High-throughput profiling of antibiotic resistance genes in wastewater: comparison between a pond system in Namibia and an activated sludge treatment in Germany

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ABSTRACT

There are increasing concerns about wastewater treatment plants (WWTPs) acting as hotspots for antibiotic resistance genes (ARG). However, their role largely depends upon the treatment methods and antibiotics in the wastewater. To better understand these influences, we compared the occurrence and fate of ARG between a pond system in a developing country (Namibia) and an advanced WWTP (activated sludge system) in a developed country (Germany). A targeted metagenomic approach was used to investigate the wide-spectrum profiles of ARGs and their co-occurrence patterns at both locations. In total, 93 ARG subtypes were found in the German influent wastewater, 277 in the Namibian influent wastewater. The abundant ARG types found in Namibia and Germany differed, especially for multidrug resistance genes. The differences in occurrence and reduction can help to understand the performance of simple WWTP such as pond systems common in Namibia, where direct contact with wastewater is a potential risk for contamination.

Key words | antibiotic resistance, Namibia, pond system, targeted metagenomics, wastewater treatment

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HIGHLIGHTS

- Targeted metagenomic analysis of the occurrence and fate of the antibiotic resistance genes (ARGs) in a pond system in Namibia.
- Low abundance of ARGs associated with tetracycline, trimethoprim, and beta-lactam in the pond effluent than in the influent.
- Lower abundance of Acinetobacter in the pond effluent than in the influent.
- Pond systems have potential in the removal of ARGs and pathogens.

INTRODUCTION

The extensive and anthropogenic use of antibiotics in human and veterinary medicine, as well as agriculture, accounts for one of the most threatening health-care problems of our time: the spread of antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARG). Many studies stated wastewater treatment plants (WWTP) as so-called hotspots for ARG and ARB due to their high bacterial densities and diversities. In WWTPs, the combination of complex networks of microbial populations and the adaptation to the selective pressure of antibiotics can accelerate the acquisition of ARG by lateral transfer from donor species (Lupo *et al.* 2012; Pärnänen *et al.* 2019). Finally, ARG and ARB are released into the environment, if no advanced treatment is implemented downstream of conventional biological processes before effluent disposal (Laxminarayan *et al.* 2013).

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Several studies investigated the abundance of ARG and ARB in WWTP with a focus on influent and effluent concentrations in industrialized countries. For example, Pallares-Vega *et al.* (2019) analyzed ARG in 62 Dutch WWTPs, still discharging on average 10^6 copies of ARG per liter of effluent into the environment from conventional WWTPs. Hembach *et al.* (2017) investigated the abundance of the colistin resistance gene (*mcr-1*) in seven different WWTPs in Germany. The *Mcr-1* gene was not eliminated during wastewater treatment, and moreover, the beta-lactam resistance genes *CTX-M-32, blaTEM, CTX-M, CMY-2* were found in high abundance in the WWTPs' effluent. Beta-lactam antibiotics represent about 50% of all prescribed antibiotics in Germany in 2018 (World Health Organization 2018). Müller *et al.* (2018) followed the dissemination of extensively drug-resistant (XDR) bacteria from clinical wastewater through a WWTP into the receiving surface waters and found that current WWT is not capable of inhibiting the dissemination of XDR genes and bacteria into these surface waters. These recent studies have raised questions with the operators of WWTP and researchers for new technologies and strategies to eliminate or reduce the spread of ARB and ARG into the environment. At present, there is no consensus about the best technology/strategy for elimination or even reduction of ARB and ARG (Rizzo *et al.* 2013), which is also economically feasible.

On one side, developed countries are discussing the implementation of additional treatment stages at WWTP to remove ARG and ARB. On the other side, developing countries are still struggling with the management and treatment of their wastewater in general, even though it carries the same threats in handling only with less investment capital in combination with a worse medical system, as it is easier to access drugs without a prescription (Laxminarayan *et al.* 2013; Nnadozie *et al.* 2017). Studies with inhabitants and medical personnel in Windhoek showed that there is a variety of first-line choice antibiotics. The most commonly used is the combination of amoxicillin with clavulanic acid, amoxicillin, and ciprofloxacin (Pereko *et al.* 2015).

In developing countries like Namibia, wastewater treatment is given a lower priority due to high costs and high maintenance. It is difficult to implement WWTP country-wide and activated sludge (AS) systems might also be unfeasible or even impossible to use due to constant- and high-energy demands and operational challenges. Besides adding further treatment steps for ARG/ARB removal, similar to the ones currently discussed in Germany, it seems impossible for Namibia because it imposes a significant financial burden. At present, mostly low-tech systems are used instead, including stabilization pond systems, septic tanks, and wetlands. The most widely used systems are stabilization ponds due to their low cost of installation and maintenance and optimum climatic conditions (Kivaisi 2001).

Additionally, Namibia is a semi-arid region dealing with increasing water scarcity with a high demand for new low-tech concepts for direct water reuse, especially for irrigation. In semi-arid regions, wastewater may constitute 25–75% (UNEP 2002) of the available irrigation water. This emphasizes that the reuse of wastewater requires consideration for health impact, among other factors. However, there is a lack of knowledge about the current spectrum of pathogens (including emerging and zoonotic) and especially ARG present in Namibian wastewater and the role of pond systems in their removal. Therefore, in this study, we focused on the comprehensive profiling of ARGs and pathogens in Namibian wastewater. Also, we compared the occurrence and fate of the ARGs and pathogens in the pond system in Namibia with the AS system in Germany.

MATERIALS AND METHODS

Sampling

Samples from the influent and effluent were collected from a pond system in Namibia and a WWTP in Germany, respectively. Physical and chemical parameters characterizing the water quality of these samples are included in the Supplementary Information (S.Table 1). The pond system is located in Northern Namibia at a town with an estimated 11,000 inhabitants in 2018 (Mwinga *et al.* 2018). In order to determine how different or similar is the composition of the ARGs and pathogens in (un)-treated wastewater in the pond system to the one in a more advanced wastewater treatment system in a developed country, we took samples from a WWTP with a conventional AS system in central Germany. The pond system was initially constructed in

2004 for 2,000–2,500 inhabitants. It consists of two parallel lines (A and B) with four ponds each, one primary facultative pond, followed by three maturation ponds and one common evaporation pond (Figure 1). Further details of both systems are provided in the Supplementary Information (S.Table 2).

For both the WWTPs, three grab samples for influent and effluent were collected. Three times 5 L wastewater was collected, in new 5 L carboys, at the entry and discharge point of the wastewater at the pond and AS system. After sampling, three 50 mL aliquots were prepared in sterile 50 mL centrifuge tubes for each grab sample. Immediately, the centrifuge tubes were centrifuged at 8,000 × g at 4 °C for 25 min. Pellets were used for DNA extraction and the supernatant was discarded. In the case of Namibia, pellets were stored at 4 °C overnight and brought back to Germany at room temperature the next day for further downstream analysis. In the case of Germany, after sampling, carboys were directly brought to the laboratory, and the pellet was obtained as mentioned above. The pellets were stored at 4 °C for DNA extraction. In the case of the Namibia samples, it took around 38 h from the time of the sample until the samples arrived in the laboratory, and immediately DNA extraction was performed. For the German sample, pellets were kept at 4 °C for the same duration till DNA extraction was performed. Total genomic DNA was extracted using the Fast DNA Spin kit for soil (MP Biomedicals) according to a modified manufacturer's protocol (Orschler *et al.* 2019). DNA concentration was analyzed using Qubit 3.0 Fluorometer with Qubit dsDNA HS kit (Thermo Fisher Scientific).

16S rRNA gene amplicon sequencing

For each sample, multiple hypervariable regions of 16S rRNA genes were amplified with the 16S Ion Metagenomics Kit™ (Thermo Fisher Scientific) by two separate PCR reactions, amplifying the V2, V4, V8 and V3, V6–7, and V9 hypervariable regions, according to the kit protocol. Both primer pools were utilized to create two unique libraries for each sample. For each sample, two PCR reactions (one for each of the two primer pool) were prepared. Each PCR reaction consists of 3 μL 10× primer mix, 6 μL of each sample, 15 μL 2× mastermix, 6 μL nuclease-free water. PCR condition was 95 °C for 10 min, 20 PCR cycles consisting of a 95 °C for 30 s; 58 °C for 30 s; 72 °C for 20 s, and 72 °C for 7 min. Equal volumes of V2, V4, V8 and V3, V6–7, and V9 amplicons were combined. 100 ng of pooled amplicons were processed to the amplicon library using the Ion Xpress Plus Fragment Library Kit™, and each sample was tagged using the Ion Xpress Barcodes Adapters™ (Thermo Fisher Scientific), according to the manufacturer's protocol. Each sample was adjusted to a 10 pM concentration. All the samples were pooled, in equal volumes, and processed with One-Touch 2 system (Thermo Fisher Scientific) according to the manufacturer's instructions. Sequencing was performed on the Ion Torrent

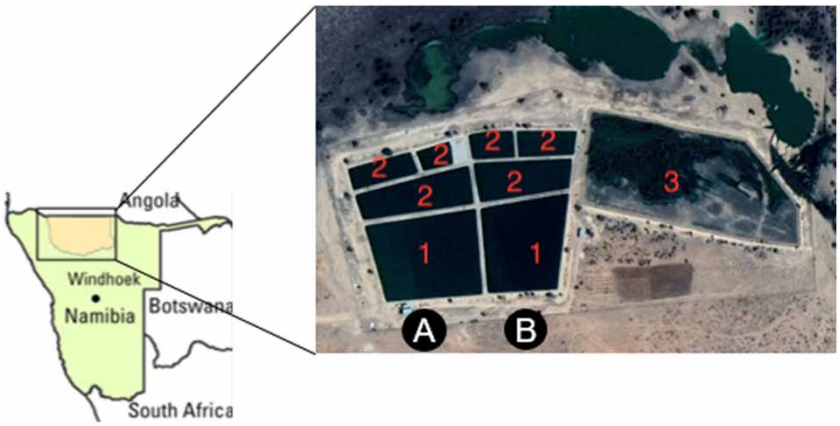

Figure 1 | Study area: pond system in Northern Namibia. It consists of two parallel lines (A and B) with four ponds each, one primary facultative pond marked as (1), followed by three maturation ponds marked as (2) and one common evaporation pond marked as (3). Left picture source: http://www.swissfot.ch/htm_public_d/wappen/world/Af/Africa_Umriss_Laender.html.

(ION Torrent Ion S5) using the 400-bp kit and 530 chip. Base calling and run demultiplexing were conducted by Torrent Suite version 4.4.2 (Thermo Fisher Scientific) with default parameters. DADA2 was implemented for separating sequences for each sample; filtering the low quality and limiting the length of sequences >260 bp; filtering sequences with potential chimera. After filtering and trimming, 482,572 high-quality reads were obtained from the samples (110,779–123,931 reads per sample). To reduce any sequencing bias, the datasets were randomly subsampled at 75,000 reads per sample and then ASV (amplicon sequence variant) picking with its default settings was performed. Overall, the *de novo* clustering of ASVs was done with 97% identity, corresponding to species level. The sequences were classified based on the taxonomy in the Silva database (97% confidence threshold, version 132). The sequencing data were analyzed in R, using ggplot2 (v0.9.3.1). For the profiling of the pathogens, we manually created a database of 1,415 known pathogens (which include known-, emerging-, and zoonotic pathogens) based on the previous study (Taylor *et al.* 2001). Then, 16S rRNA amplicon sequencing data which were annotated based on the Silva database were searched using an in-house script against the manually created database for the potential genera containing known pathogens. Hereafter in this study, we refer to genera containing known pathogens as 'pathogenic genera'. ASV representative sequences have been submitted to the GenBank under the project number PRJNA594917.

Targeted metagenomics for ARG

Targeted metagenomics was performed on the Ion Torrent (ION Torrent Ion S5) using the AmpliSeq™ Antimicrobial Resistance (AMR) panel (Thermo Fisher Scientific) and the 530 chip. The Ion AmpliSeq™ AMR panel contains 2 primer pools to assess the presence of 478 ARGs to help detect 28 different antibiotic classes. Library preparation was performed on an IonChef instrument (Thermo Fisher Scientific) which performs PCR amplification, barcoding of samples and pooling of libraries. The PCR conditions were as follows: 2× primer mix, 6 μL of each sample and 16 PCR cycles consisting of a 99 °C for 15 s; 60 °C for 4 min. Both primer pools were utilized to create two unique AmpliSeq™ libraries for each sample. The list of primers is available upon request from Thermo Fisher Scientific (https://www.thermofisher.com/), these primers result in maximum 375 bp long amplicons. Base calling, run demultiplexing, and read filtering were conducted by Torrent Suite version 4.4.2 (Thermo Fisher Scientific) based on quality score (≤0.05), ambiguous nucleotides (≤2) and filtered on read length (≥75 bp). Subsequently, all the targeted metagenomics sequencing data were searched for ARG against the CARD v 3.0.5. (Comprehensive Antibiotic Resistance Database), which includes all the targeted 478 ARGs. For annotation, the CARD database v 3.0.5 was downloaded, and DIAMOND v0.7.0.49 was used for the annotation of the sequences. Only hits with sequence identity >95% were kept. The annotation output was converted into a count table using an in-house python script.

Bioinformatics analysis

Heatmaps, barplots, and venns diagrams were all generated in R (http://www.R-project.org/). The dendrogram heatmap was created with the 'pheatmap' package, which assesses the uncertainty in hierarchical cluster analysis.

RESULTS

Occurrence and fate of pathogens

The relative abundances of different phyla in the samples are shown in Figure 2(a). It was found that the phylum Proteobacteria dominated in all samples except the AS effluent. In the pond influent, Proteobacteria accounted for 48% relative abundance, followed by Firmicutes (35%) and Actinobacteria (15%), as dominant phyla, whereas in the pond effluent, Proteobacteria (56%) and Actinobacteria (27%) were dominant. Firmicutes accounted for 5% and Cyanobacteria for 4% relative abundance in the pond effluent. In comparison to the pond samples, the relative abundance of Proteobacteria was lower in the AS influent (40%) and effluent (26%). Except for Proteobacteria, other abundant phyla were in the following descending order of abundance: Firmicutes (30%) > Bacteroidetes (12%) > Actinobacteria

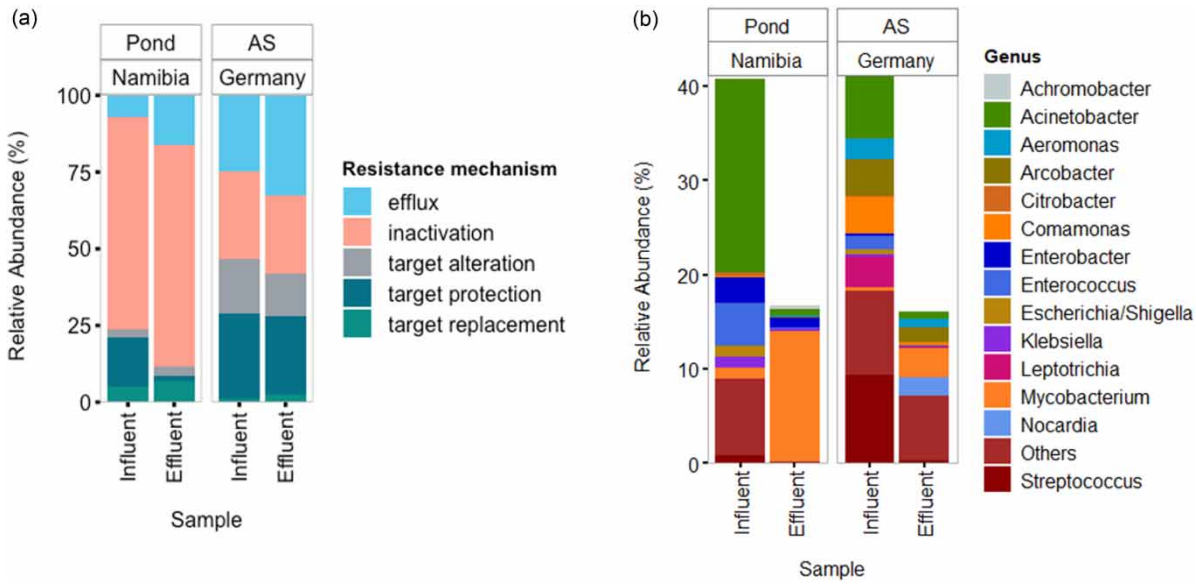

Figure 2 | (a) Microbial community structures in the samples at phylum level. Phylum having ≤1% relative abundance are grouped as 'Others.' (b) Abundance of pathogenic genera found across the samples. Pathogenic genera having ≤1% relative abundance are grouped as 'Others.' Pond: pond system in Namibia, and AS: activated sludge system in Germany.

(8%) in the AS influent; and Actinobacteria (8%) > Patescibacteria (23%) > Bacteriodetes (9%) > Firmicutes (5%) in the AS effluent.

Influent and effluent samples from the pond and AS system were analyzed for 1,415 previously reported known pathogens (which include known-, emerging-, and zoonotic pathogens) (Taylor *et al.* 2001). It is important to note that characterization of the pathogens is based on a database which was created in 2001. In total, 63 genera containing known pathogens were found across the samples (Figure 2(b)). Among the detected genera, 41 genera occurred in the AS, and 45 occurred in the pond samples. The total relative abundance of pathogenic genera in both influents was nearly the same (accounting for 41% of the total microbial population in influent of both systems) (Figure 2(b)). The majority of the detected dominant pathogenic genera (which includes *Acinetobacter, Citrobacter, Comamonas, Enterobacter, Enterococcus, Escherichia, Klebsiella, Mycobacterium*, and *Streptococcus*) were found in both the influents. *Acinetobacter* was dominant (21% of the population) in the pond influent, and *Streptococcus* was dominant (9% of the population) in the AS influent. The total fraction of pathogenic genera reduced in the pond as well as AS system, i.e. to 17% in the pond effluent and 16% in the AS effluent, but the abundance of *Mycobacterium* increased in both the effluents (from 1 to 14% in the pond; and 0.3 to 3% in the AS). Among the detected pathogens, some are zoonotic (pathogens that are naturally transmitted between humans and other vertebrates) (Woolhouse 2002) (Supplementary Information, S.Figure 1). The fraction of zoonotic pathogens was higher in the AS influent (16%) than in the pond influent (4%), but the abundance reduced in the effluents at both locations (3% in the AS and 0.1% in the pond).

Occurrence, fate, and resistance mechanism of ARGs

Through the targeted metagenomic analysis approach, a total of 1,896,047 reads for pond influent, 1,763,145 reads for pond effluent, 1,754,794 reads for AS influent, and 1,801,123 reads were annotated against CARD database (v 3.0.5.). Overall, 20 ARG types were identified in the samples (Figure 3(a)). In the pond system, ARG types encoding resistance to aminocoumarin and sulfonamide antibiotics were missing, while genes conferring resistance to carbapenem, cephamycin, fosfomycin, and nucleoside antibiotics were absent in the AS system. ARG types encoding for multidrug resistance were most abundant in the pond and AS samples. However, it was less abundant in AS effluent (35%) than in AS influent (50%), and no significant

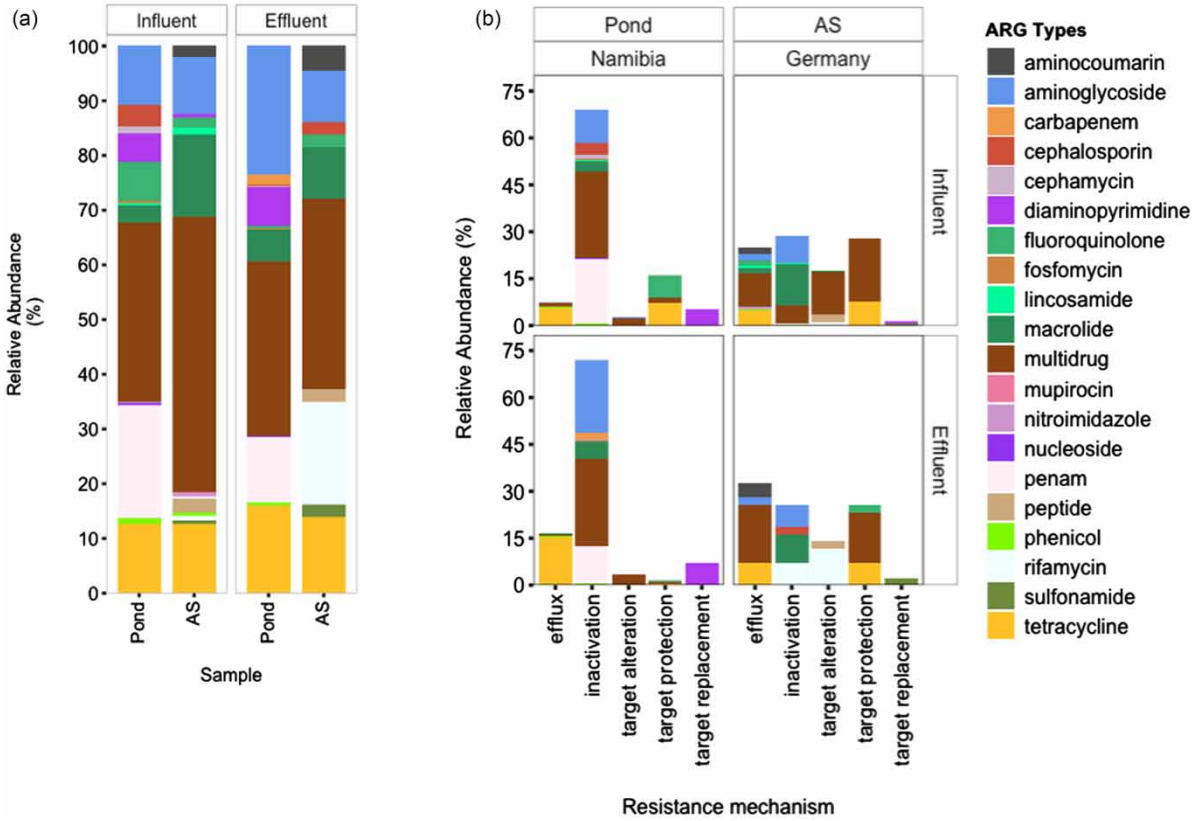

Figure 3 | (a) Composition of the ARG types encoding resistance to different drug class. (b) Relative abundance of resistant mechanisms.

difference was between the influent (33%) and effluent (32%) of the pond system. The ARG types associated with tetracycline and aminoglycoside had similar abundance in both influents. The ARG types encoding for penam (a subgroup of *β*-lactams antibiotics which is commonly known as 'penicillin') resistance were significantly abundant in the pond influent than in the AS influent, and macrolide-associated ARG types were high in abundance in the AS influent than the pond influent. In the pond effluent, the relative abundance of the five ARG types was higher than in the influent: aminoglycoside (11 → 23%); carbapenem (0 → 2%); diaminopyridimine (5 → 7%); macrolide (3 → 6%); and tetracycline (13 → 16%). In the AS effluent, a higher abundance of the ARG types encoding resistance to aminocoumarin (2 → 5%), cephalosporin (0 → 2%), and rifamycin (17 → 1%) was observed.

Figure 3(b) shows the relative abundances of resistance mechanisms in the samples. Inactivation was the predominant resistance mechanism in pond samples (relative abundance of 69% in influent and 72% in effluent), which was mainly associated with resistance to aminoglycosides, *β*-lactams, macrolides, and multidrug. In AS samples, efflux, inactivation, and target protection were the most abundant resistance mechanism, accounting for 25, 29, and 28% in the influent; and 28, 26, and 26% in the effluent of all the ARG types. Efflux resistance mechanism was associated with resistance to aminocoumarin, aminoglycosides, tetracycline, and multidrug; and target protection was associated with resistance to tetracycline and multidrug.

From a total of 478 ARG subtypes targeted with Ion AmpliSeq™ AMR panel, 363 ARG subtypes were identified in the samples. Out of the 363 ARG subtypes, 277 were found in the pond influent and 157 in the pond effluent; 93 were found in the AS influent and 23 in the AS effluent (Figure 4(a)). On comparing all the samples (i.e. influent and effluent sample of the pond and AS system), we found that only five ARG subtypes (*mphD*, *EreA2*, *tet44*, *aadA5*, and *tet(C)*) were shared across the samples (Figure 4(a)).

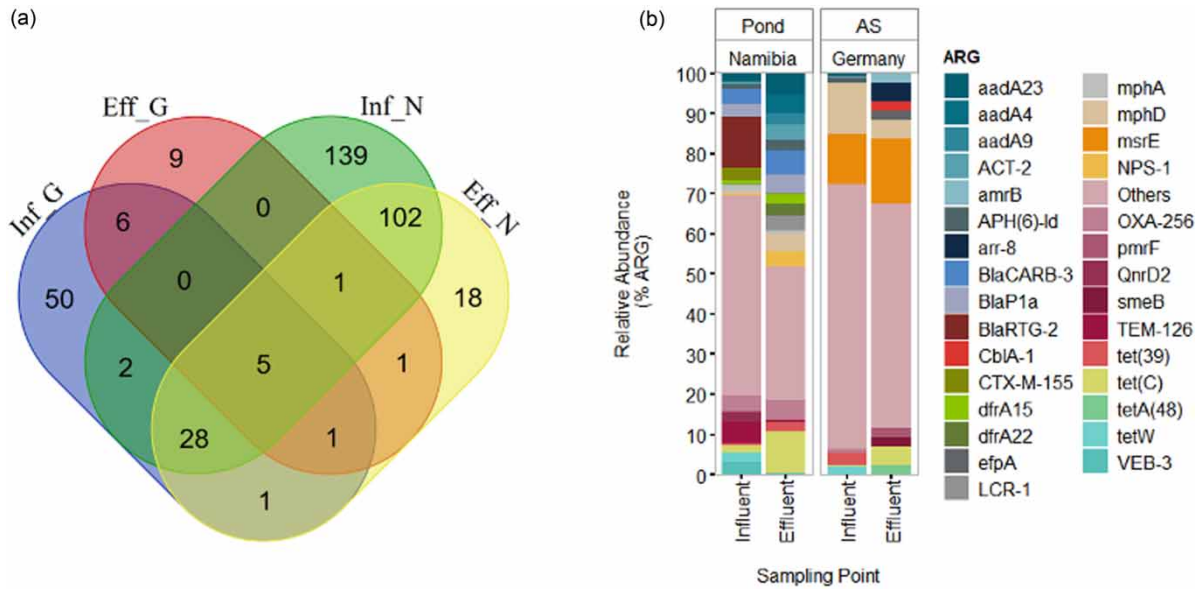

Figure 4 | (a) Venn diagram showing the number of shared and unique ARG subtypes between the pond and AS samples. (Inf_G: Influent AS; Eff_G: Effluent AS; Inf_N: Influent pond). (b) The relative abundance of the top 30 ARG subtypes found across the samples. Remaining ARG subtypes are grouped as 'Others'.

The top 30 most abundant ARG subtypes found in the samples accounted for 50% in the pond influent, 67% in the pond effluent, 34% in the AS influent, and 26% in the AS effluent of the total ARG abundance (Figure 4(b)). The influent of the pond system showed a high abundance of ARG for beta-lactam (i.e. 13% of the *BlaRTG-2* gene), whereas, with 10% abundance, the *tet(C)* resistance gene for tetracycline was dominant in the effluent. Two resistance genes for macrolide–lincosamide–streptogramin (MLS) (13% *mphD* and 13% *msrE*) were dominant in the influent, whereas in effluent of the AS system, only *msrE* (16% relative abundance) was dominant.

Figure 5 shows that the 25 most abundant ARG subtypes in the samples clustered in two groups based on the 'complete' method of the pheatmap, using Euclidean distance between the ARG subtypes as the similarity measure. Also, the relationships of the ARG among samples were assessed, and the four samples could be divided into three clusters, with the influent AS and effluent AS samples forming two individual clusters, and the pond samples forming the third cluster. Different dominant ARG associated with different antibiotic classes were observed in the pond, as well as the AS system. The ARG for tetracycline (*tetM*), trimethoprim (*dfrA14*), and beta-lactam (*OXA-164*) exhibited higher *Z*-scores in the influent of the pond than in the effluent (Figure 5). However, ARG for aminoglycoside resistance (*aadA13*, *aadA23*, *aadA6/aadA10*, and *APH(6)-Id*), MLS (*ErmF*), tetracycline (*tet(C)*, *tet(G)*, and *tet(39)*), and beta-lactam (*OXA-256*) exhibited higher *Z*-scores in the pond effluent. A similar trend was also observed in the AS system. *Z*-scores of the ARG for MLS (*mefA*, *mel*, *ErmB*, and *mphD*) and tetracycline (*tetW* and *tetQ*), which were dominant in the influent, decreased in the effluent (Figure 5). However, ARG for aminoglycoside resistance (*APH(6)-Id* and *aadA5*), tetracycline (*tet44*), rifamycin (*rpoB*), macrolide (*mtrA*, *EreA2*), quinolone (*QnrS6*) were dominant in the AS effluent.

We also compared the relative abundance of previously reported clinically relevant ARG types (Szczepanowski *et al.* 2009). We detected 14 ARG types in the samples, some being specific to the location (Figure 6). The impact of the WWTP on the removal of ARG differed for different ARG. Some ARG types were removed at both locations, but some only at a specific location. For example, the abundance of the *APH* gene decreased in the effluent of the pond (i.e. from 4% in the influent to 3% in the effluent) but increased in AS system (from 2 to 5%). The opposite was observed for the *Erm* gene. In the AS system, the *Erm* gene was not detected, whereas in the pond system, the relative abundance increased 1.5 times. Apart from removal, the

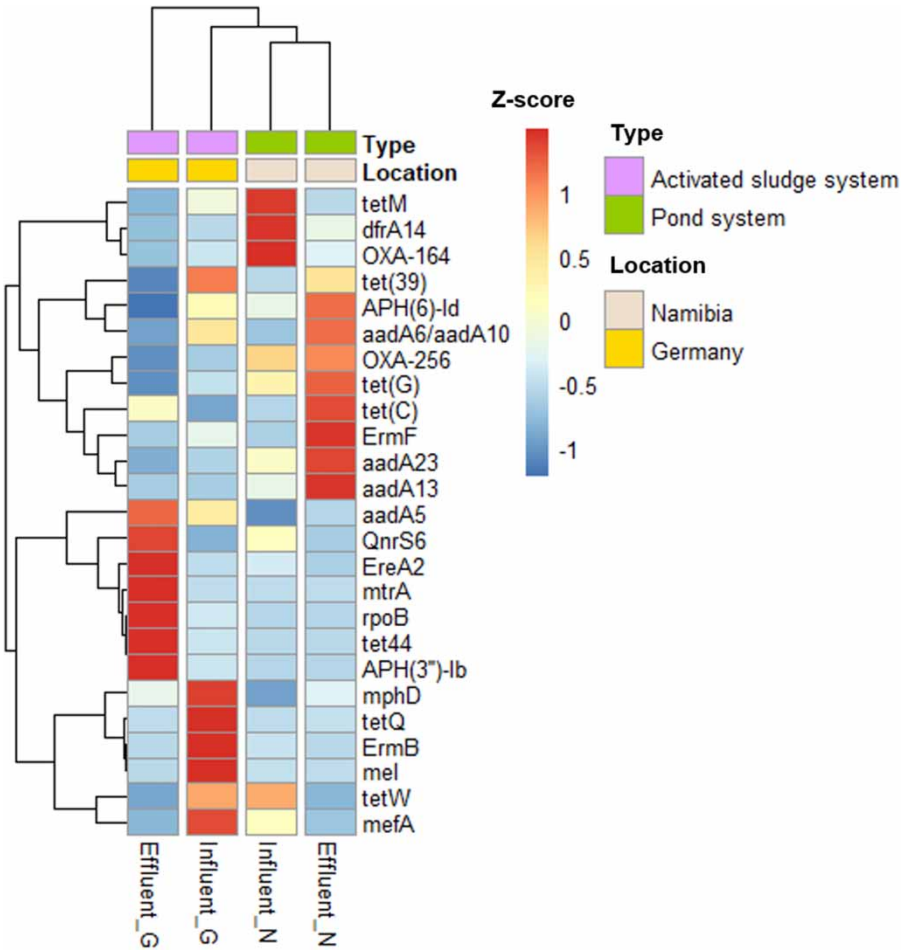

Figure 5 | Heatmap visualizing the *z*-score distribution of the top 25 ARG subtypes found across the samples. The *z*-score represents the differences of relative abundances of the top 25 ARG subtypes among the samples. *Z* is negative for any ARG subtype in any sample when the actual abundance is below the mean of the abundance of that ARG subtype across all the samples and vice versa. $Z = (x - m)/s$, where *x* is the relative abundance of ARG subtype in each sample, *m* is the mean value of relative abundances of ARG subtype in all samples, and *s* is the standard deviation of the relative abundances. Sample names are listed on the *x*-axis and ARG names on the *y*-axis.

enrichment of clinically relevant ARG was observed. In Germany, abundances of ARG for tetracycline (*tet*), sulfonamide (*sul*), quinolone (*Qnr*), beta-lactam (*CblA*), and aminoglycoside (*APH*) increased in the effluent, whereas in Namibia abundances of ARG for *tet*, and MLS (*Erm*) increased in the effluent.

DISCUSSION

The major purpose of this study was to determine the similarities and differences between the resistome and pathogen profiles of wastewater in Namibia and Germany. Moreover, we assessed whether a pond system (Namibia) can remove pathogens and ARG in comparison to an AS system (Germany). Although the relative abundance of pathogenic genera found in the influent of the AS and pond system was comparable, the relative abundance of *Acinetobacter* was distinctly higher in the influent of the pond system (Figure 2(a)). *Acinetobacter* spp. are generally considered to be part of a normal microbiome of the skin. They are also accountable for a wide variety of infections (Almasaudi 2018) and known for carrying resistance genes for amoxicillin, chloramphenicol, and colistin (Zhang *et al.* 2009). Extensive use of amoxicillin (a beta-lactam antibiotic) in Namibia (Pereko *et al.* 2015) and dominance of

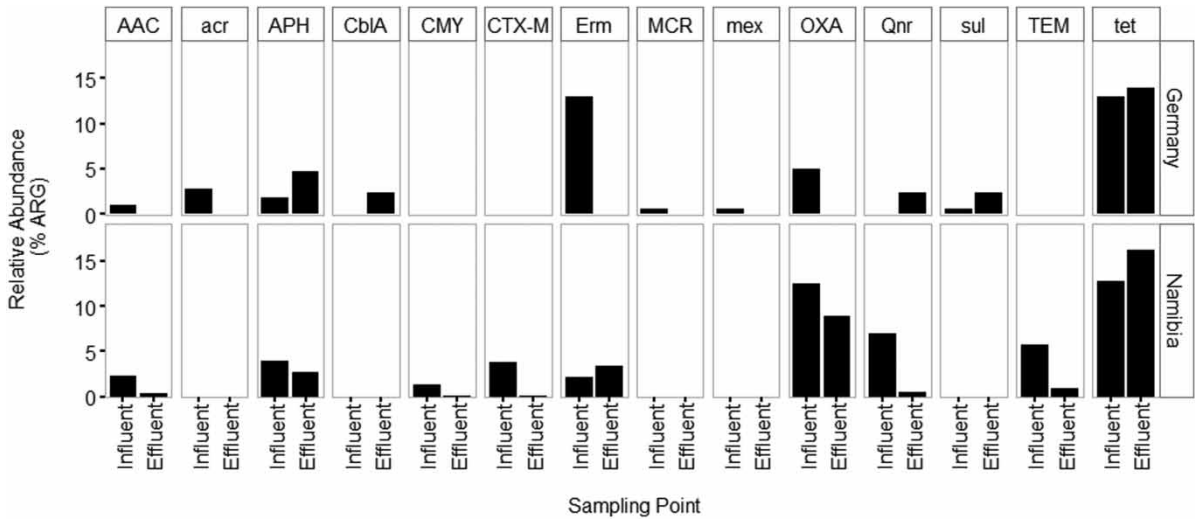

Figure 6 | Abundances of clinically relevant ARG types detected in the influent and effluent of the pond (Namibia) and the AS (Germany) system.

Acinetobacter in the pond influent might explain the high fraction of the *blaRTG-2* ARG subtype for beta-lactam (Figure 4(b)), which was found in *Acinetobacter calcoaceticus* (Choury *et al.* 2000). Nevertheless, *Acinetobacter* abundance was significantly less in the pond effluent (97% lower than in the influent) and also a very low abundance of *blaRTG-2* ARG subtype in the effluent was observed (S.Figure 2). In the German influent, the abundance of *Streptococcus* was relatively high, as well as the abundance of some multidrug resistance genes (mefA and mel) (Figures 2(b), 3, and 4(b)), which have been previously detected in *Streptococcus* (Ambrose *et al.* 2005). However, those bacteria and genes had reduced in the effluent.

Interestingly, 10 times increase in the relative abundance of *Mycobacterium* was observed in both the effluents, suggesting that the current state of both WWTP supported enrichment of *Mycobacterium*, although both WWTP employ very different technologies. Previous studies report that *Mycobacterium* can persist in wastewater (Whan *et al.* 2005) and AS (Berekaa & Steinbuchel 2000). *Mycobacterium* is a re-emerging waterborne pathogen (Taylor *et al.* 2001; Woolhouse 2002), and its discharge into the environment must be avoided to decrease the occurrence of human mycobacteriosis which can be associated with water exposure. In 2018, a global total of 186,772 cases of infections with multidrug-resistant or rifamycin-resistant *Mycobacterium tuberculosis* were detected (World Health Organization 2019). We also expected a high abundance of zoonotic pathogens in the Namibian pond system due to direct contact between the pond and animals, as it is not possible to completely prevent animals drinking from the ponds. However, the results reveal that the relative abundance of zoonotic pathogens was distinctly higher in the German WWTP. Wastewater from a nearby slaughterhouse is also treated at the German WWTP, which might explain a higher relative abundance of zoonotic pathogens in German WWTP.

This study adopted a targeted metagenomics approach for obtaining a comprehensive ARG profile of a pond system in Namibia and an AS system in Germany. Previous studies focusing only on German WWTPs were mainly based on quantitative PCR, thus limiting the knowledge to few ARG subtypes (Alexander *et al.* 2016; Caucci *et al.* 2016; Hembach *et al.* 2017). To the best of our knowledge, this is the first study giving an insight into ARG found in a wastewater treatment pond system in Namibia applying the Ampliseq AMR panel (Thermo Fisher Scientific). It is important to note that samples from Namibia were transported at room temperature, which could have an influence on the observed microbial community structure and ARG composition. The diversity of ARG subtypes in the urban resistome showed a distinct separation between Namibia and Germany (Figures 4 and 5), which is a result of varying variables: (1) types and rate of antibiotic usage

in humans (Pereko *et al.* 2015; Zweigner *et al.* 2018); (2) antibiotic use in pets and livestock; (3) temperature and precipitation; (4) eating habits; (5) wastewater composition (physical/chemical parameters). This study revealed different compositions of ARG subtypes in the influent of the pond system and the AS system, especially for ARG with a *Z*-Score higher than 1. Multidrug resistance genes (*mefA* and *mel*) were present in the German, as well as the Namibian (*mefA*) wastewater. The presence of multidrug resistance genes in German wastewaters, as well as water bodies, is well known (Exner *et al.* 2017; Müller *et al.* 2018; Pärnänen *et al.* 2019). However, there is a lack of information for Namibian wastewater. Figure 5 underlines that the pond system is also quite capable of reducing the abundant ARG present in the influent in comparison to the AS system. Also, the pond system can reduce the abundance of clinically relevant ARG types occurring in the influent, similar to the AS system (Figure 6). It is important to note that in this study, we present the relative abundance of ARG, which can only be used for relative comparison of the pond system and the AS system. Absolute abundance of pathogens and ARG is important to determine the hygienic quality of the effluents. This pond system has very high algae growth, which is very common for pond systems. The presence of algae, which alter the water quality (pH, O_2 concentrations) might contribute to the reduction of pathogens and ARG. For example, Mara (2000) reported that during high algal activity, carbonate and bicarbonate ions react to provide more carbon dioxide for the algae, which in turn leaves an excess of hydroxyl ions leading to higher pH (above 9) rapidly killing fecal bacteria. The higher pH values were one of the main distinctive features between the pond and the AS effluent (S.Table 1).

CONCLUSION

In recent years, many studies have been performed in developed countries like Germany on pathogens and ARG in the wastewater, but there is a lack of information about ARG and pathogens in countries like Namibia, where water quality is somewhat more critical due to high inclination towards direct water reuse for irrigation. Therefore, it requires special attention. Given the lack of financial and infrastructural resources, it is challenging to plan the implementation of advanced wastewater treatment systems, such as ultrafiltration, ozonation, or activated carbon filters, to inhibit the dissemination of ARG and pathogens from wastewater into the environment and onto crops. However, based on the results of this study, it seems that pond systems have the potential not just to remove indicator microorganisms, as previously reported (Mara 2000) but also to reduce ARG and pathogens. These findings provide a first basis for further studies to explore the potential of pond systems in the removal of ARGs and pathogens. Even with this first assessment of a pond system, there is a need for more research to understand the processes and the influence of, for example algae, like *Chlorella*, for removing ARG and pathogens.

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COMPETING INTERESTS

The authors declare no financial and non-financial competing interests.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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