



Multi-drug resistant Gram-negative bacteria in wastewater treatment plants

-With focus on *Acinetobacter baumannii* complex

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Abstract

Multidrug-resistant (MDR) pathogens are emerging problems humanity faces today. Increasing MDR Gram-negative bacteria is not only a threat towards humanity itself but also towards food safety. The genus *Acinetobacter* is an environmental bacterium that can be found in habitats, including aquatic environments, soil, wastewater, food, and animals. *Acinetobacter* spp. is naturally resistant to a broad spectrum of antibiotics and quickly develop resistance due to their OXA β -lactamases enzyme set. Their broad-spectrum resistance makes infections caused by *Acinetobacter* hard to treat. The study aimed to investigate if there are any *Acinetobacter* spp. in the wastewater treatment plants (WWTP) in Sweden with focus on *A. baumannii*. The study also checked for antibiotic resistance in *Acinetobacter* isolates. Four sample sites in Sweden were chosen to investigate for the persistence of *Acinetobacter* spp. in both incoming and outgoing wastewater. Through antibiotic susceptibility testing, it was confirmed that the majority of *Acinetobacter* spp. were resistant towards antibiotic groups, carbapenem, aminoglycosides, folic acid synthesis inhibitors, and suspected resistance towards cephalosporins. It was also concluded that the WWTPs might have an impact on decreasing the amount of *Acinetobacter* spp. released into the environment.

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1. Background

World Health Organization (WHO) listed ten of the global threats that face humanity (WHO, 2019). Out of the ten major threats that the human race faces, the increase of drug-resistant pathogens is one. Antimicrobial resistance is the ability of bacteria, viruses, parasites, and fungi to change in response to the use of antibiotics to treat the infectious disease. With the rise of antimicrobial resistance, it is harder to treat common infections such as pneumonia, salmonellosis, tuberculosis, and other infections. Drug-resistant bacteria are a result of the usage of antimicrobials in both humans and animals. Antimicrobial resistance is a threat not only to humans but also to food safety (WHO, 2018). The overuse of antibiotics is not only implied to humans but also livestock like cattle, pig farms, and more. However, antimicrobial resistance is a naturally occurring thing in the environment for bacteria. Over the counter antibiotics, antibiotics to promote growth in animals and, antibiotics to prevent illness in healthy animals are some ways that is accelerating the process (WHO, 2018).

A serious concern regarding the phenomenon of multi-drug resistance (MDR) in pathogens has caused concern regarding nosocomial and community-acquired infections. The increase of resistant gram-negative bacteria has increased drastically in the last two decades, causing an increase in mortality and higher healthcare costs (Eichenberger & Thaden, 2019). The acronym "ESKAPE" has been used to describe the most common and severe MDR pathogens (Dekic, Hrenivic, Ivankovic, & Wilpe, 2018). "ESKAPE" stands for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.

1.1. Gram-negative bacteria

The distinction between Gram-positive and Gram-negative bacteria can be seen in the lining of the wall (NE, 2020). In Gram-negative bacteria, the cell wall consists of a thin layer of peptidoglycan between an outer- and inner membrane wall. In Gram-positive bacteria, the peptidoglycan layer is thicker (Eichenberger & Thaden, 2019). The outer membrane wall consists of protein and lipopolysaccharides that give the bacteria its form and also functions as a support.

Infections caused by Gram-negative bacteria include bloodstream infections, pneumonia, surgical or wound site infections, and meningitis. Studies have shown that Gram-negative

bacteria are resistant towards a different wide spectrum of antibiotics due to its built-in abilities to become resistant and to pass genetic material that allows other bacteria to become drug resistant (Egervärn & Ottoson, 2016; Dekic et al., 2018). The most common bacterial infections caused by Gram-negative bacteria are those caused by *Escherichia coli*, *P. aeruginosa*, *Klebsiella*, and *Acinetobacter* (CDC, 2011).

1.2. Extended Spectrum Beta-Lactamase Resistance

Beta-lactamase is a group of enzymes that inhibits antibiotic beta-lactamases by chemical decomposition of the beta-lactam ring. There are around 100 different beta-lactamase enzymes and are mostly produced by bacteria. Important beta-lactam rings are penicillin and cephalosporins. Bacteria that produce beta-lactamases enzymes are resistant to these types of antibiotics. Some beta-lactamases have a wide spectrum that can inhibit some or all penicillins and cephalosporins; these enzymes are denominated as extended-spectrum beta-lactamase (ESBL). ESBL resistance often occurs in gut bacteria (Tortora, Funke, & Case, 2016).

1.2.1. ESBL_{CARBA}

One type of ESBL-enzyme is ESBL_{CARBA} that can break down carbapenems (Egervärn & Ottoson, 2016). Carbapenems are excellent in treating a wide spectrum of Gram-negative bacteria. As carbapenems are similar to cephalosporins and penicillin, the bactericidal agent inhibits the cell wall synthesis as it binds to the Penicillin-binding proteins (PBPs) (Bassetti, Merelli, Temperoni, & Astilean, 2013). Carbapenems are one of the last treatment alternatives in the fight against severe life-threatening infections but are the first-choice antibiotic to fight against multi-resistant *Pseudomonas* spp. and *Acinetobacter* spp. infections. Antibiotics that are used instead of the wide spectrum antibiotics, such as colistin, tigecycline, and fosfomycin, are less effective against these infections. This causes high mortality and higher healthcare costs and a prolonged hospital visit. The most common *Acinetobacter* spp. to cause severe infections in *A. baumannii*.

1.3. Acinetobacter spp.

Acinetobacter is a genus of bacteria in the Gammaproteobacterial and belongs to the family *Moraxella* (Erdem & Leber, 2018). There are around 40 known species of the *Acinetobacter* genus that can be found in different habitats, including soil, wastewater, food, animals,

etcetera (Kittinger, et al., 2018). *Acinetobacter* spp. are naturally resistant to many antibiotics, and a striking characteristic is their ability to quickly develop resistance under antibiotic pressure due to their OXA β -lactamase enzymes set. *Acinetobacter* spp. can be found in both dry and moist areas of the human body; some of these places include toe webs, groin, respiratory tract, intestine, and axilla (Wisplinghoff & Seifert, 2010). Around 43% of non-hospitalized patients were found to carry *Acinetobacter* spp. on their normal skin flora, where the most common *Acinetobacter* was *Acinetobacter lowoffii*, *Acinetobacter junii*, and *Acinetobacter johnsonii* (Erdem & Leber, 2018). In another study conducted in the US, one-third of *Acinetobacter* bloodstream infections were mostly caused by *A. baumannii* and *Acinetobacter nosocomialis* or *Acinetobacter pittii* (Erdem & Leber, 2018).

1.3.1. *Acinetobacter baumannii*

The Gram-negative coccobacillus *A. baumannii* is commonly identified as pleomorphic, strictly aerobic and non-motile and can be found in aquatic environments (drinking and surface waters), soil, sewage, different types of food, animals, and arthropods (Kitty, o.a., 2014; Tortora et al., 2016; Karumathil et al., 2014). The bacteria often form coccus pairs and is more enduring in the environment than other Gram-negative bacteria (Tortora, Funke, & Case, 2016). *A. baumannii* forms biofilms on abiotic and biotic surfaces and has shown to be resistant towards a vast variety of antibiotics (Dekic, Hrenivic, Ivankovic, & Wilpe, 2018). WHO listed *A. baumannii*, carbapenem-resistant, as priority class 1 bacteria for new research and development for new antibiotics (WHO, 2017).

A. baumannii is an opportunistic pathogen with a high incidence of immunocompromised individuals, especially those that have had a prolonged stay at the hospital (EFSA BIOHAZ Panel, 2013). *A. baumannii* is primary a respiratory pathogen but can cause severe infections to individuals who are in poor health. *A. baumannii* has also been found to colonize healthy individuals without causing any symptoms. *A. baumannii* is uncommon to colonize healthy human skin and is infrequently found outside the hospital environment.

1.3.2. Antibiotic resistance genes

ESBL_{CARBA} genes often sit on the plasmids of the bacteria that can also carry other resistance genes, such as against ciprofloxacin and aminoglycosides. As the gene sits on the plasmids, the gene is transferred horizontally (HGT) both within *Acinetobacter* spp. and among other

Gram-negative bacteria (Zhang, et al., 2013). Resistant *A. baumannii* can also transfer-resistant genes to other *A. baumannii* through genetic transformation (Tortora, Funke, & Case, 2016). This makes the treatment alternatives few when infected with ESBL_{CARBA} resistant bacteria. Some of the antimicrobial substances that *A. baumannii* is resistant to include glycopeptides, lincosamides, streptogamins, and macrolides (Eichenberger & Thaden, 2019).

Resistant *A. baumannii* strains have shown to be resistant towards heat, chemical sanitizers (commercially available disinfectants), and ultraviolet sanitizers, thus making eradicating the bacteria hard through routine sanitation (Zhang et al., 2013; Tortora, Funke, & Case, 2016). In a report from Karumathil and colleagues (Karumathil et al. 2014) regarding the effects of chlorine exposure on *A. baumannii*, they found that the recommended amount of chlorine for treating drinking water does not eradicate or decrease the amount of MDR *A. baumannii* strains in drinking water. The recommended amount of chlorine to disinfect drinking water is 1-3 ppm, but the study showed that all eight strains of *A. baumannii* survived exposure up to 4 ppm and showed no signs of decrease. This is a problem as one of the goals regarding Agenda 2030 (UNDP, 2020) is clean and safe water for consumption. This is also confirmed in a report from Zhang et al., 2013, that after the disinfection process in hospitals that *bla*NDM-1 carrying *A. baumannii* isolates were still recovered after this process and may contaminate the surface-, drinking- and groundwaters.

1.4. *Acinetobacter* spp. in the food industries

Acinetobacter spp. is typically associated with soil and water environments, but they have also been isolated from different livestock animals in southern Europe and China. In a report from EFSA (2013), they concluded that *Acinetobacter* spp. is an emerging problem in the food system. Few reports have concluded that there is a presence of carbapenems-producing bacteria in livestock animals and their environment (EFSA BIOHAZ Panel, 2013; Egervärn & Ottoson, 2016). Two of the genes identified are *bla*OXA-23 and *bla*NDM-1 in *Acinetobacter* spp., but the bacteria were only found in the animals themselves and not in the food derived from them. One of the reports identified *Acinetobacter* spp. in a dairy cattle farm in France as well as in chicken farm and a pig slaughterhouse (EFSA BIOHAZ Panel, 2013).

ESBL_{CARBA} producing *A. baumannii* bacteria has not been found in the Swedish food industries or on livestock in Sweden (Egervärn & Ottoson, 2016). There is no proof that

ESBL producing bacteria with or without the resistant genes can spread from contaminated food sources to humans, but it is still regarded as a potential risk (Egervärn & Ottoson, 2016).

1.5. Cases of *A. Baumannii* related to Sweden

In March of 2013, an outbreak of *A. baumannii* occurred at the burn unit in a University hospital in Linköping (Folkhälsomyndigheten, 2014). Two patients that had been brought from a hospital overseas carried ESBL_{CARBA} producing *A. baumannii*, later that summer, three more cases of *A. baumannii* were confirmed in the same hospital, and in the fall two more patients were confirmed to carry the same bacteria. The strain of *A. baumannii* was sensitive to the antibiotic colistin and carried the type *bla*_{OXA-23} and *bla*_{NDM-1} resistance genes. Pulse-field gel electrophoresis (PFGE) was done on the seven cases of *A. baumannii* and showed that the two first cases did not share the same PFGE pattern. However, the following five cases shared the same pattern (Folkhälsomyndigheten, 2014). There are currently no other studies that have been carried out in Sweden regarding *Acinetobacter* spp. in the general population.

2. Aim of the study

The study aimed to evaluate MDR Gram-negative bacterium in wastewater treatment plants, focusing on the genus *Acinetobacter* and *A. baumannii* in the incoming and effluent wastewater.

The aim of the study is based on four questions;

Is there any *Acinetobacter* spp. in the general population?

Is there any *Acinetobacter baumannii* in the general population?

Is there any antibiotic-resistant *Acinetobacter* spp. in the general population?

Do sewage treatment plants reduce the amount of *Acinetobacter* spp. release to the environment?

3. Methods and materials

3.1. Sample Collection

All the samples were collected during April and May 2020. The four different WWTP that the samples were collected from include; Karlskoga, Eskilstuna, Örebro, and Stockholm (Bromma). The three first cities have a population ranging from 30 to 124 thousand citizens, and lastly, Stockholm, with around 1,5 million citizens. The samples were taken at three different times during one day of collecting, where the first sample was taken in the morning, noon, and afternoon from both incoming and effluent water.

Incoming water was taken after the mechanical process but before the biological step to filter out bigger particles. The effluent water was taken from water that leaves the WWTP. In total, six bottles containing 250ml of wastewater were sampled from each treatment plant. The samples were stored in 4°C until collected for analysis.

3.2. Isolating *Acinetobacter* spp.

3.2.1. Selective media for *Acinetobacter* spp.

In order to culture the bacteria, a selective media for *Acinetobacter* spp. was used from the company CHROMagar. The selective media inhibits gram-positive bacteria's growth and

some Gram-negative bacteria but does not inhibit all Gram-negative bacteria. The media base consists of a mixture of agar, peptone, yeast extract, salts, and a chromogenic mix. With the base comes a supplement that contains inhibition factors and growth regulators. An additional supplement which contains an inhibitory substance that inhibits non-MDR growing bacteria was added, the company does not disclose the inhibitory substance.

The media was prepared according to the manufacturer's instructions for both MDR and without the MDR supplement (NON-MDR). The plates were later stored in a refrigerator (6°C) for later use. The selective media was tested with known *Acinetobacter* spp. before use in the study.

3.2.2. Membrane filtration and culturing

The samples were filtered using a membrane filtering station with a 0.45µm filter. The filter catches the bacteria as the pores are too small for bacteria to pass through when water is filtered through it. All dilutions were diluted with 100ml distilled water and later placed on the selective media plates as described before. The dilutions used to filter the wastewater was; incoming (NON-MDR) 10000 and 1000 X, incoming (MDR) 1000 and 100 X, effluent (NON-MDR) 1000 and 100 X, effluent (MDR) 200 and 20 X.

Two different filtering stations were used, one for incoming and one for effluent water. The plates were later incubated for 18-24h in 37°C, as suggested by the manufacturer. Some reports have stated that *Acinetobacter* spp. grow in 40°C (Dekic et al., 2018). The colonies were counted and documented from the selective media plates. To determine how many colonies per 100ml water for each WWTP, a standard equation for CFU/100ml was used (see equation 1). For average CFU/100ml for both incoming and effluent water for each WW TP, the function AVERAGE in excel was used (Microsoft Excel, 2010). For calculating standard deviation, SDTEV was used in Excel (Microsoft Excel, 2010).

$$\frac{CFU}{100ml} \Rightarrow \frac{\# \text{ of colonies} * 100ml}{\text{volume filtered}}$$

Equation 1. Standard CFU/100ml count.

3.2.3. Identification of bacterial isolates

The bacterial isolates were identified using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Bruker biotyper) following the manufacturer's instructions. The colonies chosen were those that produced a red to burgundy color, uniform in color, and did not merge with other colonies. Each colony from the filter was restreaked on TSA, nutrient agar, and LB agar that was marked with a grid on the plate's backside and numbered. The plates were incubated in 37°C for another 18-24h. The colonies were spotted on a MALDI-TOF target plate and covered with an HCCA-Matrix (cyano-4-hydroxy-cinnamic acid) for bacterial cell wall breakdown. The identifications of *Acinetobacter* spp. in MALDI-TOF-MS were isolated for antibiotic susceptibility testing.

3.3. Antibiotic susceptibility test

For the antibiotic susceptibility testing, the pure culture of *Acinetobacter* spp. from Örebro WWTP was taken from TSA agar and incubated for 18-24h in 37°C. The fresh cultures from an overnight incubation were suspended in phosphate buffer saline (PBS) to the same density of McFarland 0,5 standard (TM50). The McFarland standard was prepared using 9.95mL of 1% H₂SO₄ and 0.05mL of 1% BaCl₂. The inoculated PBS suspension was streaked over Mueller-Hinton agar (non-selective, non-differential agar, MHA) with sterile swabs. For each *Acinetobacter* spp. two plates of MHA was streaked. Each plate was stamped with six antibiotic discs. The plates were incubated for 24h in 37°C. The antibiotics used are in the groups' Carbapenems (Meropenem (MEM), Imipenem (IMP), and Ertapenem (ETP)), Cephalosporins (Cefoxime (CAZ), Ceftazidime (FEP), Cefepime (FOX), and Cefoxitin (CTX)), Aminoglycosides (Gentamicin (GEN), Amikacin (AMK), and Tobramycin (TOB)), Fluoroquinolones (Ciprofloxacin (CIP)) and Folic acid synthesis inhibitors (Trimethoprim-sulfamethoxazole (SXT)). After the incubation, the inhibiting zone was determined by measuring the diameter of the ring in mm. For the interpretation of the zone-diameter to see if the *Acinetobacter* spp. was either R (Resistant), I (Susceptible, increased exposure), or S (Susceptible), a standard was used provided by European Committee on Antimicrobial Susceptibility Testing (EUCAST). If no breakpoints were provided from EUCAST, then breakpoints from the Clinical and Laboratory Standards Institute (CLSI) were used instead. The Control strain for the susceptibility testing was *Escherichia coli* HB101.

4. Results

4.1. Concentration of colonies from four WWTP in Sweden

To determine how much bacteria is in the wastewater of all sample sites colony-forming units (CFU) was used. The total number of all CFU media plates were counted, and an average for NON-MDR and MDR selective media plates in incoming and effluent water was determined through the standard equation for CFU/100ml (Figure 1) (Equation 1).

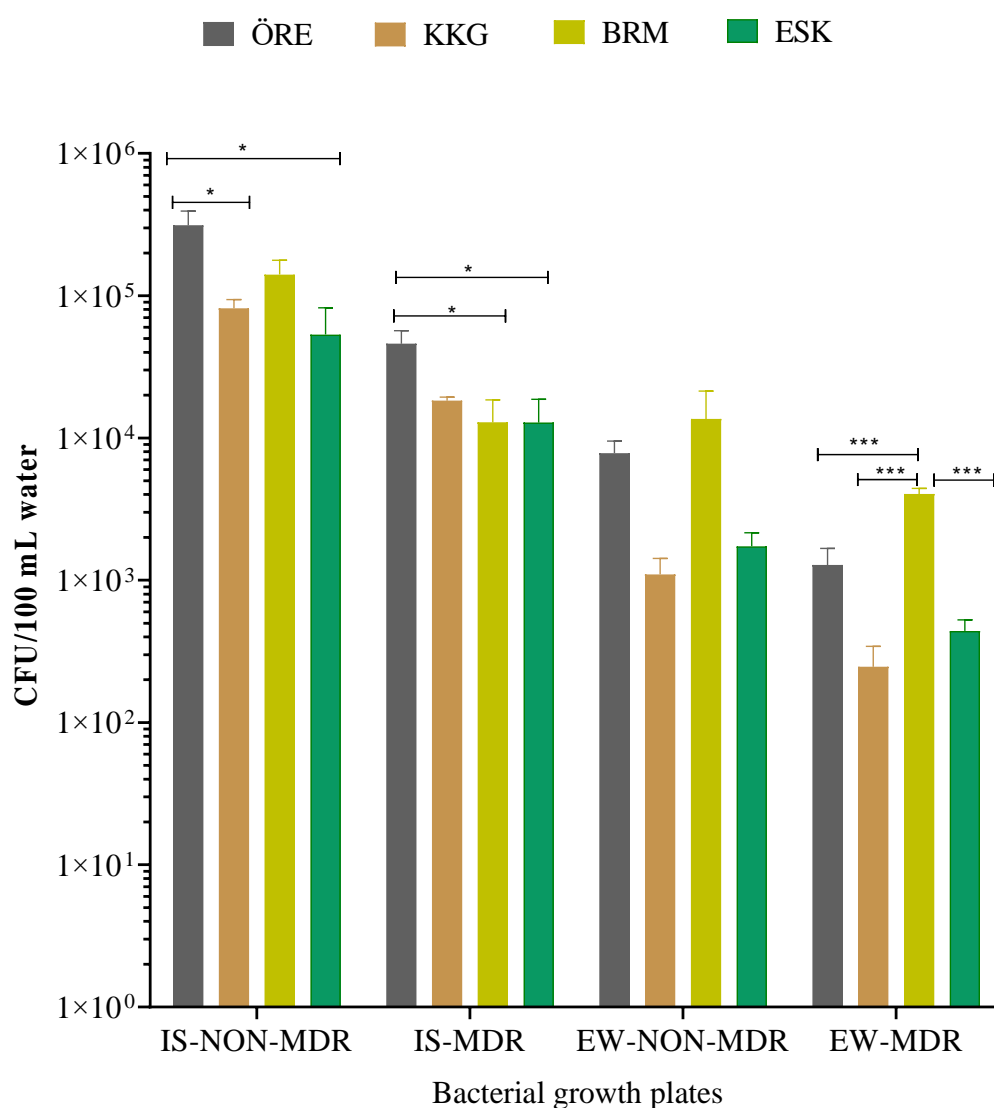


Figure 1. Comparison of total CFU in 100 mL water from Örebro, Karlskoga, Bromma and Eskilstuna wastewater treatment plant (WWTP). Statistical significance is shown by * for $P \leq 0.05$, and *** for $P \leq 0.001$ as determined by One-Way ANOVA Tukey multiple t-test. Error bars represent standard deviation of three biological replicates ($n=3$).

From all WWTP, it is shown that a decrease in colonies from incoming wastewater to effluent wastewater (Figure 1). The decrease for NON-MDR is more significant in Karlskoga and Eskilstuna WWTP compared to Bromma and Örebro WWTP. The same pattern is seen for the MDR plates. For standard deviation, effluent NON-MDR exceeded incoming MDR for

Bromma WWTP (Figure 1). For Karlskoga WWTP, no *Acinetobacter* spp. was found in incoming and effluent water. Örebro has the highest *Acinetobacter* count than the other WWTP with Eskilstuna as second and Bromma as the third (Figure 2).

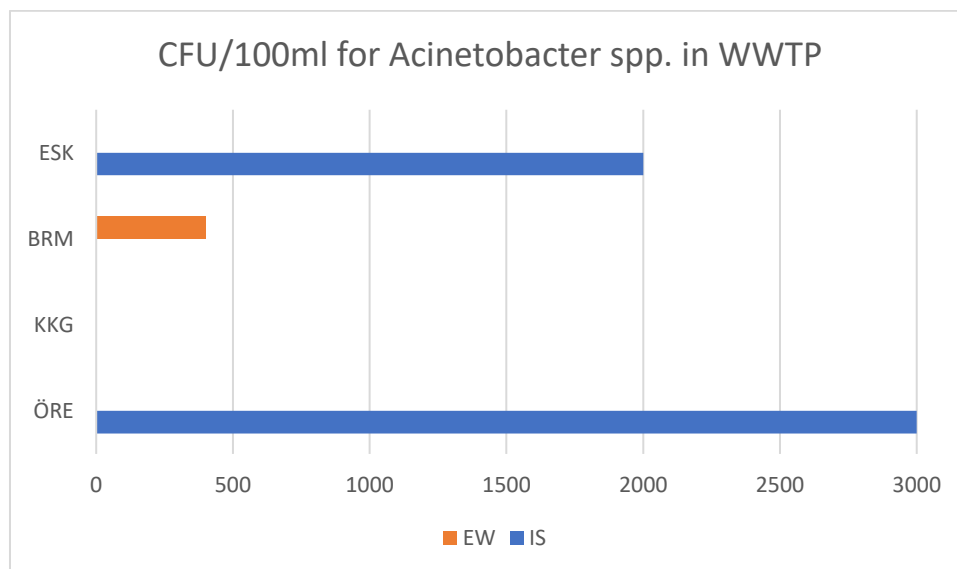


Figure 2. CFU/100ml for *Acinetobacter* spp. in WWTP. EW stands for effluent water and IS for incoming.

WWTP; Eskilstuna (ESK), Karlskoga (KKG), Bromma (BRM) and Örebro (ÖRE).

4.2. Levels of *Acinetobacter* found in WWTP in Sweden

All colonies were taken from the selective media plates that produced a red to burgundy color (Figure 3a and b) for MALDI-TOF MS identification.



Figure 3. Colonies were growing on an MDR (a) and NON-MDR (b) selective media plate. Colonies vary in shape and size, producing a red to burgundy color to a transparent color.

In a random colony testing on NON-MDR plates and MDR plates from Örebro WWTP, 32 *Acinetobacter* spp. were found. The majority of the *Acinetobacter* genus found was *A. pittii* (n=19), followed by *A. baumannii* (n=12) (Figure 4). All of the colonies came from NON-MDR plates incubated at 37°C and 40°C from incoming and effluent water.

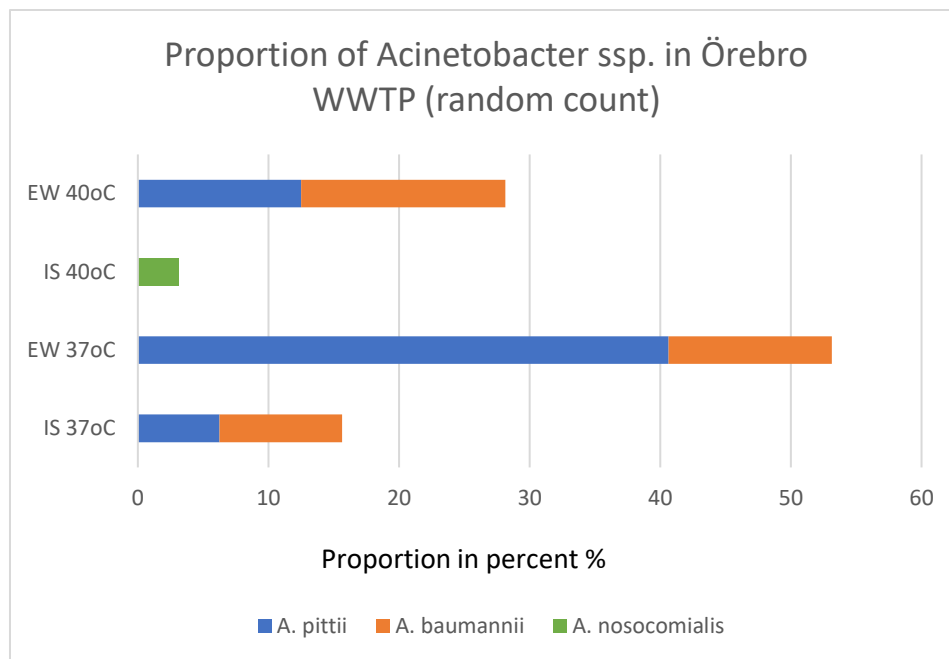


Figure 4. Proportional levels of different bacteria identified in wastewater treatment plant (WWTP). EW stands for effluent water and IS for incoming. The percentage shows the *Acinetobacter* spp. identified in each WWTP. The figure shows the most common genus of bacteria found (*Stenotrophomonas* spp. and *Acidovorax* spp.). Other minority species and colonies that could not be identified are merged into one column. WWTP; Eskilstuna (ESK), Karlskoga (KKG), Bromma (BRM) and Örebro (ÖRE).

The colonies were randomly taken from each plate, following only the color description in Figure 3a and b. Around 100 colonies from all WWTP were tested through MALDI-TOF MS, and the most common bacterial identification was *Stenotrophomonas* spp. and *Acidovorax* spp. (Figure 5).

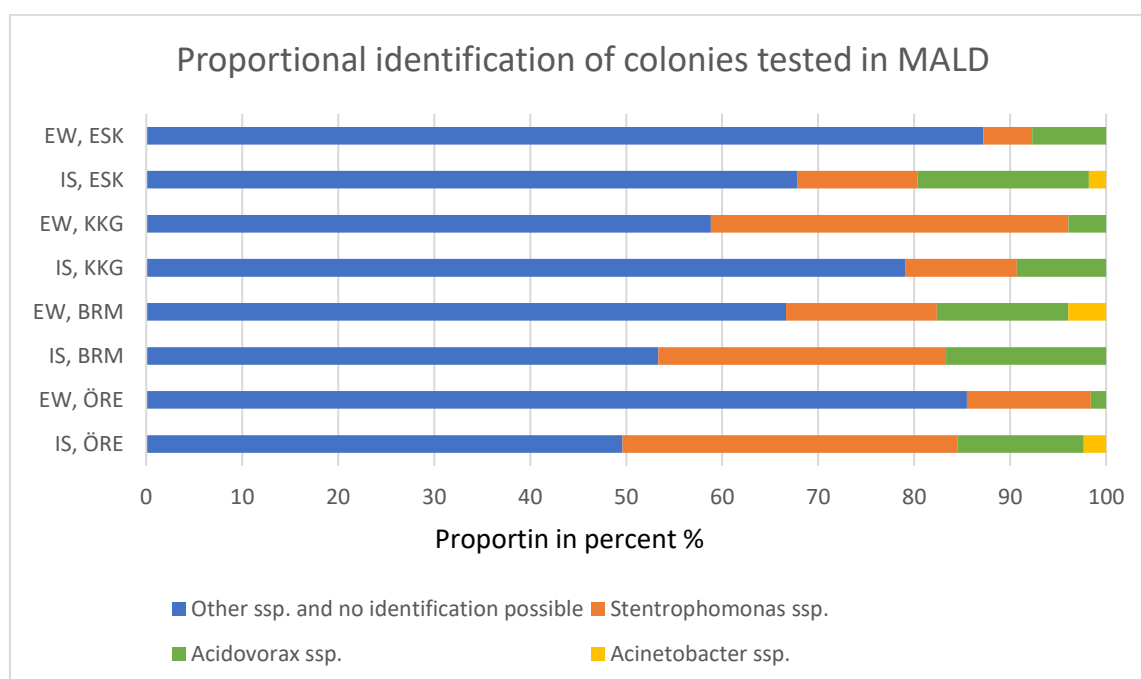


Figure 5. Proportional identification of bacteria identifies in wastewater treatment plant (WWTP). EW stands for effluent water and IS for incoming. The percentage shows the *Acinetobacter* spp. identified in each WWTP. The figure shows the

most common genus of bacteria found (*Stenotrophomonas* spp. and *Acidovorax* spp.). Other minority species and colonies that could not be identified are merged into one column.

WWTP; Eskilstuna (ESK), Karlskoga (KKG), Bromma (BRM) and Örebro (ÖRE).

No *Acinetobacter* spp. were found in Karlskoga WWTP for both incoming and outgoing wastewater. The most common *Acinetobacter* spp. found was *A. baumannii* (n=3), followed by *A. bereziniae* (n=2). All *Acinetobacter* spp. were found on NON-MDR selective media plates, but in Bromma WWTP, the *Acinetobacter* spp. found was in effluent water and not in incoming. Örebro and Eskilstuna WWTP the *Acinetobacter* spp. found was in incoming and not in effluent water.

4.3. Antibiotic resistant *Acinetobacter* isolates

Antibiotic susceptibility testing was done on isolates of *Acinetobacter* from Örebro WWTP. In total, 18 isolates were tested from both *A. baumannii* (n=10), *A. pittii* (n=5), *A. nosocomialis* (n=1), and *A. bereziniae* (n=2). The susceptibility testing revealed that MDR resistance was rare in the tested *A. baumannii* and other *Acinetobacter* spp. For antibiotics ETP, CAZ, FEP, FOX, and CTX, no breakpoints were provided from EUCAST or CLSI.

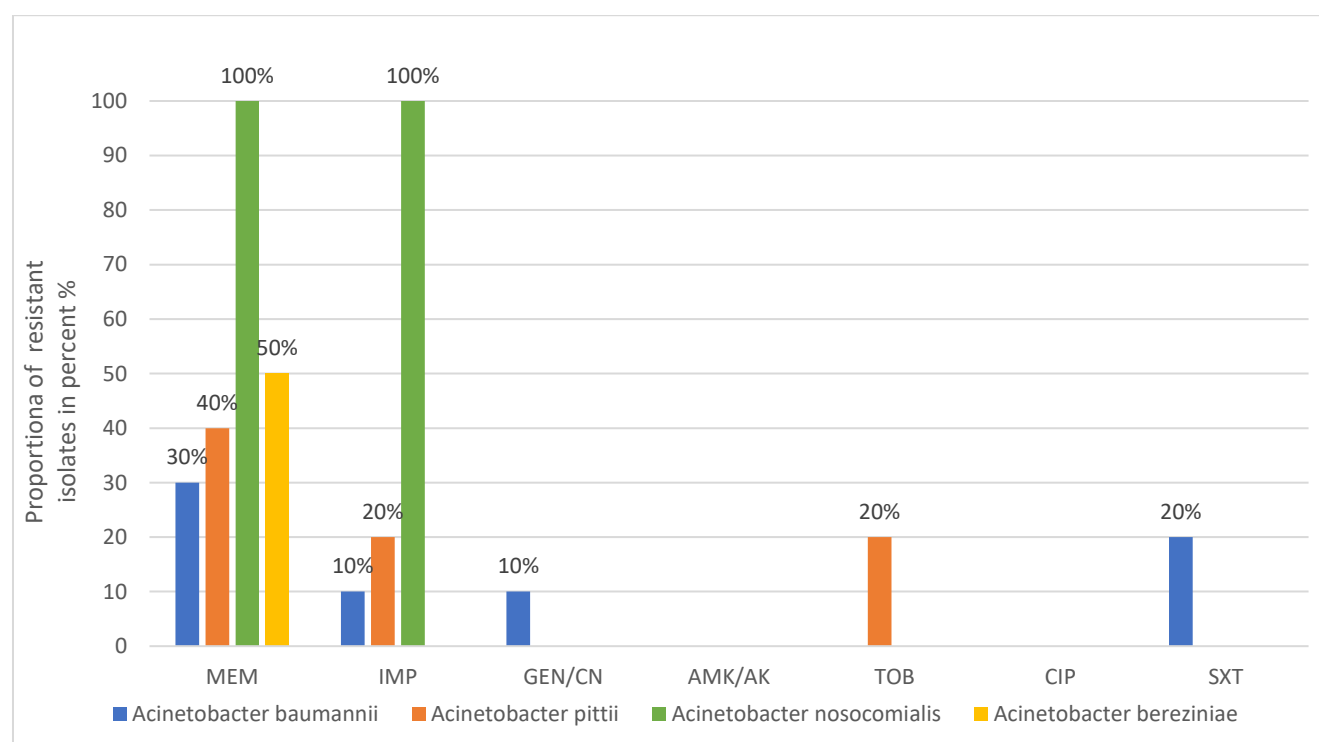


Figure 6. Percentage of resistant *Acinetobacter* spp. isolates from Örebro WWTP . Resistance based on breakpoints from EUCAST v 10.0. No breakpoints from CLSI was used. Values indicate the proportion in which the general tested *Acinetobacter* spp. is resistant (R) towards. Antibiotics: MEM meropenem, IMP imipenem, GEN gentamicin, AMK amikacin, TOB tobramycin, CIP ciprofloxacin, and STX trimethoprim-sulfamethoxazole.

The only resistance shown in all *Acinetobacter* spp. was towards meropenem with *A. baumannii* 30% (3/10), *A. pittii* 40% (2/5), *A. nosocomialis* 100% (1/1) and *A. bereziniae* 50% (1/2) (Figure 6). *A. baumannii* isolates showed resistance towards multiple antibiotics such as IMP, GEN and STX followed by *A. pittii* isolates that showed resistance towards IMP and TOB. Only one isolate of *A. nosocomialis* was found and tested, which showed resistance towards IMP and MEM. All isolates were susceptible to the antibiotic AMK, and all isolates were susceptible (but increased exposure) towards fluoroquinolones antibiotic CIP (Table 1).

Table 1. Susceptibility testing of *Acinetobacter* spp. in wastewater treatment plants.

Serial #	MEM	IMP	ETP	CAZ	FEP	FOX	CTX	GEN/CN	AMK/AK	TOB	CIP	SXT
IS0420-1	12 (R)	30 (S)	10	10	20	10	0	20 (S)	20 (S)	20 (S)	22 (I)	0 (R)
IS0420-9	12 (R)	30 (S)	18	6	14	0	10	24 (S)	24 (S)	24 (S)	30 (I)	20 (S)
IS0420-10	16 (I)	30 (S)	18	6	12	6	0	24 (S)	22 (S)	22 (S)	26 (I)	20 (S)
IS0420-14	16 (I)	28 (S)	18	0	14	0	0	22 (S)	20 (S)	22 (S)	26 (I)	18 (S)
IS0420-16	16 (I)	10 (R)	16	0	16	8	6	26 (S)	28 (S)	0 (R)	28 (I)	28 (S)
IS0420-18	14 (R)	32 (S)	20	10	18	8	0	30 (S)	28 (S)	30 (S)	30 (I)	25 (S)
IS0420-19	20 (I)	34 (S)	10,5	10	18	8	0	23 (S)	20 (S)	22 (S)	25 (I)	26 (S)
IS0420-20	20 (I)	30 (S)	24	12	19	14	17	25 (S)	25 (S)	24 (S)	29 (I)	28 (S)
IS0420-21	18 (I)	28 (S)	24	12	0	0	0	24 (S)	24 (S)	20 (S)	28 (I)	0 (R)
IS0420-22	8 (R)	15 (R)	9	6	7	6	0	22 (S)	20 (S)	22 (S)	28 (I)	20 (S)
IS0420-25	14 (R)	26 (S)	20	10	14	0	12	11 (R)	22 (S)	24 (S)	28 (I)	20 (S)
IS0420-26	22 (S)	28 (S)	32	16	22	0	12	30 (S)	28 (S)	28 (S)	30 (I)	18 (S)
EW0420-1	16 (I)	30 (S)	20	12	15	10	12	20 (S)	22 (S)	22 (S)	24 (I)	22 (S)
EW0420-2	20 (I)	34 (S)	25	16	22	9	18	28 (S)	28 (S)	24 (S)	32 (I)	24 (S)
EW0420-6	8 (R)	0 (R)	11	8	8	8	12	22 (S)	26 (S)	24 (S)	28 (I)	28 (S)
IS0420-30	15 (I)	32 (S)	18	0	18	6	12	30 (S)	32 (S)	30 (S)	28 (I)	21 (S)
IS0420-31	14 (R)	34 (S)	20	0	20	6	12	30 (S)	30 (S)	31 (S)	29 (I)	23 (S)
IS0420-45	18 (I)	28 (S)	23	14	20	12	14	24 (S)	24 (S)	24 (S)	30 (I)	24 (S)

Antibiotics: MEM meropenem, IMP imipenem, ETP ertapenem, CAZ ceftazidime, FEP ceftazidime, FOX cefepime, CTX ceftazidime, GEN gentamicin, AMK amikacin, TOB tobramycin, CIP ciprofloxacin, and STX trimethoprim-sulfamethoxazole.

For antibiotics CAZ, FEP, FOX, and CTX, some isolates showed no inhibition zone towards them, which can indicate resistance towards those antibiotics even if no breakpoints were available (Table 1). The antibiotic CIP showed that all isolates were susceptible, but with increased exposure, they are not resistant but not fully susceptible to CIP antibiotics.

For isolate, IS0420-21 (*A. baumannii*) showed resistance towards almost all cephalosporin antibiotics that were tested except for CAZ. Isolate IS0420-14 (*A. pittii*) showed as well resistance towards almost all cephalosporin antibiotics but not towards FEP, as seen in Table 1 and Figure 7.

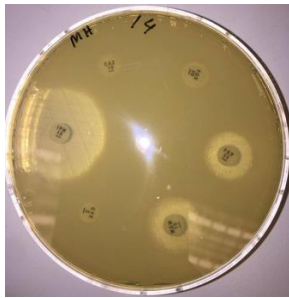


Figure 7. Disc diffusion test on isolate IS0420-14 (*A. pittii*) grown on MHA. Clear inhibition zone on antibiotics IMP, MEM, and FEP. No zone inhibition showed on antibiotics FOX, CAZ, and CTX.

5. Discussion

Only four media plates contained positive *Acinetobacter* spp. identification. The different *Acinetobacter* found was; *A. baumannii*, *Acinetobacter bereziniae*, and *Acinetobacter gernerii*. In total three *A. baumannii* was found as is classed as the most infectious species in the genus *Acinetobacter* (WHO, 2017). *A. bereziniae* is an environmental and clinical microorganism, it has been reported to cause severe sepsis in immunocompromised patients and quickly develop resistance towards antibiotics (Bonnin, Ocampo-Sosa, Poirel, Guet-Revillet, & Nordmann, 2012). *A. gernerii* is ubiquitously found in the environment and has also been isolated from activated sludge (Singh, Khatri, Subramanian, & Mayilraj, 2014). As *A. bereziniae* can quickly become resistant as well as *A. baumannii* this can be a cause of concern regarding infections caused by these bacteria.

The selective media used also supported growth of other bacteria including *Stentrophomonas* spp., *Pseudomonas* spp. and *Acidovorax* spp., all producing the same appearance as *Acinetobacter* spp. This made it difficult to differentiate between *Acinetobacter* spp. and what were the other Gram-negative bacteria, therefore the total counts may contain high levels of these bacteria. This was also mentioned in the manufacturer's instructions regarding the identification of *Acinetobacter* on the selective media plates. Both *Stentrophomonas* spp. and *Pseudomonas* spp. can cause infections in humans and can become antibiotic resistant (U.S Department of Health & Human Services, 2018; CDC, 2019). *Stentrophomonas* is usually found in wet environments and cause severe infections in immunocompromised people (U.S Department of Health & Human Services, 2018). *Acidovorax* spp. are mostly environmental and cause seldom infections in humans (Wisplinghoff & Seifert, 2010).

For the identification for *A. baumannii* and *A. pittii*, it gave a more reliable identification in MALDI-TOF (score value over two) but for the other *Acinetobacter* spp. tested the identification score was below two often contained one or more species of *Acinetobacter* environmental isolates than the first suggestion given. As discussed by Wisplinghoff and Seifert (2010), the MALDI-TOF can mostly identify all *Acinetobacter* in the *A. baumannii* complex but not all the other environmental isolates. When testing random colonies from Örebro WWTP, a high concentration of *A. pittii* and *A. baumannii* was found on plates incubated at 37°C and 40°C. *A. pittii* belongs to the *A. baumannii* complex, and both are regarded as the most infectious (Erdem & Leber, 2018; Wisplinghoff & Seifert, 2010) as both can cause bloodstream infections as well as *A. nosocomialis* (Erdem & Leber, 2018). For random colony testing *Acinetobacter* was found in both incoming and outgoing wastewater (Figure 4.). This may be of concern if the *Acinetobacter* is not reduced in outgoing waters as some species of this genus are opportunistic pathogens. As discussed in Agenda 2030, one of the main goals is clean and safe waters to all, if *Acinetobacter* are released to the environment this can cause infections in immunocompromised individuals. Also, this might indicate that the WWTP do not decrease or eradicate the *Acinetobacter*, which is also validated in Zhang, et al. report regarding *Acinetobacters* in the WWTP (2013). But when structured testing of *Acinetobacters* in all four WWTP it showed that most *Acinetobacter* spp. was found in incoming rather than outgoing wastewater. This might further be explained that the colonies were only collected from half of the plate as the total amount of colonies exceeded 30 colonies.

If *Acinetobacters* are released to the environment from the WWTP it can cause problems if farmers irrigate untreated water to crops (EFSA BIOHAZ Panel, 2013). As *Acinetobacter* spp. are environmental bacterium they can easily survive on crops and infect both humans and animals (Kitty, o.a., 2014). But this theory is not confirmed as extended research is needed. Reports have confirmed *Acinetobacters* on livestock animals but have not confirmed if the bacteria can transfeere between live animals and meat from them (Egervärn & Ottoson, 2016).

For Örebro WWTP, more colonies were taken and categorized strategically three *Acinetobacter* spp. were found. The most common *Acinetobacter* spp. found was *A. baumannii* for all tested WWTP. The *Acinetobacter* spp. identified was found in both incoming and effluent wastewater, which corresponds to Zhang et al., article (2013), that

Acinetobacter isolates can be found in effluent water as well as incoming from WWTP. As *Acinetobacters* is naturally resistant towards chemical disinfectants, especially chlorine treatment, it can survive both the biological and chemical step in WWTP (Karumathil, Yin, Kollanoor-Johny, & Venkitanarayanan, 2014; Zhang et al., 2013). *Acinetobacter* is regarded as an environmental bacterium; it is more enduring in the environment than other Gram-negative bacteria, thus surviving in a different niche (Kitty, o.a., 2014; Tortora, Funke, & Case, 2016).

Only the *Acinetobacter* isolates from Örebro WWTP were tested for antibiotics. From the 18 tested isolates, three of them showed no resistance towards any antibiotics. The other 15 showed resistance towards one or more antibiotics, which corresponds to Dekic and colleagues' findings (2018). *A. baumannii* isolates showed resistance towards more antibiotics than *A. pittii* and *A. nosocomalis* regarding EUCAST breakpoints for antibiotics MEM, IMP, GEN, AMK, TOB, CIP, and SXT. Even tho no breakpoints were provided by EUCAST and CLSI for the cephalosporins antibiotics; it can be assumed that the isolates showing no inhibition zone are resistant towards those antibiotics. Only *A. pittii* showed resistance towards the aminoglycoside antibiotic TOB.

In total, for carbapenem antibiotics, ten isolates showed resistance to either MEM or IMP or both, thus causing the concern if the isolates are ESBL_{CARBA} producers (Egervärn & Ottoson, 2016). Some of the isolates that were resistant toward MEM and IMP were also resistant towards cephalosporin antibiotics, as carbapenems are similar to penicillin and cephalosporins as it inhibits the cell-wall synthesis, can be another indicator that the isolates are ESBL_{CARBA} producers and therefor resistant towards a wide-spectrum of antibiotics and last defense in combating MDR *Acinetobacter* (Tortora et al., 2016; Egervärn & Ottoson, 2016; Bassetti et al., 2013; WHO, 2017).

Isolate EW0420-6 (*A. nosocomalis*), and IS0420-22 (*A. baumannii*) showed resistance towards MEM and IMP, but only IS0420-22 showed resistance towards one cephalosporin antibiotic (CTX). The other isolates that were resistant towards carbapenems were for either MEM or IMP. As the genus, *Acinetobacter* is naturally resistant towards many antibiotics; the bacterium can quickly develop resistance towards antibiotics under antibiotic pressure due to their OXA β -lactamases enzymes set (Kitty, o.a., 2014). The ESBL_{CARBA} gene often sits on

the plasmids of the bacteria; it is easily HGT transferred between gram-negative bacteria and within the *Acinetobacter* genus (Zhang, et al., 2013).

6. Conclusions

This study focused on the MDR Gram-negative *Acinetobacter* genus in Swedish WWTP. Throughout the investigation, it is concluded that there is *Acinetobacter* spp. in the general population as most of the *Acinetobacter* spp. are found on humans. It is also concluded that there is a presence of *A. baumannii* isolates as well as *A. pittii*, *A. nosocomialis*, *A. bereziniae*, and other environmental *Acinetobacter* spp. isolates. The study also verified that the isolates in various degrees indicate resistance towards antibiotics, carbapenems, aminoglycosides, folic acid synthesis inhibitors, and resistance towards cephalosporins. If WWTP plays a role in eradicating or decreasing the amount of *Acinetobacter* released to the environment is still uncertain, and further investigation is needed.

To better understand the effects *Acinetobacter* spp. have on the human population extensive research is needed. As the genus *Acinetobacter* is found in the human population in Sweden it is critical to find an easy way to categorize these bacteria to minimize and prevent infections caused by them. By optimizing the selective agar, a more accurate analysis will be done to see in what quantity *Acinetobacter* are in the WWTPs in Sweden. Also, it is needed to take more samples from each WWTP to get a more accurate reading in what quantity these bacteria is in the WWTPs. Further investigation is also needed in the food sector as this may be a potential risk-factor for the spread of *Acinetobacter* spp. between livestock and humans. This study will lay the foundation for extended research on the genus *Acinetobacter* in Sweden and in what ways we can prevent the spread of this super bug.

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