NH_4-N removal) and R_P (NO_2-N removal/NH_4-N removal) fluctuated slightly; however, it was close to the theoretical value (1.32 and 0.26, respectively). This indicated that anammox was the dominant nitrogen removal process in bioreactor treating medium with 1 mg L^{-1} of antibiotics. Such tolerance of anammox bacteria could benefit from the secretion of EPSs and the spread of ARGs (as discussed in Sections 3.2 and 3.4).

Increasing the concentration of antibiotics to 10 mg L^{-1} resulted in further deterioration of the anammox process performance. On day 271, the NRR of the reactor fed with OTC and CLA decreased to the lowest levels of P_3 reaching 0.134 kg N m^{-3} d^{-1} and 0.118 kg m^{-3} d^{-1}, respectively, while R-CIP reached the lowest value of NRR on day 288 (0.091 kg m^{-3} d^{-1}). In the same periods, the NRR in the Control reactor presented at a level of 0.184 kg N m^{-3} d^{-1}. Moreover, as presented in Tab. S3., the average of NRR in P_3 was 0.169 ± 0.02 kg m^{-3} d^{-1} (R-OTC), 0.156 ± 0.03 kg m^{-3} d^{-1} (R-CIP) and 0.145 ± 0.04 kg m^{-3} d^{-1} (R-CLA) representing a decrease from the initial level (P_0) by 19.9 %, 26.57 %, 29.9 %, respectively, while in the Control reactor in P_3 an increase in NRR of 1.57 % compared to the initial level was obtained. These results indicated that CIP and CLA had a significant impact on the anammox process performance at 10 mg L^{-1}. Zhang et al., [32] reported that spiramycin at 3 mg L^{-1} led to significant deterioration of the anammox process, while 5 mg L^{-1} provided to decrease nitrogen removal efficiency (NRE) to 53.08 %. Correspondingly, erythromycin at 10 mg L^{-1} caused the drop of anammox bacteria activity [18]. It has been shown that 2 mg L^{-1} of OTC could diminish the nitrogen removal capacity of anammox [33]. Additionally, operating the anammox process under 5 ± 3.5 mg L^{-1} for 35 days could completely inactivate the

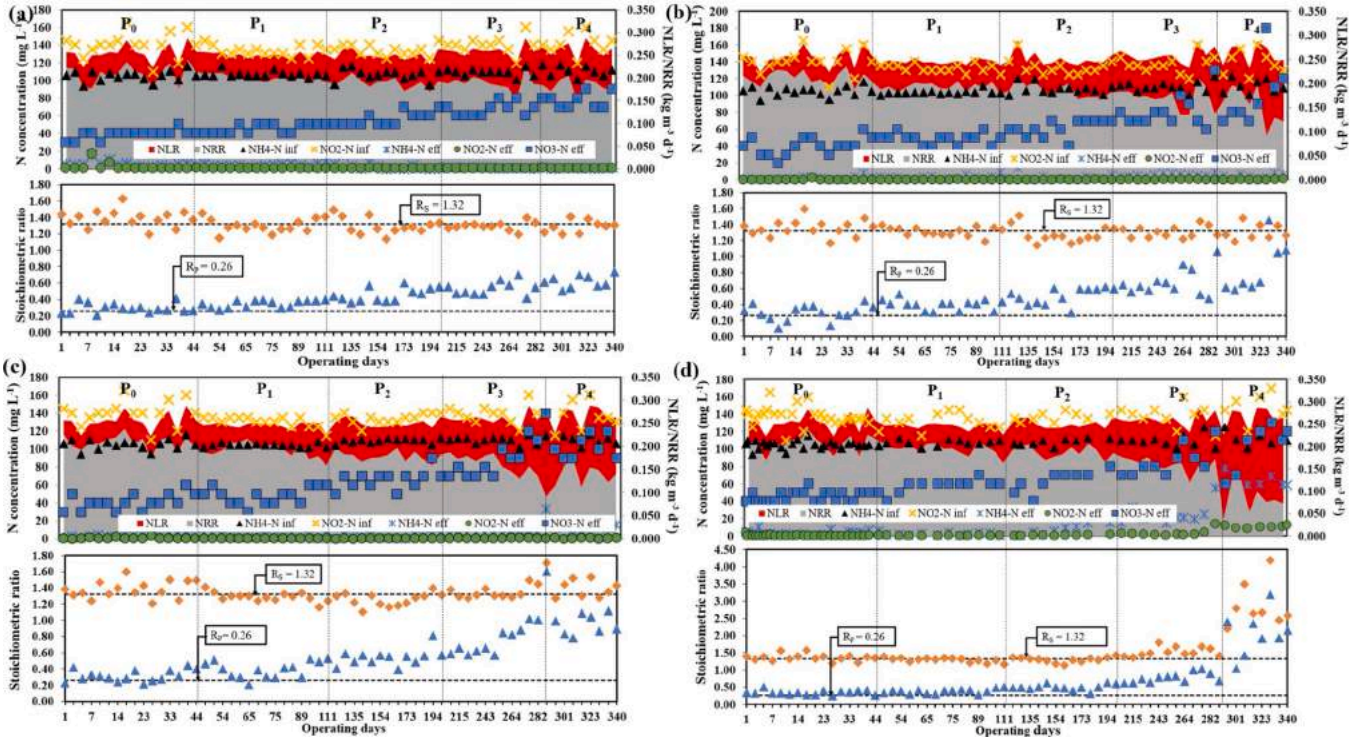

Fig. 1. The nitrogen removal performance of each SBR during operating days. The control was fed medium without antibiotics (a): R-OTC was fed with medium with OTC (b): R-CIP was fed with medium with CIP (c): R-CLA was fed with medium with CLA (d): NLR: nitrogen loading rate; NRR: nitrogen removal rate. R_P: NO_2-N rem/NH_4-N rem; R_S: NO_3-N prod/NH_4-N rem.

process performance [34]. Although the dosage of OTC used in P_3 was slightly higher, the process performance had not deteriorated dramatically. This is probably due to the high abundance of resistance genes targeted OTC in the tested biomass (Section 3.4). Zhang et al. [46] found that norfloxacin (NOR) in concentrations between 0.001 and 50 mg L^{-1} did not affect anammox bacteria, while in this study CIP caused significant deterioration of the anammox process at 10 mg L^{-1}. This suggests that the inhibitory effect of CIP on the anammox process is higher than that of NOR.

On day 289, the concentration of the antibiotic dosed to the bioreactors was changed to 100 mg L^{-1}, which was still much lower than the IC_{50} (half-maximal inhibitory concentration) value for OTC of 517.5 mg L^{-1} [36]. Unfortunately, there is no information in the literature on the IC_{50} for CIP and CLA. Nevertheless, Sguanci et al. [37] determined the value of IC_{50} for another antibiotic belonging to the fluoroquinolone family to be 157 mg L^{-1}. After 12 days of operation at 100 mg L^{-1}, the concentrations of NO_2-N and NH_4-N in the effluent of R-OTC were 0.2 and 1.0 mg L^{-1}, respectively, of R-CIP was 0.1 and 18.0 mg L^{-1}, while in the R-CLA reached 12.0 and 77.4 mg L^{-1}. At the end of the period P_4, the concentrations of NO_2-N and NH_4-N in the effluent of tested reactors were 1.3 and 6.9 mg L^{-1} (R-OTC), 0.8 and 15.3 mg L^{-1} (R-CIP), 12.3 and 58.6 mg L^{-1} (R-CLA). Correspondingly, the NRR decreased by 38.2 % (R-OTC), 10.9 % (R-CIP), and 44.3 % (R-CLA) under 100 mg L^{-1} antibiotic suppression. These results suggested that the inhibitory effect of CLA on the anammox process is higher than of CIP and CLA. Moreover, the stoichiometric molar ratios R_S and R_P values in tested reactors were much higher than the theoretical value ($R_S = 1.32$, $R_P = 0.26$). On day 330, R_S and R_P reached the highest values of 4.18 and 3.19, respectively (R-CLA), 1.24, and 1.46, respectively (R-OTC), while in R-CIP the highest value was obtained on day 337 ($R_S = 1.35$, $R_P = 1.11$). This indicates that anammox biomass underwent remodeling, and the activity of nitrifying bacteria increased. The occurrence of nitrifying bacteria in the anammox biomass was previously reported by Ziembińska-Buczyńska et al., [22] and Banach-Wiśniewska et al., [38]. Special attention should be paid to *Nitrospira*, which was been proven to be present in the test reactors (Section 3.5). The *Nitrospira* genus, belonging to NOB, is able to conduct complete nitrification (comammox) under anaerobic conditions [39]. A previous study showed that *Nitrospira* develops under a low concentration (0.001 mg L^{-1}) of OTC, CIP, and CLA [27]. Moreover, *Nitrospira* could be enriched under ampicillin (AMP), kanamycin (KAN), lincomycin (LIN), and trimethoprim (TMP) suppression [40].

Overall, the anammox process exhibited resistance OTC, CIP, and CLA under low concentrations (<1 mg L^{-1}). Although the dose of antibiotics that affected the anammox process mainly depends on the type of antibiotics, it has been suspected that 1 mg L^{-1} can be the threshold for antibiotic action [31,41]. EPS play a crucial role in the protection of anammox bacteria exposed to antibiotic concentrations below 1 mg L^{-1} [42]. As the concentration of antibiotics increased to 10 mg L^{-1}, the inhibitory effect of OTC, CIP and CLA gradually became apparent. Antibiotic inhibition achieved the highest level in all reactors in the P_4 period. Although each tested antibiotic affected the anammox process performance the most at 100 mg L^{-1}, the highest deterioration in anammox performance was caused by CLA.

3.2. EPSs production and anammox bacteria morphology changes

In terms of anammox biomass characteristics, the production of extracellular polymeric substances (EPSs) was monitored. EPSs were calculated with regard to volatile suspended solids (VSS) (Fig. 2). Production of EPSs by microorganisms is recognized as a protective barrier to prevent inhibition by pollutants [35,42]. Among all EPSs components, proteins (PN) and polysaccharides (PS) are viewed as important [27,31]. Cumulative protein and polysaccharide-based EPSs (PN + PS) in each reactor fed with medium containing antibiotics presented a similar tendency; first these decreased for 48 days, then increased at the beginning of antibiotic addition to the anammox system. A similar tendency was observed in the control, where EPSs content first decreased for 48 day, then increased between 48 and 111 day. The highest EPSs concentration was observed on the last day of the experiment when the antibiotic concentration was 100 mg L^{-1} (R-OTC – 73.7 mg g^{-1} VSS, R-CIP – 67.1 mg g^{-1} VSS, R-CLA – 55.3 mg g^{-1} VSS). Although the content in the control reactor was poorly flocculated between 201 and 340 day, the average of EPSs concentration was at 48.3 ± 2.5 mg g^{-1} VSS. Correspondingly, the EPSs content reached the highest level under OTC suppression, reflecting previous research where

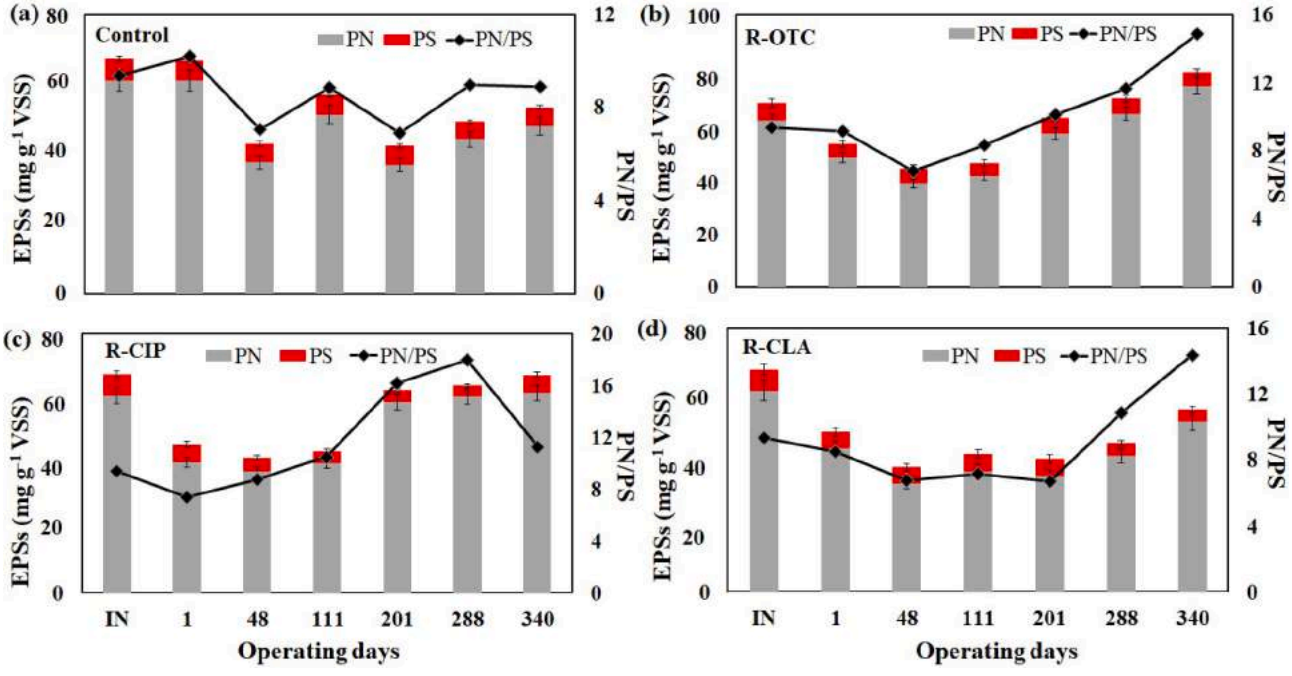

Fig. 2. Changes in EPSs concetration in the anammox biomass during the experiment period. (a): control, (b): R-OTC, (c): R-CIP, (d): R-CLA.

tetracycline antibiotics stimulated the expression of *lapA* gene responsible for protein synthesis needed in EPSs production [43]. Biomass with higher SAA tends to secrete more EPSs [44]. As shown in Fig. S3, biomass in the R-OTC reactor showed the highest SAA among all reactors dosed with antibiotics throughout all operating days. Jing-Wu et al. [31] reported that two different strategies used to dose spiramycin to the anammox systems at a maximum of 5 mg L^{-1} (elevating-concentration strategy and repeated-exposure strategy) resulted in an increase in EPSs production by 11.7 % and 34.3 %, respectively. Furthermore, other studies have shown that anammox bacteria could resist the influence of erythromycin at concentrations of 1 mg L^{-1} due to EPSs protection [19,45]. According to the PN and PS content in the EPSs, the ratio of PN/PS reflected the hydrophobicity of biomass [46]. A reduction in PN/PS ratio results in poor hydrophobicity and reduces the flocculation capacity of the biomass. As shown in Fig. 2b and c, in the R-OTC and R-CIP reactors the PN/PS ratio first decreased reaching the lowest value on day 48 (6.8 and 6.7, respectively), and then gradually increased reaching the maximum value on day 340 (14.8 and 14.3, respectively). A slightly different situation was observed in the R-CIP reactor, where the PN/PS ratio increased from day 1 (PN/PS = 9.38) until day 288 reaching 18.0, however, after operating the anammox process at 100 mg L^{-1} CIP, the PN/PS ratio decreased to 11.29 on day 340. This was probably due to the inhibition of protein synthesis caused by CIP suppression. Notably, the PN content gradually increased when antibiotics were added to the bioreactors. It was reported that the proteins in EPSs play an important role in transporting small molecules [47,48]. PN binding to antibiotics is caused by the presence of amine, hydroxyl, and carboxyl functional groups [49,50]. An increase in the PN/PS ratio causes stronger hydrophobicity inducing adsorption of antibiotics and further antibiotic suppression on the anammox bacteria.

Three basic components can be distinguished in the structure of anammox bacteria cells: paryphoplasm, riboplasm, and the anammoxosome. The last one is a structure in which biochemical processes occur [51]. Changes in anammox bacterial cell inner structure under antibiotic suppression are shown in Fig. 3. The addition of 0.001 mg L^{-1} of antibiotics did not cause significant changes in the structure of anammox bacteria cells. The anammox community identified in the TEM images displayed mostly regular shapes with clearly visible anammoxosomes in the center of the cells. In the P_2 phase, the cell structures became more irregular, especially for the cells treated with the R-OTC and R-CLA. In the next two phases, changes in cell irregularity progressed. It is worth pointing out that no lysed cells were found in the R-OTC and R-CLA. This phenomenon is probably due to the presence of EPSs production. It can be seen that the surface of the anammox cell is covered by a network-like matrix which is probably EPSs. The thickness of this matrix increased as the concentration of OTC and CLA increased (marked E in the Fig. 3). This confirmed the protective role of EPSs against antibiotic action. Similarly, Zhang et al. [10], using TEM, showed that EPSs protected anammox bacteria against cell damage under sulfamethazine exposure. In the R-CIP, it was observed that the density of anammox-like cells decreased at antibiotics concentrations above 10 mg L^{-1}. Moreover, at 10 mg L^{-1}, many lysed or irregular cells were observed. Similar changes were observed at 100 mg L^{-1} of CIP. This stays in line with the PN/PS ratio obtained for EPSs content in R-CIP. As mentioned, a decreased PN/PS ratio led to the reduction of flocculation properties, therefore, more biomass could be washed out of the reactor. Lysis of anammox bacterial cells has been observed previously under florfenicol belonging to the chloramphenicol class [10]. The irregular shape of anammox cells exposed to antibiotics has been reported in the literature [52,53]. Although OTC, CIP, and CLA do not act directly on the cell wall via blocking its synthesis, they can effectively inhibit the protein synthesis of components of the cell wall. Therefore, the integrity of the cell wall is disrupted, which can be seen in irregular morphology.

3.3. Functional gene abundance changes

The abundance of nitrogen-cycle functional genes with increasing antibiotics concentration is shown in Fig. 4. The abundance of *amoA* in the inoculum (IN) presented the highest value (1.24×10^{-3}), while for

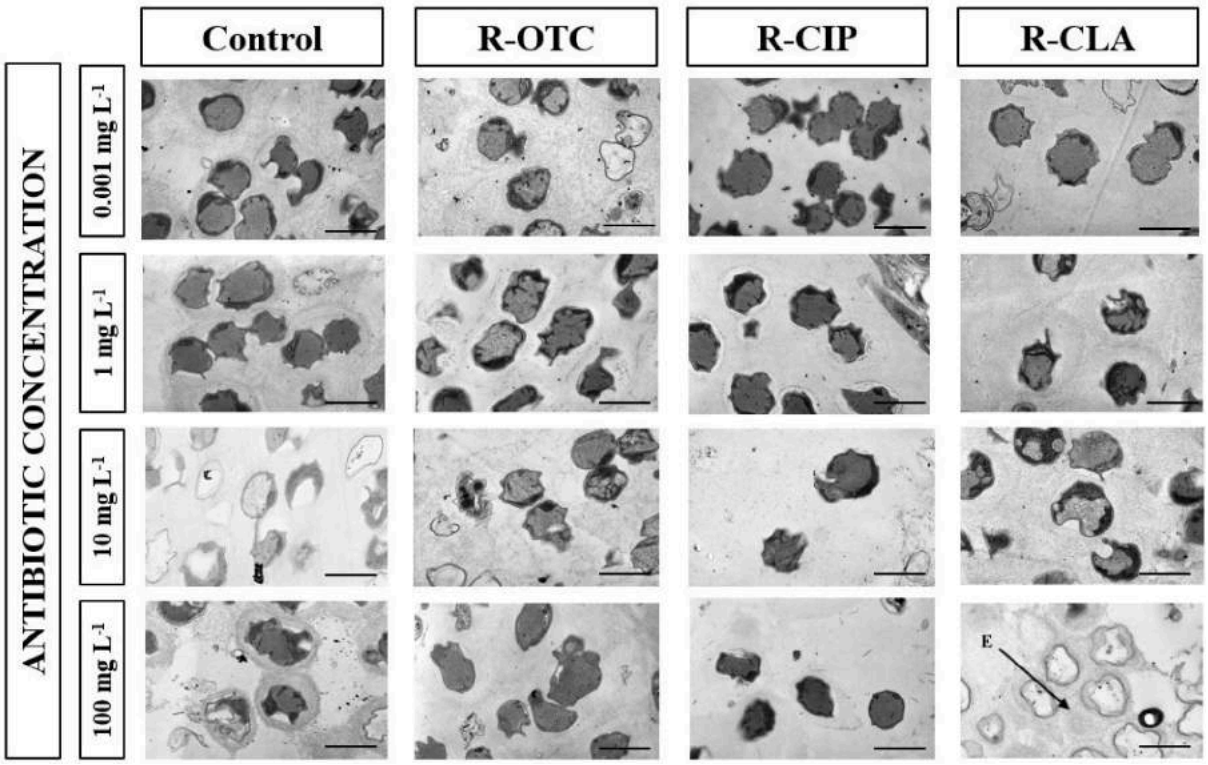

Fig. 3. Anammox bacteria structure visible under transmission electron microscopy under OTC, CIP, CLA suppression at successive concentrations. Bar = 0.85 μm.

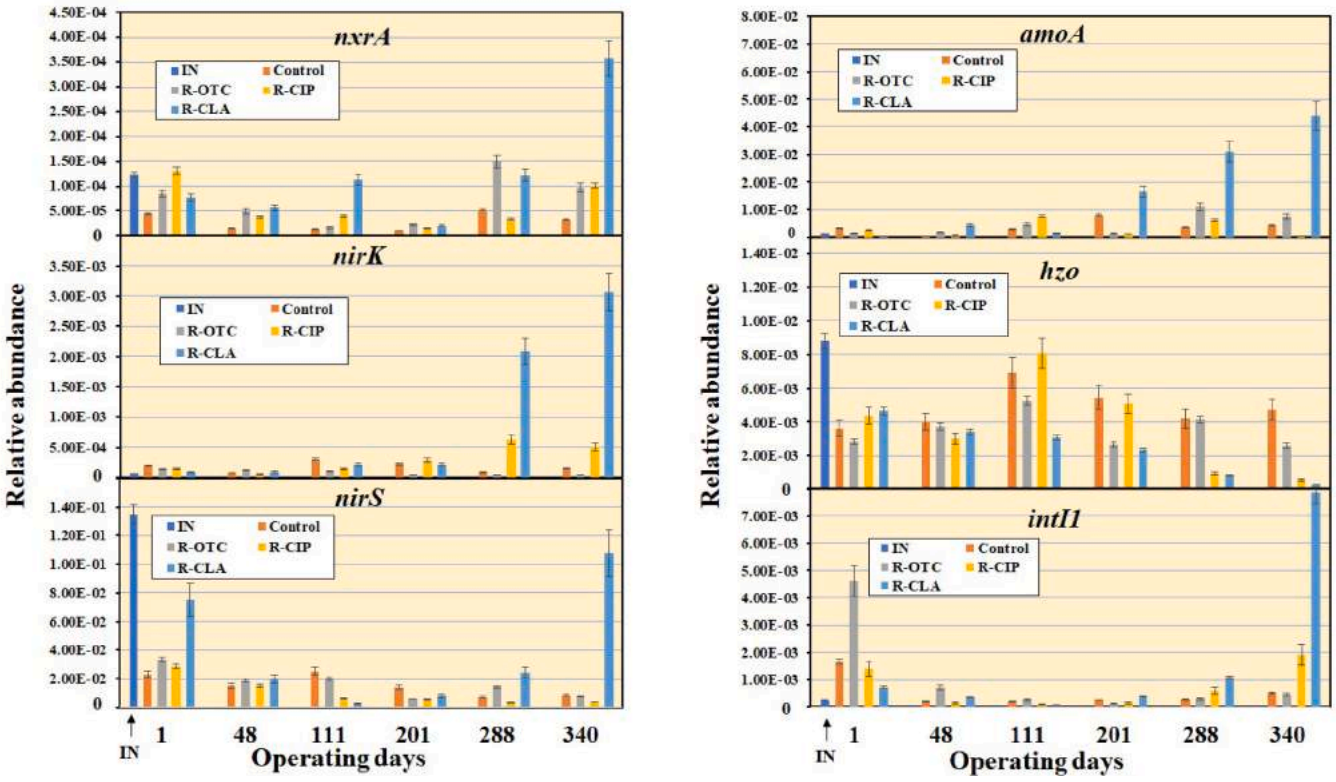

Fig. 4. Changes in the relative abundance of five nitrogen conversion genes (nxrA, amoA, nirK, nirS, hzo), and intI1 in anammox biomass in four tested SBRs.

hzo it was 8.83×10^{-3}. In general, a significant increase of *amoA*, *nxrA*, *nirK*, *nirS* abundance was observed in the experimental bioreactor fed with medium containing CLA at concentrations of 10 mg L^{-1} and 100 mg L^{-1}. For R-OTC and R-CIP there were no significant changes in *amoA*, *nxrA*, *nirK*, *nirS* gene abundance. Although anaerobic conditions prevailed in the reactors, a relatively high number of both *amoA* and *nrxA* commonly attributed to aerobic AOB and NOB, respectively was observed. The high abundance of *amoA* and *nxrA* genes may suggest the activity of *Nitrospira* species. It is worth noting that the presence of *Nitrospira* in high abundance was confirmed by metagenomic analysis (Section 3.5) As reported previously, *Nitrospira* expresses a full set of ammonium converting genes: ammonium monooxygenase (AMO), nitrite oxidoreductase (NXR), and hydroxylamine dehydrogenase (HAO) [39]. This allows *Nitrospira* to undergo complete nitrification directly. This is called the comammox process. The occurrence of comammox capable bacteria in anammox systems was previously confirmed [27]. Moreover, the increasing abundance of both *amoA* and *nxrA* under the successive concentration of CLA suggests that bacteria using these pathways might be resistant to CLA. Previous studies found that some antibiotics had a negligible effect on nitrifying bacteria i.e., OTC and ERY caused slight inhibition of nitrification at 75 mg L^{-1} [54] and 20 mg L^{-1} [55], respectively [56].

Similar to previous studies, the dominant pathway attributed to the denitrifying bacteria in the anammox system is *nirS* [27,38]. However, when the concentration of CLA reached 10 mg L^{-1} the *nirK* gene began to dominate. Pashaei et al. [57] suggest that a high diversity of denitrifying microorganisms is likely to mean that an individual antibiotic is unlikely to completely inhibit the process because inhibition of some group of denitrifiers does not mean inhibiting another group. Therefore, a concentration of CLA above 10 mg L^{-1} might not cause inhibition of denitrifiers using the *nirK* pathway. Moreover, the stable level of both *nirK* and *nirS* genes in R-OTC and R-CIP during the experimental period show that denitrifiers appear to be relatively insensitive to the presence of antibiotics.

The abundance of the *hzo* gene was relatively stable in the control reactor during the total experimental period. For bioreactors fed with antibiotic-rich medium, no changes were observed after the stabilization period (day 48). After this time in the reactor fed with CIP, first, the increase of *hzo* abundance was observed reaching 8.09×10^{-3} on day 111, this then dropped to 5.11×10^{-3} (day 201) and maintained a stable value until the end of the experiment. The increase of anammox functional gene abundance under low antibiotic concentrations was previously reported [18,31,58]. The increase in enzymatic activity at low concentrations of antibiotics may be due to the protective function of EPSs [58] or the stimulation of stress factor induced gene expression [18]. The addition of CLA leads to a continuous decrease of *hzo* gene abundance. Between day 111 and day 201 a slight decrease was observed (from 3.06×10^{-3} to 2.35×10^{-3}), while the addition of 10 mg L^{-1} of CLA caused a drop in *hzo* abundance to 8.46×10^{-4} and further decline to 2.78×10^{-3} at 100 mg L^{-1}. These results correspond to the previous study, where spiramycin (macrolide) at a concentration between 1 and 5 mg L^{-1} did not change the abundance of anammox functional genes [32]. The abundance of the *hzo* gene under OTC suppression maintained a relatively stable level, with the highest value on day 111 (5.29×10^{-3}) and the lowest value on day 340 (2.61×10^{-3}). This indicated that OTC had little impact on the expression of the *hzo* gene. These results contrast with previous studies, where 2 mg L^{-1} of OTC was able to inhibit the anammox functional genes (*hzsA*, *hdh*) [19]. It was proven that *hdh* is more sensitive to a low dose of OTC [19]. This may suggest that the *hzo* gene is more resistant to OTC than other anammox functional genes.

Integron – integrase 1 class (*intI1*) is recognized as a representative horizontal mobile element and is usually located on mobile genetic elements such as plasmids and transposons [59,60]. *IntI1* was found to have a positive correlation with some ARGs, thus playing an important role in the transfer of antibiotic resistance [31,61]. In this study, the Control bioreactor, R-OTC maintained a stable level during the operating period with an average of 5.16×10^{-4} and 1.08×10^{-3}, respectively. In the R-CIP, a slight increase was observed on day 340 from 6.02×10^{-4} to 1.91×10^{-3}. The most significant change in *intI1* abundance

was obtained for the bioreactor fed with CLA at 100 mg L^{-1}, which increased from 1.09×10^{-3} to 7.85×10^{-3}. A previous study shows a significant correlation between ARGs targeting tetracycline in the anammox system [15]. On the other hand, there was no positive interaction between *intI1* and *tet* genes studied in this research (Section 3.6). However, it is worth noting that a positive correlation was obtained for *intI1* and CLA resistance genes (*mphA*) ($r = 0.86$, $p < 0.05$). Indeed, a strong positive interaction was reported between the macrolide resistance genes *ereA* and *intI1* [61–63]. Confirmation of these results was also presented by Jing-Wu et al. [31] where a similar interaction between *ereA* and *intI1* in anammox biomass was obtained. This implies that *intI1* is involved in the spreading of ARGs in anammox biomass, especially for genes targeting macrolide resistance.

3.4. ARGs abundance changes

The expression of ARGs is recognized as one of the most important mechanisms of bacterial self-protection against antibiotics. In this study nine ARGs were investigated: three determining OTC resistance (*tetX*, *tetC*, *tetM*,), two determining CIP resistance (*qnrB4*, *qnrS*), and one determining CLA resistance (*mphA*). In terms of ARGs encoding OTC resistance, as shown in Fig. 5, exposure to 10 mg L^{-1} OTC resulted in a significant increase of t*etX* from 1.93×10^{-3} (day 201) to 1.55×10^{-2} (day 288) and then slightly decreased under 100 mg L^{-1} OTC to 1.10×10^{-2} (day 340). However, concentrations below 10 mg L^{-1} OTC did not cause significant changes in *tetX* abundance. The relative abundance of *tetW* dropped during the adaptation period from 1.40×10^{-3} (day 1) to 2.02×10^{-4} (day 48), then increased slightly to 2.79×10^{-4} at 0.001 mg L^{-1} OTC (day 111). However, when the concentration of OTC was 1, 10, and 100 mg L^{-1} the abundance of *tetW* was almost undetectable. With regards *tetC* abundance, it first dropped from 1.40×10^{-3} (day 1) to 2.91×10^{-4} (day 48), then was stable at 0.001 mg L^{-1} and when the concentration of OTC was increased to 1 mg L^{-1}, a significant increase in *tetC* abundance was observed (from 3.49×10^{-4} to 1.98×10^{-3}). It is worth noting, that all ARGs-OTC genes were also detected in the control bioreactor with relatively low and stable abundance during all experiments. In this study, the abundance of *tetX* is higher than *tetC* during the operating period. Similar results were previously reported by Zhang et al. [32], Shi et al. [33]. Both *tetX* and *tetC* induce a different resistance mechanism: enzymatic modification and on the efflux pump, respectively [15]. Therefore, it can be suspected that enzymatic modification is a favored resistance mechanism for microorganisms in the anammox community. According to *tetW* abundance, this gene occurred mainly in WWTPs and livestock [61], implying that its occurrence in laboratory systems could be negligible, as observed in this study.

The relative abundance of the *qnrB4* gene increased significantly after exposure to 0.001 mg L^{-1} of CIP from 1.57×10^{-5} (day 48) to 2.37×10^{-4} (day 111), then dropped to 7.94×10^{-5} (day 201) and it maintained at a stable level until the end of the experiment. The abundance of the *qnrS* gene was found at a negligible level between day 1 and day 201. However, under exposure at 10 mg L^{-1} and 100 mg L^{-1} CIP, the abundance of *qnrS* increased reaching 2.04×10^{-5} and 2.97×10^{-5}, respectively. Comparing the *qnrS* and *qnrB4* gene abundance we suspect that resistant bacteria might preferentially express *qnrB4* at lower concentrations of CIP (0.001 mg L^{-1}), while at concentrations above a few mg L^{-1} they express *qnrS*. Similar to *tetW*, the *qnrS* gene is mainly prevalent in wastewater treatment plant installations contaminated with antibiotics [64]. However, its presence was detected in laboratory installations for the treatment of antibiotic-contaminant wastewater [27,65].

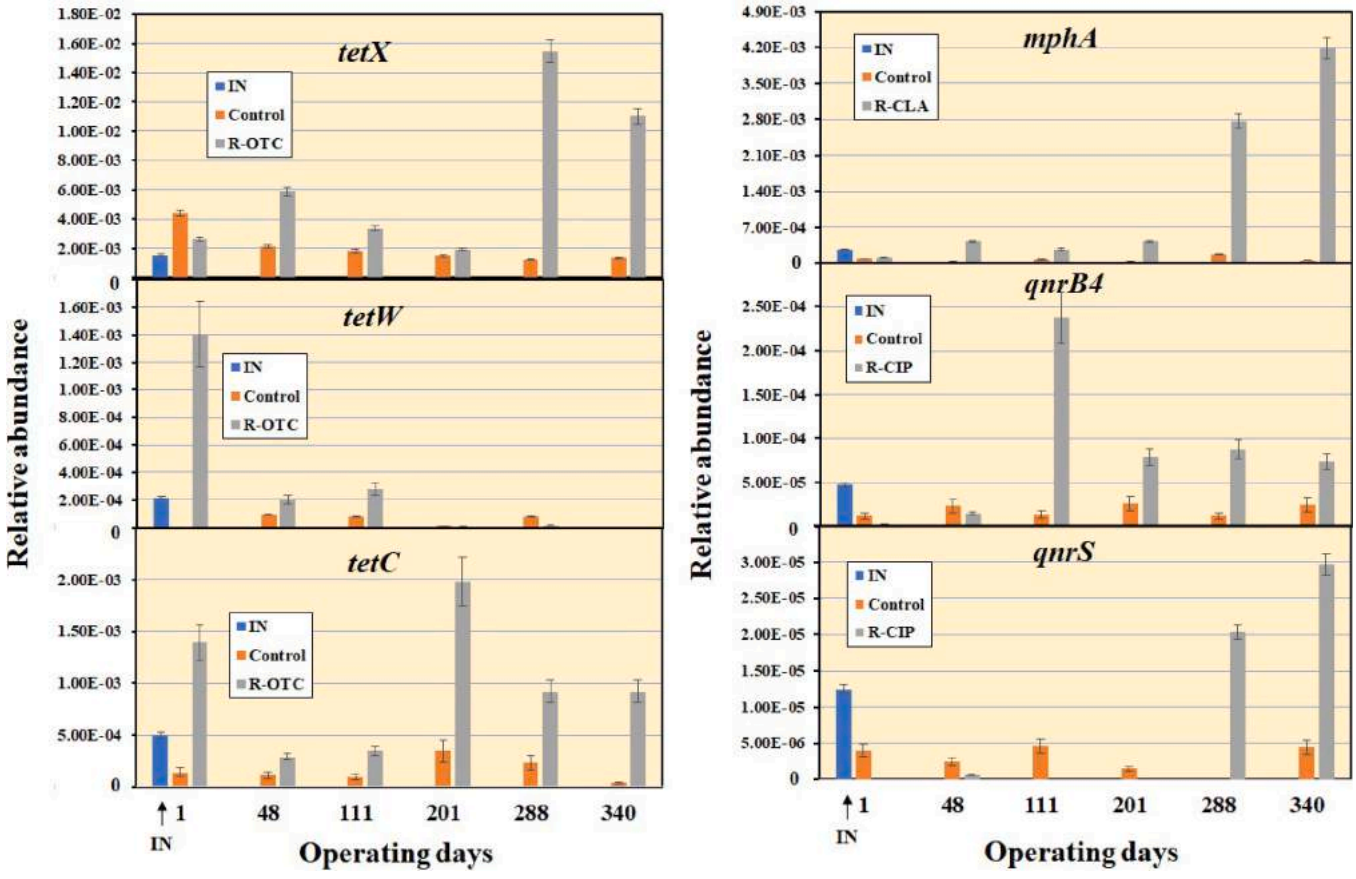

Fig. 5. Changes in the relative abundance of different ARGs determined OTC (tetX, tetW, tetC), CIP (qnrB4, qnrS), CLA (mphA) in the four SBRs during the experiment.

In our previous work [27], the abundance of *mphA* decreased from 7.45×10^{-4} to 9.0×10^{-5} under CLA stress at 0.001 mg L^{-1}. Similar results were obtained in this study, where *mphA* decreased under 0.001 mg L^{-1} from 4.34×10^{-4} to 2.72×10^{-4}. Notably, the abundance of *mphA* increased when the concentration was 10 and 100 mg L^{-1} and reached 2.76×10^{-3} and 4.19×10^{-3}, respectively. Therefore, it can be stated that concentrations above a few mg L^{-1} led to the development of *mphA* abundance in the anammox biomass. Zhang et al., [32] investigated the variation in expression of the *mphA* gene under spiramycin (macrolide) stress in anammox biomass. The authors obtained an increased *mphA* abundance from 3.60×10^{4} to 5.19×10^{6} copies ng^{-1} DNA under exposure of 1 mg L^{-1} spiramycin. Subsequently, exposure to 3 mg L^{-1} caused a decrease in *mphA* abundance, then a slight increase at 5 mg L^{-1}. They suggest that the *mphA* gene is more susceptible to macrolides under environmental conditions, due to the resistance mechanism.

3.5. Changes in the anammox community structure

Assessment of the change in community structure in response to increasing concentrations of OTC, CIP, and CLA was carried out using high-throughput sequencing of 16S rDNA. At the phylum level, *Proteobacteria*, *Planctomycetes,* and *Nitrospira* were the dominant phyla in the inoculum (Fig. 6a) occupying>70 % of the total abundance. Similarly, these three phyla presented the highest abundance on day 1 in the tested bioreactors. A significant increase in *Planctomycetes* abundance was observed in the control bioreactor after the adaptation period from 20.73 % (day 1) to 36.67 % (day 48), while in R-OTC, R-CIP, R-CLA no significant changes were observed with the level of 28.32 %, 27.71 %, 26.49 %, respectively. When the concentration of antibiotics was 0.001 mg L^{-1}, the relative abundance of *Planctomycetes* increased in tested bioreactors to 31.71 % (R-OTC), 34.54 % (R-CIP), 32.73 % (R-CLA), suggesting that trace concentration of antibiotics might contribute to the proliferation of *Planctomycetes*. The continuing increase of *Planctomycetes* abundance was observed in the R-OTC in P_2 (day 201), reaching 40.48 % while in R-CIP and R-CLA this decreased slightly to 32.75 % and 27.96 %, respectively. Similar proliferation of *Planctomycetes* abundance was obtained by Fan et al., [42] under erythromycin and sulfamethoxazole exposure at 0.1 and 1.0 mg L^{-1}, respectively. When the antibiotic concentration increased to 10 mg L^{-1} in P_3 (day 288), the abundance of *Planctomycetes* dropped to 24.67 % in R-OTC, while in R-CIP and R-CLA the abundance increased slightly to 34.99 % and 28.96 %, respectively. Interestingly, in P_4 the abundance of *Planctomycetes* in R-CIP and R-CLA with antibiotics increased reaching the highest value during the operating period of 49.20 % and 34.51 %, respectively. On the other hand, the abundance in R-OTC dropped to a level below that presented on day 1. This phenomenon suggested that *Planctomycetes* could overcome the inhibition of CLA and CIP action, while OTC significantly inhibited proliferation of *Planctomycetes* at concentrations above 10 mg L^{-1}. These results stand in contrast to nitrogen removal performance because in R-OTC the NRR in the P_3 and P_4 presented the lowest deterioration among all bioreactors fed with antibiotic containing medium. These indicated that CIP and CLA promote the development of unclassified *Planctomycetes* that have no ability to perform anaerobic oxidation processes. This statement correlates with biodiversity indexes for *Planctomycetes* phylum presented in Table S5. The Shannon index which informs on the richness of the community, in R-OTC presents a lower value in P_4 than in P_1, while in R-CIP and R-CLA increases in the index value were obtained. An opposite tendency was observed for the Simpson index which determines the probability of drawing two individuals belonging to the same species. This showed that CIP and CLA increased the biodiversity of genera belonging to *Planctomycetes*, which might be the reason for the resistance of *Planctomycetes* to these antibiotics. Under CIP and CLA stress, there may be an increased abundance of *Planctomycetes* bacteria unable to carry out the anammox process. This statement is supported by data on the *hzo* gene abundance (Section 3.3), which decreased under CIP and CLA suppression. Numerous studies have shown that *Planctomycetes* abundance decreased under suppression by various antibiotics [21,27,31].

Although the abundance of *Proteobacteria* at the end of the

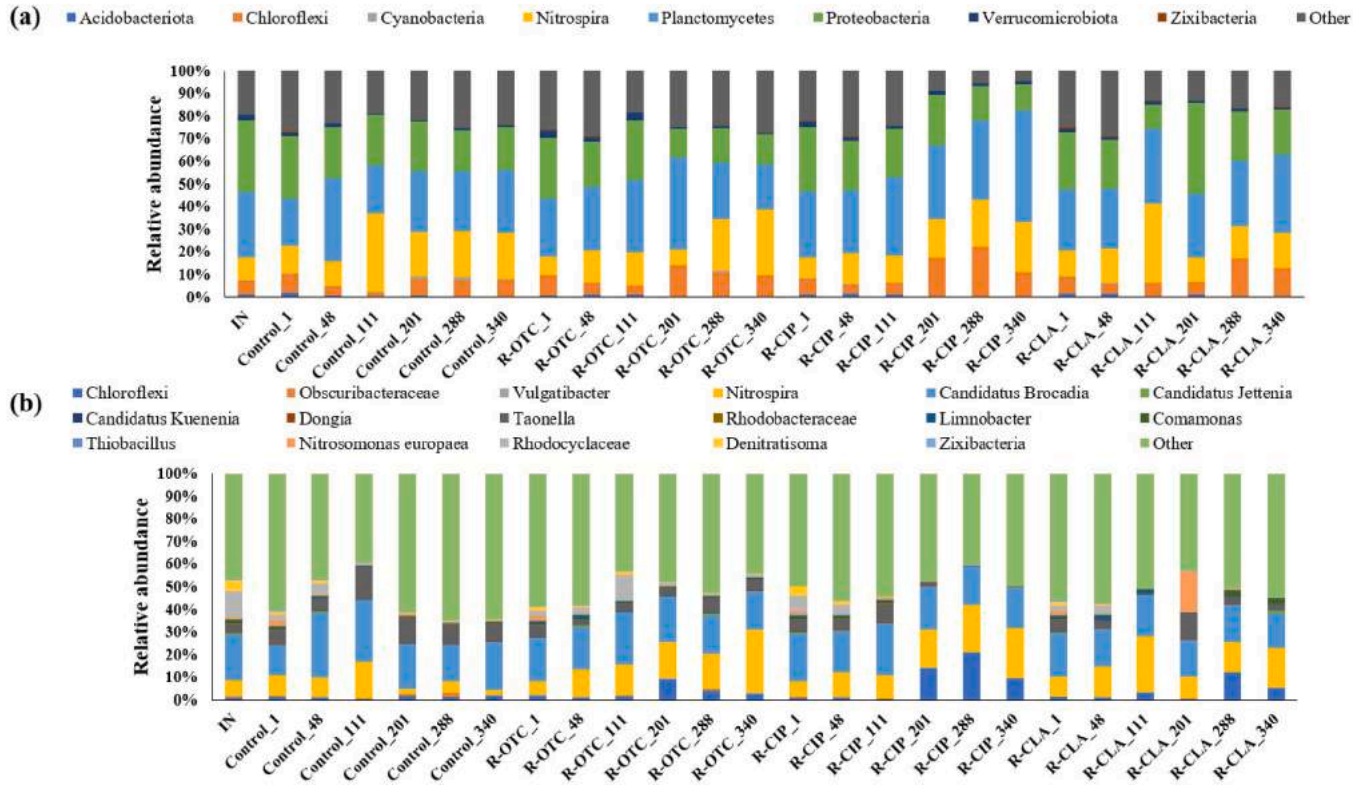

Fig. 6. Microbial community structure of the anammox bioreactors at the phylum level (a) and the genus level (b).

experiment were lower than initially they were still a dominant phylum in the biomass. As reported previously, this might be caused by the presence of dead cells of bacteria which are a source of organic carbon for *Proteobacteria* [27]. Nevertheless, *Proteobacteria* are able to utilize antibiotics as a carbon source [66]. In contrast, as the concentration of antibiotics increases the abundance of *Chloroflexi* bacteria continuously increased, reaching 9.36 %, 10.96 %, and 12.85 % in the R-OTC, R-CIP, R-CLA, respectively at the end of the experiment. *Chloroflexi* co-occurs with anammox bacteria in the technical anammox system, as well as laboratory-scale bioreactors. As reported previously, *Chloroflexi* can utilize EPSs produced by microorganisms as a substrate for growth [27,67]. Therefore, the increase in EPSs production obtained in this work promotes the growth of *Chloroflexi*. Although lack of organic carbon in the synthetic medium promotes autotrophic bacteria, the heterotrophic bacteria may survive by consuming EPSs and soluble microbial products [42,68]. Furthermore, it is possible to use antibiotics as a source of carbon, but this is difficult due to their chemical structure [69].

The community structure at the genus level is shown in Fig. 6b. The major anammox genera in tested bioreactors was Candidatus *Brocadia*. In the control bioreactor, the abundance of Candidatus *Brocadia* first increased reaching the highest level on day 48 (28.58 %) then continued to decrease to 21.35 % on day 340. In the bioreactors fed with antibiotic-rich medium, a similar trend was observed, firstly, the abundance of Candidatus *Brocadia* increased for 111 days reaching 23.15 % (R-OTC), 22.85 % (R-CIP), 18.55 % (R-CLA), then declined to 19.84 % (R-OTC), 19.49 % (R-CIP), 16.52 % (R-CLA) on day 201. However, in concentrations above 10 mg L^{-1}, the abundance maintained at a stable level until the end of the experiment with an average of 17.03 % (R-OTC), 17.66 % (R-CIP), 15.48 % (R-CLA), showing that incubation in increasing concentration of antibiotics allows the adaptation of Candidatus *Brocadia* to antibiotic suppression. This phenomenon could lead to observation that Candidatus *Brocadia* may be able to protect itself against antibiotic action. A similar statement was presented previously, where Candidatus *Brocadia* was able to oppose sulfamethazine at 7 mg L^{-1} [21]. On the other hand, the high sensitivity of Candidatus *Brocadia* to erythromycin was presented by Zhang et al., [12]. The ability of anammox bacteria to resist antibiotics was also confirmed by Zhang et al., [32], where an increase of Candidatus *Kuenenia* abundance was observed under exposure to 50 mg L^{-1} of streptomycin. Numerous studies have indicated that Candidatus *Kuenenia* could be the potential host of ARGs in the anammox system [27,30,41]. In this study however, Candidatus *Kuenenia* shows a negligible abundance during the experimental period. However, as reported by Bi et al [17], Candidatus *Brocadia* could be a host of sulfadiazine and chlortetracycline resistance genes.

The *Nitrospira* genus presented a high abundance in tested bioreactors. Among all bioreactors, the lowest level of *Nitrospira* was presented in the Control. On day 1 the abundance of *Nitrospira* was 9.36 %, then gradually decreased reaching 5.21 % on day 340. The opposite tendency was observed in bioreactors fed with OTC, CIP, and CLA. In R-OTC, the initial abundance of *Nitrospira* was 6.32 % (day 1) and significantly increased to 28.28 % at the end of the experiment. Similar, in the R-CIP and R-CLA the *Nitrospira* abundance increased by 15.67 % and 8.7 %, respectively. These results might suspect that *Nitrospira* was resistant to the tested antibiotics. *Nitrospira* is well-known as nitrite-oxidizing bacteria (NOB), converting nitrite into nitrate under aerobic conditions. Therefore, the anaerobic condition presented in the reactors could lead to *Nitrospira* abundance reduction. Nevertheless, in 2015 *Nitrospira* was reported to perform both stages of nitrification in one step under anaerobic conditions in a process called comammox [70]. Previous studies suggested that *Nitrospira* supports anammox bacteria during nitrogen treatment performance [27,71]. Moreover, as reported previously, the comammox process is highly resistant to antibiotics [40] Therefore, *Nitrospira* was able to proliferate in the tested systems with the addition of antibiotics because the anammox process was gradually inhibited which increased the availability of substrate to carry out the comammox process by *Nitrospira*.

At the genus level, OTC, CIP, and CLA inhibited the proliferation of *Limnobacter*, *Comamonas* and *Denitrosoma*. Both *Denitrosoma* and *Comamonas* are denitrifying bacteria, which often exist in anammox systems [72]. *Limnobacter* was reported to play a protective role for anammox bacteria by utilizing nitrate produced in the anammox process and reducing the inhibitory effect of oxygen and organic matter [73]. The *Nitrosomonas* genus is mainly responsible for the first step of nitrification, converting ammonium to nitrate. The occurrence of AOB was previously reported in both lab- and full-scale anammox systems [22,38,74]. *Nitrosomonas* was also detected in the on-step nitritation-anammox system distributed in the inside of the anammox granule [75]. As reported, *Nitrosomonas* play a special role in anammox biomass protecting anammox bacteria against the inhibitory effect of oxygen [22]. As reported previously by Sepehri and Sarrafzadeh [87] AOB and NOB may interact with other microorganisms via the production of EPSs and soluble microbial products (SMP). Moreover, changes in their production through antibiotics action may result in modification of the abundance of AOB and NOB in the biomass. The development of AOB and NOB microorganisms in the antibiotic-containing reactors is confirmed by the increasing abundance of *amoA* and *nxrA* genes (Section 3.3.), as well as nitrogen removal efficiency (Section 3.1.). Both R_p and R_s ratios increased as the concentration of antibiotics increased exceeding the stoichiometric values for these coefficients, which inform about the higher contribution of nitrifiers to nitrogen removal from wastewater. On the other hand, despite the increasing abundance of genes determining the denitrification process in the R-CLA, the results of nitrogen removal do not indicate a significant contribution of denitrifiers to the wastewater treatment process, because of the accumulation of NO_3-N in the effluent.

PCA was used to visualize and elucidate the impact of environmental factors on changes in dominant genera. In the bioreactor treated with R-OTC, R-CIP, and R-CLA (Fig. S3b–d), the addition of antibiotics led to clear separation along with the Dim2 vector (6.6 %, 10.2 %, 12.0 %, respectively), which was mainly due to proliferation of *Nitrospira* and Candidatus *Brocadia*. Moreover, these two genera have a prominent influence on separation along the Dim1 vector (89.8 %, 87.7 %, 84.0 %, respectively), due to their dominance in community structure. Similar separation was observed in the Control (Fig. S3a). Notably, in R-OTC and R-CIP Candidatus *Brocadia* showed a positive correlation on days 1, 48, and 111, when the antibiotic concentration increased to 10 mg L^{-1} and then to 100 mg L^{-1} *Nitrospira* began to play a crucial role in the anammox system. These results correlate to process performance. As shown in Fig S3d, CLA mostly promotes *Nitrospira* for almost the entire operating period, while Candidatus *Brocadia* showed a positive correlation mainly at the beginning of the experiment. Finally, PCA analysis at the genus level indicated that each studied antibiotic influenced the community structures of anammox biomass promoting the development of *Nitrospira* as the antibiotic concentration increased.

3.6. Co-occurrence of ARGs and bacteria in the anammox systems

Co-occurrence network analysis was performed to explain the interactions among the most abundant genera, functional genes (including *intI1*), and ARGs (Fig. 7a–d), while correlation analysis is shown in Fig. 8. In the R-OTC a significant interaction was observed between *hzo* and *amoA* ($r = 0.98$, $p < 0.05$), as well as *hzo* and *nxrA* ($r = 0.95$, $p < 0.05$), which confirmed previous knowledge about co-existing anammox bacteria with AOB and NOB in the anammox systems and their mutual interaction [22,38,75]. At the genus level, a strong connection between *tetW* and *Taonella* ($r = 0.97$, $p < 0.05$), *Denitratisoma* ($r = 0.92$, $p < 0.05$) and *Nitrosomonas europaea* ($r = 0.97$, $p < 0.05$) was observed, suggesting these genera as potential antibiotic resistance bacteria. At the functional genes level, genes *amoA* and *nirS* were positively correlated with *tetC* ($r = 0.82$, $p < 0.05$ and $r = 0.82$, $p < 0.05$, respectively), proving the role of

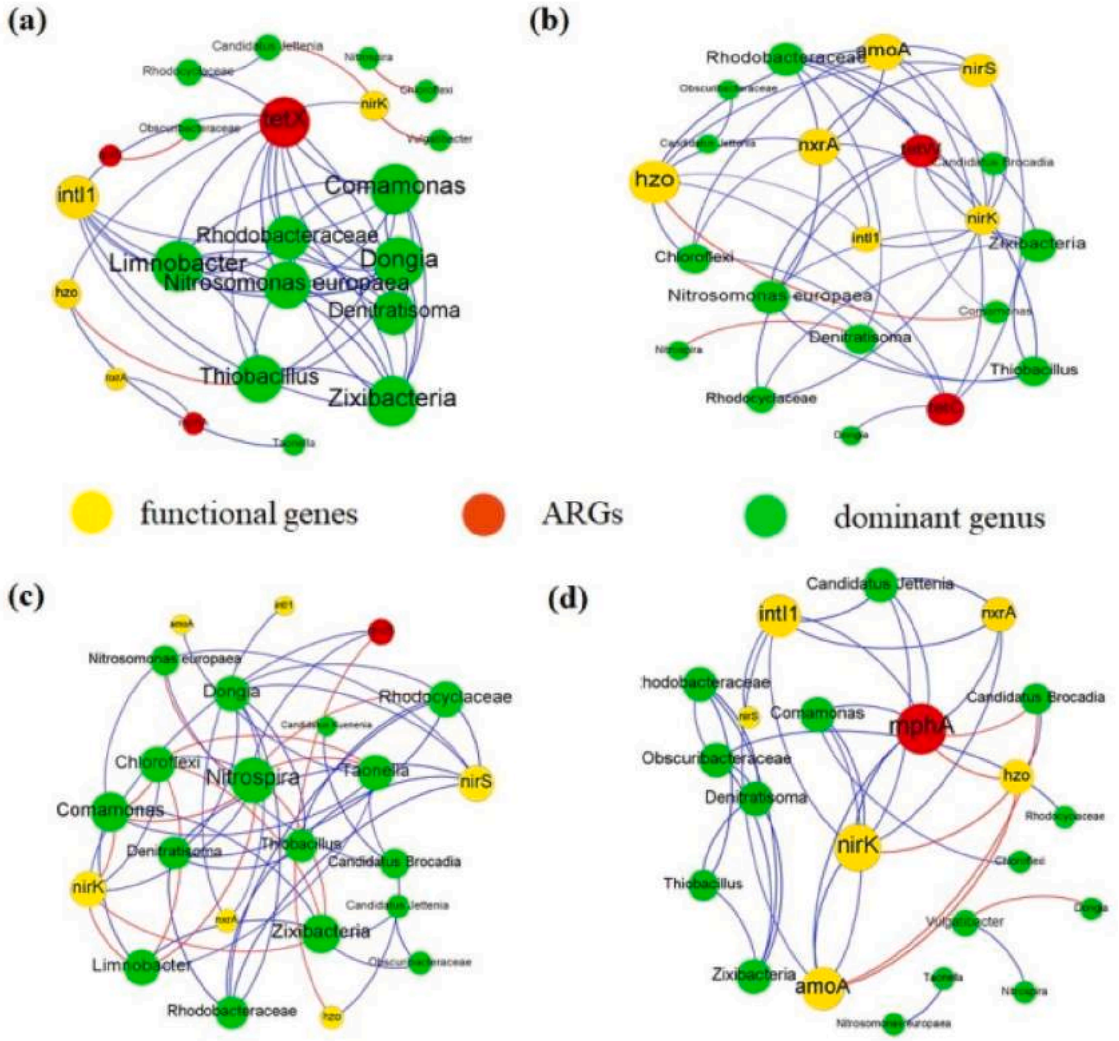

Fig. 7. Co-occurrence network revealing the complex interaction between ARGs, functional genes, intI1 and dominant genera in control (a), R-OTC (b), R-CIP (c), R-CLA (d). The positive interactions are indicated by thin blue curves; negative interactions between genes are indicated by thick red curves.

Denitratisoma and *Nitrosomonas europaea* in ARGs hosting. The correlation between denitrification functional genes, including *nirS* and *nirK*, and sulfonamide resistance genes (*sul1*, *sul2*) have been previously reported [72]. It is worth noting that a strong positive correlation between sulfonamide targeting ARGs and tetracycline targeting ARGs was found by He et al. [76]. Taking all this into account, denitrifiers can be considered to be hosts of ARGs under OTC pressure. Fan et al. [41] found a significant positive correlation between ARGs targeting tetracycline in anammox biomass under tetracycline stress. In contrast, there was no interaction between tetracycline resistance genes researched in this study. This is probably due to different resistance mechanisms encoded by these genes.

In the R-CIP, *Nitrospira* was the only genus that showed a significant positive correlation ($r = 0.86$, $p < 0.05$) with *qnrS*, while *qnrB4* had no interactions. It is worth noting that *Nitrospira* becomes the most abundant genus in R-CIP during the operating period. This suggests that *Nitrospira* plays a crucial role in alleviating CIP stress via the *qnrS* pathway. The confirmation of this statement can be found in Shi et al. [77], where a positive correlation was found between *Nitrospira* and *qnrS* in the conventional nitrification–denitrification system. In the same paper, a strong interaction between denitrifiers and *qnrS* was presented. Despite that no correlation occurs between dominant denitrifiers and *qnrS* in this research, there is however a positive relationship ($r = 0.86$, $p < 0.05$) between *nirK* and *qnrS*, suggesting denitrifiers using the *nirK* pathway as resistant bacteria. At the community structure level, a positive connection between anammox bacteria (Candidatus *Brocadia*) and *Nitrosomonas europaea* ($r = 0.81$, $p < 0.05$) confirms that AOB supports anammox bacteria in wastewater systems [22].

A strong interaction was found between Candidatus *Jettenia* and *mphA* under CLA stress (Fig. 8d). On the other hand, *mphA* had a negative correlation ($r = -0.90$, $p < 0.05$) with *hzo*. Moreover, a significant correlation was obtained between *intI*1 and *mphA* ($r = 0.86$, $p < 0.05$), as well as *intI*1 and Candidatus *Jettenia* ($r = 0.99$, $p < 0.05$). This might suggest that Candidatus *Jettenia* is the main anammox bacteria that presents resistance against CLA stress in opposition to other bacteria using the *hzo* pathway. Similarly, Zhang et al. [32] obtained a positive correlation between spiramycin resistance genes and *intI1* in anammox biomass showing the importance of *intI1* in the spread of macrolide-ARGs. Moreover, a positive correlation was observed between *intI1* and *nirS* ($r = 0.83$, $p < 0.05$) and *nirK* ($r = 0.85$, $p < 0.05$) implying denitrifiers as the host bacteria.

To sum up, co-occurrence networking analysis was performed between the most abundant genera, functional genes (including *intI1*), and ARGs. Statistical analysis indicated the important role of denitrifiers, to be hosts of ARGs in the anammox systems. Moreover, performing networking analysis allowed us to examine the relationship between active functional genes and ARGs and *intI1* gene, which allowed us to determine that anammox bacteria (Candidatus *Jettenia*) may participate

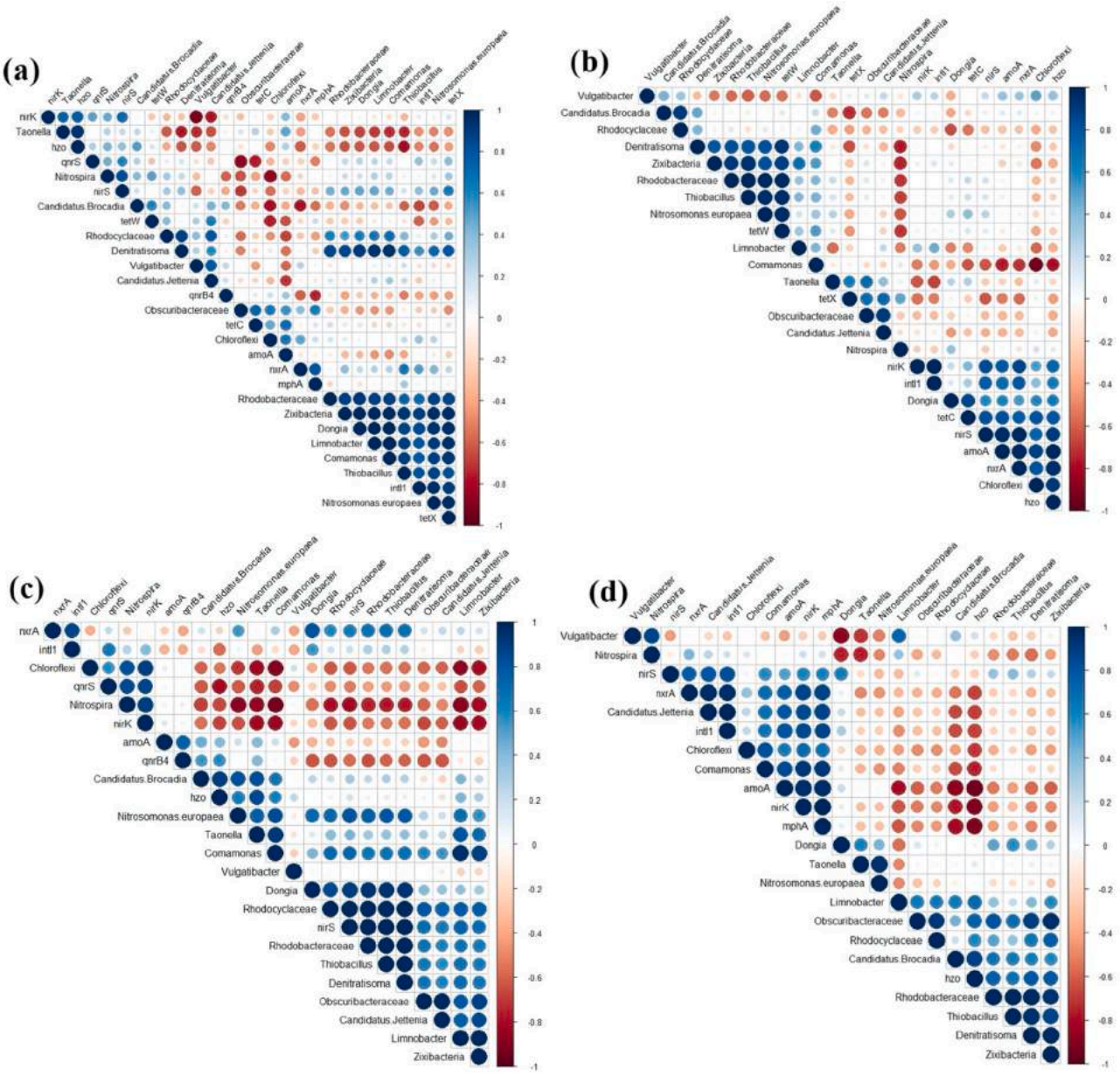

Fig. 8. Correlation analysis between genes and bacteria for control (a), R-OTC (b), R-CIP (c), R-CLA (d).

in the transfer of CLA-targeted ARGs. Thanks to the analysis of correlation, it was possible to demonstrate ecological relationships between anammox bacteria and nitrifiers and denitrifiers under antibiotic suppression.

3.7. Mechanism of OTC, CIP, CLA inhibition on the anammox bacteria

Owing to active transport, OTC is transferred to anammox bacteria cells, then bound with the 30S small ribosomal subunit to inhibit protein synthesis and hence cell growth [78]. CIP is an antibiotic that targets gyrase DNA - the enzyme responsible for introducing negative supercoils into DNA during replication and transcription. This antibiotic tapped gyrase DNA on genetic material by creating drug/enzyme/DNA complexes that held double-strand DNA breaks [80]. CLA as a macrolide antibiotic, after entering the cytoplasm, bound with the 50S ribosomal subunit limiting the transposition of tRNA, which results in inhibition of the synthesis of proteins [79]. Finally, as shown in Fig. 9 each antibiotic causes a decrease in the activity of anammox functional enzymes, leading to the deterioration of anammox process performance.

Wang et al. [45] proposed that after antibiotics enter the anammox cells, they affect the tricarboxylic acid cycle (TCA). Because the TCA affects protein metabolism, it can participate in the production of reactive oxygen species (ROS) which damage DNA, lipids, and proteins. Production of O_2^- results in the release of Fe (II) from heme c [45] which is important for anammox functional key enzymes [15]. As proven previously, O_2^- can be converted to H_2O_2 in the anammox bacteria metabolic system [81]. Further, H_2O_2 and released Fe (II) are available for the Fenton reaction whereby hydroxyl radicals ($OH\cdot$) are formed inducing cell damage.

Antibiotic resistance genes can enter bacteria in different ways: injection by phage (transduction), uptake from the environment (transformation), and transferred from another bacterium by indirect cell to cell contact (conjugation) [82]. Moreover, it was found that ARGs can be transferred directly to nucleoids through mobile elements [83]. In addition, when antibiotics enter the cytoplasm, expression of ARGs occurs. Mostly, ARGs are associated with mobile genetic elements (MGEs) such as plasmids, transposons, and integrons, thus accumulation of microbial consortia in wastewater systems enhances ARG transfer [45].

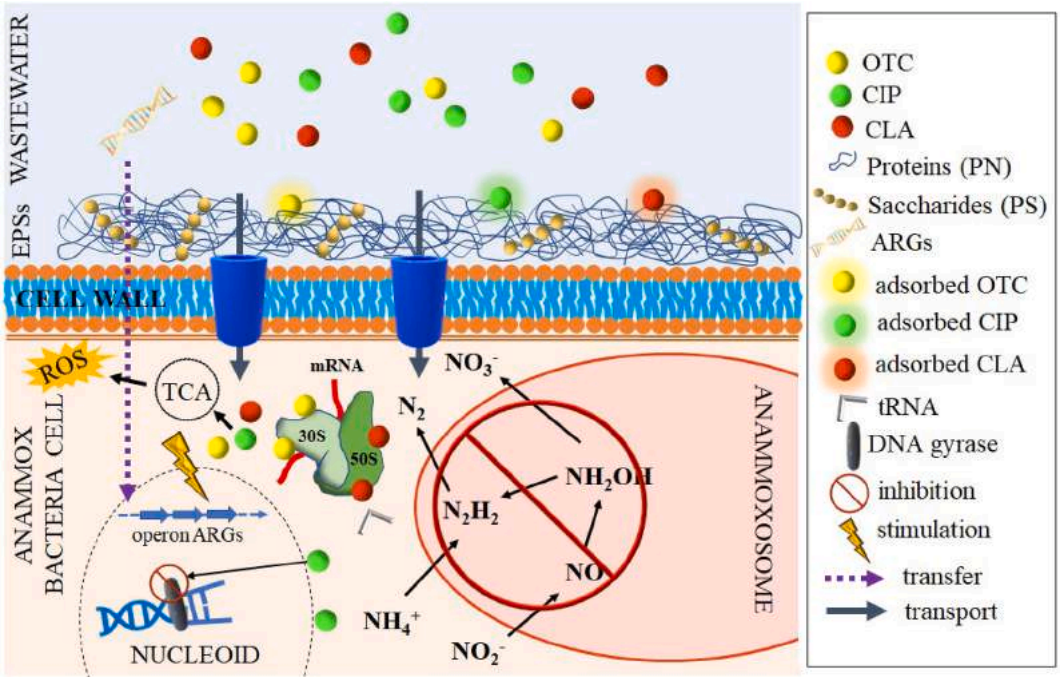

Fig. 9. Potential mechanism of OTC, CIP, and CLA influence on the anammox bacteria.

The development of ARGs in anammox biomass can maintain their stability when OTC, CIP, and CLA are introduced to the system. Resistance development depends on the concentration of antibiotics. At relatively low antibiotics concentration (<1 mg L^{-1}) anammox bacteria are able to protect themselves by expressing ARGs and maintain the stability of nitrogen removal performance, while at higher concentrations, there is a deterioration of the anammox process performance due to the antibiotics overwhelming the ability of bacteria to protect against antibiotic action. It is worth noting that the concentration that is effective on anammox bacteria is different for each antibiotic. Such a conclusion derived from the studies shows that despite increased expression of ARGs at higher doses of antibiotics, there is a decrease in the efficiency of the anammox process [30,42,72]. Moreover, different antibiotic resistance genes are expressed depending on the antibiotic concentration, as demonstrated in this study.

Another protective mechanism of anammox bacteria is the production of EPSs. Before reaching the cell, antibiotics must overcome the first barrier provided by the EPSs. Anammox bacteria during long-term cultivation synthesize EPSs to protect the cells against the impact of various substances. Moreover, EPSs may also be connected with the expression of ribosomal protective genes resulting in stimulation of the anammox bacteria to secrete more EPSs [45]. To highlight, such a gene was detected in the anammox community in the case of ARGs targeting OTC (Section 3.4). The presence of proteins in the EPSs structure confers their adsorptive ability due to the presence of hydroxyl and carboxyl groups [49]. It is worth noting that each tested antibiotic has the ability to be sorped on activated sludge. As reported by Felis et al. [1], all macrolides are characterized by a high log K_{ow} value parameter (>3), which increases the affinity for sorption on activated sludge. Similarly, fluoroquinolones, which, despite low log K_{ow} value parameter (<1), are adsorbed into activated sludge. Tetracyclines exhibit chelating properties, therefore in the presence of calcium, iron or magnesium, they may form a stable complex with a high affinity for activated sludge [84]. However, the mineral medium used in this study includes chemical compounds containing calcium, iron, or magnesium, so it can be suspected that OTC was adsorbed on anammox biomass including EPSs.

3.8. *Implication of this work*

Constantly increasing concentrations of antibiotics in wastewater make it necessary to expand the knowledge of the impact of these substances on the wastewater treatment process and to look for antibiotic-resistant treatment systems. The anammox process showed stable operation even at antibiotic concentrations reaching a few mg L^{-1}, while only at higher (>10 mg L^{-1}) concentrations of antibiotics caused deterioration of the anammox process. However, such high concentrations are rather undetected in the environment. Therefore, this study presents anammox as a good technology for antibiotic-reach wastewater treatment the anammox process for the antibiotic-containing wastewater, at least wastewater containing tetracycline, quinolone, and macrolide antibiotics. As mentioned in Section 1, CIP and CLA are recognized by European Union to be hazardous to the environment and their impact on the environment should be monitored. On the other hand, OTC presented a high concentration in wastewater in China as one of the biggest producer and consumer of tetracycline antibiotics. Therefore, investigating the response of the anammox process to numerous antibiotics is helpful to optimize the process of changing operating modes to adjust for different types of wastewater including different antibiotics in practical application. Additionally, this research confirmed that sequencing batch technology is suitable to conduct the anammox process. On the other hand, the outcomes of this study suggest that antibiotic resistance is developed during long-term exposure to antibiotics. Therefore, it is necessary to consider a thorough understanding of the mechanism of antibiotic resistance gene transfer and look for methods to mitigate or eliminate the dangerous effects of their spread in the system,

4. Conclusion

This work has presented the effect of the anammox system under the stress of successive concentrations of three antibiotics (OTC, CIP, CLA). The anammox process had higher tolerance to OTC and CIP than CLA. Nevertheless, a gradual increase in antibiotic concentrations provides a possibility for the anammox bacteria to acclimatize and then reduce the toxic effect of high concentrations of antibiotics. Exposure to CIP and CLA led to the increase of *Planctomycetes* number, while OTC resulted in

a decrease in the abundance of *Planctomycetes*. However, a comparison of the biodiversity indexes value for the *Planctomycetes* and *hzo* gene abundance has shown that CIP and CLA may encourage the development of *Planctomycetes* unable to conduct the anammox process. On contrary, antibiotic resistance bacteria gradually became the dominant part of the community in the reactor with increasing OTC content, accompanied by an increase in the relative abundance of ARGs. Exposure to antibiotics induced an increase in the abundance of corresponding resistance genes which might be the strategy for the bacteria community in the biomass to relieve antibiotics stress. Network analysis revealed that Candidatus *Jettenia* and CLA resistance gene (*mphA*) were positively correlated with *intI*1, highlighting the role of anammox bacteria in the spreading of the macrolide resistance gene. However, the mechanism for the exchange of ARGs by anammox bacteria needs to be clarified. The present study provides clue knowledge about the effect of OTC, CIP, and CLA on the anammox process and provides some guidelines to use the anammox process to treat antibiotic-containing wastewater. One of the crucial aspects that need to be research further. Due to the fact that antibiotics cooccur in the wastewater, the simultaneous effect of these antibiotics on process anammox should be studied in the future. Moreover, it would also be worth to pay attention to the study of quorum sensing in anammox sludge under antibiotics pressure, which would explain in detail the part of this phenomenon in the development of resistance of the anammox system to antibiotics.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2022.138546.

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Supplementary materials for

Insight into the microbial and genetic response of anammox biomass to broad range concentrations of different antibiotics: Linking performance and mechanism

Gamoń Filip[1*], Banach-Wiśniewska Anna[2], Poprawa Izabela[3], Cema Grzegorz[1], Ziembińska-Buczyńska Aleksandra[1]

[1]*Department of Environmental Biotechnology, Silesian University of Technology, Akademicka 2A, 44-100 Gliwice, Poland*

[2]*Regional Center for Water and Wastewater Management Co., Tychy, Poland*

[3] *Institute of Biology, Biotechnology and Environmental Protection, Faculty of Natural Sciences, University of Silesia in Katowice, Bankowa 9, 40-007 Katowice, Poland*

**Corresponding author e-mail: filip.gamon@polsl.pl*

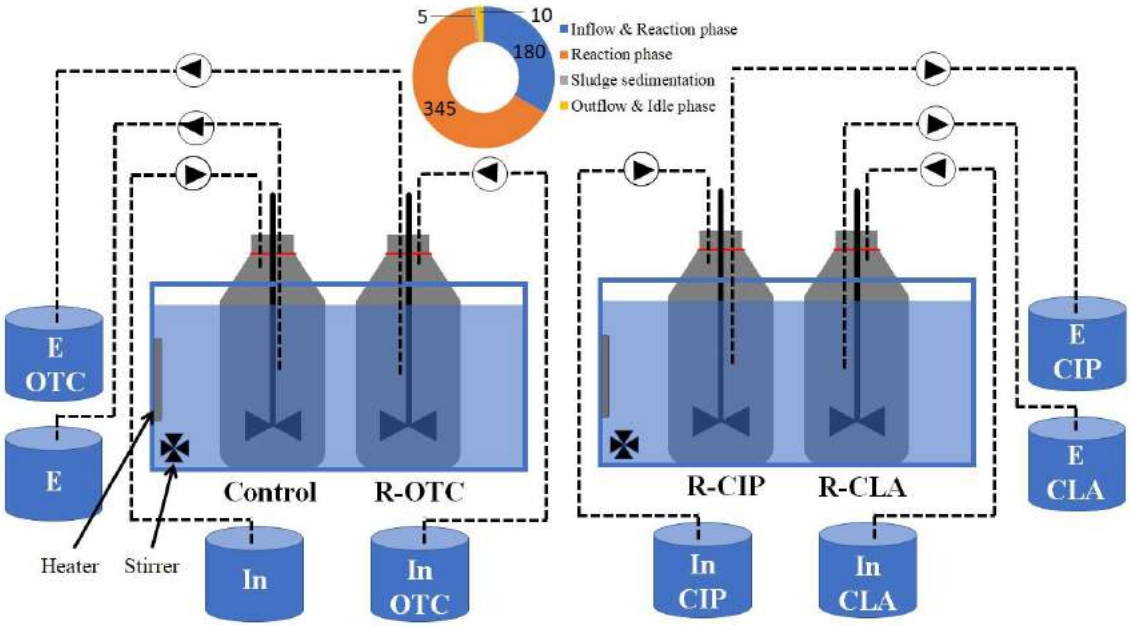

Figure S1 Experimental set-up of the four SBR reactors and their cycle schedule and time of cycle steps (in minutes). In: influent, E: effluent.

The SBR reactors used in this experiment were constructed from 5 L glass bottles (Simax, Czech Republic) made from borosilicate glass (type 3.3.). The reactors were inoculated with 5 L of anammox biomass. The reactors were capped using multiport caps (DWK Life Sciences, Germany) with a center connector through which placed stirrers. To create anaerobic conditions, all ports in the cap were sealed and the stirrer was placed in a guide (Bionovo, Poland) to provide limited air exchange. Stirrers (Aqua-Trend, Poland) were used to provide mixing in the reactors. The mineral medium was dosed to the reactor using a peristaltic pump (Ismatec, Germany), while influent was pumped out using a peristaltic pump (Aqua-Trend, Poland) with higher efficiency, due to shorter time of outflow as shown in Fig S1. Reactors were placed in two glass aquariums (2 reactors per aquarium) and then flooded

with distilled water up to the active volume of the reactors. The water in the aquarium was heated to 32.0 °C using an aquarium heater with a thermostat (CoralHouse, Poland). To provide an equal temperature throughout the volume of the aquarium, the water was constantly stirred. Control of the reactors cycles was provided using a digital timer programmer (BEMKO Sp. z o.o., Poland).

Table S1. Primers used for qPCR analysis of nitrogen cycle bacteria functional genes.

Specificity	Target gen	Primers	Sequence 5'-3'
Total bacteria	16S rRNA	1055F	ATGGCTGTCGTCAGCT
		1392R	ACGGGCGGTGTGTAC
Ammonia oxidizers	*amoA*	amoA-1-F	GGGGTTTCTACTGGTGGT
		amoA-2R	CCCCTCKGSAAAGCCTTCTTC
Nitrite oxidizers	*nxrA*	nxrA-RT-F	GTGGTCATGCGCGTTGAGCA
		nxrA-RT-R	TCGGGAGCGCCATCATCCAT
Denitrifiers	*nirS*	nirS 1f	TACCACCCSGARCCGCGCGT
		nirS 3r	GCCGCCGTCRTGVAGGAA
	nirK	nirK876	ATYGGCGGVCAYGGCGA
		nirK1040	GCCTCGATCAGRTTRTGGTT
All known Planctomycetes	*hzo*	hzoCl1f1 1f	TGYAAGACYTGYCAYTGG
		hzoCl1r2	ACTCCAGATRTGCTGACC
All bacteria	*IntI1*	*int*I1_F	GGCTTCGTGATGCCTGCTT
		*int*I1_R	CATTCCTGGCCGTGGTTCT

Table S2. Primers used for ARGs analysis in anammox biomass.

Targeted antibiotic	Targeted gen	Primers	Sequence 5'-3'	Resistance mechanism
OTC	*tetC*	tetCf	GCGGGATATCGTCCATTCCG	Efflux pump
		tetCr	GCGTAGAGGATCCACAGGACG	
OTC	*tetX*	tetXf	GAAAGAGACAACGACCGAGAG	Drug modification
		tetXr	ACACCCATTGGTAAGGCTAAG	
OTC	*tetM*	tetMf	ACAGAAAGCTTATTATATAAC	Ribosomal protection protein
		tetMr	TGGCGTGTCTATGATGTTCAC	
CLA	*mphA*	mphAf	CTGACGCGCTCCGTGTT	Drug modification
		mphAr	GGTGGTGCATGGCGATCT	
CIP	*qnrB4*	qnrB4f	AGTTGTGATCTCTCCATGGC	DNA gyrase protection
		qnrB4r	CGGATATCTAAATCGCCCAG	
CIP	*qnrS*	qnrS_F	ACGACATTCGTCAACTGGAA	DNA gyrase protection
		qnrS_R	TTAATTGGCACCCTGTAGGC	

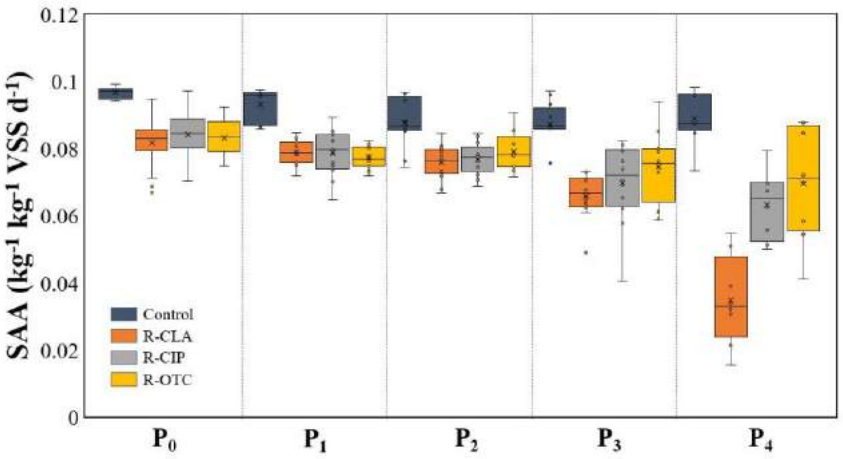

Figure S3 The SAA values of the anammox process performance in each phase of experiment.

Table S3 Performance of the SBRs under antibiotics stress. NLR: nitrogen loading rate, NRR: nitrogen removal rate, VSS: volatile suspended soil.

Phase		**P_0**		**P_1**		**P_2**		**P_3**		**P_4**	
Experiment day		1-48		49-111		112-201		202-288		289-340	
Antibiotic concentration (mg L^{-1})		no		0.001		1		10		100	
		Average	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**
Control	NLR (kg m^{-3} d^{-1})	0.255	0.02	0.249	0.01	0.249	0.01	0.259	0.01	0.263	0.02
	NRR (kg m^{-3} d^{-1})	0.208	0.02	0.249	0.02	0.189	0.01	0.187	0.02	0.184	0.02
	VSS (mg L^{-1})	1246.7	2.05	1209	11.00	1185	8.29	1128.7	1.89	1112	14.00
R-OTC	NLR (kg m^{-3} d^{-1})	0.254	0.02	0.245	0.005	0.250	0.01	0.256	0.01	0.263	0.02
	NRR (kg m^{-3} d^{-1})	0.211	0.01	0.193	0.01	0.185	0.01	0.169	0.02	0.154	0.04
	VSS (mg L^{-1})	1232	8.29	1221.5	3.50	1161.3	17.13	1124	9.42	1095	7.00
R-CIP	NLR (kg m^{-3} d^{-1})	0.254	0.02	0.244	0.01	0.248	0.01	0.256	0.02	0.259	0.02
	NRR (kg m^{-3} d^{-1})	0.207	0.02	0.193	0.02	0.179	0.01	0.154	0.03	0.144	0.02
	VSS (mg L^{-1})	1200	10.2	1205.5	4.50	1139.7	12.04	1103.3	10.21	1071.5	3.50
R-CLA	NLR (kg m^{-3} d^{-1})	0.254	0.02	0.246	0.01	0.251	0.01	0.257	0.02	0.269	0.02
	NRR (kg m^{-3} d^{-1})	0.207	0.02	0.193	0.01	0.188	0.01	0.145	0.04	0.091	0.03
	VSS (mg L^{-1})	1202	2.83	1208	4.00	1163.7	16.82	1128.7	1.89	1106.5	8.50

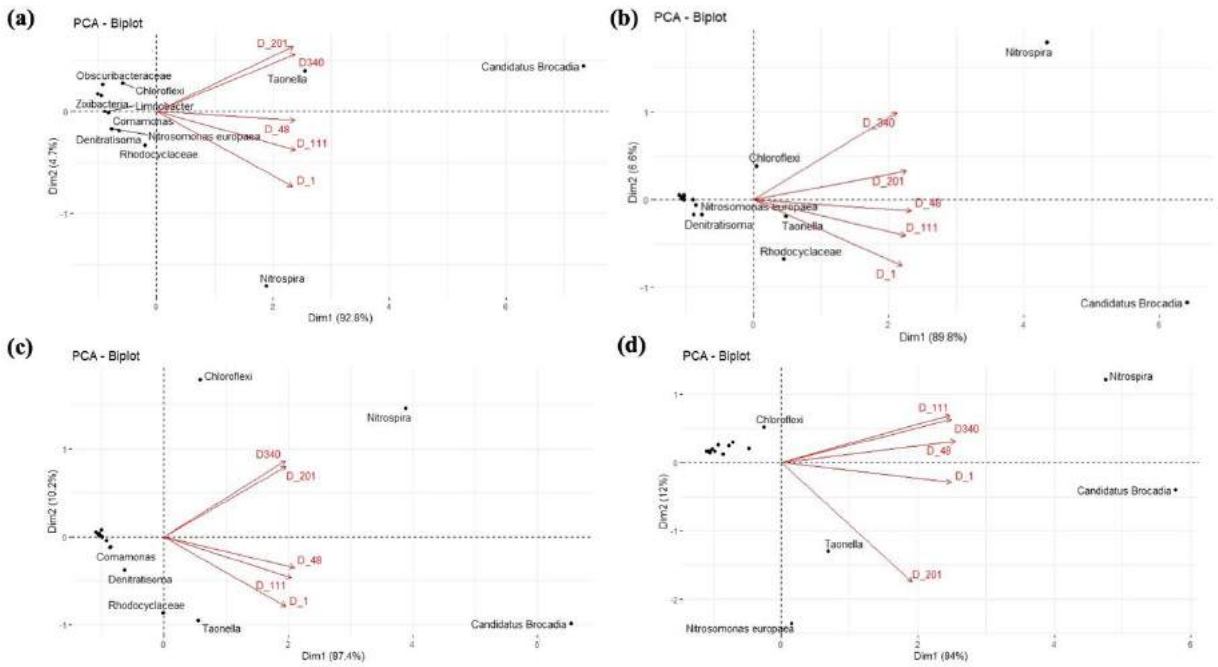

Figure S3 Principal component analysis (PCA) on the composition of dominant genera in Control (a), R-OTC (b), R-CIP (c), R-CLA (d).

Table S4 Shannon and Simpson indexes for all tested SBR reactors during each phase.

	Shannon Index			
	Control	**R-OTC**	**R-CIP**	**R-CLA**
$\mathbf{P_0}$	1.498014	1.610525	1.651698	0.938694
$\mathbf{P_1}$	1.179202	1.394261	1.4517	0.832776
$\mathbf{P_2}$	1.413405	1.385838	1.205381	0.905582
$\mathbf{P_3}$	1.543684	1.552842	1.092097	0.925227
$\mathbf{P_4}$	1.4159	1.290332	1.020864	0.922199
	Simpson Index			
$\mathbf{P_0}$	0.897531	0.93416	0.939795	0.938694
$\mathbf{P_1}$	0.852359	0.902991	0.913779	0.832776
$\mathbf{P_2}$	0.90595	0.885157	0.887538	0.905582
$\mathbf{P_3}$	0.928447	0.928903	0.859801	0.925227
$\mathbf{P_4}$	0.903447	0.87011	0.831837	0.922199

Table S5 Shannon and Simpson indexed calculated for species belonging to *Planctomycetes* for all tested SBR reactors during each phase.

	Shannon Index			
	Control	**R-OTC**	**R-CIP**	**R-CLA**
$\mathbf{P_0}$	0.301034	0.290789	0.298398	0.29186
$\mathbf{P_1}$	0.21973	0.279753	0.309982	0.320948
$\mathbf{P_2}$	0.235042	0.306871	0.302532	0.275047
$\mathbf{P_3}$	0.26506	0.258387	0.331381	0.310364

P_4	0.239912	0.191973	0.35997	0.34851
Simpson Index				
P_0	0.915999	0.960062	0.964109	0.967926
P_1	0.977709	0.943184	0.941435	0.956652
P_2	0.956162	0.905924	0.953289	0.965664
P_3	0.96683	0.968719	0.953538	0.966889
P_4	0.951965	0.969917	0.893222	0.929535

8. Authors contribution statements

PUBLICATION 1

Gamoń F., Cema G., Ziembińska-Buczyńska A., The influence of antibiotics on the anammox process - a review. Environ Sci Pollut Res 29, 8074–8090 (2022) DOI: https://doi.org/10.1007/s11356-021-17733-7

Filip Gamoń.....70%

Grzegorz Cema.....15%

Aleksandra Ziembińska-Buczyńska.....15%

Gliwice, 23.01.2023

Author contribution statement

I declare that my percentage contribution to the preparation of the publication 'The influence of antibiotics on the anammox process — a review' (Environmental Science and Pollution Research, 29, 8074–8090 (2022), DOI: https://doi.org/10.1007/s11356-021-17733-7) has been estimated at 80%. It involved: conceptualization, data curation, formal analysis, methodology, visualization, validation, writing – original draft.

mgr inż. Filip Gamoń

Gliwice, 23.01.2023

Author contribution statement

I declare that my percentage contribution to the preparation of the publication 'The influence of antibiotics on the anammox process — a review' (Environmental Science and Pollution Research, 29, 8074–8090 (2022), DOI: https://doi.org/10.1007/s11356-021-17733-7) has been estimated at 10%. It involved: conceptualization, formal analysis, supervision, validation, writing -review & editing.

dr inż. Grzegorz Cema

Gliwice, 23.01.2023

Author contribution statement

I declare that my percentage contribution to the preparation of the publication 'The influence of antibiotics on the anammox process — a review' (Environmental Science and Pollution Research, 29, 8074–8090 (2022), DOI: https://doi.org/10.1007/s11356-021-17733-7) has been estimated at 10%. It involved: conceptualization, formal analysis, supervision, validation, writing -review & editing.

dr hab. Aleksandra Ziembińska-Buczyńska, prof. PŚ

PUBLICATION 2

Gamoń F., Banach-Wiśniewska A., Jaspreet Jandoo Kaur, Cema G., Ziembińska-Buczyńska A., Microbial response of the anammox process to trace antibiotic concentration. Journal of Water Process Engineering, Volume 46, 102607 (2022), DOI: https://doi.org/10.1016/j.jwpe.2022.102607

Filip Gamoń.....70%

Anna Banach-Wiśniewska.....5%

Jaspreet Jandoo Kaur.....5%

Grzegorz Cema.....10%

Aleksandra Ziembińska-Buczyńska.....10%

Gliwice, 23.01.2023

Author contribution statement

I declare that my percentage contribution to the preparation of the publication 'Microbial response of the anammox process to trace antibiotic concentration' (Journal of Water Process Engineering, 46, 102607 (2022), DOI: https://doi.org/10.1016/j.jwpc.2022.102607) has been estimated at 70%. It involved: conceptualization, data curation, formal analysis, methodology, visualization, validation, writing – original draft.

mgr inż. Filip Gamoń

Gliwice, 23.01.2023

Author contribution statement

I declare that my percentage contribution to the preparation of the publication 'Microbial response of the anammox process to trace antibiotic concentration' (Journal of Water Process Engineering, 46, 102607 (2022), DOI: https://doi.org/10.1016/j.jwpe.2022.102607) has been estimated at 5%. It involved: data curation, formal analysis, visualization, validation.

dr inż. Anna Banach-Wiśniewska

Gliwice, 23.01.2023

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I declare that my percentage contribution to the preparation of the publication 'Microbial response of the anammox process to trace antibiotic concentration' (Journal of Water Process Engineering, 46, 102607 (2022), DOI: https://doi.org/10.1016/j.jwpe.2022.102607) has been estimated at 5%. It involved: conceptualization, methodology.

mgr inż. Jaspreet Kaur Jandoo

Silesian University of Technology
Faculty of Energy and Environmental Engineering
Department of Environmental Biotechnology
Akademicka 2, 44-100 Gliwice
+48 32 237 29 15

Gliwice, 23.01.2023

Author contribution statement

I declare that my percentage contribution to the preparation of the publication 'Microbial response of the anammox process to trace antibiotic concentration' (Journal of Water Process Engineering, 46, 102607 (2022), DOI: https://doi.org/10.1016/j.jwpe.2022.102607) has been estimated at 10%. It involved: conceptualization, methodology, validation, , supervision, writing -review & editing.

dr inż. Grzegorz Cema

Gliwice, 23.01.2023

Author contribution statement

I declare that my percentage contribution to the preparation of the publication 'Microbial response of the anammox process to trace antibiotic concentration' (Journal of Water Process Engineering, 46, 102607 (2022), DOI: https://doi.org/10.1016/j.jwpe.2022.102607) has been estimated at 10%. It involved: conceptualization, methodology, validation, supervision, writing -review & editing.

dr hab. Aleksandra Ziembińska-Buczyńska, prof. PŚ

PUBLICATION 3

Gamoń F., Banach-Wiśniewska A., Poprawa I, Cema G., Ziembińska-Buczyńska A. Insight into the microbial and genetic response of anammox biomass to broad range concentrations of different antibiotics: Linking performance and mechanism. Chemical Engineering Journal, 451(1), 138546 (2023). DOI https://doi.org/10.1016/j.cej.2022.138546

Filip Gamoń.....60%

Anna Banach-Wiśniewska.....5%

Izabela Poprawa.....5%

Grzegorz Cema.....15%

Aleksandra Ziembińska-Buczyńska.....15%

Gliwice, 23.01.2023

Author contribution statement

I declare that my percentage contribution to the preparation of the publication 'Insight into the microbial and genetic response of anammox biomass to broad range concentrations of different antibiotics: Linking performance and mechanism' (Chemical Engineering Journal 451, 138546 (2023) DOI: https://doi.org/10.1016/j.cej.2022.138546) has been estimated at 60%. It involved: conceptualization, data curation, formal analysis, methodology, visualization, validation, writing – original draft.

mgr inż. Filip Gamoń

Gliwice, 23.01.2023

Author contribution statement

I declare that my percentage contribution to the preparation of the publication 'Insight into the microbial and genetic response of anammox biomass to broad range concentrations of different antibiotics: Linking performance and mechanism' (Chemical Engineering Journal 451, 138546 (2023) DOI: https://doi.org/10.1016/j.cej.2022.138546) has been estimated at 5%. It involved: data curation, formal analysis, visualization, validation.

dr inż. Anna Banach-Wiśniewska

Izabela Poprawa PhD, DSc, Associate Professor
University of Silesia in Katowice
Faculty of Natural Sciences
Institute of Biology, Biotechnology and Environmental Protection
Bankowa 9,
40-007 Katowice, Poland

Katowice, 23.01.2021

Author contribution statement,

I declare that my percentage contribution to the preparation of the publication 'Insight into the microbial and genetic response of anammox biomass to broad range concentrations of different antibiotics: Linking performance and mechanism' (Chemical Engineering Journal 451, 138546 (2023) DOI: https://doi.org/10.1016/j.cej.2022.138546) has been estimated at 5%. It involved: TEM methodology, visualization, validation and manuscript editing.

Izabela Poprawa

Gliwice, 23.01.2023

Author contribution statement

I declare that my percentage contribution to the preparation of the publication ' Insight into the microbial and genetic response of anammox biomass to broad range concentrations of different antibiotics: Linking performance and mechanism' (Chemical Engineering Journal 451, 138546 (2023) DOI: https://doi.org/10.1016/j.cej.2022.138546) has been estimated at 15%. It involved: conceptualization, methodology, validation , supervision, writing -review & editing.

dr inż. Grzegorz Cema

Silesian University of Technology
Faculty of Energy and Environmental Engineering
Department of Environmental Biotechnology
Akademicka 2, 44-100 Gliwice
+48 32 237 29 15

Gliwice, 23.01.2023

Author contribution statement

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dr hab. Aleksandra Ziembińska-Buczyńska, prof. PŚ

SUPPORTING ARTICLES

Supporting articles published during doctoral school related to the presented research area, but not part of the doctoral dissertation.

1. **Gamoń F**, Tomaszewski M, Ziembińska-Buczyńska A. Ecotoxicological study of landfill leachate treated in the ANAMMOX process. Water Qual. Res. J. Can. 2019, 54, 3, s. 230-241 IF = 0.600, MEiN = 20 points.
2. Tomaszewski M., **Gamoń F.,** Cema G., Ziembińska-Buczyńska A., Manganese dioxide (MnO2) nanoparticles influence on the nitrification and anammox activity. Arch. Environ. Prot. 2020 vol. 46 no. 4 s. 54-58. DOI 10.24425/aep.2020.135764 IF = 1.489, MEiN = 100 points.
3. Banach-Wiśniewska A., **Gamoń F.**, Ziembińska-Buczyńska A., DNA vs RNA based studies of nitrogen removal bacteria genes via qPCR. Arch. Environ. Prot. 2021 vol. 47 no. 1 s. 19-2. DOI 10.24425/aep.2021.136444 IF = 1.489, MEiN = 100 points.
4. **Gamoń F.**, Tomaszewski M., Cema G., Ziembińska-Buczyńska A., Adsorption of oxytetracycline and ciprofloxacin on carbon-based nanomaterials as affected by pH. Archives of Environmental Protection, 48(2), 34-41, (**2022**). DOI: 10.24425/aep.2022.140764 IF = 1.8 MEiN = **100** points.
5. **Gamoń F.**, Ziembińska-Buczyńska A., Łukowiec D., Tomaszewski M. Ecotoxicity of selected carbon-based nanomaterials. International Journal of Environmental Science and Technology (2022). https://doi.org/10.1007/s13762-022-04692-w IF = 3.519, MEiN = **70** points