Removal of antibiotic resistant *E. coli* in two Norwegian wastewater treatment plants and by nano- and ultra-filtration processes

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Abstract

The effectivity of different treatment stages at two large wastewater treatment plants (WWTPs) located in Oslo, Norway, to remove antibiotic resistant *Escherichia coli* from municipal wastewater was investigated. The WWTPs were effective in reducing the total cultivable *E. coli*. The *E. coli* in WWTP samples were mainly resistant to ampicillin (6–27%) and trimethoprim-sulfamethoxazole (5–24%), and, to a lesser extent, tetracycline (3–14%) and ciprofloxacin (0–7%). In the first WWTP, a clear decrease in the percentage of *E. coli* resistant to these antibiotics was found, with the main removal occurring during physical/chemical treatment. In the second WWTP, the percentage of cultivable resistant *E. coli* did not display a considerable change. During lab-scale membrane filtration of WWTP effluents using ultrafiltration (UF) and nanofiltration (NF) membranes, all *E. coli*, including those resistant to antibiotics, were removed completely. The results imply that UF and NF processes are potent measures to remove antibiotic resistant bacteria (ARB) during post-treatment of WWTP effluents, thus reducing the potential spread of antibiotic resistance in the receiving aquatic environment.

Keywords

Antibiotic resistant *E. coli*; contaminants of emerging concern (CEC); membrane filtration; wastewater treatment plant (WWTP) effluent polishing; wastewater treatment

Introduction

Antimicrobial resistance (AMR) is a major emerging threat to water quality and human health globally (WHO 2014). Yet, in Norway it is still regarded as a limited problem with respect to clinically important microorganisms, and at this point, is considered under control (ECDC 2014; NORM/NORM-VET 2015). Urban wastewater treatment plants (WWTPs) in which sub-therapeutic concentrations of resistance-driving antibiotics, biocides, and metals continuously co-occur with a high density of diverse microorganisms, are potential hotspots for antibiotic-resistant bacteria (ARB) as well as for horizontal gene transfer (Michael *et al.* 2013; Rizzo *et al.* 2013). Thus, the ecologically competitive and challenging environment in biological treatment stages of WWTPs potentially contributes to (i) the selection of present antibiotic resistance genes (ARGs); (ii) the creation of novel ARGs; (iii) the release of resistance-driving chemicals; (iv) the dispersal of AMR into the receiving water (Michael *et al.* 2013; Berendonk *et al.* 2015). It is increasingly recognized that WWTP discharges pose a major anthropogenic source of ARGs being released into the environment. Concurrently, WWTPs are important nodes where the spread of antibiotic resistance can be controlled/improved before the effluent is disposed to the water body or reused (Riquelme Breazeal *et al.* 2013).

Due to the lack of routine monitoring, little is known about the abundance, fate, and removal of both ARB & ARGs in full scale WWTPs (Rizzo *et al.* 2013; Colque Navarro *et al.* 2014; Berendonk *et al.* 2015).

Abbreviations: Amp, ampicillin; AOP, advanced oxidation processes; ARB, antibiotic resistant bacteria; ARG, antibiotic resistance gene; BEVAS, Bekkelaget Vann AS; CEC, contaminants of emerging concern; CFU, colony forming unit; Cip, ciprofloxacin; COD, chemical oxygen demand; Da, Dalton; DDD, defined daily doses; DN, denitrification; K_D, sorption coefficient; LOQ, level of quantification; MF, microfiltration; MIC, minimal inhibitory concentration; MPN, most probable number; N, nitrification; NF, nanofiltration; RO, reverse osmosis; SP, Sampling point; Tet, tetracycline; Tmp/Smx, trimethoprim-sulfamethoxazole; UF, ultrafiltration; VEAS, Vestfjorden Avløpsselskap; WWTPs, wastewater treatment plants.

In Norway, antibiotic resistance in human health care and veterinary medicine has been stringently monitored for many years, and is regulated through national strategies and action plans (NIPH 2015; NMHCS 2015; NORM/NORM-VET 2015). Despite antimicrobial policies in healthcare and food production seeming to appear successful, this situation is believed to rapidly change if antibiotic consumption and import of ARB from abroad increases (ECDC 2014; NORM/NORM-VET 2015). In fact, increase of resistant pathogens in Norway is already registered (ECDC 2014). However, due to focus on clinical microbes, the role, relevance, and potential risks of antibiotic resistance in environmental settings, including WWTPs, has received very little attention. This may also explain the lack of research related to this topic in Norway. While a few pharmaceuticals were quantified in the effluent of two major Oslo City hospitals, along with influent, sludge, and final effluent at the effluent receiving WWTP (Thomas *et al.* 2007b; Langford & Thomas 2009), systematic studies on ARB & ARGs, most of which are part of international antibiotic resistance screening programs (NORMAN Network[1], NEREUS COST Action[2], StARE project[3]), have only recently been initiated (Tiodolf *et al.* 2013).

The recent implementation of Europe's One Health action plan (COM 2017) that recognizes the close interconnection of human and animal health acknowledges the environment as another important contributor to the development and spread of AMR in humans and animals. To close knowledge gaps on the role of AMR in the environment, the action plan calls for an increased effort into monitoring AMR in environmental settings, and development of risk assessment methodologies that evaluate risks of AMR to human and animal health. In addition, it requests the development of technologies that reduce the spread of AMR in wastewater (COM 2017).

Until recently, the research focus of WWTPs has been describing the abundance and relative change of antibiotic resistance in raw and treated wastewater. Little is known about how the treatment process and operational conditions in WWTPs influence ARB removal and ARG transfer. Like other contaminants of emerging concern (CEC), including pharmaceuticals and personal care products, the fate and spread of ARB & ARGs is expected to be dependent on the type of treatment process/technology applied at each plant (Rizzo *et al.* 2013). It will also be influenced by other factors such as water quality, seasons, climate conditions, and geographical location. Thus, the improvement or upgrading of WWTPs to minimize AMR contamination of the receiving water calls for an understanding of what degree the concentration of ARB & ARGs is decreased in WWTPs, or whether they might even proliferate in such plants. Assuming that 85% of all antibiotics used by humans occurs at private households (NORM/NORM-VET 2015), of which most end up into the municipal sewage network, this calls for measures to eliminate antibiotic resistance from wastewater at WWTPs. Such measures are currently not in place because the actual risk resulting from ARB & ARGs is basically unknown. Moreover, conventional WWTPs are not designed to completely remove antibiotics and ARB & ARGs.

Advanced treatment technologies and disinfection downstream of the conventional biological process could provide further inactivation of ARB and removal of ARGs from WWTP effluents. Those technologies include the addition of chemical oxidants and disinfectants, UV-C irradiation, ozonation, advanced oxidation processes (AOP), adsorption, and membrane filtration processes. The latter include microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis (RO), which may provide a potent alternative for ARB & ARG removal. While only a few studies have investigated the effect of MF or UF on the removal of ARB & ARGs from real wastewater (Bockelmann *et al.* 2009;

[1] Network of reference laboratories, research centers and related organizations for monitoring of emerging environmental substances, http://www.norman-network.net

[2] New and emerging challenges and opportunities in wastewater reuse (ES 1403), http://www.nereus-cost.eu

[3] Water JPI Stopping antibiotic resistance revolution, https://stareeurope.wordpress.com

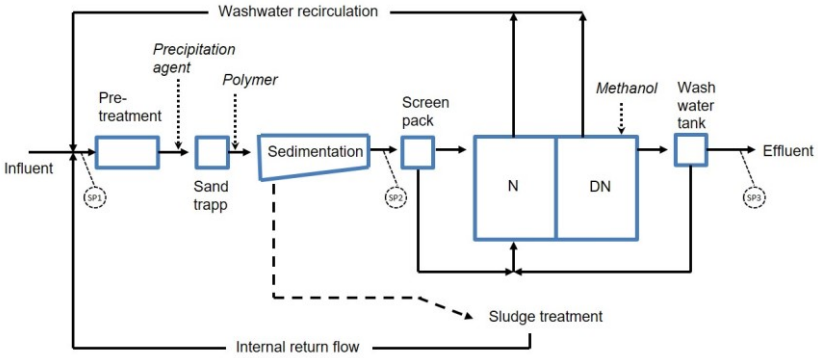
Riquelme Breazeal *et al*. 2013), the effects of NF and RO membrane filtration, either alone or combined with other methods, on ARB & ARG removal from WWTP effluent has not been explored. The first objective of this study was to quantify cultivable *Escherichia coli* exhibiting resistance to four selected antibiotics commonly used for medication at Norwegian hospitals; namely ampicillin (Amp), ciprofloxacin (Cip), tetracycline (Tet), and trimethoprim-sulfamethoxazole (Tmp/Smx), in samples collected at different treatment stages from two Oslo City WWTPs. This allows to evaluate the effectivity of the treatment stages to decrease the concentration of ARB, and ultimately on the risk potential of spread of antibiotic resistance to the Oslofjord. The implementation of tertiary disinfection technologies to prevent ARB release by conventional WWTPs requires the investigation of the potential effectiveness, amongst other factors. In the search of feasible methods, membrane filtration processes pose a potent alternative worthy of further exploration. Hence, the second objective was to evaluate the efficiency of UF and NF in removal of cultivable *E. coli* resistant to the selected antibiotics from WWTP effluents. Based on these results, the feasibility of UF and NF for ARB & ARG removal during post-treatment at full-scale can be explored.

Methods

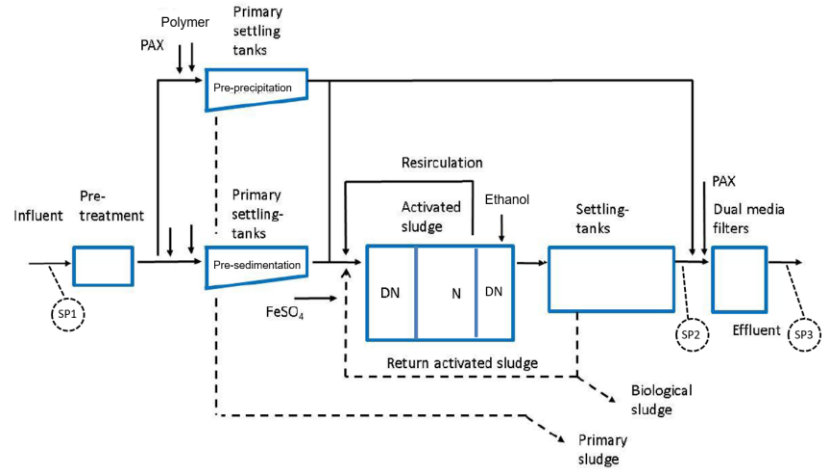
Description of WWTPs. Water samples were collected at two full-scale municipal WWTPs in Oslo, Norway, where wastewater was treated mechanically, chemically, and biologically. As the final biological treatment step, Vestfjorden Avløpsselskap (VEAS) WWTP applied a biofilm process while Bekkelaget Vann AS (BEVAS) WWTP used an activated sludge process and dual media filtration (Figure 1).

VEAS WWTP is Norway's largest WWTP receiving municipal wastewater from a population of 600,000 in both the Oslo and Akershus county areas. The plant receives 100–110 million m^3 of urban wastewater annually, including sewage from five major hospitals in the Oslo area. Coagulant and polymer are added during the chemical precipitation-sedimentation process. The chemically enhanced primary treatment is followed by a two-stage biofilm process with post-denitrification (Figure 1). The biological system consists of nitrification and denitrification fixed-film processes (BIOFOR®, Degremont), using expanded clay aggregates (Leca, Norway) as medium, with methanol addition to the denitrification stage. The total hydraulic retention time in the plant is 4 h. The sludge is treated by anaerobic digestion and drying. The effluent water is discharged into the Oslofjord at a depth of 30–55 m.

BEVAS WWTP is Norway's second largest WWTP serving a population of about 290,000 person equivalents living in the eastern and south eastern parts of Oslo. The plant has an average daily flow of 100,000 m^3/d and a maximum capacity of 260,000 m^3/d. The plant annually receives about 40 million m^3 of urban (70% of chemical oxygen demand [COD] load) and light industrial wastewater (30% of COD load; brewery, abattoir, dairy). The raw influent is pre-treated by 3 mm sieving screen, sand- and fat-trap and pre-sedimentation (Figure 1). The chemically enhanced precipitation-sedimentation process is applied only at higher flow rates, i.e., above the dry weather flow of 2.0 m^3/s. Biological treatment, based on activated sludge process combined with simultaneous precipitation with iron sulfate, is followed by dual media sand filters. The dual media filters contain Filtralite MC size 2.5–4 mm (top-layer) and fine-grained sand with particle size of 1.2–2.0 mm (bottom-layer). The hydraulic retention time in the biological treatment unit is approximately 16 h, with 23 h total hydraulic retention time. The plants effluent is discharged at a 50 m depth into the Oslofjord.



a) VEAS WWTP



b) BEVAS WWTP

Figure 1. Simplified flow sheet of (a) VEAS and (b) BEVAS WWTPs. SP = Sampling point; N = Nitrification; DN = Denitrification (Figure b was adapted and modified from Storhaug 2014).

Sample collection. Automated 24 h composite samples were collected at the WWTPs from the influent, after the sedimentation/activated sludge and settling step, and the final effluent (Figure 1). Influent water at VEAS WWTP contained backwash water from biofilters and from internal return flow from the sludge treatment. Samples were taken during October 2014 (VEAS) and February 2015 (BEVAS). Samples were transported to the laboratory for immediate experimental analyses.

Membrane filtration tests. WWTP effluents were subjected to membrane filtration to elucidate the impact of membrane filtration on ARB removal. A bench scale membrane testing apparatus was used to evaluate three commercially available membranes in the UF and NF range (Table 1). An effective membrane area of 99.4 cm^2 was used by cutting pieces of different flat sheet and spiral wound membranes obtained from the manufacturer. Test were done in cross-flow mode at constant pressure of 1–2 bar (UF) and 6–7 bar (NF) until a volume of 1.4 L of permeate was obtained. Details about the test system and experimental conditions are described elsewhere (Krzeminski *et al.* 2017).

Table 1. Specifications of assessed membranes. UF = Ultrafiltration; NF = Nanofiltration; Da = Dalton.

Membrane	Filtration spectrum	Molecular weight cut off (Da)	Producer and brand name	Material
UF	UF	10.000	Alfa Laval, UFX-10pHt	Polysulphone permanently hydrophilic
NF#1	NF	200–400	DOW, NF270	Polyamide thin-film composite
NF#2		150	Toray, TM600	Piperazine polyamide composite

***E. coli* quantification and antimicrobial susceptibility assay.** *E. coli* were cultured on Difco MI agar plates with and without added antibiotics. Difco MI agar was prepared in sterile Milli-Q water according to the manufacturer's instruction (Becton Dickinson). The agar was autoclaved (121°C, 15 min) and cooled to 45°C in a water bath. The respective antibiotic compound (all purchased at Sigma-Aldrich) was added to the agar from stock solutions (dissolved in either sterile Milli-Q water, Dimethyl sulfoxide, or methanol) to the final concentrations stated in Table 2. These antibiotic concentrations represent the minimal inhibitory concentration (MIC) breakpoint concentrations for testing with *E. coli* recommended by the Clinical and Laboratory Standards Institute (CLSI 2003, 2012) and as reported elsewhere (Watkinson *et al.* 2007). In addition, Cefsulodin, an inhibitor of gram-positive and some non-coliform gram-negative organisms, was added (5 µg/mL) to all plates (Brenner *et al.* 1996). The medium was mixed well and the agar was instantly dispensed into sterile petri dishes. Control agar plates contained no antibiotics except for Cefsulodin.

Table 2. Antibiotics being tested and minimal inhibitory concentration (MIC) breakpoint concentrations used. ATC = Anatomical Therapeutic Chemical classification

Antibiotics	**ATC group**	**MIC breakpoint (µg/mL)**	**Sorption coefficient K_D (L/kg) primary sludge[(4)]**
Trimethoprim/ Sulfamethoxazole (CAS 738-70-5/723-46-6)	J01EE Combinations of sulfonamides and trimethoprim	4/76 [(1)]	427/3.2
Ciprofloxacin (CAS 85721-33-1)	J01MA02 Fluoroquinolones	4 [(2)]	2512
Ampicillin (CAS 69-53-4)	J01CA01 Penicillins with extended spectrum	32 [(2)]	--
Tetracycline (CAS 60-54-8)	J01AA Tetracyclines	16 [(2)]	8400
Cefsulodin (CAS 52152-93-9)	J01DD03 Third-generation cephalosporin antibiotic	No breakpoint concentration. Added to 5 µg/mL final concentration[(3)].	--

[1]CLSI 2012; [2]CSLI 2003; [3]Watkinson *et al.* 2007; [4]Eslamian

The antibiotic susceptibility analysis was carried out as reported elsewhere (Watkinson *et al.* 2007). For each water sample, two parallel dilution series (in phosphate buffered saline) were filtered through cellulose nitrate membrane filters (Sartorius, Göttingen, Germany) with 0.22 µm pore size. Dilutions between 10^{-1} and 10^{-4} were filtered together with 10 mL of sterile peptone water (10 g peptone/L and 5 g NaCl/L). The membrane filters were transferred onto dishes with and without (control; cefsulodin) antibiotics, followed by incubation for 24 h at 35°C. Blue colonies were then counted under ambient light, and the results were confirmed at 366 nm UV light. The total concentration of cultivable *E. coli* was obtained from control dishes. The percentage of resistance for each antibiotic was calculated by relating the colony forming unit (CFU) counts on antibiotic-containing plates with the CFU counts on the control plates without antibiotics according to equation 1. The limit of detection was 10 CFU/mL.

$$\% \text{ resistance} = \frac{\text{CFU/mL in medium with antibiotics}}{\text{CFU/mL in medium without antibiotics}} \times 100 \qquad \text{Eq. 1}$$

In addition to the plating method, the total concentration of viable *E. coli* was quantified using the most probable number (MPN) Colilert Quanti-Tray/2000 method (LOQ: 1 organism/100 mL; IDEXX Laboratories, Inc.) according to ISO 9308-2:2012.

Results and Discussion

Abundance of *E. coli* in WWTPs. The total concentration of viable *E. coli* decreased considerably (> 2.2 log) across the treatments at both WWTPs (Figure 2). Most *E. coli* entering VEAS WWTP were removed by the biofilm process (ca. 2 log), while at BEVAS WWTP, they were gradually removed across the entire treatment process. However, as expected, no full disinfection was achieved at either plant. Results obtained by the plating method (LOQ: 10 CFU/mL) were within the 95% confidence interval of the Colilert MPN method (LOQ: 1 CFU/100mL) (Figure 2).

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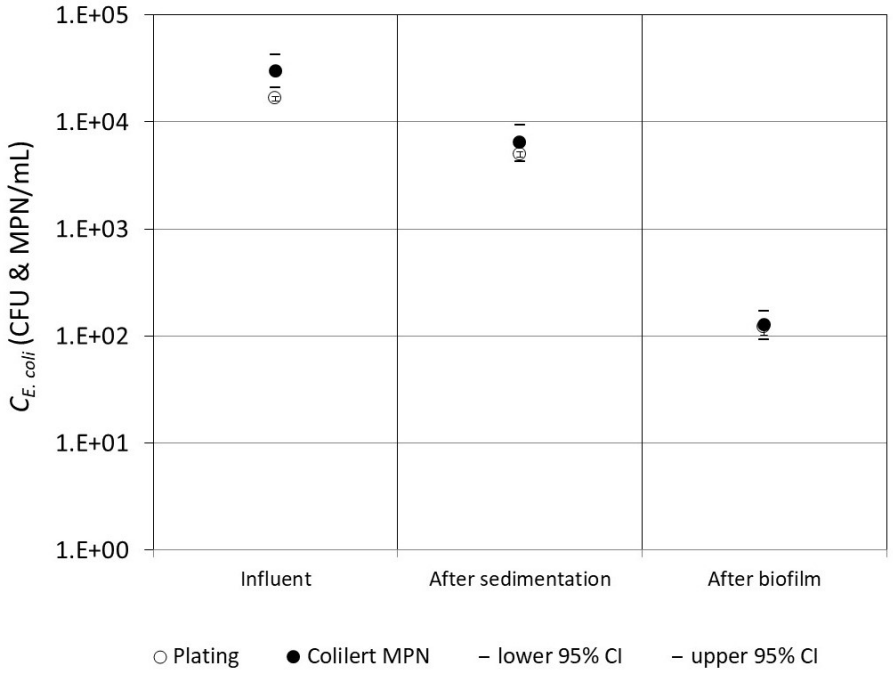
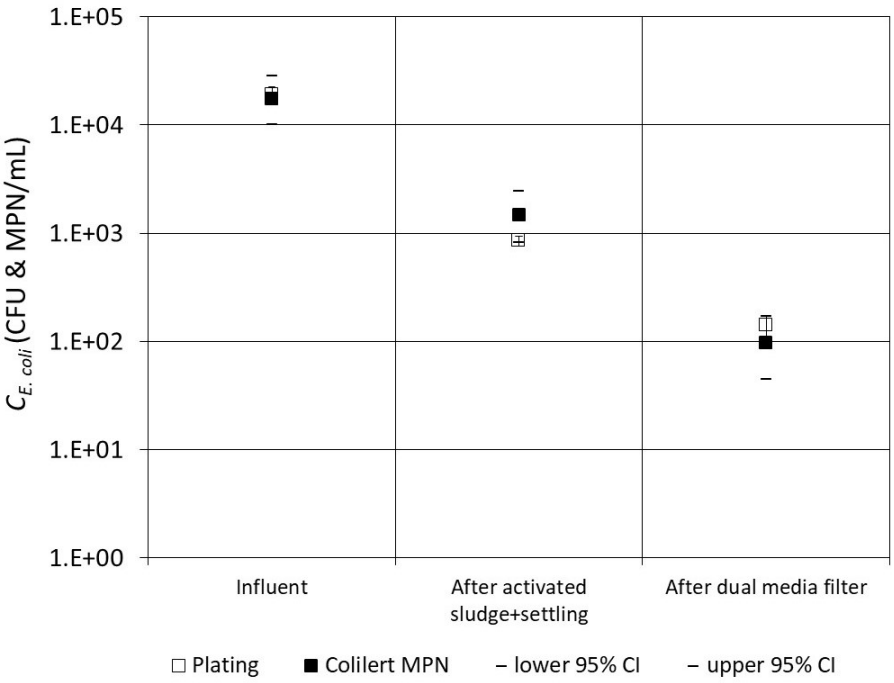


a) VEAS WWTP



b) BEVAS WWTP

Figure 2. Concentration of total viable *E. coli* in samples collected at (a) VEAS and (b) BEVAS WWTPs. Concentrations were measured by the plating method (open symbols) and by Colilert MPN (closed symbols). Error bars represent 33% confidence interval, CI, (n=2) for plating method; 95% CI for a single measurement, as given by the manufacturer, for the Colilert method.

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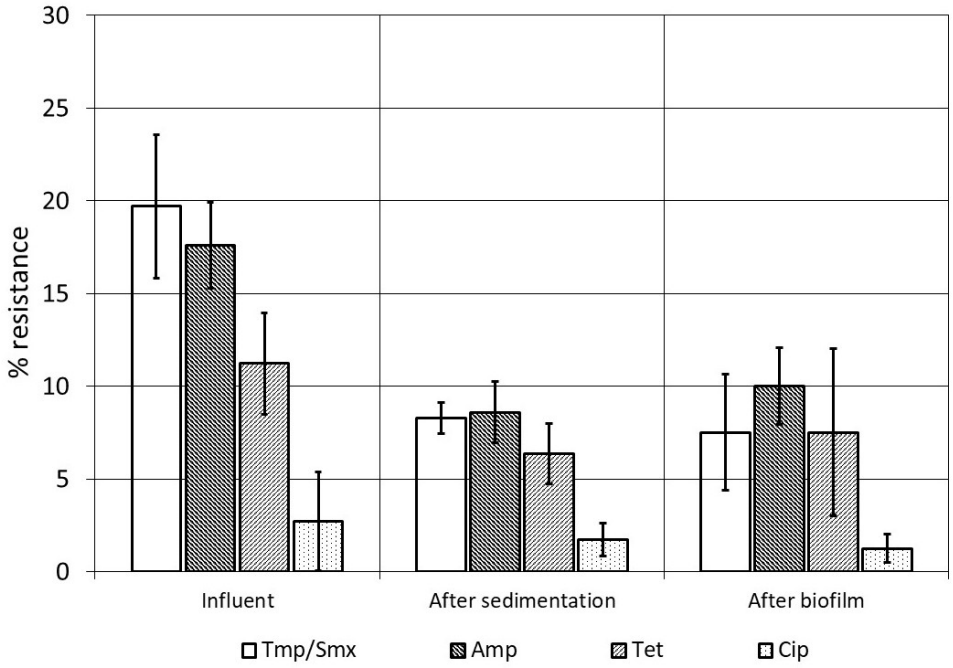
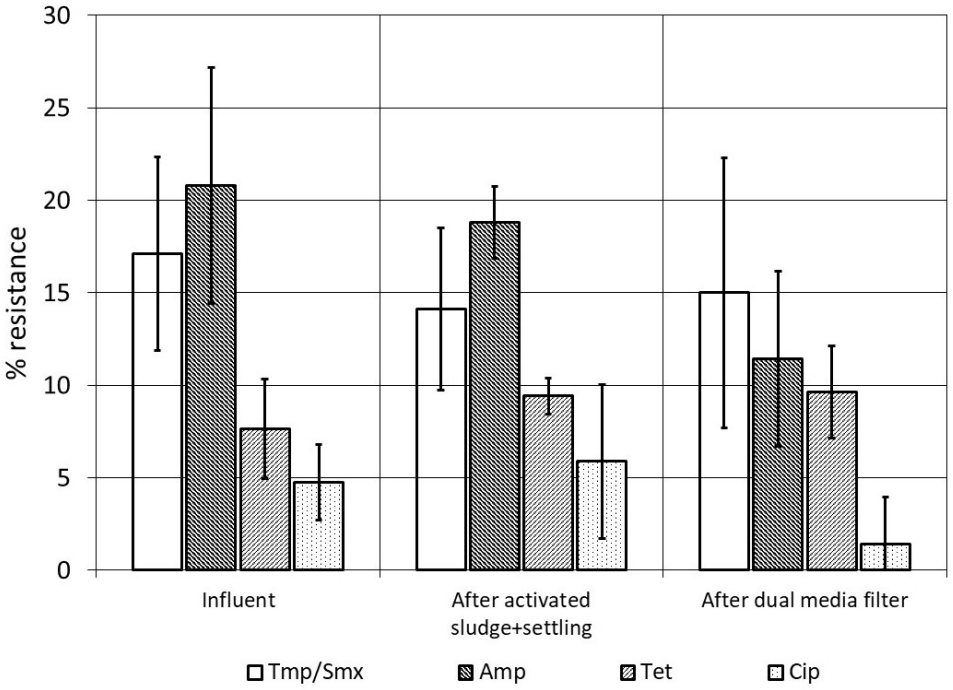
Decrease in the concentration of antibiotic resistant *E. coli* in WWTPs. The percentage of cultivable *E. coli* resistant to the four investigated antibiotics in the influent was comparable in both WWTPs (Figure 3).



a) VEAS WWTP



b) BEVAS WWTP

Figure 3. Percentage of antibiotic resistant *E. coli* in samples collected at (a) VEAS and (b) BEVAS WWTPs. Columns represent average measurements with error bars representing 33% confidence interval (n=2).

Given that VEAS WWTP receives sewage from several hospitals in the Oslo area (with total capacity of ca. 2100 beds) and BEVAS WWTP receives no hospital sewage, the comparable percentage of antibiotic resistant *E. coli* in the inlet of both facilities implies that the main source of resistance to all

antibiotics may not be linked to hospital discharges but rather, other sources. In fact, diffuse sources, and mainly urban household effluent, have been reported to be the major source of ARB in municipal WWTP influents, while hospital effluents contribute usually less than 1% of the total amount of municipal sewage (Kümmerer 2004). Hospital inputs of resistance-driving substances to the municipal sewers system are relatively small (Verlicchi *et al.* 2012), with the exception of a very limited number of compounds and sporadic incidences of elevated concentrations in hospital discharged effluents, as described for VEAS WWTP (Thomas *et al.* 2007a; Langford & Thomas 2009). Thomas and coauthors (2007a) showed that two large Oslo City hospitals, Rikshospitalet and Ullevål, only contribute to the general pharmaceutical load from domestic effluent received at VEAS WWTP. On the other hand, 85% of the total sales of human antibiotics in Norway are used in primary care, i.e., in the community outside hospitals (Figure 4); in addition, the contribution of the veterinary sector in total antibiotics consumption is marginal (ECDC 2014; NORM/NORM-VET 2015). This leads to the assumption that urban households play a major role in the induction or spread of antibiotic resistance in the municipal sewage network being detected at the inlet of both WWTPs investigated.

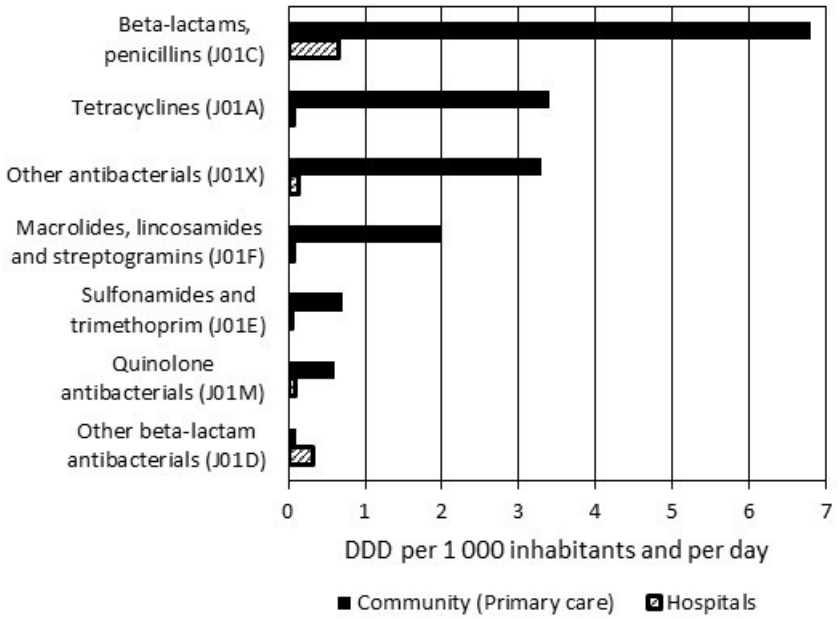


Figure 4. Human usage (Defined Daily Doses, DDD, per 1000 inhabitants and per day) of antimicrobial agents (ATC group J01) for systemic use in Norway between 2008 and 2012 (source: ECDC 2014).

WWTPs display nodes where multiple wastewater streams from different sources with loads of resistance-driving compounds and ARB & ARGs merge, and where the spread of anthropogenic-derived antibiotic resistance to the environment can be controlled/improved, provided this is technically feasible.

With caution, our limited set of results suggests that removal of ARB from wastewater could be performed at the WWTPs rather than at hospitals. We also propose the removal of antibiotics at the WWTPs as they are not currently removed by the present conventional treatment processes at the investigated WWTPs (Thomas *et al.* 2007b). However, this may not necessarily exclude consideration of implementing effluent point-treatment locally at the hospitals of multi-resistant ARB & ARGs, specific clinical pathogens, and certain resistant-driving compounds, which are primarily hospital based and prevail there at elevated concentrations (Kümmerer 2004; Thomas *et al.* 2007a; Langford & Thomas 2009). Despite this knowledge, none of the hospitals in Oslo presently treats or separates its wastewater effluent streams, even though much effort has been made during the past few years

to implement the ISO-14001 ecological standard that targets to minimize environmental pollution from hospitals.

For both WWTPs, the percentage of *E. coli* resistant to Tmp/Smx and Amp in the influent water is about two-fold higher than for Tet, while the percentage of Cip resistant *E. coli* is by far the lowest (Figure 3). Assuming a causal relationship of antibiotic concentration and resistance, this may explain the difference in the rate of antibiotic resistance observed. Related to antibiotic concentrations, Thomas and colleagues (2007b) detected lower concentrations of Tet and Cip in the influent of VEAS WWTP during 5 of 7 measurement incidences, while Tmp and Smx levels were always high. This behavior was explained by the properties of the antibiotics. Tet and Cip are more hydrophobic and tend to rapidly sorb to negatively charged particles compared to the more hydrophilic Tmp and Smx, which are less likely to absorb to particles, and therefore remain in the water phase (Thomas *et al.* 2007b) (adsorption coefficients are given in Table 2). Tet and Cip will then primarily accumulate in the sludge, while the other aqueous phase antibiotics are mobile through the downstream WWTP process, if they are not biodegraded or removed by other physical or chemical means (Thomas *et al.* 2007b). It is therefore assumed that the lower aqueous phase concentrations of Tet and Cip may pose a lower selective pressure to develop resistance than the other two antibiotics. Moreover, ARB resistant to Tet and Cip will mainly be removed by the sludge sedimentation, leading to lower resistance rates for those agents. Due to sporadic peaks in Tet and Cip concentrations at the influent of WWTPs (Thomas *et al.* 2007b), occasionally elevated antibiotic resistance rates for those compounds could be anticipated. However, to solidify this assumption, more systematic investigations of the causal relationship of antibiotic concentration and resistance over time are needed.

In VEAS WWTP, the percentage of cultivable antibiotic resistant *E. coli* decreases in the physical and chemical treatment, while it does not decrease further by the biofilm process (Figure 3). Hence, it seems that the fraction of non-resistant *E. coli* is removed to a somewhat lower extent than the fraction of antibiotic resistant *E. coli*. Yet, due to the small number of samples, it remains unclear whether the decreased percentage of antibiotic resistant *E. coli* is significant or not. The total concentration of viable *E. coli* decreased by 2.4 log during the biofilm process and the percentage of resistant *E. coli* mostly remained unchanged. For BEVAS WWTP, the percentage of cultivable antibiotic resistant *E. coli* did not change considerably during the treatment processes, and this was independent of the antibiotic compound (Figure 3).

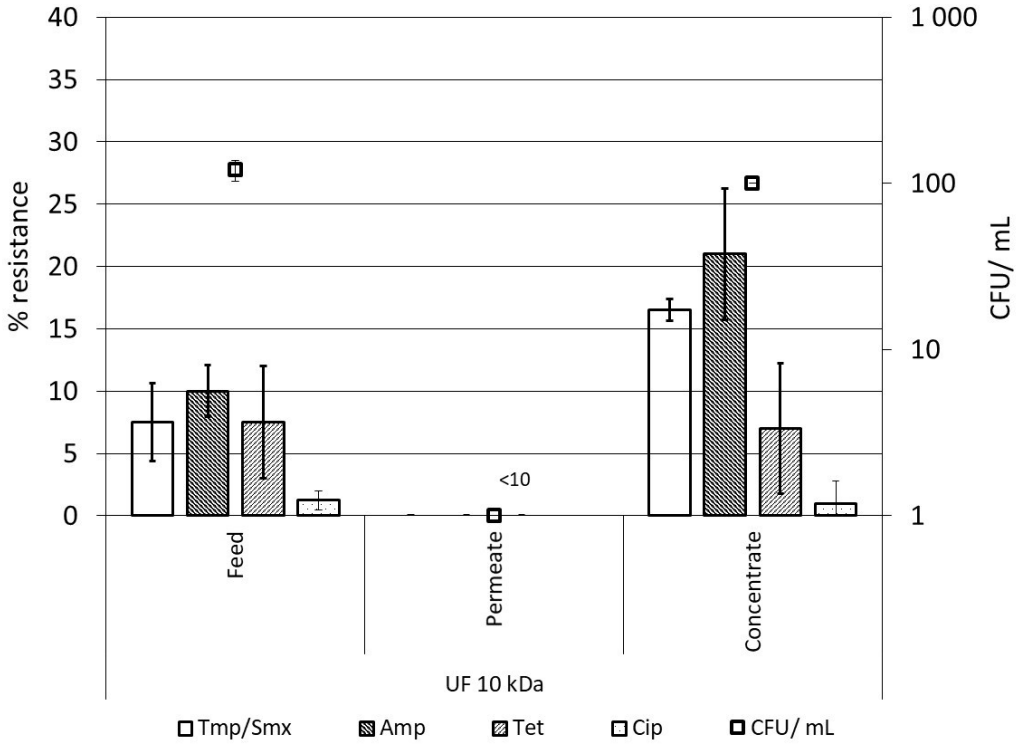
In spite of WWTPs significantly reducing the total concentration of *E. coli*, and consequently the relative fraction of antibiotic resistant organisms, data shows that full disinfection was not achieved. Therefore, it is assumed that the WWTPs release ARB to the receiving water body, the Oslofjord, to which WWTPs effluent are discharged at 30 to 55 m depths. The environmental impact on this ecosystem of ARB & ARGs and periodically high levels of some resistance-driving compounds, such as Tmp and Cip, being released into the fjord (Thomas *et al.* 2007b), is currently unknown due to lack of systematic and long-term studies. While a simple risk assessment has revealed that Cip containing effluent discharges by VEAS WWTP may at times pose an acute risk to certain aquatic organisms in the Oslofjord (Thomas *et al.* 2007a), uncertainty prevails if this is also true for ARB & ARGs, particularly due to the occurrence of Cip resistance in effluent samples from both WWTPs. Depending on the quantity and risk of WWTP discharges, they may pose a serious threat to the ecosystem, and may lead to a rising conflict with various other users potentially affected, such as bathing, fishing, and recreation.

Membrane filtration removal effectivity. UF and NF membranes were investigated by means of the membrane filtration test unit for their efficiency to remove antibiotic resistant *E. coli* from WWTP effluents. All membranes assessed removed viable *E. coli* completely below the limit of quantification

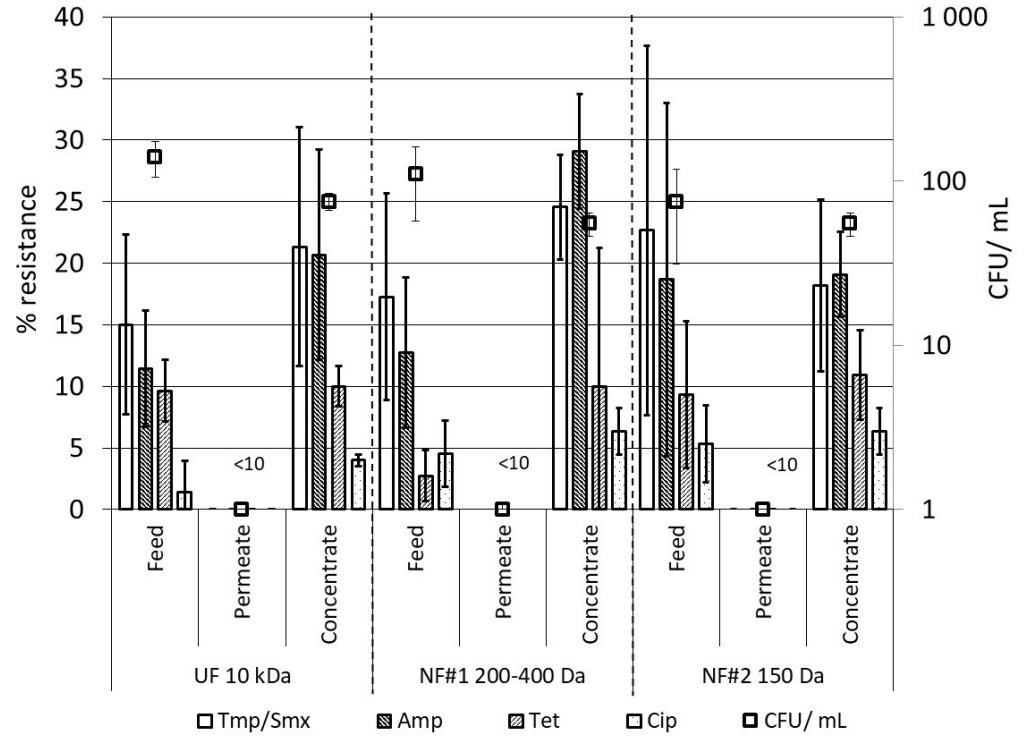
(10 CFU/mL) of the plating method illustrating that the membranes provide a potent hygienic barrier, as was expected (Figure 5, Table S1). The results from plating were confirmed by the MPN method, with no *E. coli* being detected (LOQ: 1 MPN/100 mL) in permeates of the UF (10 kDa) and the NF#2 (150 Da) membranes (Krzeminski *et al.* 2017). The total removal effectiveness of viable *E. coli* in the untreated raw water by WWTP treatment combined with UF was > 4.2 log for both WWTPs. For the NF#1 membrane (200–400 Da), *E. coli* was found in the permeate (Krzeminski *et al.* 2017), but that was attributed to a sample contamination. The concentrate streams of membrane filtration contained almost the same concentration of *E. coli* as the feed, implying that those bacteria were accumulating in the concentrate streams during operation. Differences are attributed to the method's precision (Figure 5).

While the data suggest that ARB in WWTP effluent can be controlled by UF and NF, no conclusions can be made with regard to the destruction or removal of ARGs. However, ARGs are the main targets for disinfection as they display the main risk for spread of antibiotic resistance and are more difficult to destruct than ARB. For UF and NF membrane processes, ARG removal could be challenging because DNA is able to penetrate even through UF membranes due to its size, shape, and flexibility (Arkhangelsky *et al.* 2008; Arkhangelsky *et al.* 2011; Riquelme Breazeal *et al.* 2013). Riquelme and colleagues (2013) reported significant removal of ARGs spiked to WWTP effluents by means of membranes of 100 kDa and smaller. Interestingly, the interaction of DNA with wastewater colloidal particles enhanced the ARG removal by 10 kDa and 1 kDa membranes. The removal of *E. coli* during the present study and under the applied operational conditions is assumed to be due to size exclusion and cell-colloid interactions. However, for the NF experiments, other mechanisms such as electrostatic interactions with the membrane, may also play a role.

The results indicate that membrane filtration provides an additional barrier for ARB in wastewater treatment. Membrane filtration for ARB control may provide several key advantages compared to other methods as it removes particles and a range of other pollutants, including CECs (Krzeminski *et al.* 2017); it provides stable and high quality effluent that can be tailored to the needs enabling fit-for-purpose approach; there is no need for continuous addition of disinfectants; no selection of resistance; it shows no formation of disinfection by-product; it has a small footprint, plant flexibility, is field proven, has long-term stability, and robustness. Conversely, based on current research, the challenges of membrane processes with regard to ARB & ARG removal include (i) possible penetration of DNA through the UF and NF membranes; (ii) unknown interaction of ARB & ARGs with biofilms developed on the membrane; (iii) handling of the waste stream containing ARB & ARGs in up-concentrated form; (iv) high energy consumption at large-scale application. Given that the presented results focused on ARB, future investigations need to clarify if and to what extent membranes provide a barrier for ARGs. Further research is required to confidently draw conclusions on whether membrane processes can provide a sufficient barrier for ARB & ARGs, either as stand-alone technology or as part of a multi-barrier treatment train.



a) VEAS WWTP



b) BEVAS WWTP

Figure 5. Concentrations of total viable *E. coli* (CFU/mL; boxes) and percentage (columns) of antibiotic resistant *E. coli* in the feed (i.e., WWTP effluent), permeate, and concentrate post-treatment with different membranes (UF = ultrafiltration; NF = nanofiltration). Feed samples were collected from (a) VEAS and (b) BEVAS WWTPs at different dates. Error bars represent 33% confidence interval (n=2).

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Conclusions

The results of this study highlight that comparably high concentrations of viable *E. coli* resistance to the four antibiotics investigated occur in the effluent of both WWTPs. This suggests that urban households in the Oslo City area significantly contribute to the spread of antibiotic resistance in the municipal sewage network, which was detected at the inlet of the WWTPs. The relevance of these findings will need to be confirmed further by future investigations with more frequent sampling over longer terms including more statistical analysis. With regard to the relevance of WWTPs in the spreading of antibiotic resistance, it is important to unravel the causal relationship between antibiotic consumption, the antibiotics concentration in the wastewater streams of the entire WWTP (water and sludge), and the rate of antibiotic resistance amongst the prevailing populations. Although this relationship is described for clinical settings, this is not the case for the environment and requires further investigation.

Besides a significant overall reduction of viable *E. coli* congruent to the reduction in the fraction of resistant bacteria across the treatment at both WWTPs, full disinfection of the final effluent by conventional treatment was not achieved and ARB were detected in the WWTP effluents. This may be critical, considering the release of ARB along with certain antibiotic resistance-driving compounds to the Oslofjord ecosystem. To ensure effective removal of ARB and ARG destruction in particular, adequate tertiary treatment methods will need to be assessed and verified for efficient functioning at full-scale.

Consideration to the implementation of measures against ARB at WWTPs should include UF and NF, which may provide effective alternatives for the post-treatment of WWTP effluent to reduce the risk of ARB release to the receiving aquatic environment. Fortunately, this can be done in parallel with the removal of other pollutants. Yet, there is still uncertainty if this is also true for the removal of ARGs, thus further research is required given that ARGs are more difficult to remove and may require additional treatment of the permeate.

Even though WWTPs are major hotspots for the spread of antibiotic resistance, to date no technical measures have been introduced at WWTPs to minimize the problem. This may be due to the current lack of knowledge. In order for decision makers to judge the implementation of measures against the anthropogenic-induced spread of antibiotic resistance at WWTPs and relevant point-sources, an improved understanding of (i) the causal relationship of driving factors and organisms responsible for the spread of antibiotic resistance in full-scale WWTPs, (ii) the effect of the conventional and advanced treatment on those factors, and (iii) the fate and risk of ARB & ARGs spreading into the downstream environment, is required. In conclusion, further monitoring data, such as presented in this study and as outlined in the One Health approach (COM 2017), is required to better assess the risk of ARB & ARGs in wastewater treatment processes and to develop an action plan to manage the impact on human and animal health.

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Supplementary Material

Table S1. Concentrations of total viable *E. coli* from membrane experiments determined by the agar plating method (LOQ: 10 CFU/mL). Numbers state average values ± 33% confidence interval (n=2).

WWTP	Membrane	Sample location	cfu/mL
VEAS	UF (10 kDa)	Feed	120±18
		Permeate	<10
		Concentrate	100
BEVAS	UF (10 kDa)	Feed	140±35
		Permeate	<10
		Concentrate	75±9
	NF#1 (200-400 Da)	Feed	110±53
		Permeate	<10
		Concentrate	55±9
	NF#2 (150 Da)	Feed	75±44
		Permeate	<10
		Concentrate	55±9

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