Abstract

INTRODUCTION

The global consumption and demand for energy continue to increase, but fossil fuels such as coal, natural gas, and oil are not sustainable energy sources. The supply of energy carriers has to increase to cater to rapid population growth, which is directly responsible for the recent interest in renewable energy. Biogas produced during anaerobic digestion is one of the most promising alternatives to fossil fuels in energy generation (Holm-Nielsen et al., 2009; Scarlat et al., 2018). Biogas is environmentally friendly, and it can be converted to biomethane, which is the most efficient type of biofuel (Bowe, 2013). The number of biogas plants continues to increase around the world, and the biogas sector has a massive growth potential in all countries. At present, nearly 50 million micro-scale digesters and 132,000 small, medium, and large-scale digesters are operated around the world (World Biogas Association, 2019).

Anaerobic digestion is an attractive waste processing method that promotes effective waste management and energy recovery. This four-stage processes takes place in the presence of various microbial populations, including hydrolyzing, acidogenic, acetogenic, and methane-producing bacteria, which are responsible for different stages of fermentation (Świątczak et al. 2017). However, the efficiency of anaerobic digestion is difficult to optimize, which prevents the widespread implementation of this process. Anaerobic digestion is inhibited mainly by substances that are present in large quantities in stabilized waste, including antimicrobial compounds (Rusanowska et al. 2019; Meegoda et al. 2018; Scarlat et al. 2018). Global antibiotic consumption increased by 65% between 2000 and 2015, and a further increase of 200% is expected by 2030 (Klein et al., 2018). High antibiotic consumption in human medicine and the overuse of antimicrobials in the veterinary sector promote the spread of the parent compounds and their metabolites in the environment. As a result, antimicrobials can accumulate in substrates that are subjected to anaerobic digestion.

Anaerobic digestion is one of the key strategies for stabilizing sewage sludge from wastewater treatment plants (WWTPs) (Grobelak et al. 2019). Wastewater treatment plants produce increasing quantities of sewage sludge, and hundreds of millions tons of sewage sludge are being generated each year around the world. Incoming raw wastewater contains various pollutants, including microbial contaminants and drug residues, which are accumulated in sewage sludge. Antibiotic concentrations in sewage sludge can range from <1 to several

thousand μg kg^{-1} (Czatzkowska et al., 2022). In addition, both wastewater and sewage sludge are also massively colonized by microorganisms, including pathogens. Bacteria harboring mechanisms of antimicrobial resistance (AR) pose a particular threat (Manaia et al. 2018). The misuse of antibiotics in human and veterinary medicine leads to the emergence of bacteria with one or more antibiotic resistance genes (ARGs). The exchange of genetic structures, including ARGs, between microorganisms contributes to the spread of AR in the environment. Due to the widespread use of antibiotics in recent decades, ARGs have been classified as a new source of pollution that poses a threat to public health and safety (Becerra-Castro et al. 2015; Xu et al. 2017; Barancheshme and Munir 2019). In addition, wastewater and sewage sludge contain subinhibitory concentrations of antimicrobials, and WWTPs promote increased selection pressure on bacteria harboring specific ARGs (Sun et al. 2019). The types and concentrations of antibiotics and ARGs, and the abundance and composition of microbial communities in sewage sludge can vary, depending on the quality of incoming raw wastewater and the applied wastewater treatment technology.

As previously mentioned, anaerobic digestion is an effective method of managing sewage sludge, a by-product of the wastewater treatment process which is generated in massive quantities around the world. The energy accumulated in sewage sludge is recovered during anaerobic digestion. The efficiency of this process can be compromised by the presence of antimicrobial substances in the substrate. Decreased activity of different microbial groups that participate in anaerobic digestion can also undermine digestion efficiency (Chen et al., 2008; Scarlat et al. 2018). The presence of inhibitors in sewage sludge can destabilize anaerobic digestion and decrease methane output. Nearly half of the sewage sludge produced in the EU is used in agriculture as organic fertilizer and is introduced to the soil environment (Campo et al., 2021). Digestate-based fertilizers can promote the spread of ARGs in cultivated fields; ARGs are then transferred from the soil to groundwater and crops, and they ultimately reach humans and animals (Xiao et al., 2021). The pollutants present in the substrate should be controlled, and processing parameters in reactors should be closely monitored to maximize the efficiency of anaerobic digestion. In turn, the quality of the resulting digestate, including the presence of ARGs, should be analyzed to reduce the spread of AR in the environment.

AIM OF THE STUDY

The aim of the study, whose results constituted the basis for this doctoral dissertation, was to determine the effect of antibiotics present in sewage sludge subjected to anaerobic

digestion on (1) efficiency of methane production, (2) fate of ARGs and the spread of AR, (3) qualitative and quantitative changes in the consortia of microorganisms that participate in anaerobic digestion, and (4) prevalence of methanogens.

RESEARCH HYPOTHESES

1. Exposure of sewage sludge to antibiotics affects the efficiency of methane production during anaerobic digestion.
2. Anaerobic digestion of sewage sludge does not eliminate ARGs.
3. The presence of antibiotics in sewage sludge subjected to anaerobic digestion induces changes in the structure of Eubacteria communities.
4. Antimicrobials present in sewage sludge affect the activity and biodiversity of methanogens that participate in anaerobic digestion.

SCOPE OF THE STUDY, RESEARCH METHODS AND RESULTS

Inhibitors of anaerobic digestion may affect the activity of both methanogens and other groups of microorganisms that participate in anaerobic digestion. Each year, thousands of millions tons of antibiotics, classified as non-specific organic inhibitors, are detected in wastewater around the world. Due to the continuous supplementation of wastewater with antimicrobials, these substances are also identified in sewage sludge. The inhibition of anaerobic digestion of sewage sludge by antibiotics is affected by their type and concentration, and the co-occurrence of other antimicrobial agents in the substrate. Recent research findings regarding anaerobic digestion and its inhibitors, including antibiotics, were summarized in a review based on 168 articles (**Appendix 1**). Microbial groups involved in each stage of biomethanation, inhibitors of the process and mechanisms of their action were identified and characterized. The aim of the review was to identify the inhibitors of anaerobic digestion, which remain insufficiently investigated in the scientific literature. Particular emphasis was placed on a knowledge gap regarding the inhibition of the activity of the microbiome involved in each stage of anaerobic digestion. The review provided a basis for verifying the research hypotheses formulated in the dissertation, and paved the way for future research whose stages are presented in Figure 1. An experiment conducted as part of doctoral research was divided into three stages:

I. The aim of the first stage of research was to identify antimicrobials that exert the most significant effect on anaerobic digestion. For this purpose, individual antibiotics were added to the feedstock in bioreactors. The efficiency of methane production, the structure of

microbial communities and the fate of ARGs during the anaerobic digestion of sewage sludge were determined;

II. Three antimicrobials, selected in the first stage of the study, were simultaneously applied to the substrate subjected to anaerobic digestion. The second stage of research was divided into experimental series, and the concentrations of all antibiotics in the mixture were increased in each series. The aim of this stage was to determine the long-term impact of increasing concentrations of the tested antibiotic mixture on the efficiency of methane production, structure of microbial populations and ARG profiles;

III. The last stage of research focused on complementing previous findings with the results of an analysis of variation in the prevalence of methanogens and their characteristic functional genes during long-term exposure of the substrate in a bioreactor to increasing concentrations of the tested antibiotic mixture.

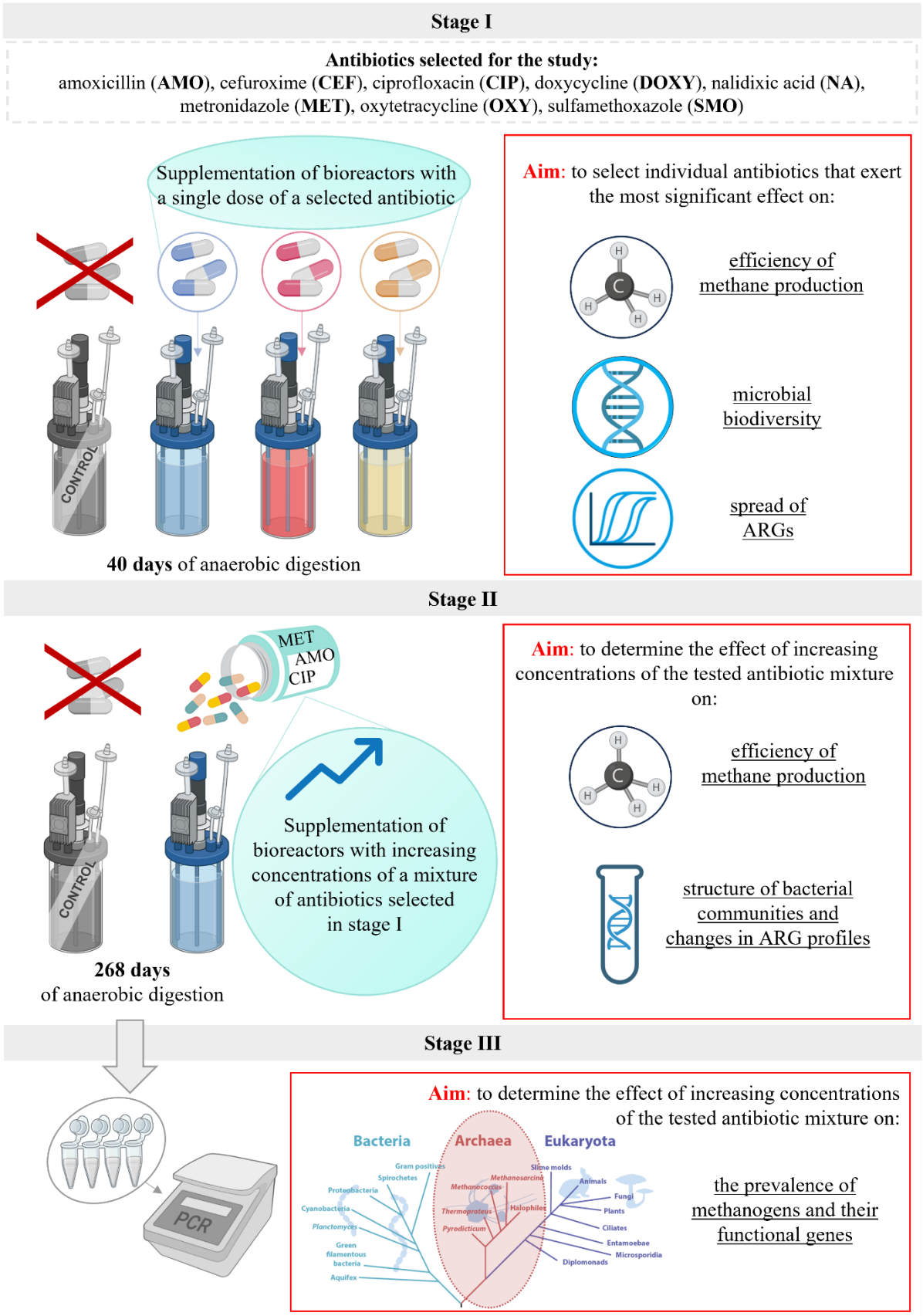


Figure 1. Stages of research described in the doctoral dissertation.

Stage I

Based on a report of the European Center for Disease Prevention and Control (ECDC 2018), antimicrobial drugs that are most widely used in human medicine were selected for the study: metronidazole (MET), amoxicillin (AMO), cefuroxime (CEF), oxytetracycline (OXY), doxycycline (DOXY), sulfamethoxazole (SMO), ciprofloxacin (CIP) and nalidixic acid (NA). In order to determine their individual effects on the anaerobic digestion of sewage sludge, bioreactors were filled with 25 g of the substrate and 175 g of the inoculum, i.e. sewage sludge and anaerobically digested sludge from a fermentation tank, respectively, collected in the "Łyna" Municipal WWTP in Olsztyn. Selected antimicrobials at doses determined experimentally during a preliminary study were added to substrates in bioreactors. The experiment was performed in duplicate. In the control bioreactor, the substrate was not exposed to antibiotics. Anaerobic digestion was conducted for 40 days under mesophilic conditions at a temperature of 37°C. Methane production was measured with the AMPTS II. Biogas quality was analyzed in a gas chromatograph equipped with a thermal conductivity detector. After anaerobic digestion, representative digestate samples were collected from each bioreactor. The samples were analyzed to determine the concentrations of volatile fatty acids (VFAs). In addition, DNA was isolated from digestate samples with the use of the FastDNA™ Spin Kit for Soil (MP Biomedicals). The genetic material was subjected to sequencing of the hypervariable region V3-V4 of the *16S r*RNA gene with the use of the Illumina MiSeq instrument, to determine microbial diversity. Real-time quantitative polymerase chain reaction (qPCR) was applied to analyze the concentrations of selected ARGs, genes encoding class 1 and 2 integrases, genes specific to the families of methanogens that predominate in anaerobically digested sludge (*Methanosarcinaceae* - *MSC* and *Methanosaetaceae* - *MST*), and the functional methyl-coenzyme M reductase gene (*mcr*A), which catalyzes the final step of anaerobic digestion - methanogenesis. The results of this experiment were presented in a research article (**Appendix 2** to this dissertation), and all research hypotheses were verified.

In the described study, anaerobic digestion was significantly inhibited in the bioreactors where the substrate was supplemented with MET, AMO, OXY, DOXY and CIP. Metronidazole, which is used to treat infections caused by anaerobic bacteria, was the strongest inhibitor, and its introduction to the bioreactor decreased the methane content of biogas to 12.8±4.0% and reduced the methane production rate to 21.85 L kg^{-1} d^{-1}. The inhibition of methanogenesis led to the accumulation of VFAs. The concentrations of seven out of the nine analyzed VFAs were significantly higher in the digestate supplemented with MET than in the sample from the control

bioreactor. Amoxicillin and CIP were also potent inhibitors of anaerobic digestion of sewage sludge. The addition of AMO to the bioreactor significantly reduced the methane content of biogas (by approx. 44%), whereas the introduction of CIP decreased methane production (by approx. 40%, relative to the control bioreactor).

An analysis of DNA sequences in the samples from all bioreactors revealed that *Bacteroidetes*, *Firmicutes* and *Proteobacteria* were the dominant bacterial phyla in the digestate. In comparison with the control treatment, the greatest decrease in the number of *Bacteroidetes*-specific reads was noted in digestate samples exposed to MET. A significant increase in the proportions of *Firmicutes* and *Proteobacteria* (by 6% and 6.8% relative to the control treatment, respectively) was also observed in these samples. The exposure of the substrate to AMO and CIP significantly decreased the proportion of Syntrophobacteriales, which may point to the inhibition of acetogenesis.

Analyses involving qPCR demonstrated that the concentration of the *mcr*A gene in digestate samples exposed to MET, CEF and NA was lower than in the control treatment. However, it was found that the abundance of this functional gene does not fully reflect the actual efficiency of methane production in the presence of antimicrobials. The total concentration of genes specific to the families *Methanosarcinaceae* and *Methanosetaceae* decreased significantly (relative to the control treatment) in digestate samples exposed to MET, CEF and NA, which could suggest that methanogens are somewhat sensitive to these antimicrobials.

The presence of antibiotics affected the prevalence of the analyzed ARGs, leading to an increase in the copy numbers of genes encoding resistance to β-lactams, tetracyclines and fluoroquinolones. It was found, however, that the selective pressure exerted by antimicrobials was not specific to the corresponding ARGs. The correlation analysis revealed that genes encoding class 1 and 2 integrases play an important role in the transfer of ARGs during anaerobic digestion.

Stage II

The research described in stage I was continued, and another experiment was conducted to determine a long-term, simultaneous impact of antimicrobials on the efficiency of anaerobic digestion of sewage sludge. Three antibiotics, MET, AMO and CIP, which individually exerted the most significant effect on anaerobic digestion, including the microbiological aspects of the process, were analyzed. The results of this experiment were presented in a research article (**Appendix 3** to this dissertation), and the first three research hypotheses were verified.

In the second stage of the study, sewage sludge was inoculated with anaerobically digested sludge from a fermentation tank, collected in the same WWTP, and it was subjected to anaerobic digestion in semi-continuous flow digesters, under mesophilic conditions (37°C). The digesters were fed substrates containing a mixture of MET, AMO and CIP or sewage sludge not exposed to antibiotics (control). The experiments were performed in duplicate for each substrate. The study consisted of six experimental series that differed in the concentrations of antibiotics applied to the substrate. The initial concentration of each of the three antibiotics in the mixture dosed to sewage sludge during the first experimental series was similar to the concentrations of the respective drugs in the wastewater flowing into the WWTP from which the sludge was obtained. The concentrations of the antibiotics added to the digester were increased after doubling the hydraulic volume of each digester. Each of the experimental series lasted 45 days on average, and the entire study lasted 268 days. Throughout the study, methane production was measured (AMPTS II) every week, and biogas quality was analyzed in a gas chromatograph equipped with a thermal conductivity detector. The concentrations of VFAs in the collected samples were also determined at weekly intervals. Samples for DNA isolation and sequencing were collected from process bioreactors (containing substrate supplemented with antibiotics) at the beginning, in the middle and at the end of each experimental series, whereas one sample was collected from the control bioreactor during the first, fourth and last experimental series. Microbial biodiversity and prevalence of ARGs were determined using a sensitive molecular method of metagenomic sequencing (Illumina NovaSeq).

An analysis of methane production revealed that prolonged exposure to a combination of antimicrobials could influence microbial acclimation inside the bioreactor, thus contributing to the adaptation of microorganisms. Methane production in process and control bioreactors followed a similar trend, but its efficiency was significantly lower in the process bioreactor where the substrate was supplemented with a mixture of MET, AMO and CIP than in the control bioreactor. No significant differences in the concentrations of individual VFAs were found between sewage sludge samples collected from process and control bioreactors.

The sequencing analysis revealed significant differences in the structure of microbiota between samples of control and supplemented sewage sludge during the experiment. Exposure to increasing doses of the tested antibiotics induced changes in the structure of microbial populations. Bacteria of the phylum *Acidobacteria* were predominant in samples of anaerobically digested sewage sludge collected from both control and process bioreactors, but their abundance differed significantly between substrates exposed to antibiotics and the control

substrate. It was found that substrate exposure to AMO (36 µg/mL), CIP (16 µg/mL) and MET (16 µg/mL) significantly inhibited the growth of *Acidobacteria*. During the entire process, the prevalence of *Candidatus Cloacimonetes* and *Proteobacteria* was similar in control and antibiotic-supplemented substrates, whereas the abundance of operational taxonomic units (OTUs) characteristic of *Bacteroidetes* differed significantly between samples collected from process and control bioreactors. The high initial abundance of *Firmicutes* in the process bioreactor decreased under exposure to a mixture of the tested antibiotics.

Exposure to a mixture of the tested antibiotics significantly increased the abundance of OTUs characteristic of *Archaea* in the analyzed samples, but these changes did not affect the efficiency of biogas production. An analysis of the abundance of OTUs characteristic of methanogens supported the identification of four main orders of methanogens in substrate samples: *Methanosarcinales* and *Methanomicrobiales* of the class *Methanomicrobia*, *Methanomassiliicoccales* of the class *Thermoplasmata*, and *Methanobacteriales* of the class *Methanobacteria*. Throughout the experiment, significant differences were observed in the abundance of *Methanosarcinales* and *Methanomassiliicoccales* in samples collected from both process and control bioreactors. The abundance of OTUs characteristic of these methanogens was bound by a significant negative correlation. It was concluded that dynamic changes in microbial communities and their adaptation to changing environmental conditions are essential for the stable operation of anaerobic digesters.

Substrate samples collected from both process and control bioreactors were characterized by a predominance of genes conferring resistance to multiple drugs and MLS antibiotics (>20 ppm). Tetracycline and bacitracin resistance genes were also abundant (5-15 ppm) in the analyzed samples. It was confirmed again that selective pressure exerted by antibiotics added to the substrate in process bioreactors did not specifically target genes encoding resistance to various classes of antimicrobials. Total ARG abundance decreased in both process and control digestates at the end of anaerobic digestion. However, it should be noted that the prevalence of multidrug resistance genes increased in samples of antibiotic-supplemented samples, whereas a reverse trend was observed in control samples. It should also be stressed that the transfer of multidrug resistance genes between bacteria poses a serious public health threat around the world.

Stage III

Although the metagenomic sequencing of samples collected in the previous stage of the study supported the identification of sequences characteristic of *Archaea*, their number was significantly lower than the number of Eubacteria-specific reads. Therefore, the last stage of research focused on expanding the existing knowledge on the effect exerted by long-term supplementation of sewage sludge with a mixture of antibiotics at increasing concentrations on methanogenic microorganisms.

The genetic material isolated in the second stage of the study was evaluated to determine the prevalence of genes characteristic of methanogens of the families *Methanosarcinaceae* (*MSC*) and *Methanosaetaceae* (*MST*), belonging to the order *Methanosarcinales*. The analyses were performed using the qPCR technique. The results of a preliminary study enabled to exclude a significant proportion of methanogenic families of other orders in the domain *Archaea* in anaerobically digested sludge. The activity of methanogenic microorganisms was assessed based on the concentration of the methyl-coenzyme M reductase gene (*mcr*A), which catalyzes the final step of anaerobic digestion - methanogenesis. The results of this experiment were presented in a research article (**Appendix 4** to this dissertation), and the fourth research hypothesis was verified.

A molecular analysis of the efficiency of anaerobic digestion, including the concentration of the functional *mcr*A gene, demonstrated that the concentration of this gene in substrate samples collected from process and control bioreactors varied throughout the entire experimental series. It was found that methane production was not correlated with the prevalence of the *mcr*A gene during long-term anaerobic digestion, regardless of exposure to antibiotics. It was confirmed again that changes in methane yields cannot be reliably measured based on the abundance of the *mcr*A gene despite the fact that this gene has been proposed by other researchers as a bioindicator for monitoring methanogen activity.

In both experimental and control substrate samples, the copy numbers of genes characteristic of *Methanosarcinaceae* remained stable during the experiment. The only exception was the last, sixth experimental series, where the abundance of *MSC* genes increased by two orders of magnitude in 1 gram of digestate. This points to a stable increase in *Methanosarcinaceae* counts during the anaerobic digestion of sewage sludge, regardless of antibiotic supplementation. In turn, highly significant ($P \leq 0.001$) differences were noted in the abundance of *MST* genes, characteristic of the family *Methanosaetaceae*, between antibiotic-

supplemented and control samples. It should be stressed that in the last experimental series, the abundance of *MST* genes was similar in experimental and control substrate samples, which suggests that microbial consortia adapted to the increasing concentrations of antibiotics. It was concluded that both methanogen families well adapted to the conditions in the process bioreactor, but irrespective of antibiotic exposure, *Methanosaetaceae* predominated in the digestate.

SUMMARY AND CONCLUSIONS

The results of the study provided new information on the impact of antimicrobials on anaerobic digestion, in both technological and microbiological contexts. The research objectives were achieved, and all hypotheses formulated in the doctoral dissertation were positively verified. It was found that:

(1) some antimicrobials, widely used in human medicine, may decrease the efficiency of anaerobic digestion of sewage sludge.

(2) the presence of antimicrobials during anaerobic digestion exerts a significant effect on ARG profiles. Exposure to MET induced the most significant changes in ARG concentrations, in particular through increasing the concentrations of genes encoding resistance to β-lactams, tetracyclines and fluoroquinolones, and decreasing the concentrations of genes encoding resistance to MLS antibiotics. The selective pressure exerted by antimicrobials was not specific to the corresponding ARGs. Moreover, the study highlighted the problem associated with ineffective elimination of ARGs during the anaerobic digestion of sewage sludge and threats posed by the spread of AR, which are very important considerations.

(3) the exposure of sewage sludge to antimicrobials during anaerobic digestion significantly affects the structure of microbial communities, including methanogens. The greatest changes in microbial diversity were induced by MET, which decreased the proportion of *Bacteroidetes*, and increased the abundance of OTUs characteristic of *Firmicutes* and *Proteobacteria*. *Methanosaetaceae* dominated among methanogenic microorganisms during supplementation with individual antibiotics and during simultaneous exposure to MET, AMO and CIP.

(4) the results of a quantitative analysis of genes specific to methanogenic microorganisms, such as the functional *mcr*A gene, do not fully reflect the parameters of anaerobic digestion, i.e. the actual efficiency of methane production in the presence of antimicrobials.

The conducted experiments revealed the need for further research to determine the effect of inhibitors, such as antibiotics, on the activity of microorganisms responsible for each stage of anaerobic digestion, which in the future will make it possible to ensure optimal conditions for their growth and development. Such research should involve the use of modern molecular techniques, including metagenomic sequencing.