Evidence of mutations conferring resistance to clarithromycin in wastewater and activated sludge

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Abstract

The occurrence of clarithromycin in wastewater samples and of the activated sludge bacteria possibly resistant to this pharmaceutical was the object of the study. Samples of wastewater or activated sludge were taken from a municipal wastewater treatment plant in summer and winter and characterised regarding their clarithromycin concentrations and the presence of nucleic acid fragments (Cla-sequences) known to be responsible for clarithromycin resistance in *Helicobacter pylori*. The concentrations of clarithromycin in raw wastewater were about 1086–2271 ng/L. Around 50–60% less of the pharmaceutical was found in treated wastewater. The concentrations were much higher in winter samples, as compared to summer samples. The clarithromycin resistance markers in *H. pylori* were detected by fluorescence in situ hybridisation in activated sludge bacterial cells. Cla-sequences were found in all the detected *Proteobacteria*, independently of the sampling season. Among nitrifying or phosphate or glycogen accumulating bacteria only *Nitrosomonas* spp. revealed presence of the clarithromycin sequences.

Keywords Clarithromycin · Clarithromycin resistance mutation · Activated sludge · Nitrifying bacteria · Wastewater treatment

Introduction

Consumption of antibiotics and antibiotic resistance are increasing from year to year. Data on microbial drug resistance are frightening. The extensive and often reckless use of pharmaceuticals poses serious problems. Among pharmaceuticals, antibiotics are gaining more and more attention due to their potential genotoxic effect and their spread in the aquatic environment, in particular, the spread of antibiotic resistance. The primary source of antibiotics in the environment is their natural production by soil bacteria and some

fungi. Thousands of tons of pharmacologically active substances are produced annually and mainly used in medicine, veterinary medicine, animal husbandry, agriculture, and aquaculture. Most of these substances are not metabolised in 100% (Liu et al. 2017; Terzic et al. 2018). The degree of their metabolism is reflected by the half-time value, which is a period in which any chosen concentration of a given drug will be halved. Thus, the concentration of a pharmaceutical in human excrements depends on the substance type, a form of its use, and the time after consumption. Individual features are also of great importance for the pharmacokinetics of the drug being taken (Lebel 1993). It has been demonstrated that 40–90% of tetracyclines, sulphonamides, and macrolides are excreted in an unchanged form (Liu et al. 2017; Terzic et al. 2018). Non metabolised antibiotics, antibiotic metabolites, and drug-resistant microbes are discharged together with urine and faeces through wastewater treatment plants, where they often are not eliminated (Kang and Kang 2016; Liu et al. 2017). In wastewater treatment plants (WWTP) they pass through successive stages of the purification process, and if not removed, they get to the environment, mainly into surface water (McArdell et al.

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2003; Ahn and Choi 2016; Barancheshme and Munir 2018). Municipal sewage treatment plants are a major source of drugs in the environment, as most conventional treatment technologies are not designed to eliminate this type of pollution (Bouki et al. 2013; Liu et al. 2017). As a result, sewage treatment plants are a potential place for the dissemination of resistant bacteria (Bellanger et al. 2014; Gupta et al. 2018; Barancheshme and Munir 2018), sometimes to several antibiotics simultaneously (Barancheshme and Munir 2018).

Macrolides are important first-choice antibiotics, which have become gradually ineffective due to increasing bacterial resistance (Prestinaci et al. 2015; Terzic et al. 2018). They are used to treat infections of the upper and lower respiratory tract, as well as skin and soft tissue. They exhibit a broad spectrum of activity against Gram-positive and, to a lesser extent against Gram-negative bacteria and are used as a substitute for penicillin (Zuckerman 2000; McArdell et al. 2003). Clarithromycin is a drug belonging to a new generation of the macrolide class and a semi-synthetic derivative of erythromycin (the oldest antimicrobial substances in this class), introduced into medical practice in 1990 (Zuckerman 2000). Clarithromycin is a semisynthetic 14-membered ring macrolide antibiotic. It reveals stability in an acidic environment and good antibacterial and pharmacokinetic properties (Hardy et al. 1992; Lebel 1993). The mechanism of this substance activity is the inhibition of the bacterial protein synthesis at the ribosomal level (Hardy et al. 1992; Lebel 1993). Clarithromycin is rapidly absorbed from the gastrointestinal tract and metabolised in liver by the cytochrome P-450. The main metabolite of the antibiotic is a 14-hydroxycarithromycin, which is active a few hours after drug ingestion and, together with an additive or clarithromycin acts synergistically against certain microorganisms. The antibiotic and its metabolites are excreted by kidneys in urine (Hardy et al. 1992; Lebel 1993). McArdell et al. (2003) report that clarithromycin is initially secreted from the body via bile and faeces (50–67%), while only 10–20% of the non-metabolised substance is eliminated with urine. Clarithromycin interacts reversibly with the 23S ribosomal RNA, a part of the 50S subunit of the bacterial ribosome, and it inhibits the bacterial protein synthesis. The antibiotic binds to the adenine nucleotide at position 2058 and 2059 domain V 23S rRNA subunits and to a lesser extent—to the adenine nucleotide position 752 in domain II. As a result of the antibiotic binding, the protein comes to the output channel blocking the 50S ribosome subunits and thus leads to the translocation of peptidyl transferase and the termination of the peptide chain elongation (Lebel 1993; Polikanov et al. 2018).

The effect of the presence of antibacterial agents in the environment is to produce resistance produced through mutations in the bacterial genetic material or acquiring resistance genes from other bacteria. Resistance is the ability

of a microorganism to survive in the presence of an antibiotic, which interferes with the processes of life, and thus inhibits growth and multiplication (Prestinaci et al. 2015). Spontaneous genetic mutations (errors in the DNA synthesis during replication and in the DNA repair) and recombination of genetic material often lead to the emergence and proliferation of antibiotic-resistant strains. Mutations occur spontaneously, albeit very rarely. Their rate for a single pair of nucleotides is about 10^{-10} (Pray 2008). Nevertheless, in light of the speed of bacterial fission the possibility of the emergence of antibiotic-resistant cells is large (Prestinaci et al. 2015). The antimicrobial resistance is a natural phenomenon that appears in microorganisms when they are exposed to antimicrobial substances (Prestinaci et al. 2015). In general, the resistance mechanism can be divided into three main categories: *intrinsic resistance*—a naturally occurring phenomenon as well as *acquired* and *adaptive resistance*, when the acquisition of a resistance phenotype requires a genetic change (e.g. mutation or horizontal transfer) (Prestinaci et al. 2015; Von Wintersdorff et al. 2016; Hughes and Andersson 2017). The presence of antimicrobial substances in the environment results in selective pressure on sensitive bacteria and privileges bacteria possessing natural or acquired resistance. Thus, the presence of antibiotics leads to the selection of the fittest strains. As the resistant bacteria have a better ability to grow in adverse conditions, they can be found in both clinical and environmental niches with higher than natural concentrations of antimicrobials (Prestinaci et al. 2015; Von Wintersdorff et al. 2016).

Wastewater treatment plants have been identified as a source of antibiotic-resistant bacteria and antibiotic resistance genes (Rizzo et al. 2013; Jäger et al. 2018). Although a significant number of bacteria, including antibiotic-resistant ones, are removed from treated wastewater, many bacteria still survive and are discharged with the effluent into the receiver (Bai et al. 2016; Jäger et al. 2018). The relative abundance of antibiotic-resistant bacteria in wastewater effluents can be even higher in treated wastewater than in raw wastewater (Ferreira Da Silva et al. 2007; Gupta et al. 2018). Moreover, the biofilm taken from the effluent sewerage had a higher number of antibiotic resistance bacteria, mostly coliforms, than the biofilm from the sewerage bringing sewage to the treatment plant (Lépesová et al. 2018). To avoid the release of ARBs and ARGs to the environment advanced treatment scenarios are tested (Jäger et al. 2018; Barancheshme and Munir 2018). Diseases caused by antibiotic-resistant bacteria are difficult to treat, last longer and pose a risk of death. Also, they contribute to increased costs of treatment because often their eradication requires to use alternative and more expensive drugs to be used (WHO 2018).

However, resistance is not always associated with the presence of antibiotics in the environment. Sometimes

resistant bacteria that transmit resistance genes to other bacteria are more important for its emergence and spread (Hughes and Andersson 2017). The spread of resistance poses a significant threat to health (WHO 2018; Li et al. 2019b). Resistance also leads to a reduction or loss of the effectiveness of antibiotics in the treatment or prophylaxis of infections caused by bacteria (WHO 2018).

Several mechanisms of resistance to antibiotics are described in the literature (Schmieder and Edwards 2012; Von Wintersdorff et al. 2016): inactivation or modification of the antibiotic, alteration in the target site of the antibiotic that reduces its binding capacity, modification of metabolic pathways to circumvent the antibiotic effect or reduced intracellular antibiotic accumulation by decreasing permeability and/or increasing active efflux of the antibiotic.

The mechanism of clarithromycin resistance is based on a mutation and it relies on the modification of a point that is a target of the antibiotic activity (Trebesius et al. 2000). The resistance of *Helicobacter pylori* to this antibiotic drug is the most important cause of treatment failure. In clinical *H. pylori* isolates, resistance to clarithromycin is caused predominantly by three distinct point mutations within the peptidyl transferase region of the 23S rRNA (A2143G, A2144G, and A2143C) (Hakemi Vala et al. 2016). The mechanism comprises the production of methyltransferase enzymes (coded by an *erm* gene), which after transcription modifies the 23S rRNA. Those enzymes transport one or two methyl residues ($-\text{CH}_3$) to adenine in the 23S rRNA thus N^6 -methyladenine or N^6,N^6 -dimethyladenine is produced. As a result, a conformation change occurs in rRNA that enables the antibiotic molecule to be fixed. Genes that encode this mechanism of antibiotic resistance can be located at a chromosome or be transferred by plasmids or transposons. Clarithromycin resistance is usually detected through molecular methods, such as polymerase chain reaction (PCR) and fluorescence in situ hybridisation (FISH), with the use of oligonucleotide probes. The probes ClaR1 and ClaR2 were designed to detect the most prevalent 23S rRNA mutations responsible for clarithromycin resistance. ClaR1 and ClaR2 detect the mutation resulted from the transition of adenine to guanine in position 2143 and 2144, respectively. Hence the probe ClaR3 serves to identify the mutation resulted from the transversion of adenine to cytosine in position 2143 (Trebesius et al. 2000; Hakemi Vala et al. 2016).

There is a lack of information about the presence of clarithromycin resistance bacteria and the transfer of clarithromycin resistance among environmental bacteria. There is little information in the literature on the incidence of antibiotic resistance in isolates from environmental communities. Most of the research has been done on clinical isolates (Trebesius et al. 2000; Iamsawat et al. 2010;

Alfaresi and Elkoush 2010; Hakemi Vala et al. 2016), but much less has been done concerning antibiotic-resistant organisms in the environment.

The study aimed to detect the sequences known to be clarithromycin resistance mutations in *H. pylori* (Cla sequences) in activated sludge and to check if the mutations appear in bacterial species that are not known to have them naturally.

Materials and methods

Sampling and sampling points

Samples of wastewater and activated sludge were taken from “Zabrze Śródmieście” wastewater treatment plant located in Zabrze, Poland in six sampling series—three of them in the winter period (February) and the remaining of them in the summer period (June) of the same year. During these months, the average air temperature was 0 °C and 20 °C for February and June, respectively. Within a particular season/month the samples were taken three times, in ca. 1 week intervals. Activated sludge samples were taken from the nitrification chamber. The configuration of the WWTP in the study with the sampling points and the main characteristics of the WWTP are presented in Fig. 1 and Table 1, respectively. The WWTP treated municipal sewage with a small share of industrial or hospital sewage.

Samples of activated sludge were immediately fixed with 4% paraformaldehyde (incubation time of 90 min), washed with 1xPBS and stored at $-20\text{ }^{\circ}\text{C}$. These samples were used to determine the presence of selected bacterial groups and to detect CLA-sequences.

Samples of raw (influent) and treated (effluent) wastewater were taken on the same days as the samples of activated sludge, frozen and stored in dark glass bottles at $-20\text{ }^{\circ}\text{C}$ until the analysis. These samples were used for the clarithromycin concentration analysis.

Presence of clarithromycin in wastewater samples

After defrosting, the samples were filtered through glass fibre filters (pore size of $<1\text{ }\mu\text{m}$, diameter 55 mm; Dassel, Germany). One hundred mL of the influent and 200 mL of the effluent were spiked with a mixture of internal standards (IS). The pH of all the samples was adjusted to 7.5. (E)-9-[O-(2-methyloxime)]-erythromycin was used as the internal standard. The concentration of clarithromycin was measured by SPE-HPLC–MS–MS, that is described in detail by Ziemińska-Buczyńska et al. (2015).

Fig. 1 The scheme of WWTP Zabrze “Śródmieście” with the indication of the sampling points for analytical procedure and molecular analysis; 1—screens, 2—grit removal, 3—primary settling, 4—pre-

denitrification, 5—biological dephosphatation, 6—denitrification chamber, 7—nitrification, 8—secondary settling

Table 1 Raw wastewater characteristics of the municipal wastewater treatment plants WWTP Zabrze “Śródmieście”

Parameter(s)	WWTP Zabrze “Śródmieście”
Max flow, m ³ /d	63,300
PE (approx.)	100,000
COD, mg/L	305–810
BOD ₅ , mg/L	190–506
NH ₄ -N, mg/L	44.4–89.6
P _{tot} , mg/L	14.9

Detection of activated sludge bacteria

The detection/identification of the activated sludge bacteria was carried out using fluorescence in situ hybridisation (FISH) with oligonucleotide 16S rRNA-targeted probes. In situ hybridisation was performed as described previously by Daims et al. (2005). The following activated sludge bacteria groups were screened (probe used): *α-proteobacteria* (ALF968), *β-proteobacteria* (BET42a together with a competitor), *γ-proteobacteria* (GAM42a together with a competitor), nitrifying bacteria—mostly halophilic and halotolerant *Nitrosomonas* spp. (NEU and CTE as a competitor), *Nitrosomonas oligotropha* lineage (Cluster6a192 together with a competitor), *Nitrosococcus mobilis* lineage (Ncmob), *Nitrospira* sp. (Nsv443), *Nitrosomonas communis* (Ncom1025), *Nitrobacter* sp. (NIT3 together with a competitor), *Nitrospira* sp. (Ntspa663 together with a competitor), representatives of phosphate accumulating bacteria (PAO)—mostly *Accumulibacter* (PAO651) and glycogen accumulating bacteria (GAO)—*Competibacter phosphatis* (GAOQ431). The probes EUB338, EUB338 II, and EUB338 III were mixed in a 1:1:1 ratio and used as a set (EUBmix) to detect all the bacteria (Wagner et al. 2003). Detailed information about the probes can be found elsewhere (Trebesius et al. 2000; Loy et al. 2003; Wagner et al. 2003).

The probes and optimal hybridisation conditions (hybridisation and the wash buffer composition) were chosen according to the probeBase database (Loy et al. 2003). Both the probes and unlabelled competitor oligonucleotides were obtained from Biomers (Ulm, Germany). Detection of the bacteria was performed using a fluorescence microscope Motic400T. Before microscope observations, samples were embedded in Citifluor (Citifluor Ltd, United Kingdom) to reduce fluorochrome fading. Images were made using means of the MotiCam Pro-285A color camera and the Motic Images Plus 2.0 software.

Detection of ClaR1, ClaR2, and ClaR3 sequences

Detection of ClaR1, ClaR2 and ClaR3 sequences (known and used for the detection of clarithromycin resistance in *H. pylori*) was carried out by means of fluorescence in situ hybridisation (FISH) in the activated sludge bacteria. Three oligonucleotide probes ClaR1, ClaR2, and ClaR3 were hybridised with the activated sludge bacteria using the formamide concentration of 30% in the hybridisation buffer (Trebesius et al. 2000). Microscopic observations were performed as described previously.

Detection of the sequences in different groups of the activated sludge bacteria was made by means of hybridisation of the activated sludge genetic material with the Cla probes and specific 16S rRNA-targeted probes simultaneously. Microscopic observation of both the red and green signal (originated from the Cy3 (Cla-probes) and 6-FAM (specific 16S rRNA) dyes, respectively) in the same place of the microscopic slide was assumed as the presence of the sequences in the cell of a particular species.

Results

Presence of clarithromycin in raw and treated wastewater

The concentrations of clarithromycin in raw wastewater were 1836 ± 435 ng/L and 1329 ± 243 ng/L in winter and summer, respectively. The clarithromycin effluent concentrations in winter were 928 ± 65 ng/L and in summer 457 ± 82 ng/L.

Composition of activated sludge bacterial biocenosis

“Zabrze Śródmieście” Wastewater Treatment Plant treats typical municipal wastewater on the basis of predenitrification—dephosphatation—denitrification—nitrification system. In such a treatment nitrifying, denitrifying, and polyphosphate accumulating bacteria were expected. The bacterial strains and groups identified in activated sludge are presented in Table 2.

Among the tested proteobacterial groups, β -proteobacteria were the most abundant group. All the groups were present regardless of the season. *Nitrosomonas* spp., *N. mobilis* and *Nitrospira* sp. were detected among the ammonia-oxidising bacteria (AOB) group, while among nitrite-oxidising bacteria (NOB) were represented only by *Nitrospira* sp. There was no *Nitrobacter* sp. in all the tested samples of activated sludge. The composition of the nitrifying bacteria biocenosis represents the same pattern in winter and summertime.

Phosphate accumulating organisms (PAO) represented by *Accumulibacter* sp. were present in the tested activated sludge in every sample and had to compete with glycogen accumulating organisms (GAO) only in summertime. GAO

represented by *Competibacter phosphatis* were detected only in the warm season, and in small amounts.

Presence of ClaR1, ClaR2, ClaR3 sequences in activated sludge

The probes tested in the study for the clarithromycin resistance (ClaR1, ClaR2, ClaR3) detection represent three mutation points resulting in resistance to clarithromycin in *H. pylori*. In the investigated activated sludge, each of the three Cla-sequences was detected, which is demonstrated in Table 3. There was no difference if the samples were taken in winter or summertime.

To apply the probes to the biocenosis of different bacterial strains, which the biocenosis of activated sludge is, the Cla-sequences were previously checked in the genomic database to assess their distribution among microorganisms. The oligonucleotide sequences were searched for in the microbial nucleotide database by means of the NCBI BLASTn software to identify sequences of high identity (Zhang et al. 2000). The database was searched for microorganisms possessing complementary sequences with no more than five mismatches.

The results of the BLASTn analysis based on the ClaR1 or ClaR2 or ClaR3 sequence showed that the ClaR1 or ClaR2 or ClaR3 probes might hybridise with some species from many of the bacterial groups, but only in *Proteobacteria* were there species with a similar sequence found in rRNA. Table 4 presents several species that might be detected in the proteobacterial group, as well as nitrifying bacteria or bacteria found in dephosphatation units. The results of the presence of clarithromycin resistance in bacteria responsible for nitrification or phosphorus removal are presented in Table 5. Among *Proteobacteria*, all the tested Cla sequences were detected. Besides the fact that many nitrifying bacteria belong to *Proteobacteria* (*Nitrobacter* belongs to α -proteobacteria, *Nitrosomonas* and *Nitrospira* to β -proteobacteria, *Nitrococcus* to γ -proteobacteria (Wagner and Loy 2002) hybridisation with most of them did not occur. Clarithromycin resistance mutations were detected only in *Nitrosomonas* spp. (except for at least *N. oligotropha*). *Nitrosomonas* spp. exhibited the obtainment of the fragment of ClaR1 and ClaR2 only, and the absence of the

Table 2 Organisms detected in activated sludge originated from WWTP “Zabrze Śródmieście”

Target	Wintertime	Summertime
α -proteobacteria	+	+
β -proteobacteria	+	+
γ -proteobacteria	+	+
<i>Nitrosomonas</i> sp.	+	+
<i>Nitrosomonas oligotropha</i>	+	+
<i>Nitrosococcus mobilis</i>	+	+
<i>Nitrospira</i> sp.	+	+
<i>Nitrospira</i> sp.	+	+
<i>Nitrobacter</i> sp.	–	–
<i>Accumulibacter</i>	+	+
<i>Competibacter phosphatis</i>	–	+

+ presence; – absence

Table 3 Clarithromycin resistance mutations found in activated sludge of WWTP “Zabrze Śródmieście”

Probe name	Wintertime	Summertime
ClaR1	+	+
ClaR2	+	+
ClaR3	+	+

+ presence; – absence

Table 4 Number of species where Cla-sequences were found in genome (or chromosome) by BLASTN analysis

Species or bacterial group	ClaR1	ClaR2	ClaR3
α -proteobacteria	4 (2)	20 (12)	3 (2)
β -proteobacteria	3 (3)	5 (3)	12 (11)
γ -proteobacteria	6 (5)	10 (3)	70 (28)
δ/ϵ -proteobacteria	9 (4)	20 (10)	2 (2)
<i>Nitrosomonas</i> sp. (β -proteobacteria)	–	–	–
<i>Nitrosococcus mobilis</i> (γ -proteobacteria)	–	–	–
<i>Nitrospira</i> sp. (β -proteobacteria)	–	–	–
<i>Nitrospira</i> sp. (Nitrospiraea)	–	–	–
<i>Accumulibacter</i> (β -proteobacteria)	–	–	–
<i>Competibacter phosphatis</i> (γ -proteobacteria)	–	–	–

Table 5 Cla-sequences found in activated sludge bacteria

Target	ClaR1	ClaR2	ClaR3
α -proteobacteria	+	+	+
β -proteobacteria	+	+	+
γ -proteobacteria	+	+	+
<i>Nitrosomonas</i> sp.	+	+	–
<i>Nitrosomonas oligotropha</i>	–	–	–
<i>Nitrosococcus mobilis</i>	–	–	–
<i>Nitrospira</i> sp.	–	–	–
<i>Nitrospira</i> sp.	–	–	–
<i>Accumulibacter</i>	–	–	–
<i>Competibacter phosphatis</i>	–	–	–

+ presence; – absence

fragment of ClaR3 in its nucleic material. The examples of detection of the CLA-sequences in activated sludge bacteria are presented in Fig. 2.

Discussion

Presence of clarithromycin in raw and treated wastewater

The amount of clarithromycin in influent wastewater varies from 330 to 2750 ng/L, whereas in treated wastewater from 185 to 1920 ng/L (Łuczkiwicz et al. 2013; Liu et al. 2017; Lépesová et al. 2018). However, the UNESCO reports (UNESCO and HELCOM 2017) average concentration of clarithromycin of 1 μ g/L, and a maximum of 10 μ g/L in the municipal WWTP of the Baltic Sea region countries. The concentration of clarithromycin stated in our study can be considered as higher than the typical average. It can be due to the fact that Finland, Poland, and Malta are the

highest consumers of that compound among EU countries (ECDPC 2018). The observed differences of clarithromycin concentrations in winter and summer (both in influent and effluent) results mainly from seasonal fluctuations in the antibiotic usage. The consumption of such medicaments in the cold season was 25% higher in comparison to the summer period (Goossens et al. 2005; Coenen et al. 2006). This can be explained by a higher consumption of macrolides in this period, which is understandable, as they are recommended in the treatment of upper respiratory tract infections, which occur more frequently in winter. Similar trends related to the high variability of this antibiotic, depending on the season, were observed in other wastewater treatment plants (McArdell et al. 2003; Łuczkiwicz et al. 2013). A load of macrolides in winter may be even twice as high as in summer (McArdell et al. 2003). Despite the satisfactory effectiveness of nutrients removal, both at the investigated WWTP and other plants (McArdell et al. 2003; Łuczkiwicz et al. 2013; Lépesová et al. 2018), the total elimination of this substance is not reached.

The elimination of antibiotics in wastewater treatment plants in the winter is probably even lower due to the lower biological activity of the activated sludge bacteria. Moreover, Novo et al. (2013) report that resistance prevalence of some antibiotics is higher not only after wastewater treatment but it is also increased when the temperature decreases (a stress agent). However, the obtained average removal of the compound of 27% is typical for municipal activated sludge systems that have been usually around 30–50% (Łuczkiwicz et al. 2013; Liu et al. 2017; Lépesová et al. 2018).

Composition of the activated sludge bacterial biocenosis

Proteobacteria are Gram-negative mostly pathogenic microorganisms possessing nitrogen fixation capabilities (Ahn and Choi 2016). Together with *Actinobacteria* and *Bacteroidetes*, they are regarded as the dominated phyla in activated sludge processes and have been found to play a crucial role in nitrification, denitrification and organic compounds transformations (Wang et al. 2013; Ahn and Choi 2016; Gupta et al. 2018). Representatives of *Proteobacteria* in activated sludge belong mostly to α , β , and γ -*proteobacteria* (Wang et al. 2013; Ahn and Choi 2016). In activated sludge from the hospital WWTP, the amount of *Proteobacteria* was 50% higher than in the domestic WWTP (Ahn and Choi 2016). In addition, it has been found that resistance genes are better retained in *Proteobacteria* (Hu et al. 2013). Thus, *Proteobacteria* seems to be important not only from the technological point of view but also regarding the dissemination of antimicrobial resistance. In our study, activated sludge is represented

Fig. 2 Examples of activated sludge after fluorescence in situ hybridisation; **a** alphaproteobacteria, **b** gammaproteobacteria; **c** *Nitrosomonas* spp.; **d** Cla1; **e** Cla3, **f** Cla2 (photos by Katarzyna Kunda)

by all the three subgroups of *Proteobacteria* (Table 2). According to the literature, nitrification is typically carried out by autotrophic *Proteobacteria* as *Nitrosomonas* spp., and *N. mobilis* and the presence of the genus *Nitrospira* is rather sporadic (Wagner and Loy 2002). More than two decades ago *Nitrobacter* sp. was regarded as the principal bacterial strain responsible for the oxidation of nitrites. However, it occurs in municipal treatment plants in quantities much smaller or insignificant in comparison to the amount of *Nitrospira* sp. (Wagner and Loy 2002). *Nitrospira* sp. is considered to be the most widespread NOB in natural conditions and it occurs in WWTPs, together with *Nitrobacter* sp. or alone (Wagner and Loy 2002). In the activated sludge under study, the AOB group was quite diverse, represented by at least four species: *N. oligotropha*, *N. communis*, *Nitrospira* sp. and *N. mobilis*. Hence the NOB group was represented only by one of the two known species responsible for nitrification.

Among bacteria concerned with enhanced biological phosphorus removal, there was a difference between the warm and cold season. In wintertime polyphosphate accumulating organisms could exist without competition for volatile fatty acids since GAOs are not present in that season. More PAOs than GAOs in municipal wastewater treatment systems were also observed by López-Vázquez et al. (2008), who found GAOs in amounts ca. 4-times lower than PAOs. Moreover, temperatures lower than 20 °C might have

favoured PAOs over GAOs as lower temperatures limit the proliferation of GAOs (López-Vázquez et al. 2008).

Detection of ClaR1, ClaR2, ClaR3 sequences

Each of the subgroups of *Proteobacteria* revealed the presence of the CLA-sequences. Moreover, each of the three CLA-sequences was found in each of the studied *Proteobacterial* group. Although there are many publications on the identification and removal of antibiotic resistance genes, to the best of our knowledge, there is no confirmation that the CLA-sequences are so common in activated sludge. However, approximately 25% of the antibiotic resistance genes in activated sludge have been found to be responsible for macrolide resistance (Yang et al. 2013; Terzic et al. 2018). As municipal wastewater is composed largely of human excreta, it was not a surprise that oligonucleotides used for the detection of the clarithromycin resistance point mutation were detected among activated sludge bacteria since the community can be composed of the bacteria from the gastrointestinal tract known to possess the clarithromycin resistance mutations.

Clarithromycin is active in vitro against a variety of aerobic and anaerobic Gram-positive and Gram-negative microorganisms. It inhibits the growth of Gram-positive cocci, especially *Streptococcus pneumoniae* and *Streptococcus pyogenes*, *Streptococcus viridans*, and most strains

of *Staphylococcus aureus* (*S. aureus*) and certain strains of enterococci. Clarithromycin is also active against such bacteria as *Haemophilus influenzae*, *Moraxella catarrhalis*, *Moraxella pneumoniae*, *Legionella pneumophila*, *Chlamydia pneumoniae* and atypical mycobacteria such as *Mycobacterium avium*, and in the elimination of *H. pylori*. Among the species that possess the clarithromycin resistance ability the following are mentioned: *H. pylori* (Trebesius et al. 2000), *M. avium* and *Mycobacterium intracellulare* (Iamsawat et al. 2010) or *Streptococci* (Goossens et al. 2005). *Mycobacterium* spp. belong to *Actinomycetes* and *Streptococcus* spp. to *Firmicutes*. In the literature, among *Proteobacteria* resistant to clarithromycin, *H. pylori* (ϵ -*proteobacteria*) (Trebesius et al. 2000; Kargar et al. 2013; Prestinaci et al. 2015; Bai et al. 2016) and *Haemophilus influenza* (γ -*proteobacteria*) (Seyama et al. 2016) are only mentioned. Meanwhile, heterotrophic *Proteobacteria* such as *Rhodocyclus Pseudomonas*, *Burkholderia*, *Bacteroides*, *Clostridium* and *Sphingobacteria*, *Clostridium* and *Desulfovibrio* dominate usually in activated sludge (Wagner and Loy 2002; Wang et al. 2013). Due to the commonly assessed frequency of the occurrence of the CLA-sequences in activated sludge (Fig. 2), it is assumed that there is an intense transfer of these sequences between the activated sludge bacteria. Such a transfer has been found as a frequent manner in activated sludge (e.g. Von Wintersdorff et al. 2016; Zhou et al. 2018; Li et al. 2019a) and can be extended in optimal conditions (e.g. Shoemaker et al. 2001). The simultaneous detection of fluorescence signals of *proteobacteria*-targeted probes and Cla-probes may result from the presence of sequences similar to Cla-sequences found by the BLASTn analysis.

It has to be marked that the detection proclaims the occurrence of the fragments in *Nitrosomonas* spp., but it does not confirm clarithromycin resistance of the species. There were only the Cla1 and Cla2 sequences found in *Nitrosomonas* spp., and there were no Cla3 sequences. The location and function of the fragments in *Nitrosomonas* should be verified in future research. However, this result indicates the presence of the fragment in the genetic material of *Nitrosomonas* spp. that has never been detected in the fully sequenced genome of that species and may suggest the possibility of obtainment of this fragment from another individual. In our study, among the nitrifying bacteria that revealed the presence of some Cla-sequences there were *Nitrosomonas* spp. but not *N. oligotropha*. *Nitrosomonas* spp. also includes *Nsm. eutropha*, *Nsm. europaea*, *Nsm. groningensis*, *Nsm. javenensis* and *Nsm. monocella*, hence it is stated that at least one of these species had the Cla-sequences. However, among them *Nsm. eutroph* and *Nsm. europaea* are those frequently present (Wagner and Loy 2002). There are several reports suggesting that the transfer of genes in the 16S rRNA in nature is possible despite the apparent conservative nature of rRNA (Kitahara et al. 2012; Prestinaci et al. 2015;

Von Wintersdorff et al. 2016; Hughes and Andersson 2017). Kitahara, et al. (2012) found unexpected plasticity of the 16S rRNA and asserts that the evolution of both 16S rRNA and 23S rRNA is possible and relies on the recognition mechanism of ribosomal proteins.

There is little knowledge about antibiotic resistance in environmental bacteria, even nitrifying bacteria. The research procedures for the detection of antibiotic resistance in environmental samples can be threefold. First, the identification of the species possessing antibiotic resistance result from the molecular analysis of concrete isolates received by culturing at the beginning of the experiment (e.g. West et al. 2011). Second, they rely on the sequencing of the 16S rRNA encoding genes obtained from the same DNA extract as the particular resistance genes (Novo et al. 2013; Kargar et al. 2013). Despite the fact that these methods are very accurate there is no guarantee that the sequenced fragments used for further species identification are derived from the same species since the analysis is performed on extracts from environmental samples of a diverse community. Thirdly, they make use of simultaneous hybridisation of the searched resistance genes or mutations and the 16S rRNA enabling identification (Trebesius et al. 2000; Alfaresi and Elkoush 2010; Hakemi Vala et al. 2016). For bacteria that are unculturable, only in situ methods allow to confirm particular resistance in a particular cell/species. Unfortunately, there are not so many examples of the detection of resistance genes or mutations by FISH as it usually uses short oligonucleotides. Researchers reviewing the current knowledge and assessment methods of the antibiotic resistance transfer are consistent that in complex environments, it remains a difficult task requiring further research (Rizzo et al. 2013; Bouki et al. 2013).

We tried to assess the presence of clarithromycin resistance genes in activated sludge bacteria as clarithromycin is commonly detected in the inflow and outflow of wastewater treatment plants (Łuczkiwicz et al. 2019). Additionally, clarithromycin, like other antimicrobial agents from the macrolide family, mainly selects against Gram-positive bacteria, which were noted to be very active within activated sludge flocs (Forster et al. 2002).

The nitrifying bacteria group was taken under special focus as it is quite a narrow group of species for identification, and they commonly exist in activated sludge. Three major factors are presented to have an influence on the antibiotic resistance bacteria and their fate. These are abiotic conditions (technical and technological parameters of wastewater treatment), bacterial biocenosis composition and presence of selective pressures (Novo et al. 2013). Other researchers add other factors that may have an influence on resistant bacteria and resistance genes or plasmids behaviour in environmental samples. These factors are a type of environmental matrix or eukaryotic predation (Bellanger

et al. 2014). Special focus was also given to the potential of a higher nutrients level as a factor improving the transfer of genes (West et al. 2011). All the factors can be encountered in WWTP; thus, the place is considered as very favourable for the proliferation of antibiotic-resistant bacteria and resistance transfer (Bouki et al. 2013).

Moreover, several reports confirm the impact of antibiotics presence on the nitrification efficiency and the conversion rates of nitrogen reduction (e.g. Halling-Sørensen 2001; Costanzo et al. 2005). This suggests that nitrifying bacteria can be injured by the presence of antibiotics so they may seek an effective defence against the influence of antibiotics. Naturally, the nitrifying bacteria do not exhibit the presence of antibiotic resistance genes, which is reflected by the inhibition of nitrification process caused by the presence of antibiotics in the soil or water environment (Halling-Sørensen 2001). Interestingly, not all nitrifying bacteria and not all *Nitrosomonas* species obtain the *Cla*-sequences in activated sludge. It has to be highlighted that the BLASTn analysis did not reveal any similarity of the *Cla*-sequences to any of the nitrifying bacteria genome. Thus, it looks rather like a transfer of a longer fragment to the genome of *Nitrosomonas*. However, determination of the origin of the *Cla*-sequences and their function in nitrifying bacteria was not the objective of the study. The plasmid-borne resistance genes are the most prolific mechanism of resistance transfer between bacteria. By this mechanism, many faecal bacteria (resistant to antibiotics in the gut of humans or animals) reaching the aquatic environment can distribute resistant genes to the native bacterial population (Hughes and Andersson 2017; Barancheshme and Munir 2018).

In the ammonia-oxidising autotrophic bacterium *Nitrosomonas* spp. (strain ENI-11) isolated from activated sludge two plasmids were discovered, having no detectable plasmid-linked antibiotic resistance. They are designated as pAYS and pAYL, relatively small, and approximately 1.9 kb long (Yamagata et al. 1999).

Moreover, in the genome of *Nitrosomonas europaea*, two integrase-like genes were identified. The integrase gene is a part of a gene capture system known as integron, in which gene cassettes are mobile elements responsible for a gene transfer (Leon and Roy 2003). These facts suggest that the mechanism of obtainment of resistance genes in *Nitrosomonas* spp. is by means of gene transfer rather than mutation, but further research needs to be performed.

In addition, studies on *Nitrosomonas eutropha* confirm high tolerance of this species for clarithromycin. This species was able not only to exist in the presence of 200 µg of clarithromycin/L, but also to degrade it in 40%. Concentrations of 1 µm/L did not change the activity of this bacterium compared to those without this antibiotic (Terada et al. 2017).

It also has to be reminded that nonpathogenic bacteria (such as nitrifying bacteria) do not constitute a direct threat to humans or animals, but they may be a reservoir of resistance genes that can be potentially forwarded (Hughes and Andersson 2017; Barancheshme and Munir 2018).

Conclusions

The level of clarithromycin concentration in raw and treated wastewater proclaims its rather pure removal in WWTP and its discharge to the environment. The constant presence of the drug in wastewater resulting in permanent exposition of the activated sludge bacteria to this pharmaceutical and the presence of clarithromycin resistance bacteria in activated sludge resulting mainly from the origin of municipal wastewater may be an enhancer of some mutation or transfer of nucleic acid between different species. Fragments constituting the mutation points have been detected in activated sludge bacteria by fluorescence in situ hybridisation. It was observed that the bacteria that possessed the fragments belonged to all the tested groups: α -proteobacteria, β -proteobacteria, γ -proteobacteria. Among selected nitrifying and polyphosphate or glycogen accumulating bacteria only *Nitrosomonas* spp. obtained two from the three known *Cla*-sequences. Moreover, the fragments have not been observed in *N. oligotropha* and *N. mobilis*—the latter known to be closely related to *Nitrosomonas* spp.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

References

- Ahn Y, Choi J (2016) Bacterial communities and antibiotic resistance communities in a full-scale hospital wastewater treatment plant by high-throughput pyrosequencing. *Water* 8:580. <https://doi.org/10.3390/w8120580>
- Alfaresi MS, Elkoush AA (2010) Characterization of clarithromycin resistance in isolates of *Helicobacter pylori* from the UAE. *Indian J Gastroenterol* 29:116–120. <https://doi.org/10.1007/s12664-010-0034-z>
- Bai X, Xi C, Wu J (2016) Survival of *Helicobacter pylori* in the wastewater treatment process and the receiving river in Michigan, USA. *J Water Health* 14:692–698. <https://doi.org/10.2166/wh.2016.259>

- Barancheshme F, Munir M (2018) Strategies to combat antibiotic resistance in the wastewater treatment plants. *Front Microbiol.* <https://doi.org/10.3389/fmicb.2017.02603>
- Bellanger X, Guilleto H, Bonot S, Merlin C (2014) Demonstrating plasmid-based horizontal gene transfer in complex environmental matrices: a practical approach for a critical review. *Sci Total Environ* 493:872–882. <https://doi.org/10.1016/j.scitotenv.2014.06.070>
- Bouki K, Venieri D, Diamadopoulos E (2013) Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: a review. *Ecotoxicol Environ Saf* 91:1–9. <https://doi.org/10.1016/j.ecoenv.2013.01.016>
- Coenen S, Ferech M, Malhotra-Kumar S, Hendrickx E, Suetens C, Goossens H (2006) European Surveillance of Antimicrobial Consumption (ESAC): outpatient macrolide, lincosamide and streptogramin (MLS) use in Europe. *J Antimicrob Chemother* 58:418–422. <https://doi.org/10.1093/jac/dkl184>
- Costanzo SD, Murby J, Bates J (2005) Ecosystem response to antibiotics entering the aquatic environment. *Mar Pollut Bull* 51:218–223. <https://doi.org/10.1016/j.marpolbul.2004.10.038>
- Daims H, Stoecker K, Wagner M (2005) Fluorescence in situ hybridization for the detection of prokaryotes. In: Osborn AM, Smith CJE (eds) *Advanced methods in molecular microbial ecology*. Bios-Garland, Abingdon
- European Centre for Disease Prevention and Control (ECDC) (2018) Antimicrobial consumption. In: ECDC. Annual epidemiological report 2017. ECDC, Stockholm
- Ferreira Da Silva M, Vaz-Moreira I, Gonzalez-Pajuelo M, Nunes OC, Manaia CM (2007) Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban wastewater treatment plant. *FEMS Microbiol Ecol* 60:166–176. <https://doi.org/10.1111/j.1574-6941.2006.00268.x>
- Forster S, Snape JR, Lappin-Scott HM, Porter J (2002) Simultaneous fluorescent gram staining and activity assessment of activated sludge bacteria. *Appl Environ Microbiol* 68:4772–4779. <https://doi.org/10.1128/AEM.68.10.4772-4779.2002>
- Goossens H, Ferech M, Vanderstichele R, Elseviers M (2005) Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. *Lancet* 365:579–587. [https://doi.org/10.1016/S0140-6736\(05\)70799-6](https://doi.org/10.1016/S0140-6736(05)70799-6)
- Gupta SK, Shin H, Han D, Hur HG, Unno T (2018) Metagenomic analysis reveals the prevalence and persistence of antibiotic- and heavy metal-resistance genes in wastewater treatment plant. *J Microbiol* 56:408–415. <https://doi.org/10.1007/s12275-018-8195-z>
- Hakemi Vala M, Eyvazi S, Goudarzi H, Sarie HR, Gholami M (2016) Evaluation of clarithromycin resistance among Iranian *Helicobacter pylori* isolates by E-test and real-time polymerase chain reaction methods. *Jundishapur J Microbiol.* <https://doi.org/10.5812/jjm.29839>
- Halling-Sørensen B (2001) Inhibition of aerobic growth and nitrification of bacteria in sewage sludge by antibacterial agents. *Arch Environ Contam Toxicol* 40:451–460. <https://doi.org/10.1007/s002440010197>
- Hardy DJ, Guay DRP, Jones RN (1992) Clarithromycin, a unique macrolide. *Diagn Microbiol Infect Dis* 15:39–53. [https://doi.org/10.1016/0732-8893\(92\)90055-X](https://doi.org/10.1016/0732-8893(92)90055-X)
- Hu Y, Yang X, Qin J, Lu N, Cheng G, Wu N, Pan Y, Li J, Zhu L, Wang X, Meng Z, Zhao F, Liu D, Ma J, Qin N, Xiang C, Xiao Y, Li L, Yang H, Wang J, Yang R, Gao GF, Wang J, Zhu B (2013) Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. *Nat Commun* 4:3151. <https://doi.org/10.1038/ncomms3151>
- Hughes D, Andersson DI (2017) Environmental and genetic modulation of the phenotypic expression of antibiotic resistance. *FEMS Microbiol Rev* 41:374–391. <https://doi.org/10.1093/femsre/fux004>
- Iamsawat S, Surawut S, Prammananan T, Leelaporn A, Jearanaissilavong J (2010) Multiplex PCR for detection of clarithromycin resistance and simultaneous species identification of *Mycobacterium avium*. *Southeast Asian J Trop Med Public Heal* 41:590–601
- Jäger T, Hembach N, Elpers C, Wieland A, Alexander J, Hiller C, Krauter G, Schwartz T (2018) Reduction of antibiotic resistant bacteria during conventional and advanced wastewater treatment, and the disseminated loads released to the environment. *Front Microbiol* 9:1–16. <https://doi.org/10.3389/fmicb.2018.02599>
- Kang J, Kang D (2016) International Journal of Food Microbiology Enhanced antimicrobial effect of organic acid washing against foodborne pathogens on broccoli by vacuum impregnation. *Int J Food Microbiol* 217:85–93. <https://doi.org/10.1016/j.ijfoodmicr.2015.10.004>
- Kargar M, Doosti A, Ghorbani-Dalini S (2013) Detection of four clarithromycin resistance point mutations in *Helicobacter pylori*: comparison of real-time PCR and PCR-RFLP methods. *Comp Clin Path* 22:1007–1013. <https://doi.org/10.1007/s00580-012-1519-1>
- Kitahara K, Yasutake Y, Miyazaki K (2012) Mutational robustness of 16S ribosomal RNA, shown by experimental horizontal gene transfer in *Escherichia coli*. *Proc Natl Acad Sci* 109:19220–19225. <https://doi.org/10.1073/pnas.1213609109>
- Lebel M (1993) Pharmacokinetic properties of clarithromycin: a comparison with erythromycin and azithromycin. *Can J Infect Dis* 4:148–152
- Leon G, Roy PH (2003) Excision and integration of cassettes by an integrase of *Nitrosomonas europaea*. *J Bacteriol* 185:2036–2041. <https://doi.org/10.1128/JB.185.6.2036-2041.2003>
- Lépesová K, Kraková L, Pangallo D, Medvedová A, Olejníková P, Mackuľak T, Tichý J, Grabic R, Bírošová L (2018) Prevalence of antibiotic-resistant coliform bacteria, *Enterococcus* spp. and *Staphylococcus* spp. in wastewater sewerage biofilm. *J Glob Antimicrob Resist* 14:145–151. <https://doi.org/10.1016/j.jgar.2018.03.008>
- Li B, Qiu Y, Zhang J, Liang P, Huang X (2019a) Conjugative potential of antibiotic resistance plasmids to activated sludge bacteria from wastewater treatment plants. *Int Biodeterior Biodegrad* 138:33–40. <https://doi.org/10.1016/j.ibiod.2018.12.013>
- Li R, Jay JA, Stenstrom MK (2019b) Fate of antibiotic resistance genes and antibiotic-resistant bacteria in water resource recovery facilities. *Water Environ Res* 91:5–20. <https://doi.org/10.1002/wer.1008>
- Liu H-Q, Lam JCW, Li W-W, Yu H-Q, Lam PKS (2017) Spatial distribution and removal performance of pharmaceuticals in municipal wastewater treatment plants in China. *Sci Total Environ* 586:1162–1169. <https://doi.org/10.1016/j.scitotenv.2017.02.107>
- López-Vázquez CM, Hooijmans CM, Brdjanovic D, Gijzen HJ, van Loosdrecht MCM (2008) Factors affecting the microbial populations at full-scale enhanced biological phosphorus removal (EBPR) wastewater treatment plants in The Netherlands. *Water Res* 42:2349–2360. <https://doi.org/10.1016/j.watres.2008.01.001>
- Loy A, Horn M, Wagner M (2003) Probebase: an online resource for rRNA-targeted oligonucleotide probes. *Nucleic Acids Res* 31:514–516. <https://doi.org/10.1093/nar/gkv1232>
- Łuczkiwicz A, Felis E, Ziembinska A, Gnida A, Kotlarska E, Olanczuk-Neyman K, Surmacz-Gorska J (2013) Resistance of *Escherichia coli* and *Enterococcus* spp. to selected antimicrobial agents present in municipal wastewater. *J Water Health* 11:600–612. <https://doi.org/10.2166/wh.2013.130>
- Łuczkiwicz A, Fudala-Książek S, Szopinska M, Jankowska K, Svahn O, Björklund E (2019) Fate of pharmaceuticals—via wastewater to the Baltic Sea. In: Proceedings of IWA 11th micropol and eco-hazard conference, Seoul, Korea
- McArdell CS, Molnar E, Suter MJF, Giger W (2003) Occurrence and fate of macrolide antibiotics in wastewater treatment plants and

- in the Glatt Valley watershed, Switzerland. *Environ Sci Technol* 37:5479–5486. <https://doi.org/10.1021/es034368i>
- Novo A, André S, Viana P, Nunes OC, Manaia CM (2013) Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater. *Water Res* 47:1875–1887. <https://doi.org/10.1016/j.watres.2013.01.010>
- Polikanov YS, Aleksashin NA, Beckert B, Wilson DN (2018) The mechanisms of action of ribosome-targeting peptide antibiotics. *Front Mol Biosci* 5:48. <https://doi.org/10.3389/fmolb.2018.00048>
- Pray L (2008) Antibiotic resistance, mutation rates and MRSA. *Natur Educ* 1:30
- Prestinaci F, Pezzotti P, Pantosti A (2015) Antimicrobial resistance: a global multifaceted phenomenon. *Pathog Glob Health* 109:309–318. <https://doi.org/10.1179/2047773215Y.0000000030>
- Rizzo L, Manaia C, Merlin C, Schwartz T, Dagot C, Ploy MCC, Michael I, Fatta-Kassinos D (2013) Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. *Sci Total Environ* 447:345–360. <https://doi.org/10.1016/j.scitotenv.2013.01.032>
- Schmieder R, Edwards R (2012) Insights into antibiotic resistance through metagenomic approaches. *Future Microbiol* 7:73–89. <https://doi.org/10.2217/fmb.11.135>
- Seyama S, Wajima T, Nakaminami H, Noguchi N (2016) Clarithromycin resistance mechanisms of epidemic β -lactamase-nonproducing ampicillin-resistant *Haemophilus influenzae* strains in Japan. *Antimicrob Agents Chemother* 60:3207–3210. <https://doi.org/10.1128/AAC.00163-16>
- Shoemaker NB, Vlamakis H, Hayes K, Salyers AA (2001) Evidence for extensive resistance gene transfer among *Bacteroides* spp. and among bacteroides and other genera in the human colon. *Appl Environ Microbiol* 67:561–568. <https://doi.org/10.1128/AEM.67.2.561-568.2001>
- Terada A, Usui H, Bao Q, Nakai S, Hosomi M (2017) Feasibility of biodegradation of clarithromycin by *Nitrosomonas europaea*. *Kagaku Kogaku Ronbunshu* 43:264–270. <https://doi.org/10.1252/kakoronbunshu.43.264>
- Terzic S, Udikovic-Kolic N, Jurina T, Krizman-Matasic I, Senta I, Mihaljevic I, Loncar J, Smilaj T, Ahel M (2018) Biotransformation of macrolide antibiotics using enriched activated sludge culture: kinetics, transformation routes and ecotoxicological evaluation. *J Hazard Mater* 349:143–152. <https://doi.org/10.1016/j.jhazmat.2018.01.055>
- Trebesius K, Panthel K, Strobel S, Vogt K, Faller G, Kirchner T, Kist M, Heesemann J, Haas R (2000) Rapid and specific detection of *Helicobacter pylori* macrolide resistance in gastric tissue by fluorescent in situ hybridisation. *Gut* 46:608–614. <https://doi.org/10.1136/gut.46.5.608>
- UNESCO and HELCOM (2017) Pharmaceuticals in the aquatic environment of the Baltic Sea region—a status report. France, Paris
- Von Wintersdorff CJH, Penders J, Van Niekerk JM (2016) Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Front Microbiol* 7:1–10. <https://doi.org/10.3389/fmicb.2016.00173>
- Wagner M, Loy A (2002) Bacterial community composition and function in sewage treatment systems. *Curr Opin Biotechnol* 13:218–227. [https://doi.org/10.1016/S0958-1669\(02\)00315-4](https://doi.org/10.1016/S0958-1669(02)00315-4)
- Wagner M, Hornt M, Daims H (2003) Fluorescence in situ hybridisation for the identification and characterisation of prokaryotes. *Curr Opin Microbiol* 6:302–309. [https://doi.org/10.1016/S1369-5274\(03\)00054-7](https://doi.org/10.1016/S1369-5274(03)00054-7)
- Wang Z, Zhang XX, Huang K, Miao Y, Shi P, Liu B, Long C, Li A (2013) Metagenomic profiling of antibiotic resistance genes and mobile genetic elements in a tannery wastewater treatment plant. *PLoS One* 8:1–9. <https://doi.org/10.1371/journal.pone.0076079>
- West BM, Liggit P, Clemans DL, Francoeur SN (2011) Antibiotic resistance, gene transfer, and water quality patterns observed in waterways near CAFO farms and wastewater treatment facilities. *Water Air Soil Pollut* 217:473–489. <https://doi.org/10.1007/s11270-010-0602-y>
- WHO (2018) Antibiotic resistance. <https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance>. Accessed 21 Nov 2019
- Yamagata A, Kato J, Hirota R, Kuroda A, Ikeda T, Takiguchi N, Ohtake H (1999) Isolation and characterization of two cryptic plasmids in the ammonia-oxidizing bacterium *Nitrosomonas* sp. strain ENI-11. *J Bacteriol* 181:3375–3381
- Yang Y, Li B, Ju F, Zhang T (2013) Exploring variation of antibiotic resistance genes in activated sludge over a four-year period through a metagenomic approach. *Environ Sci Technol* 47:10197–10205. <https://doi.org/10.1021/es4017365>
- Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. *J Comput Biol* 7:203–214. <https://doi.org/10.1089/10665270050081478>
- Zhou Z-C, Feng W-Q, Han Y, Zheng J, Chen T, Wei Y-Y, Gillings M, Zhu Y-G, Chen H (2018) Prevalence and transmission of antibiotic resistance and microbiota between humans and water environments. *Environ Int* 121:1155–1161
- Ziemińska-Buczyńska A, Felis E, Folkert J, Meresta A, Stawicka D, Gnida A, Surmacz-Górska J (2015) Detection of antibiotic resistance genes in wastewater treatment plant – molecular and classical approach. *Arch Environ Prot* 41:23–32. <https://doi.org/10.1515/aep-2015-0035>
- Zuckerman JM (2000) The newer macrolides: azithromycin and clarithromycin. *Infect Dis Clin North Am* 14:449–462. [https://doi.org/10.1016/S0891-5520\(05\)70257-9](https://doi.org/10.1016/S0891-5520(05)70257-9)