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Research article

Calculating expected effects of treatment effectivity and river flow rates on the contribution of WWTP effluent to the ARG load of a receiving river

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ABSTRACT

Concentrations of genetic markers for antibiotic resistance genes (ARGs) were measured in the effluents of three Norwegian wastewater treatment plants (WWTPs) and in a receiving river upstream and downstream of the discharge point of one WWTP. Calculations based on mass balances were carried out to evaluate the impact of river flow rates and treatment effectivity on the WWTP's contribution to the load of genetic markers in the river. At average river flow rates, the WWTP effluent contributes 5–15% to the genetic marker load of the respective river. However, at minimum river flow rates, the WWTP effluent contributes 22–55% to the loads of different genetic markers. Scenarios of an improved or worsened removal of genetic markers in the WWTP showed that a further 1-log removal using additional treatment would be sufficient to improve considerably the river water quality with respect to genetic markers. Then, at an average flow rate, the contribution of the WWTP effluent to the load of the river would be less than 2%. However, in the case of low treatment effectivity or malfunction of the WWTP, the marker load of the river would increase dramatically. Even at average flow rate, 75–92% of the marker load would then originate from the WWTP. The results demonstrate the importance of considering the flow rates and hydrologic characteristics of the recipient water body when deciding on priorities regarding the upgrade of WWTPs for further removal of ARGs.

1. Introduction

The global dissemination of antimicrobial resistance (AMR), one of today's major health challenges, is mainly due to the emission to the environment of antibiotic resistant bacteria (ARB), antibiotic resistance genes (ARGs) and antimicrobials contained in human and animal wastes. Meanwhile, national and international action plans targeting AMR, along with countermeasures, aim at an increased understanding of the mechanisms and pathways of the dissemination of AMR through the implementation of surveillance and monitoring programmes (WHO, 2015). In the 'One Health' approach the close interconnection of the spread of AMR to environment and human and animal health is considered (EC, 2017).

The threat of AMR in environmental settings is due mainly to environmental bacteria that contribute to the development of antimicrobial resistance in clinically associated bacteria through the mobilisation of novel ARGs and the transmission back to commensals and pathogens in humans and animals. However, the extent of ARG dissemination in environmental bacteria, how this is influenced by anthropogenic inputs, and how ARGs are shared between human-associated and environmental bacteria, is currently unknown. In addition to this indirect pathway, AMR may pose health risks in humans and animals through direct exposure to ARB and ARGs contained in food and water (Leonard et al., 2015; Wellington et al., 2013). Anthropogenic inputs to the environment in the form of discharge from urban wastewater treatment plants (WWTPs), hospitals, industries and application of manure to the land, in addition to inputs from diffuse pollution such as agricultural run-off, are thought to be the major streams influencing this process (Amos et al., 2014a, 2014b).

Urban WWTPs are considered among the most important receptors and point sources of ARB and ARGs disseminated to the environment (Amos et al., 2014a, 2018; Berendonk et al., 2015; Martínez, 2008, 2009; Rizzo et al., 2013). This is due to the presence of diverse intestinal microorganisms containing a wide array of sharable resistance determinants, which coexist with a mixture of sub-therapeutic concentrations of resistance-driving substances, along with a multitude of

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organics and nutrients, as well as other hydrological and physical conditions favourable for proliferation, cell-to-cell interactions and the survival of microorganisms (Wasteson et al., 2020). Contrary to the current opinion that WWTPs present hotspots for the selection of antibiotic resistance, recent findings suggest that the prevalence of ARGs in a WWTP strongly correlates with the presence of faecal pollution and that selection would not play a role under normal circumstances (Karkman et al., 2019). It is known that urban sewage systems mirror the behaviour of society in the consumption and excretion of metabolites and chemicals. As they are large reservoirs of human faeces, they present complex ecosystems and reflect the microbiome and resistome of the human population (Joseph et al., 2019; Pärnänen et al., 2019). Quintela-Baluja et al. (2019) describe the sewage network, which is comprised of the sewage pipe network, the treatment plant and the effluent-receiving water body, as home to three main evolutionary distinct ecosystems with different habitats and selection factors housing different bacterial microbiomes and antibiotic resistomes. Those ecosystems include (i) the raw wastewater from, e.g., households, hospitals, nursing houses, industry and WWTP influent; (ii) the WWTP's biological treatment step, with microorganisms bound in biofilm or sludge and as free-living organisms; and (iii) the receiving water body upstream of the WWTP effluent discharge point (Quintela-Baluja et al., 2019).

Urban WWTPs also play a critical role in the dissemination of ARB and ARGs to the receiving environments. However, the scale of this is currently unknown. Conventional wastewater treatment processes usually have limited capacity to reduce ARB and ARGs to negligible concentrations, as large numbers survive the treatment and are released to the environment (Alexander et al., 2015; Exner et al., 2018; Hembach et al., 2017; Schwermer et al., 2018). The abundance of ARB and ARGs has been shown to increase even across the treatment process (Alexander et al., 2015). In other cases, increased abundance of some but not other genes that were investigated was reported (Pallares-Vega et al., 2019). An evaluation of the relevance of WWTPs in the discharge of ARB and ARGs to the environment and its implications requires (i) the identification and mapping of the dominant ARB and ARGs prevailing in the different treatment stages, (ii) the determination of the loads of the relevant ARB and ARGs, and (iii) an assessment of the impact of treatment processes and operational parameters on AMR.

Many rivers impacted by urban wastewaters are contaminated with ARB, ARGs and antibiotics, while their pristine origins are free of such pollutants (Barancheshme and Munir, 2017 and references therein). Concerns have lately been raised about the elevated ARB and ARG concentrations downstream of WWTP final effluent discharge points (Brown et al., 2019 and references therein). This implies that WWTPs are sources of this contamination, although antibiotic resistance is known to be present ubiquitously in nature. In fact, rivers are regarded as possible hotspots of antibiotic resistance dissemination due to the exchange of resistance between WWTP effluent bacteria and environmental bacteria (Karkman et al., 2019). ARGs being discharged from WWTPs are associated either with settleable or suspended wastewater particles (Brown et al., 2019), contained in bacteria and bacteriophages, or as cell-free (extracellular) DNA. ARGs associated with settleable particles were found to contribute to the increase in the ARG load in river and lake sediments and play a crucial role in the spread of ARGs in downstream sediments (Brown et al., 2019; Czekalski et al., 2014). Concurrently, due to their small size and characteristics, ARGs are more difficult to remove from wastewater than are ARB (Krzeminski et al., 2020; Schwermer et al., 2018).

The discharge of ARB, ARGs and antibiotics from WWTPs is believed to cause ecological stress in the water body and result in the disruption of the natural conditions, shifting them to alternative equilibria (Corno et al., 2019). The discharge of these contaminants into rivers is currently uncontrolled. This is particularly critical for the use of reclaimed water for irrigation, fishing, recreational activities and drinking water and for other purposes that may directly or indirectly impose risks to human health. Reasons for the uncontrolled release of these pollutants include the current lack of knowledge on the scale and impact of the emission of these contaminants due to the absence of routine monitoring at WWTPs, the absence of discharge regulations and restrictions, and the absence of obligations for their removal.

The absolute load of ARB and ARGs released from WWTPs and transported into the receiving water body has been shown to be the main mechanism responsible for the on-site selection and proliferation of ARB and ARGs in the river, while antibiotic concentrations in the river, which are usually low, do not affect gene selection (Brown et al., 2019; Karkman et al., 2019). In addition, the prevalence and distribution of ARB and ARGs in WWTPs and rivers have been associated with the disposal of faeces (Brown et al., 2019; Karkman et al., 2019; Zhu et al., 2018). Thus, WWTPs are both major point sources for AMR and concurrently nodes where the spread of antibiotic resistance can be controlled before the effluent is discharged to the water body or reused (Riquelme Breazeal et al., 2013).

The major scientific questions related to the spread of AMR associated to WWTP discharges that are currently unsolved are: (i) what are the scale and dynamics of ARB and ARG loads in the receiving water body, (ii) what are the means of distribution (hydraulic transport, horizontal gene transfer among bacteria), (iii) what is the fate of ARB and ARGs in the receiving water body (accumulation, survival capacity, DNA decomposition), and (iv) what risks does this impose on health and ecology.

Despite the relevance and increased focus on the topic of global AMR spread, few investigations have addressed these questions (for example, Cacace et al., 2019; Czekalski et al., 2014; Hess et al., 2018; Jäger et al., 2018a; Khan et al., 2019; Pallares-Vega et al., 2019; Quintela-Baluja et al., 2019). However, decision makers and plant operators need information and advice regarding the identification and implementation of proper AMR management strategies, including effective technical measures. Thus, holistic models describing the dissemination of ARB and ARGs and their effects are needed.

The effectiveness of ARG removal by state-of-the-art wastewater treatment and advanced methods, combined with its impact on the receiving water, has barely been studied systematically. Jäger et al. (2018b) calculated the daily ARG charges and calculated ARG distribution in the receiving river considering different river flow scenarios. However, this was limited to an extrapolation of ARG concentrations in WWTP effluent and was not supported by quantitative data on ARG concentrations in the river.

2. Objectives and approach

2.1. The objectives of the study were

i To determine the load of genetic markers for ARGs being disseminated to the environment by three large Norwegian WWTPs. Two are treating wastewater from the City of Oslo and the third is treating wastewater from four peri-urban municipalities near Oslo.
ii. To determine differences in the genetic marker loads arising from WWTPs receiving no wastewater from hospitals at all and those receiving wastewater partly from hospitals.
iii. To understand whether the genetic marker loads from WWTPs in urban and peri-urban settings are different.
iv. To determine the contribution of the genetic marker load from a WWTP to a receiving river.
v. To calculate the impact of typical seasonal variations in the flow rate of the river on the contribution of the WWTP effluent to the genetic marker load of the river.
vi. To calculate the effects that can possibly be achieved on the ARG load of the river downstream of a WWTP by the use of additional or advanced wastewater treatment.

Using qPCR, the abundance of nine different ARGs and a class 1

Table 1
WWTP characteristics.

WWTP	Source of wastewater	Person equivalents (1000 pe	Treatment capacity (million m^3 yr^{-1})	Hospital beds/ 1000 pe	Biological treatment process
1	Urban, industry, five hospitals (2100 beds) from Oslo-Viken area	600	105	3.5	Biofilm (BIOFOR); fixed film for nitrification and denitrification
2	Urban (70%), industry (30%) from Oslo city; no hospitals	290	37	0	Activated sludge; denitrification, nitrification and denitrification
3	Urban (80%), industry (20%), two hospitals (664 beds) from Lillestrøm, Lørenskog, Nittedal and Rælingen areas	130	19	5.1	MBBR process; combined pre- and post-denitrification

integron-associated integrase gene, referred to as 'genetic markers' in the following, and 16 S rRNA as a measure for the total concentration of DNA, respectively, were quantified in the treated final effluents from the three WWTPs and in the river into which one of the treatment plants discharges directly. Two of the WWTPs receive wastewater from an urban environment. While one of the two WWTPs receives wastewater partly from hospitals, the other does not. These two urban plants discharge to a fjord (the Inner Oslofjord), i.e., the sea, and therefore their contribution to the receiving environment was not evaluated due to too high a complexity. The third WWTP, in the peri-urban setting, discharges to a river. The flow rates of the river are measured and documented with relatively high resolution. Thus, this setting is less complex and is well described, and the ARG load of the WWTP to the river can be calculated for different hydraulic situations.

To assess the potential effect of advanced wastewater treatment (or malfunctions) on the genetic marker load in the river downstream, mass balances were developed to calculate different scenarios. These included different river flow rates (minimum, annual average, and maximum flow), and stipulated treatment effectivities from advanced treatment. The effects of scenarios such as maintenance shutdowns and failures in operation were also calculated.

3. Material and methods

3.1. WWTP characteristics

The specifications of the three WWTPs investigated are given in Table 1. Two of the plants (WWTPs #1 and #3) receive wastewater from hospitals and make use of a biofilm process during biological treatment. WWTP #2 applies an activated sludge process. A detailed process description of WWTPs #1 and #2 can be found elsewhere (Schwermer et al., 2018). At WWTP #3, the raw water (ca. 19 million m^3 y^{-1}) originates from four peri-urban municipalities and is composed of urban (80%) and industrial (20%) wastewater (in% of COD). The raw water passes through a grid chamber, a sand- and fat-separator and pre-sedimentation. It is then treated in a moving-bed biofilm reactor (MBBR) process (combined pre- and post-denitrification), with ethanol as substrate. A coagulant (PAX-18, polyaluminium chloride hydroxide) is added and, after post-sedimentation, the treated water runs through a 700-m tunnel and is discharged into the river.

3.2. Characteristics of the receiving river

The catchment area of the river Nitelva, into which WWTP #3 discharges, has an area of 486 km^2. It consists mainly of forest, some agriculture and some scattered housing. The river is home to Norway's most species-rich stocks of freshwater fish. In the lower section near the WWTP, the river is polluted with phosphorus, nitrogen and faecal bacteria, resulting in moderate-to-bad ecological water quality status (DHI, 2014; Holm et al., 2014; Lindholm et al., 2011). Further downstream the river merges with two other rivers: one of them, the Glomma, is Norway's largest river. Finally it empties into northern Europe's largest inland river delta, the Øyeren.

3.3. Sample collection

3.3.1. WWTP effluent sampling

On three consecutive days, 1-L flow-proportional composite samples of WWTP effluents were collected over 24 h by means of an autosampler. At WWTP #1 and WWTP #2, samples were collected on Okotober 14th, 15th, and 16th, 2014, and at WWTP #3 they were collected on September 8th, 9th and 10th, 2015. Subsamples from the autosamplers were placed in sterile 0.5-L plastic bottles and transported cooled to the laboratory.

3.3.2. River water sampling

From the river, three grab water samples were collected approximately 500 m downstream of the WWTP #3 discharge point (downstream sample; *do*) on September 8th, 9th and 10th, 2015. Integrated samples of volume 1 L were prepared by mixing two 0.5-L samples collected at the left and right riverbanks. Other samples were collected approximately 125 m upstream of the WWTP discharge point (upstream sample; *up*) on the same days. Samples were placed in sterile 1-L bottles, cooled to 4 °C in the dark, and transported to the laboratory for immediate analysis.

3.4. Molecular biological analysis

Water samples (0.1 or 0.15 L) were filtered in triplicate through polycarbonate membrane filters (pore size 0.22 μm; Whatman), and DNA was extracted using the PowerWater DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA) and stored at −20 °C. Due to the pore size of 0.22 μm, cell-free DNA was probably not fully captured, but only DNA associated with bacteria or bound to particles. Total DNA extracted from the three filters was pooled in 100 μL of elution buffer. Selected genetic markers, i.e., the intron intl1 as a proxy for total AMR load (Amos et al., 2015), nine ARGs (bla_{TEM-1D}, $bla_{CTX-M-15}$, $bla_{CTX-M-32}$, bla_{KPC-3}, bla_{OXA-48}, bla_{OXA-58}, sul1, tetM, and mcr-1), and 16 S rRNA as an estimate for the total abundance of bacteria, were quantified by qPCR (Universal Master Mix M3003; New England BioLabs Inc.) at Technische Universität Dresden, Institut für Hydrobiologie, Germany, as described elsewhere (Cacace et al., 2019). These ARGs and the intron had been selected with regard to persistence, abundance in WWTPs, risk potential, and abundance in untouched nature (more details are given in the Supplementary Materials). The limit of quantification (LOQ) was determined for each genetic marker and was related to the sample volume, the detection limit and the amount of DNA. For practical reasons, gene abundance was then normalised to 100 mL.

3.5. Balancing and calculating loads of genetic markers in WWTP effluent and in the receiving river

3.5.1. General remarks

To calculate the contribution of the investigated genetic markers released from the WWTP into the river requires knowledge about several characteristics and the behaviour of the receiving river. This includes flow rate dynamics, marker gene dilution and up-concentration and WWTP overflow events, all of which are unique for individual water bodies. While volumetric WWTP effluent flow rates usually vary little

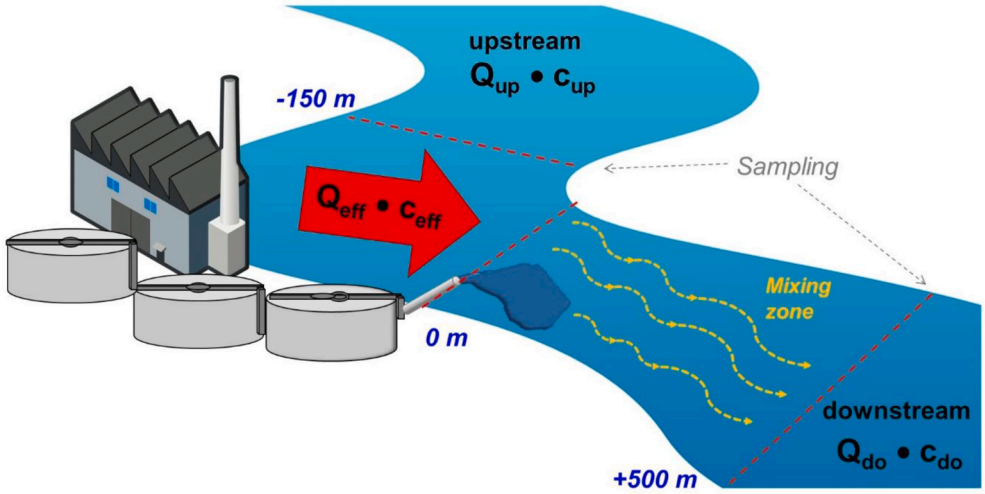

Fig. 1. Illustration of the contribution of the genetic marker load emitted from WWTP final effluent to the receiving river. Distances in blue indicate the position of sample collection locations relative to the WWTP effluent discharge point. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

over time, the water level and flow rate of rivers may vary widely between seasons, due mainly to rain events and droughts. These factors may affect genetic marker deposition and transport rates in the water body and the transfer among prevailing bacteria.

3.5.2. Calculating the loads of genetic markers

The loads of the nine ARGs, intl1 and 16 S rRNA contributed by the WWTP and passing upstream and downstream of the WWTP were calculated, as illustrated in Fig. 1. The load n_i of each genetic marker i passing the respective point at a certain time is calculated according to Eq. (1):

$$n_i = Q \cdot c_i \quad (1)$$

where Q is the volumetric flow rate of the WWTP effluent or of the river upstream or downstream of the discharge point and c_i is the concentration of genetic marker i at the respective point.

3.5.3. Determination of the volumetric river flow rate

For mass balance calculations, the volumetric flow rate Q_r of the river receiving wastewater from WWTP #3, at the respective sampling location upstream or downstream, was determined by Eq. (2):

$$Q_r = w_r \cdot d_r \cdot v_r \cdot f, \quad (2)$$

where w_r denotes the width of the river, d_r the average river depth, v_r is the distance flow rate (ADEQ, 2018) and f is a correction factor allowing to correct for the fact that water at the surface travels faster than near the stream bed due to resistance from gravel and stones. In USEPA (2020), values of f of 0.8 and 0.9 are used for rocky-bottom and muddy-bottom stream beds, respectively. Here, 0.83 was chosen for f, taking into account the rather rocky bottom of the river.

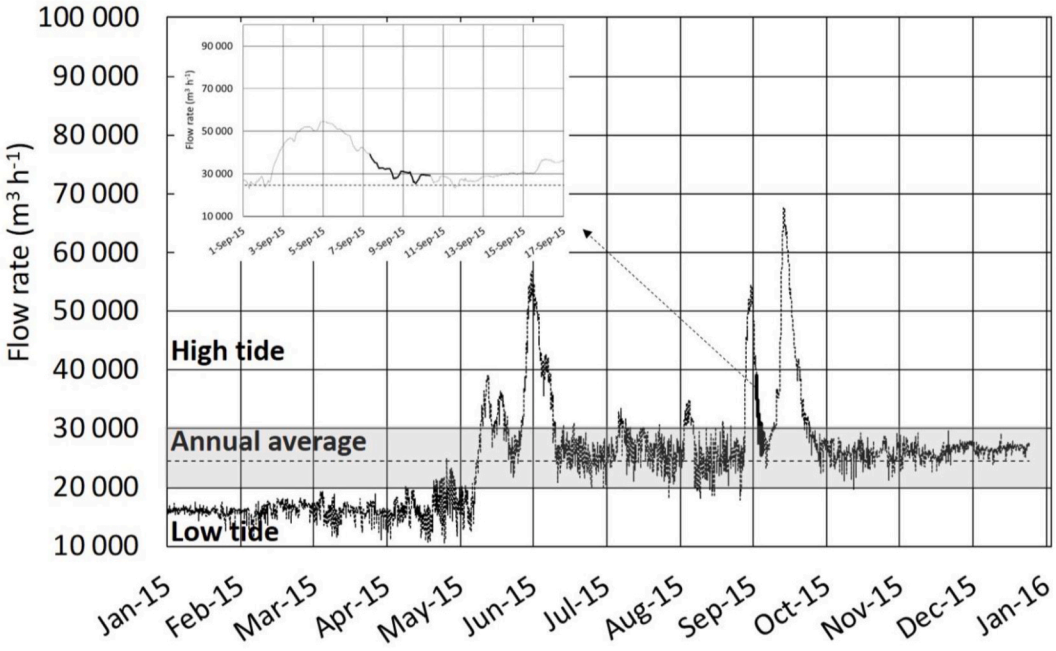

Fig. 2. River flow rates downstream of the WWTP discharge in 2015. The cutout shows flow rates in the sample collection period (bold). The dotted horizontal line represents the annual average flow rate in 2015 of 24,000 m^3 h^{-1}.

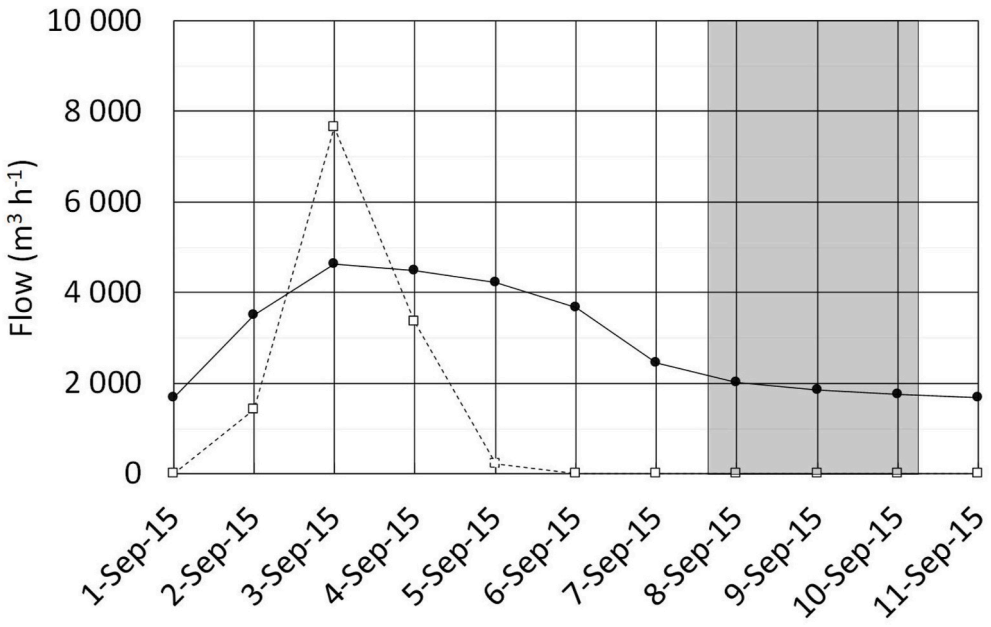

Fig. 3. Flow rates of treated effluent (solid line) and WWTP #3 overflow (dashed line) directly into the receiving river before and during (grey area) the sampling campaign.

As described in ADEQ (2018), the river depth d_r was averaged from measurements at several locations across its width. As the width was different between the sampling points, three measurements were made at the narrow location upstream and 10 measurements at the wide location downstream of the WWTP discharge point. The flow speed v_r at the respective locations was determined using the float method by measuring the time needed for an object to float a given distance of 10 m in the middle of the river.

3.5.4. Calculation of historical volumetric river flow rates

The flow rate of a river is subject to considerable variation, while the flow rates of WWTP effluent vary comparatively little. Consequently, for a receiving river it is important to know the typical variations in flow rate in order to calculate and understand the contribution of WWTP effluent to the load of genetic markers to the river.

The flow in the river to which WWTP #3 discharges is regulated artificially downstream of the plant. This strongly affects the river's nature and, supposedly, marker gene abundance. The river is characterised by flooding in spring (April–May), mainly due to snow melt, and in autumn (September). The riverbank has a fixed, permanent water-level indicator, and the level was noted on the first sampling day when the flow rate was determined as described above. Historical data give the water level at this indicator. The water level at the indicator was noted on the consecutive sampling days. Volumetric flow rates for the following days, as well as historical flow rates, were then calculated by converting the water level to volumetric flow rates according to Eq. (3):

$$Q_{r,i} = v_{r,ref} \cdot \left[\left(\overline{d}_{d,ref} + \Delta l_i \right) \right] \cdot w_{r,\ ref} \tag{3}$$

where $Q_{r,i}$ is the river flow rate on the sampling day or a historical day I, $v_{r,ref}$, $\overline{d}_{d,ref}$, and $w_{r,ref}$ are the flow velocity, the average water depth and the river bed width, respectively, on the reference day September 08, 2015, and Δl_i is the difference in height of the water surface on day i relative to the level at the level indicator on the reference day September 09, 2015 (i.e., $\Delta l_i = l_i - l_{08/09/15}$) or from the annual average level. Fig. 2 shows the strong variations of the river's water level, given as the deviation from the annual average water level (determined from the entire year 2015).

Using the relationships presented above, the calculated volumetric flow rates of the river in 2015 at 500 m downstream of the WWTP discharge point varied between ca. 10,600 $m^3\ h^{-1}$ at minimum level, to 24,000 $m^3\ h^{-1}$ at annual average level, and 68,000 $m^3\ h^{-1}$ at maximum level (Fig. 2).

3.5.5. WWTP discharge rates

Data on the flow rate for WWTP #3 effluent and overflow were provided by the WWTP. During the sampling period, the treated wastewater discharge was about 2000 $m^3\ h^{-1}$, which is equivalent to the annual average flow in 2015, and it was decreasing slightly (Fig. 3). Overflow of the WWTP and discharge of untreated wastewater happens occasionally due to stormwater events. For the respective plant, the average rate for overflow accounts only for about 4% of the total annual wastewater discharge. About one week prior to the sampling period, flooding had occurred, and the WWTP overflow had accounted for 1/3 of the total annual volume overflow (100 $m^3\ h^{-1}$) in 2015.

3.5.6. Calculating the contribution of genetic markers in WWTP effluent to the load in the river downstream

During flooding, genetic markers released from the WWTP are diluted in the river. In contrast, at low water levels during winter/spring (January to April), the contribution of WWTP effluents to the total volume in the river is high. Minimum river flow rates at low tide present a 'worst-case scenario', because genetic marker dilution during a high flow contribution by the WWTP to the river is reduced and, instead, genetic marker concentrations increase, which may enhance AMR spread. To calculate the contribution of genetic markers in WWTP effluent released to the total load of genetic markers in the river downstream, mass balances were set up as described above. Rearrangement provides Eq. (4):

$$\frac{n_{eff}}{n_{do}} = \frac{n_{eff}}{n_{up} + n_{eff}} = \frac{Q_{eff} \cdot c_{eff}}{\left(Q_{up} \cdot c_{up}\right) + \left(Q_{eff} \cdot c_{eff}\right)} \tag{4}$$

where n_{eff}, n_{do}, and n_{up} are the load of genetic markers per unit time in the effluent (*eff*) in the river downstream (*do*) and upstream (*up*) of the WWTP discharge point; Q_{eff} and Q_{up} are the respective volumetric flow rates of the WWTP effluent and of the river upstream of the discharge point; and c_{up} and c_{eff} are the genetic marker concentrations measured upstream and in the WWTP effluent.

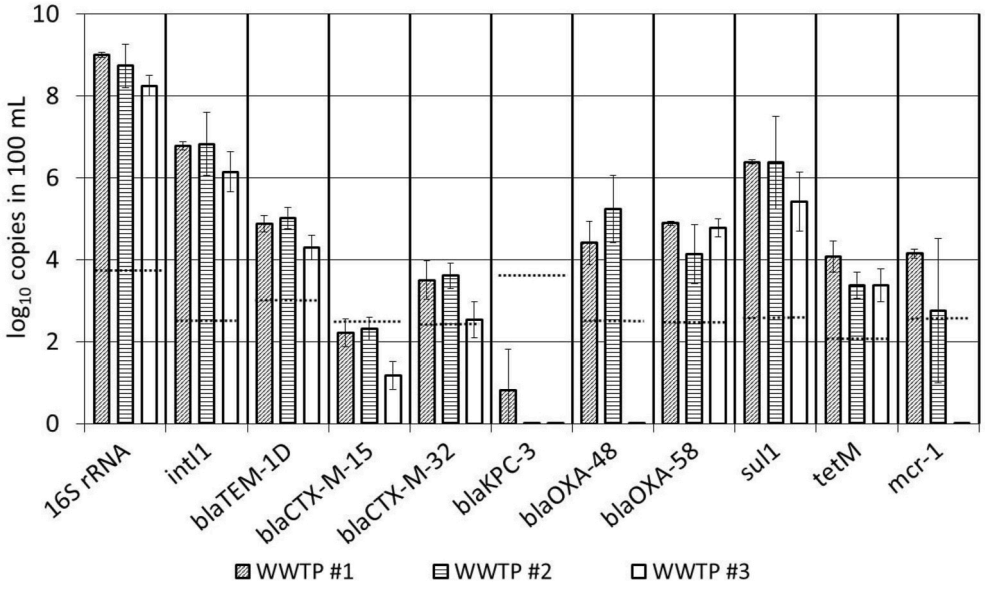

Fig. 4. Absolute abundance (normalised to 100 mL) of the measured genetic markers in WWTP final effluents. Error bars indicate 67% confidence intervals of n = 3 measurements made on the three subsequent sampling days. Dotted lines indicate the LOQ for each marker. For resistance/function, see Table 2.

3.5.7. Calculating the impact of WWTP treatment effectivity on the load in the river

The potential effect of improved or worsened performance of the WWTP in the removal of genetic markers on the contribution to the load in the river was taken into account by introducing into Eq. (4) a factor *y* for improved removal effectivity (10, 100, 1000 – corresponding to 1, 2 and 3 log improvement, respectively) and for worsened removal or higher influent concentrations to the WWTP (0.1 corresponding to 10 times higher effluent concentrations), yielding Eq. (5) for the calculations:

$$\frac{1/y \cdot n_{eff}}{n_{up} + 1/y \cdot n_{eff}} = \frac{1/y \cdot Q_{eff} \cdot c_{eff}}{(Q_{up} \cdot c_{up}) + (1/y \cdot Q_{eff} \cdot c_{eff})} \quad (5)$$

3.5.8. Calculating the effect of river flow rates on genetic marker concentrations downstream of the WWTP discharge point

As discussed above, the volumetric discharge from the WWTP Q_{eff} can be considered as almost constant. Regarding the concentration of ARG, Pallares-Vega et al. (2019) found that rainfall in the WWTP's catchment area increased the hydraulic load and decreased the ARG concentrations but did not affect the load of ARG to the WWTP in a statistically significant way. They explained this by the homogenising effect of the 24-h composite samples. For increased hydraulic load they also showed that the removal effectivity decreased slightly; i.e., the product ($Q_{eff} \cdot c_{eff}$) can be treated as constant. For removal effectivity, to calculate the impact of the discharge on the ratio of the genetic marker concentration in the river downstream to the concentration upstream, Eq. (4) was rearranged to yield Eq. (6):

$$\frac{c_{do}}{c_{up}} = \frac{\left(\frac{Q_{eff}}{Q_{up}} \cdot \frac{c_{eff}}{c_{up}}\right) + 1}{\frac{Q_{eff}}{Q_{up}} + 1} \quad (6)$$

Values of the c_{do}/c_{up} ratios were computed for different river flow rate scenarios, including low tide, average annual tide and high tide, for several genetic marker concentrations in WWTP effluents. All calculations assumed complete mixing (i.e., homogeneous distribution) of the effluent with the river flow and the absence of other sources and sinks for DNA markers. Furthermore, the flow direction of the river was assumed to be unchanged, and no resuspension of bottom sediment was assumed. All calculations used the average marker gene concentrations measured upstream of the discharge point (c_{up}) and in the WWTP effluent (c_{eff}) during the three sampling days. Confidence intervals for these concentrations were calculated using the t-distribution, the number of samples (n = 3), the standard deviation and a confidence level of 67%.

4. Results and discussion

4.1. Genetic marker emission from WWTPs

The concentration of bacteria, measured as copies of 16 S rRNA per 100 mL, was comparatively high in the effluents of the three WWTPs (Fig. 4). Regarding the concentration of genetic markers for studying ARGs, intI1 and sul1 were the most dominant, which correlates with results observed at other urban WWTPs (Cacace et al., 2019; Rocha et al., 2018). Except for mcr-1 and bla_{OXA-48}, the concentrations of genetic markers analysed were comparatively high at all three plants. This indicates that their abundance is independent of the type of treatment process and is in accordance with findings by other researchers. In a pan-European survey on ARB abundance involving many WWTPs (Cacace et al., 2019), no clear relationship between ARG abundance and WWTP characteristics (such as the number of biological stages and plant size) was found.

Besides the abundance of genetic markers for the presence of resistance against commonly used antibiotics in Norway, including penicillins and tetracyclines, WWTP and river samples contained genes coding for resistance against compounds which are seldom used in Norway, including sulfonamides, trimethoprim and aminoglycosides.

The total concentrations of all ARGs and intl1 detected in the effluents of all three WWTPs were lower by factors of 10–1000 than those measured in many other European WWTPs (Cacace et al., 2019). One of the reasons might be the low abundance of antibiotic resistance in patients in Norway compared to in other European countries, resulting from a comparatively low consumption of antibiotics and strict strategies to combat AMR spread that have successfully been implemented in the health sector in Norway for several years (NM, 2015).

The concentrations of most of the analysed genetic markers in the effluent from WWTP #2, not receiving hospital wastewater, showed the same pattern as effluents from WWTPs #1 and #3, both receiving hospital wastewater. This supports findings by Paulshus et al. (2019), who compared antibiotic resistance in hospital wastewater and in the sewer

Table 2
Genetic markers analysed for, resistance, function and relevance.

Marker genes	Product	Resistance/Function	Relevance[d]
16S rRNA	Ribosomal proteins	–	2
intl1	Integrase gene of class 1 integrons, platform for different resistance genes; a proxy for total AMR load[a]	Various antibiotics (aminoglycosides, trimethoprim, β-lactamase and erythromycin)	3
bla_{TEM-1D}	Extended-spectrum β-lactamase (ESBL) class A	Penicillin	1
$bla_{CTX-M-15}$		β-lactams, penicillins and cephalosporins[b]	3
$bla_{CTX-M-32}$			
bla_{KPC-3}	Carbapenem-hydrolysing class A β-lactamase	Carbapenem-antibiotics[c], penicillins	3
bla_{OXA-48}	Carbapenem-hydrolysing class D β-lactamase (oxacillinases)		
bla_{OXA-58}			
sul1	Dihydropteroate synthase	Sulfonamides	2
tetM	Tetracycline resistance protein	Tetracycline	2
mcr-1	Phosphatidylethanolamine transferase	Polymyxins such as colistin[b]	3

[a] Amos et al., 2015, 2018; Gatica et al., 2016
[b] Antibiotics of last resort (WHO, 2017)
[c] WHO watch group antibiotics (WHO, 2017)
[d] 1 = persistent; 2 = abundant; 3 = problematic.

systems receiving the respective hospital wastewater. From their results the authors concluded that the relative contribution of the hospital wastewater was low in terms of dissemination of ARB to the WWTP. This might be typical for Norway, where the use of antibiotics for humans is generally lower than in other countries (e.g., 6.1 g/inhabitant in Norway but 9.1 g/inhabitant in Germany) and the percentage of hospital use is much lower, at 9%, compared to 25% in Germany (Wasteson et al., 2020). But also for the Netherlands, a detailed study by Pallares-Vega et al. (2019) found that the presence of hospitals was not significant for the concentrations of ARGs in the wastewater treatment plant influents.

The resistance, function and relevance of the genes represented by the specific markers that were analysed are given in Table 2. Relatively high concentrations of sul1 and the β-lactamase-genes bla_{TEM-1D}, bla_{OXA-48}, bla_{OXA-58} and $bla_{CTX-M-32}$ were found in all WWTP effluents. This is in accordance with the findings of Schwermer et al. (2018), who cultivated *E. coli* resistant against compounds of these groups from the effluent of WWTPs #1 and #2. Also, clinical studies in Norway had shown that 20–25% of *E. coli* isolated from urine were resistant against sulfonamides (FUNL, 2018).

The abundance of OXA-58 in the effluents of WWTPs #1 and #2 indicated the presence of carbapenemases posing resistance against carbapenems, which represent last-option treatments in many patients (WHO, 2017). The finding of bla_{OXA-48} and bla_{OXA-58} in the effluents is consistent with their rapid global spread in the environment, such as in bathing water (Bakthavatchalam et al., 2016; Mahon et al., 2017). In all WWTPs, bla_{TEM-1D} coding for ESBL class A hydrolysing penicillin, and $bla_{CTX-M-32}$ coding for ESBL with activity against cephalosporins were detected. This implies the prevalence of resistance against important penicillin β-lactam antibiotics and cephalosporins. This has not been reported in environmental settings in Norway before.

In WWTPs #1 and #2 effluents, mcr-1, which codes against colistin, a antibiotic of last resort that is very seldom used in Norway but intensely consumed in other countries, was detected. Our findings agree with those of Jørgensen et al. (2017), who also found mcr-1 on beaches along the receiving water body (Inner Oslofjord) into which WWTPs #1 and #2 discharge. Mcr-1 was recently found in high concentrations in WWTPs and receiving water bodies in Germany, where colistin is heavily used in domesticated animals (Hembach et al., 2017).

4.2. Contribution of WWTP #3 effluent flow to the river

The contribution of WWTP #3 effluent to the total volumetric flow rate of the river flow was calculated using Eq. (2). Over the three subsequent days during the sampling campaign and as a result of the decreasing water level after a flood had occurred prior to the campaign, the proportion of WWTP effluent to the total flow in the river increased with the decreasing river flow rate (Fig. 5). The same reciprocal relationship was also evident for river flow rates and the contribution of WWTP effluent discharge to the total river flow for the entire year of 2015. During average river flow, WWTP effluent discharges contribute 12% of the total flow and decrease to 4% during flooding due to dilution. Notably, at minimum river flow rates WWTP effluent discharges are shown to contribute 45% of the total flow in the river. Therefore, low-tide events might be critical regarding the contribution of ARGs in WWTP effluents to the river when considering hydraulic conditions

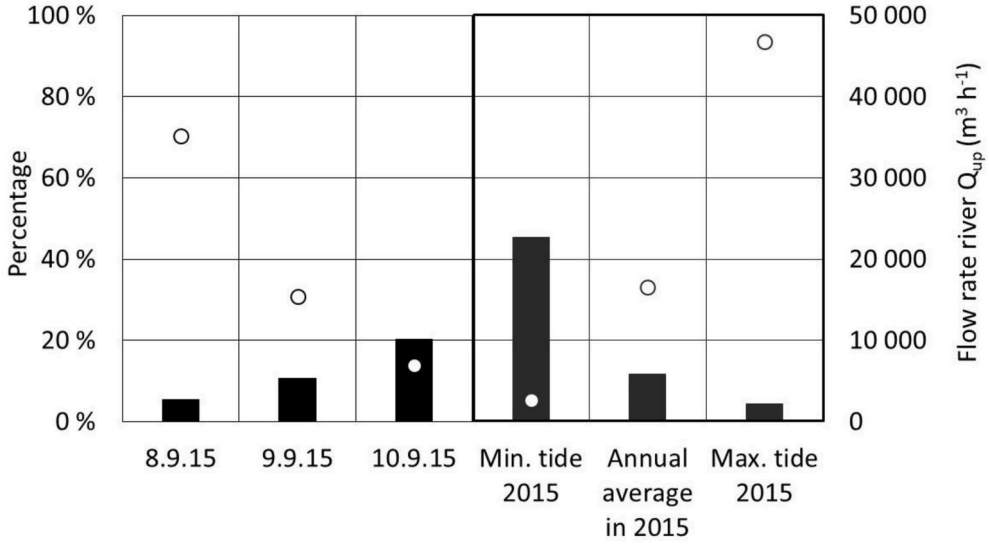

Fig. 5. Contribution of WWTP #3 effluent to the total flow in the river downstream of the discharge point (bars) and the volumetric flow rate of the river upstream (dots) for the three sampling days and for minimum, annual average and maximum water levels in the river during 2015.

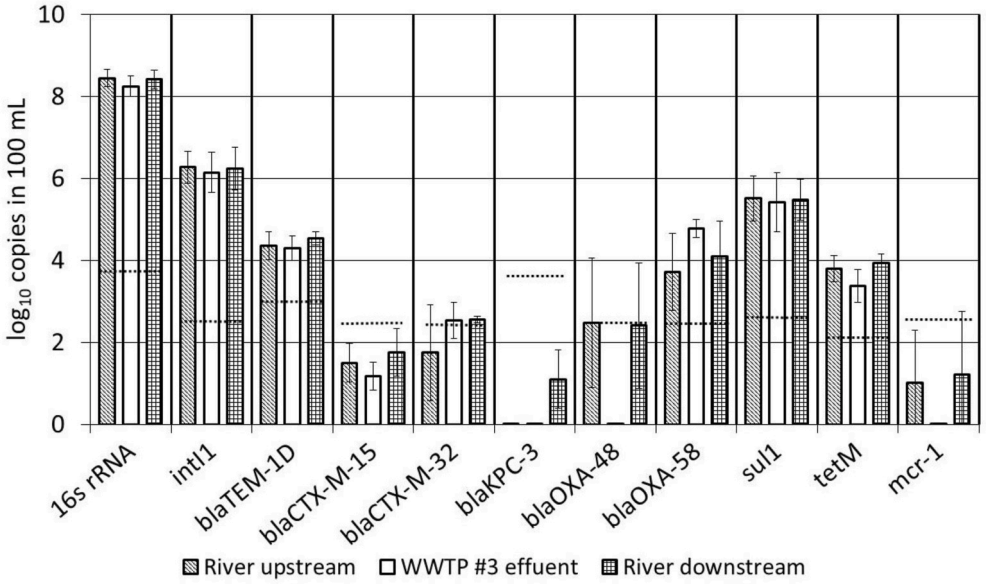

Fig. 6. Concentration of the measured genetic markers (in log copies per 100 mL) in WWTP #3 effluent and in samples collected from the river upstream and downstream of the WWTP #3 discharge. Each column represents average results from samples collected on three subsequent days. Error bars indicate 67% confidence intervals of n = 3 measurements made on the three consecutive sampling days. Dotted horizontal lines indicate the LOQ for each marker.

only. This problem may play a major role, particularly in rivers in warmer countries that receive WWTP discharges.

4.3. Load of genetic markers in the river and the effluent of WWTP #3

4.3.1. Concentration of genetic markers in the effluent, and in the river upstream and downstream of the discharge point

Considering LOQ and confidence intervals, samples collected in the river upstream and downstream of the WWTP #3 effluent discharge point contained the same concentrations of all genetic markers analysed. Also, the concentrations in the WWTP #3 effluent were equal to those in the river, except for bla_{OXA-48}, which was found exclusively in samples from the river but not in WWTP #3 effluent samples (Fig. 6). At first glance, the equal abundance of genetic markers at the sampling locations upstream and downstream of the discharge point is surprising, as other studies had shown ARG loads in the receiving water body to be lower upstream of the WWTP discharge point and higher downstream of the discharge point due to ARG emissions in WWTP effluents (Amos et al., 2018; Cacace et al., 2019; Jäger et al., 2018b). However, the volumetric contribution of the WWTP effluent to the river flow rates was not given in these publications, and ARG concentrations in the receiving rivers were lower than in the WWTP effluents. However, in our setting the ARG concentrations in the river were already high upstream of the discharge point. The river Nitelva is known to be polluted (characterised as of bad chemical and ecological status) by faecal bacteria [mostly from animals, such as aquatic birds (Vingerhagen et al., 2020);], along with organic matter and particles. Contamination derives from other highly polluted rivers that empty into the river upstream and downstream of the WWTP effluent discharge point and from diffusive pollution, including leakage from small WWTPs (Bjørndalen et al., 2011; DHI, 2014; NRA, 2017). According to Karkman et al. (2019), high genetic marker loads correspond to a high concentrations of faecal bacteria.

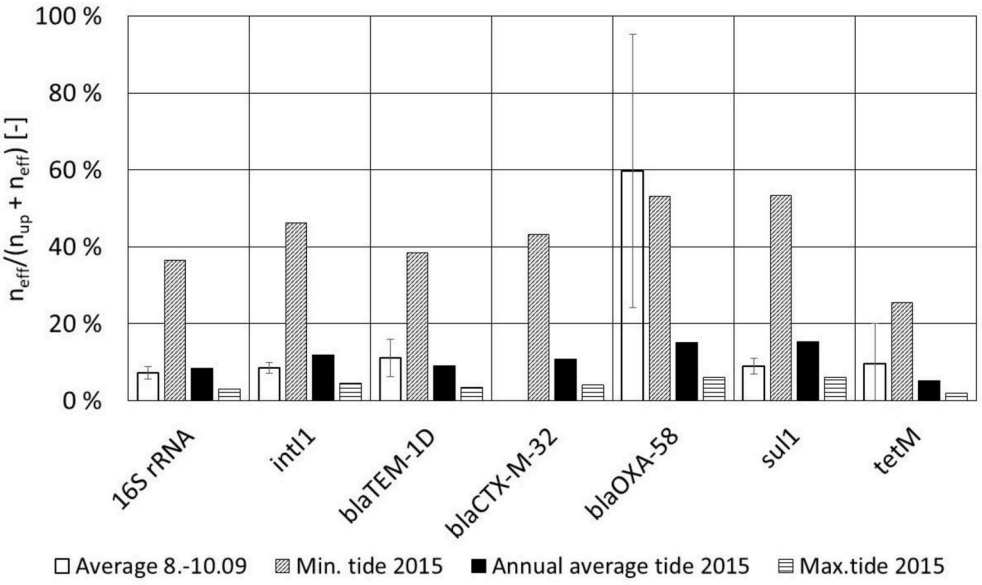

Fig. 7. Contribution of the load of genetic markers from the effluent (n_{eff}) to the total load of genetic markers in the effluent and river ($n_{up} + n_{eff}$) at the time of sampling and calculated for different river flow rates in 2015 measured at average flow in the river during the measurement campaign (white), and calculated results for annual average river flow (diagonal pattern), low tide (black) and maximum tide (horizontal pattern). Error bars indicate 67% confidence intervals of n = 3 measurements made on the three sampling days. Markers bla_{KPC-3}, bla_{OXA-48}, $bla_{CTX-M-15}$ and mcr-1 are not shown, as their concentrations in the WWTP effluent were below the LOQ.

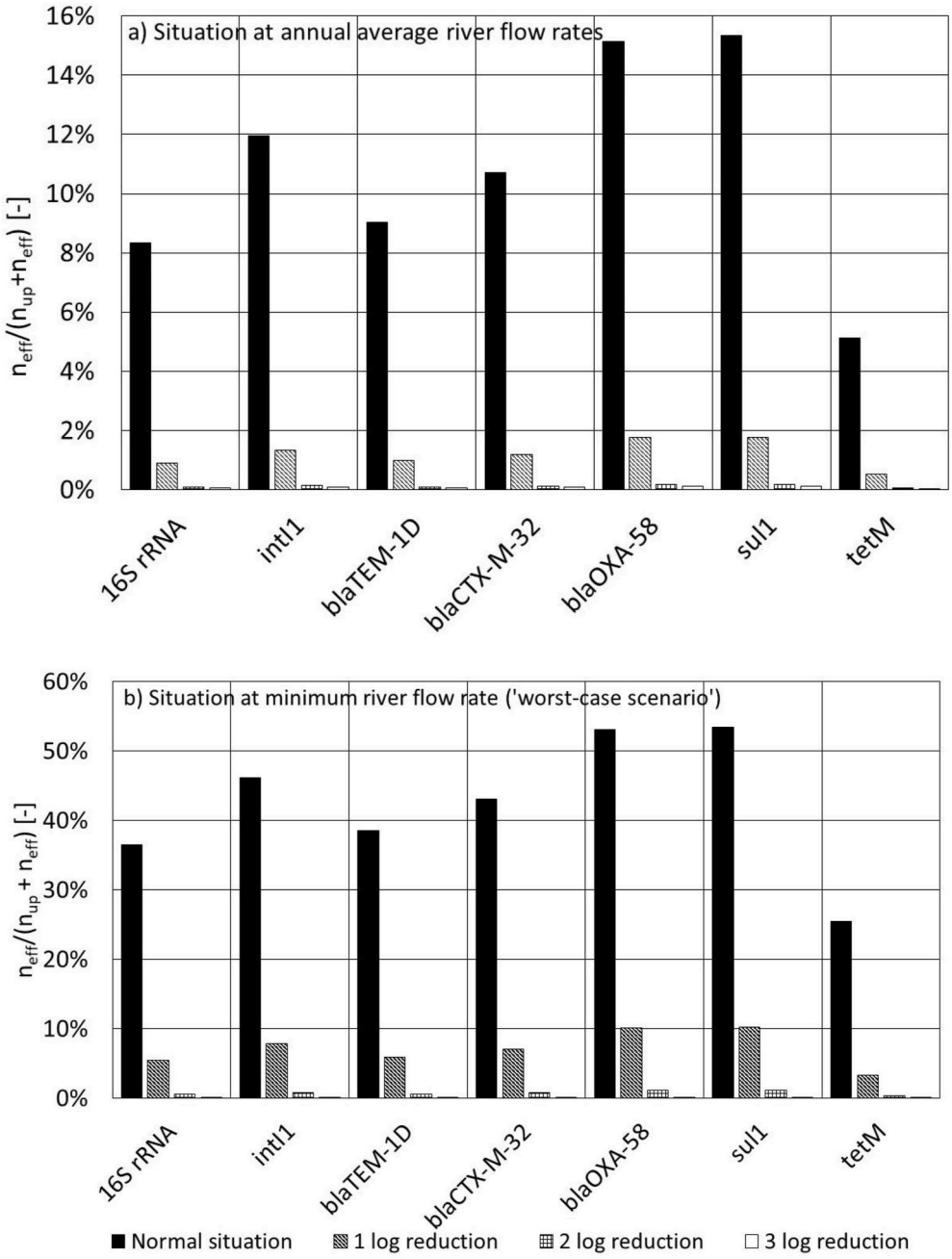

Fig. 8. Calculation of the effect of improved genetic marker removal by improved wastewater treatment: Contribution of the load of genetic markers from the effluent (n_{eff}) to the total load of markers in the effluent and river ($n_{up} + n_{eff}$), for current, 1-log, 2-log and 3-log additional marker removal effectivity. At annual average flow rate (**a**) and minimum flow rates (**b**) in the river.

Additionally, shortly before the sampling campaign a flood, due to heavy rainfall, had occurred, which might have resulted in additional genetic marker contamination of the river water from surface run-off (Almakki et al., 2019) and combined sewer overflows (Garner et al., 2017).

4.3.2. Calculation of the contribution of the genetic markers load from WWTP #3 effluent to the load in the river at different flow rates

The relative contribution of genetic markers in WWTP #3 effluent discharges to the total load of genetic markers in the river was calculated for different river flow rate scenarios by means of Eq. (4). For those genetic markers that were detected above the LOQ (see Figs. 6), Fig. 7 shows the contribution of genetic markers from the WWTP to the load of the respective markers in the river downstream of the discharge point at the time of sampling and calculated for different flow rates using the approach described above in Section 3.5.6. Data obtained during the sampling campaign and river flow rates at minimum, average and maximum were used for the calculation.

At the time of the sampling campaign, the wastewater treatment plant effluent contributed <12% to the load of genetic markers in the river for most markers, except for bla_{OXA-58} with 60%. This was evident for both the annual average river flow rate and at the flow rate occurring during the measurement campaign. However, the percentage contribution at low tide was calculated to increase significantly to between 23% ($bla_{CTX-M15}$) and 53% (bla_{OXA-58} and sul1) depending on the genetic markers, while at maximum tide this decreased to <6% for all genetic markers. In this 'worst-case scenario', the order of the most dominant genetic markers (in decreasing order of presence) was bla_{OXA-58} > sul1

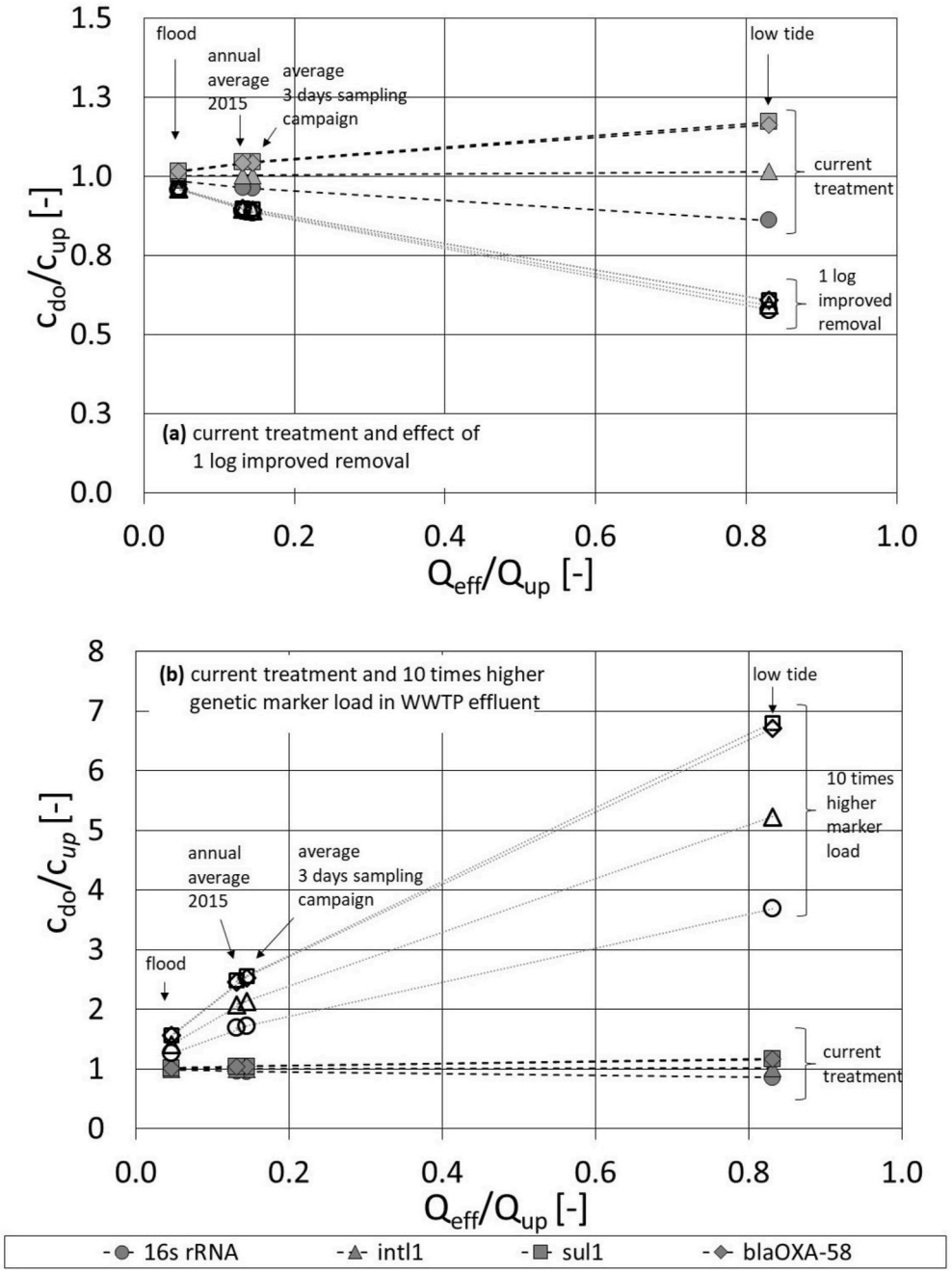

Fig. 9. Calculation (a) of the effect of improved (1-log) marker removal and (b) of 10-fold higher marker load in WWTP effluent on genetic marker concentrations in the river water downstream and upstream of the WWTP #3 discharge point. Calculations were done for annual average flow rates, high tide, low tide and average flow rates that occurred during sampling.

> intl1 > bla_{CTM-32}.

4.3.3. Calculation of the effect of improved final effluent treatment

The relative contribution of genetic markers in WWTP #3 effluent discharges to the total number of genetic markers in the river was modelled using Eq. (5) for scenarios of improved genetic marker removal (factors 10, 100 and 1,000, corresponding to 1-log, 2-log and 3-log lower effluent concentrations), as opposed to the current treatment effectivity (Fig. 8). Assuming the **average annual river flow rates** during 2015, at a 1-log reduction of the genetic markers in WWTP effluent the relative contribution of all genetic markers from WWTP effluent to the total marker genes in the river was reduced to approx. 2% in the case of bla_{OXA-58} and sul1, and to <1% for all other genetic markers. When assuming a 2-log and 3-log reduction, the relative contribution of all genetic markers are further reduced to <0.2% (Fig. 8a). Assuming the **minimum river flow rates** during 2015, the ratio of the genetic markers load from WWTP effluent in the resistome of the river was shown to range between 23 and 53% (Fig. 8b). As calculated for average annual flow rates, treatment improved by 1 log would reduce the proportion of genetic markers from WWTP effluent in the river to below 10%. Thus, treatment improvement will lead to a significant increase of genetic markers removal effectivity in the 'worst-case scenario'.

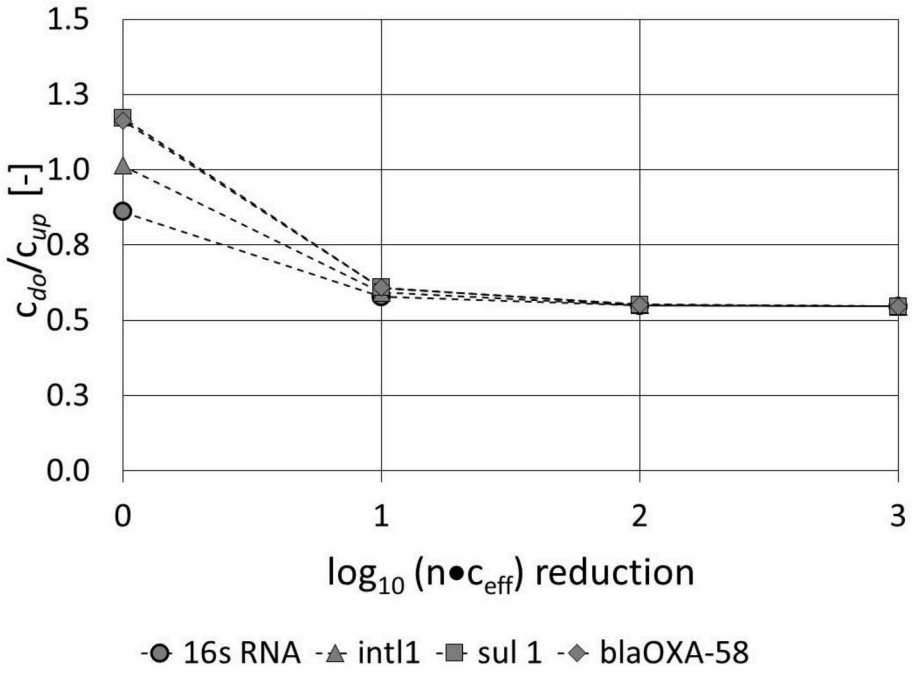

Fig. 10. Calculation of the effect of increased treatment effectivity (additional log reduction of genetic marker concentration in WWTP effluent) at low tide. Zero represents the treatment effectivity as of 2015.

4.4. Calculating the effect of WWTP #3 genetic marker removal effectivity at low and high river flow rates

The expected effect of improved or worsened genetic marker removal in wastewater treatment on the ratio of the genetic marker concentrations in the river downstream to those upstream (c_{do}/c_{up}) was calculated for different flow rate scenarios using Eq. (6). In Fig. 9, ratios of c_{do}/c_{up} above 1.0 indicate an increase in the concentration of genetic markers due to the flow from, and the concentration in, the WWTP #3 effluents. With the current treatment and at the annual average river flow rate of 2015, the contribution of markers in WWTP #3 effluent discharge is low (bold symbols in Fig. 9a and b). In this flow rate situation, the volumetric contribution of the WWTP #3 effluent (Q_{eff}/Q_{up}) is approximately 15%. With increasing volumetric river flow rate (i.e., decreasing Q_{eff}/Q_{up}), the proportion of genetic markers from WWTP #3 effluents decreases and the markers are increasingly diluted in the river. However, with a decreasing river flow rate (i.e., increasing Q_{eff}/Q_{up}), sul1 and $bla_{OXA\text{-}58}$ are increasingly added to the river due to the higher contribution of WWTP #3 effluent discharge to the river.

Calculating a scenario where the effectiveness of the WWTP #3 treatment for the removal of bacteria and genetic material is improved by 1 log, Fig. 9a shows that the ratio c_{do}/c_{up} will decrease for all the investigated genetic markers. This effect is far more pronounced at low river flow rates, as, for example, in a typical low-tide situation. Then, because of improved wastewater treatment, the river water quality will be improved, as the WWTP effluent will have a lower concentration of genetic markers than the river water upstream, and the WWTP effluent will be diluted in the river water. In contrast, at higher river flow rates, improving the wastewater treatment has a very little effect on the water quality in the river.

A scenario where the genetic marker concentration in WWTP #3 effluent discharges is increased by a factor of 10 (e.g., in the case of malfunctions, sewer overflow, or increased marker gene concentrations in the influent of the plant), is shown in Fig. 9b. Such a situation will result in increases in the c_{do}/c_{up} ratios for intl1, sul1 and $bla_{OXA\text{-}58}$. At the annual average river flow, this would result in a doubling of the c_{do}/c_{up} ratio. In low-tide situations, five-to seven-fold higher genetic marker concentrations in the river downstream than upstream are predicted. Thus, treating ARG-containing effluent to combat AMR spread in the river is particularly important in periods or cases with low river flow rates.

The calculations also suggest that a further improvement in the removal of genetic markers beyond 1 log will not notably improve the c_{do}/c_{up} ratios (Fig. 10), while the effect of further improvements in the removal of genetic markers (i.e., by 2 log or 3 log) will be very limited.

5. Summary and conclusions

In this study, the concentrations of genetic markers for studying ARGs in the effluents of three large Norwegian WWTPs were investigated. In addition, the concentrations of the respective genetic markers in the river upstream and downstream of the discharge point of one of the three WWTPs were measured. Using the data obtained, mass balance-based calculations were carried out to show the impact of WWTP effluents on the concentration of the respective genetic markers in the river. For the calculations, average annual, maximum and minimum flow rates were considered. Furthermore, calculations were done to show the potential effects of an upgrading of WWTPs for improved marker removal. Also, a scenario of 10-fold higher effluent marker concentrations, which might arise due to treatment malfunctions, sewage overflow or higher concentrations in the influent for any reason, was calculated. The main outcomes and conclusions are as follows:

- The total concentrations of all genetic markers in both the three investigated WWTP effluents and in the river were found to be quantitatively low compared to sites in other European countries (Amos et al., 2014a; Cacace et al., 2019; Pärnänen et al., 2019). However, all three monitored WWTPs were shown to emit the representative genetic markers analysed.
- Equally high concentrations of the genetic markers, considering the precision of the method, were found in river samples from upstream and downstream of the WWTP effluent discharge point. High loads of genetic markers in the river upstream are explained by pollution with intestinal bacteria [most likely arising from animals such as aquatic birds (Vingerhagen et al., 2020)], from other connected rivers and from surface run-off.

- At annual average river flow rates, the WWTP #3 effluent contributes between 5 and 15% to the load of these genetic markers in the river, depending on the type of marker.
- Mass balance-based calculations showed that for the annual minimum flow rate the situation turns dramatic. Then, in the river downstream of the discharge point, between 22 and 55% of the load of the respective genetic markers will originate from the WWTP #3 effluent.
- Scenarios of improved or worsened removal of genetic markers in WWTP #3 showed that a 1-log removal will considerably improve the river water quality. Then, at average flow rate, the contribution to the genetic marker load in the river originating from WWTP #3 will be less than 2%, compared to 5–15% under the current treatment. Even at the minimum annual flow rate of the year of sampling, 1-log improved removal would decrease the WWTP #3 contribution to the load in the river to less than 10% (from 22 to 55%). Malfunctions of the WWTP #3 in removal effectivity by 1 log, or higher influent concentrations of genetic markers, would result in a considerable contribution of the WWTP #3 effluent to the genetic marker concentration in the river. Especially at very low flow rates, the contribution of the WWTP #3 effluent to the genetic marker concentration in the river is expected to be between 75 and 92%.
- Considerations of implementing an advanced treatment stage at WWTPs for the polishing of the final effluent for contaminants of emerging concern (CECs) should also include the removal of ARGs, due to their possible high risk potential.

Generally, the results demonstrate the importance of considering the flow rates and hydrologic characteristics of the recipient water body when judging the contribution of WWTP effluents to the load of genetic markers in the recipient. Flow rates or other patterns might have been untypical at the times of sampling. This is even more relevant when decisions on further treatment of the WWTP effluent are to be made.

Credit author statement

C. U. Schwermer: Project administration, conceptualization, investigation, formal analysis, programming, writing, review & editing, funding acquisition, **W. Uhl**: Methodology, formal analysis, writing, review & editing, quality assurance.

Declaration of competing interest

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

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Abbreviations, acronyms and symbols

16 S rRNA	16 S Ribosomal ribonucleic acid
AMR	Antimicrobial resistance
ARB	Antibiotic resistant bacteria
ARG	Antibiotic resistance gene
c	Concentration
COD	Chemical oxygen demand
d	Depth
$\overline{d}$	Average water depth
l	Length
log	Logarithm
LOQ	Limit of quantification
MBBR	Moving-bed bioreactor
MF	Membrane filtration
n	Load of marker genes per time
NF	Nanofiltration
OZ	Ozonation
PAX	Polyaluminium chloride hydroxide
Q	Volumetric flow rate
RO	Reverse osmosis
UV	Ultraviolet irradiation
v	Distance flow rate
w	Width
WW	Wastewater
WWTP	Wastewater treatment plant
Y	Factor for improved removal effectivity
subscripts	
i	sampling day
eff	effluent
up	upstream
do	downstream
r	river

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