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A multi-omics study on quantifying antimicrobial resistance in European freshwater lakes

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ABSTRACT
The surveillance of wastewater for the Covid-19 virus during this unprecedented pandemic and mapped to the distribution and magnitude of the infected in the population near real-time exemplifies the importance of tracking rapidly changing trends of pathogens or public health problems at a large scale. The rising trends of antimicrobial resistance (AMR) with multidrug-resistant pathogens from the environmental water have similarly gained much attention in recent years. Wastewater-based epidemiology from water samples has shown that a wide range of AMR-related genes is frequently detected. Albeit sewage is treated before release and thus, the abundance of pathogens should be significantly reduced or even pathogen-free, several studies indicated the contrary. Pathogens are still measurable in the released water, ultimately entering freshwaters, such as rivers and lakes. Furthermore, socio-economic and environmental factors, such as chemical industries and animal farming nearby, impact the presence of AMR. Many bacterial species from the environment are intrinsically resistant and also contribute to the resistome of freshwater lakes. This study collected the most extensive standardized freshwater data set from hundreds of European lakes and conducted a comprehensive multi-omics analysis on antimicrobial resistance from these freshwater lakes. Our research shows that genes encoding for AMR against tetracyclines, cephalosporins, and quinolones were commonly identified, while for some, such as sulfonamides, resistance was less frequently present. We provide an estimation of the characteristic resistance of AMR in European lakes, which can be used as a comprehensive resistome dataset to facilitate and monitor temporal changes in the development of AMR in European freshwater lakes.

1. Introduction

The surveillance of wastewater for Covid-19 virus in our current pandemic and mapped to the distribution and magnitude of the infected in the population near real-time exemplifies the importance of tracking rapidly changing trends of pathogens or public health problems such as antimicrobial resistance that may involve large populations in a large-scale (Ktnajima et al., 2020). According to the World Health Organization (WHO), the resistance to antibiotic agents is one of the major threats to modern society (de Kraker et al., 2016; UN Interagency Coordination Group (IACG) on Antimicrobial Resistance, 2019). As of 2019, there are already around 700,000 deaths annually, with a potential increase to 10 Million in the next decades without appropriate measurements (UN Interagency Coordination Group (IACG) on Antimicrobial Resistance, 2019). Thus, it is crucial to understand how environmental pressure gives rise to new resistance mechanisms. It has been shown that the over- and misuse of antibiotics are a significant contribution to AMR (Holmes et al., 2016; Singer et al., 2016). Moreover, Hendriksen et al. observed a strong correlation between sanitation standards and health care conditions by analyzing global AMR distribution in urban sewage (Hendriksen et al., 2019). Even indications for the contamination of community sewage by hospital wastewater burdened with antibiotic-resistant bacteria is present (Hocquet et al., 2016). These epidemiological approaches measure the collective signature across a community, and they have the potential to enhance detection, contain, and mitigate an outbreak. At the same time, the application may be deployed within...
monitoring networks to provide inter-comparable data across countries (Daughton, 2020).

However, hospitals, urban transit, and sewage share a significant commonality: they pool sources of health risk, e.g., the risk of contagion through human interchange or the spread of diseases through human waste, respectively. Such urban sewage and hospital wastewater are more likely to be contaminated with multi-resistant pathogens, and the search for AMR in this direction is quite natural. Other studies investigated AMR distribution in the environment, e.g., the abundance of antimicrobial resistance genes (ARGs) in 21 Swiss lakes (Czekalski et al., 2015). Freshwaters are the recipients of the effluent of wastewater treatment plants (WWTP). Consequently, several studies proved the presence of pathogens in natural surface waters (Blaak et al., 2015; Franz et al., 2015; Yang et al., 2017). Accordingly, Czekalski et al. revealed a 200-fold increase of ARGs in the sediment close to sewage release points of freshwater lakes (Czekalski et al., 2014).

However, the dissemination of AMR is not solely associated with human behavior, as various dispersal processes in most ecosystems also contribute to the spread of AMR (Berendonk et al., 2015). Two-thirds of the global antibiotic usage is associated with treating farm animals and agriculture, having a significant effect on the rise of AMR (Done et al., 2015; Van Boeckel et al., 2017). Most of these antibiotics belong to the class of so-called “uncritical” agents, e.g., tetracyclines and penicillins (Annual report on antimicrobial agents intended for use in animals, 2018). Concerningly, other studies reported similar indications, i.e., isolates from indicator bacteria reveal medium to high AMR levels to tetracyclines, sulfonamides, and quinolones (European Union Summary Report on antimicrobial resistance, 2013). Most notably, restricted antibiotics, such as colistin and third- and fourth-generation cephalosporins, are widely applied in poultry farming (European Union Summary Report on antimicrobial resistance, 2013). Wang et al. (2020) even conclude that reducing antibiotic contamination and eutrophication reduces the risk of AMR (Wang et al., 2020).

To this end, we collected samples from multiple freshwater lakes following a standardized protocol to detect AMR levels within microbial communities and quantify the resistome of the environment. Whereas our findings suggest that all possible AMR classes can be observed within the samples, we focused on four important classes of antibiotics in animal husbandry and human healthcare, i.e., tetracyclines, cephalosporins, quinolones, and sulfonamides, for quantifying the resistance in European freshwater lakes.

2. Materials and methods

2.1. Sampling and sample preparation

The dataset consists of standardized samples from 274 lakes for which 16S rRNA has been sequenced. Moreover, for 39 of these lakes, shotgun metagenomic sequencing was performed. For homogeneity, all samples were collected within one month and followed a standardized protocol to detect AMR levels within microbial communities and quantify the resistome of the environment. Whereas our findings suggest that all possible AMR classes can be observed within the samples, we focused on four important classes of antibiotics in animal husbandry and human healthcare, i.e., tetracyclines, cephalosporins, quinolones, and sulfonamides, for quantifying the resistance in European freshwater lakes.
protocol for sampling and analysis. Sampling sites are summarized in Fig. 1a. GPS coordinates of the different sampling sites are shown in Supplement 1. The sampling sites include lakes in 13 countries all across Europe. 274 European freshwater lakes were sampled, covering a broad latitudinal gradient ranging from Scandinavia to Spain. We have chosen a gradient design to cover a broader range of sampling points under varying environmental variables, including altitude or physicochemical factors, e.g., temperature, pH value, or chemical composition, instead of a replicated design, which is why no biological replicates were collected per lake. Samples were taken from the shore of each lake or pond, collecting epilimnetic water up to 0.5 m depth. For genomic DNA extraction, samples were filtered onto 0.2 μm nucleopore filters until the filters were blocked to obtain similar amounts of biomass. Biomass filters were subsequently air-dried and preserved below -80 °C in a cryoshipper (Chart/MVE, Ball Ground, USA).

2.2. DNA extraction, PCR, and sequencing

For the amplicon analysis, genomic DNA was extracted from biomass filters using the my-Budget DNA Mini Kit (Bio-Budget Technologies GmbH, Krefeld, Germany) following the manufacturer’s protocol with minor adaptations. We changed the protocol as follows: Except that filters were homogenized in 800 μl Lysis Buffer TLS within lysing Matrix E tubes (MP Biomedicals, Santa Ana, California, USA) and homogenized three times for 45 s using FastPrep (MP Biomedicals, Santa Ana, California, USA) at 6 m/s followed by incubation for 15 min at 55°C. The DNA quality was checked using a NanoDrop™ ND-2000 UV–Vis spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and on 1% agarose gels. PCR amplifications targeted the V2-V3 region of the bacterial 16S rRNA gene using the primers 104F (5′-GGC GVA CGG GTG MGT AA-3′) and 515R (5′- TTA CCG CGG CKG CTG GCA C-3′) (Lange et al., 2015). The selected forward primer contains two wobble positions in order to cover a broad taxonomic spectrum. For each sample, two technical replicates of the extracted DNA were independently amplified using primers with different sample identifiers (Lange et al., 2015). For the PCR reaction, 1 μl of DNA template in 25 μl PCR reaction with 0.4 units of Phusion DNA polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 0.25 μM primers, 0.4 mM dNTPs, and 1× Phusion buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used. The PCR protocol consisted of 35 cycles, including a denaturation step at 98°C for 30 s, an annealing step at 72°C for 45 s (Lange et al., 2015), and an elongation step at 72°C for 30 s. Finally, the PCR was completed by a final extension step at 72°C for 10 min. Samples were pooled in equimolar ratios and sequenced using paired-end (2 × 300 bp) HiSeq 2500 Illumina sequencing in “rapid-run” mode at a sequencing provider (Fasteriis, Geneva, Switzerland). Clean sequencing reads were in total 731,842,882 or on average 2,670,959 reads per lake. Finally, clean and demultiplexed samples were provided for further analyses.

Besides the amplicon analysis, we also carried out metagenomics analysis on 39 samples. The metagenomic samples were sequenced at BGI on an Illumina HiSeq XTen machine producing 150 bp paired-end samples. At BGI, genomic DNA was quality tested, and the qualified samples were used to construct the sequencing library. Therefore, purified DNA samples were first sheared into smaller fragments with the desired size by Covaris S/E210 or Bioruptor. Then the overhangs resulting from fragmentation were converted into blunt ends using T4 DNA polymerase, Klenow Fragment, and T4 Polynucleotide Kinase. Adapters were ligated onto both ends of the DNA fragments. The desired fragments were purified through gel-electrophoresis, then selectively enriched and amplified by PCR. Index tags were introduced into the adapter sequence to allow pooling. Finally, the libraries were quality tested and sequenced. Clean and demultiplexed samples were provided.

2.3. Amplicon analysis workflow

For the amplicon analysis, we used the standardized workflow Natrix, including (i) quality filtering, (ii) clustering, and (iii) taxonomy annotation (Welzel et al., 2020). The quality of the sequencing reads was re-checked using FastQC (v0.11.8), and low-quality tails were removed from the reads using PRINSEQ (Schmieder and Edwards, 2011) (v0.20.4). Trimmed reads with an average Phred quality score of less than 25 were discarded. Additionally, we removed all reads at least one base with a quality of less than 15 and all reads that contained errors in the primer regions. Adapters containing primer, barcode, and poly-N sequences were removed, and the paired-end reads were subsequently assembled using PANDAseq (Masella et al., 2012) (v2.10). Chimeras were removed using UCHIME (usarch v7.0.1090) (Edgar et al., 2011). Subsequently, the sequences that passed quality and AmpliconDuo filtering (Lange et al., 2015) were clustered into Operational Taxonomic Units (OTUs) with SWARM (Mahe et al., 2015) (v2.1.9), using a local threshold since lineages evolve at variable rates. The local clustering threshold d was set to 1. For all OTUs, we used BLASTn (Altschul et al., 1990) (v2.7.1 + ) with the NCBI nt and the Taxonomy Database (Dec 5, 2017) to annotate the OTUs with taxonomic information (see supplement 2 and 3).

2.4. Amplicon abundance analysis

We used the R package phyloseq (McMurdie and Holmes, 2013) for the relative abundance analysis of operational taxonomic units (OTUs). Specifically, the principal coordinate analysis has been conducted with the ordinate function using the “PCoA” method and Bray-Curtis dissimilarity as the distance metric. The OTU table is used for the principal coordinate analysis (PCoA). In order to statistically revise the PCoA, we used the non-parametric multivariate analysis of variance (MANOVA) (Anderson, 2001). Subsequently, the sequences that passed quality and AmpliconDuo filtering were clustered into Operational Taxonomic Units (OTUs) with SWARM (Lange et al., 2015) (v2.1.9) through the adonis function with Bray-Curtis dissimilarity (Oksanen et al., 2019).

For the bar chart, the following genera containing strains with literature-known antimicrobial resistance were filtered out from the original dataset: Enterococcus, Mycobacterium, Staphylococcus, Streptococcus, Campylobacter, Neisseria, Escherichia-Shigella, Klebsiella, Enterobacter, Salmonella, Acinetobacter, and Pseudomonas. This list is based on a reference list of pathogens of the Pathosystems Resource Integration Center (PATRIC) (Wattam et al., 2017). The visualization has been carried out with phyloseq’s barplot function, using the genera for color filling. The final version of the plot is crafted with Altair, a visualization library for the Python programming language (VanderPlas et al., 2018).

2.5. Metagenomic analysis of antimicrobial resistance

Antimicrobial resistance (AMR) was analyzed using the resistance Gene Identifier (RGI) tool of the Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2017), which can be used to predict the resistome from raw genome sequences. CARD is a database containing AMR drug classes and resistance mechanisms and intrinsic mutation-driven and acquired resistances. The basis consists of antibiotic resistance ontologies (ARO term), a networked and hierarchically controlled system of terms (Jia et al., 2017). Internally, the RGI tool uses Bowie2 to align the metagenomic reads against CARD. We used the default settings for the analyses. For further evaluations, the focus was set on the AMR gene family and drug classes to achieve comparability and practical relevance. In addition, only those reads that have been entirely mapped to genes encoding for AMR factors were used for the subsequent analysis.

2.6. Metagenomic taxonomic analysis

In our analyses, we focused on reads related to resistance. Thus, only
those reads, which could be associated with AMR gene families or drug classes listed by the CARD database (see above), were used for further analysis. We used Centrifuge (Kim et al., 2016) to perform taxonomic analyses using Burrows-Wheeler transform (BWT) and Ferragina-Manzini (FM) index. The taxonomic analysis of the filtered FASTQ files was analyzed with the index containing all complete bacterial genomes (Kim et al., 2016). In order to cope with different sequencing depths among the samples, we normalized the mapped reads (numFragments) for gene length (geneLength) and sequencing depth (totalNumReads) (Chen et al., 2021):

$$\text{FPKM} = \frac{\text{numFragments}}{\frac{\text{geneLength}}{1000} \times \text{totalNumReads}}$$

### 2.7. Data visualization and statistics

We used the function clustermap from the Python package seaborn for drawing the heatmaps (Waskom, 2021). All fragments, derived from the mapped reads of the CARD output, were displayed in one heatmap, each for the AMR gene families and the drug class resistance. We restricted the analyses and visualization to those gene families and drug class resistances that were most common among the lakes. We then clustered the lakes based on the country. AMR gene families or drug class resistances with over 500 fragments per lake accounted for less than 2% of all samples. Therefore, we set the limit for visualization to a maximum of 500 fragments per AMR gene family or drug class resistance, respectively, i.e., AMR gene families or drug class resistance with more than 500 fragments were capped. To finalize the heatmaps, we utilized Altair (VanderPlas et al., 2018). For Fig. 2, only the most common taxa are shown for the comparison. In order to analyze the pathogenic taxa, we used a filtered list of pathogens from PATRIC (Wattam et al., 2017).

Correlation analyses were carried out based on Pearson correlation to detect associations between resistance genes and taxa. To this end, we correlated the number of reads found by centrifuge with all mapped reads found by CARD and calculated the coefficient of determination. Moreover, we analyzed the association between resistance and farmland. We used SEDE-GPS for gathering socio-economic data (Sperlea et al., 2018). That is, we collected all data related to the term agriculture as defined by Eurostat (https://ec.europa.eu/eurostat/en/web/agriculture/data), for instance, agricultural products and organic farming, among others. SEDE-GPS takes a table with the GPS coordinates as input and collects information from different databases, such as Eurostat, within a user-specified radius. In our study, we used 20 km as the radius for SEDE-GPS. Correlations were calculated and reported based on the Pearson’s product-moment correlation coefficient:

$$r_{XY} = \frac{\sum_{i=1}^{n} (x_i - \overline{x})(y_i - \overline{y})}{\sqrt{\sum_{i=1}^{n} (x_i - \overline{x})^2} \sqrt{\sum_{i=1}^{n} (y_i - \overline{y})^2}}$$

with $n$ the sample size, $x_i$ and $y_i$ the sample points, and $\overline{x}$ and $\overline{y}$ the corresponding mean; $p$ values are calculated based on Student’s t-distribution with $n-2$ degrees of freedom.

![Fig. 2. Stacked bar charts of genera found in the metagenomic taxonomy classification. Top: The fragments mapped to a particular genus (y-axis) and for a sample site (x-axis). Only non-pathogenic Pseudomonads were found. Center: Non-AMR-related genera are removed, and only the top-10 of pathogenic genera are kept. Bottom: Total distribution of fragments mapped to pathogenic genera across all 39 lakes.](image-url)
3. Results

3.1. Relative abundance of taxa

In total, we generated 731,842,882 clean sequencing reads from 274 freshwater lakes, i.e., 2,670,959 reads on average per lake, which we employed for subsequent 16S rRNA amplicon sequencing. Based on the 16S rRNA amplicon sequencing results, we analyzed the relative abundance of operational taxonomic units (OTUs) for each sample site (Fig. 1a). A summary of the lakes can be found in supplement 1. Moreover, a principal coordinate analysis reveals that seemingly no country-specific differences can be observed across the samples since only 5.2 and 4.7 % variation can be explained for the first and second components, respectively (Fig. 1b). However, a non-parametric multivariate analysis of variance unveils a significant difference across the samples ($p = 0.001$). We then focused on the genera known for antimicrobial resistance (AMR). The results indicated the presence of multiple genera, including species with known AMR (Fig. 1c). Representatives of the genus *Mycobacterium* can be observed in large quantities in almost all samples since it is widespread in aquatic ecosystems and is likely dominated by nontuberculous mycobacteria (NTM) (Roguet et al., 2018). Roguet and the coworkers pointed out that the

Fig. 3. Heatmaps are depicted of the respective lakes (x-axis) and resistances to drug classes (a) and AMR gene families (b) on the y-axis, respectively. In addition, the color density is determined by the quantity of fragments mapped against the respective category.
flowing of rivers into lakes appeared to strongly increase NTM densities, as opposed to lakes without connected rivers (Roguet et al., 2018). *Acinetobacter* species were also present in many samples, albeit in smaller quantities. Shao et al. (2019) suspected inflows polluted by stormwater runoff of sewage posed a possible source for *Acinetobacter* contamination (Shao et al., 2019). In addition, one lake in Germany seems to host larger quantities of *Pseudomonas* species (Fig. 1c). Species of these abundant genera are often associated with environmental waters and may be a source of opportunistic infections. The OTU table and the taxonomy data can be found in supplement 4 and 5.

3.3. Antimicrobial resistance levels

We analyzed metagenome samples from 39 of the 274 freshwater lakes to identify AMR-related genes. The lakes were selected as a representative subset of the lakes analyzed in the first step, i.e., considering their geographical distribution to limit country-specific findings. Our results show indications towards various AMR genes in low to moderate quantities in the different samples (Fig. 3a). Specifically, we found a cluster of lakes exhibiting resistance against, e.g., β-lactam antibiotics, such as monobactams, cephalosporins, and penems, including lakes in France, Romania, and Germany. Genes encoding potential drug class resistances for quinolones and tetracyclines, as well as sulfonamides, can also be detected in the samples, albeit in smaller quantities (Fig. 3a). Moreover, an analysis on the abundance of AMR gene families reveals higher quantities of genes acting as antibiotic targets, mainly involved in protein biosyntheses, such as the elastin–resistant elongation factor thermo unstable (EF-Tu) gene (Parmeggiani and Nissen, 2006), the rifamycin–resistant β subunit encoding RNA polymerase (rpoB) gene (Goldstein, 2014), as well as the fluoroquinolone–resistant gyrase A and B (van der Heijden et al., 2012). Again, distinct clusters based on the quantity of mapped fragments can be observed for lakes in France, Germany, and Romania (Fig. 3b). Considering all mapped fragments for resistance against tetracyclines, cephalosporins, quinolones, and sulfonamides, it turns out that precisely, lakes in these countries exhibit diverse levels of putative resistance to drug classes stated above. Moreover, individual lakes in Germany, Italy, France, and Romania show a higher amount of mapped fragments to AMR-related genes than other countries (Fig. 4). We investigated a potential correlation with surrounding farmland or other possible factors in the following section.

3.4. Association to agriculture

As stated above, livestock farming is one of the main fields for applying antibiotics (Done et al., 2015; Van Boeckel et al., 2017). We employed SEDE-GPS to retrieve information on agriculture in an area of 20 km around the GPS coordinates of the lakes (Sperlea et al., 2018). Antibiotics or resistant bacteria from sewage with human excreta, in general, can enter freshwater in many ways. In a study conducted by the German Environment Agency, three possible pathways were identified: (i) the straight entering into surface water via excretion, for instance, sewage carrying human excreta with resistant bacteria, e.g., from stormwater runoff, (ii) the detour via the soil or (iii) via manure, supplied on fields and meadows (German Environment Agency, 2015). Our findings indicate only a low, non-significant correlation (R = 0.28, p = 0.08) between agricultural use and the frequency of antimicrobial resistance genes, particularly for sulfonamides. We also found non-significant correlations for the other three antibiotics investigated, i.e., tetracyclines, cephalosporins, and quinolones, which show no significant correlation with adjacent agriculture. Our findings suggest that human-made agricultural influences are low in Europe. However, we observed indications for genes encoding for resistance to one of the four drugs mentioned above, which will be discussed.

3.5. Tetracyclines

It has been reported that tetracyclines are among the most popular antibiotics in animal husbandry, with a share of around 30% (Annual report on antimicrobial agents intended for use in animals, 2018). Our findings are generally in support of this. Thus, resistance to this drug class can be observed in several European lakes in Austria, Germany, and Poland (Fig. 4). Furthermore, tetracyclines belong to frequently observed drug classes compared to others across all lakes (Fig. 3a).

3.6. Cephalosporins

In contrast to tetracyclines, cephalosporins, starting from the third generation on, are considered as critical antimicrobials (including carbapenems which are drugs of last resort), respectively, according to the WHO (Critically important antimicrobials for human medicine, 2019). However, their application is widespread, particularly in poultry farming (Annual report on antimicrobial agents intended for use in animals, 2018). Our study indicates the presence of resistance for this drug.
class. In particular, specific sample sites show higher exposure to microbes, potentially carrying associated resistance genes, compared to resistance against the remaining drug classes (Fig. 3a). If one considers the fragments mapped for individual countries, again lakes in Romania, France, and Germany reveal a descending order of fragments of genes encoding for resistance against this drug class (Fig. 4). The indications can be further validated by regarding individual lakes as shown in Fig. 5, where increased fragments mapped to genes encoding for cephalosporin resistance can be observed for lakes in Romania, Italy, and France.

3.7. (Fluoro)quinolones

These belong to another important class of antimicrobial agents, as stated by the WHO (Critically important antimicrobials for human medicine, 2019). The results also show resistance across individual lakes (Fig. 3a) as well as for specific countries (Fig. 4). Redgrave et al. (2014) observed a strong correlation between fluoroquinolones resistance and antibiotic consumption in Greece, France, and Sweden, i.e., the higher the intake, the higher the percentage of resistant Escherichia coli isolates (Redgrave et al., 2014). We endorse the findings of Redgrave et al.; i.e., fluoroquinolones resistance can be found from lakes in Germany, Italy, Romania, and France. Even for the sole Swedish lake, for which metagenomic samples have been sequenced, we observed indications for resistance to this drug class (Fig. 4), supporting the results from Redgrave et al. Interestingly, we observed a correlation between fluoroquinolones resistance and the presence of Streptomyces albus, a bacterial strain known for non-pathogenicity ($R = 0.52$, p less than 0.001). However, this might be due to cryptic gene clusters that are not expressed but are frequently found in Streptomyces (Xu et al., 2017).

3.8. Sulfonamides

Finally, the mapped fragments to genes encoding for sulfonamide resistance are lower than the other drugs but still present (see Fig. 3a). In addition, the quantity for individual countries is lower than the remaining drug classes (Fig. 4).

4. Discussion

We collected samples from 274 European lakes for a large-scale study on quantifying antimicrobial resistance (AMR) in freshwaters. To the best of our knowledge, this is the largest, standardized data set so far, employing 16S rRNA amplicon and metagenomic analyses on the bacterial composition and the resistome of these lakes. The standardized approach clearly distinguishes our study from others, relying heavily on non-standardized metagenomic data collected from public databases, differing in sampling protocols and analytic procedures, for instance, studies dealing with environmental or agricultural resistomes (Durso et al., 2012; Pal et al., 2016).

The present study used an integrative multi-omics approach using...
16S rRNA amplicon sequencing for a first-glance taxonomy identification, followed by a shotgun metagenomics analysis. Both approaches have strengths and weaknesses: taxonomical classification using 16S rRNA is more appropriate for various samples but offers a limited resolution in taxonomic classification depth. Contrarily, shotgun metagenomics provides a detailed taxonomic resolution and the functional annotation of sequences, e.g., AMR genes (Jovel, 2016), however, at higher costs. Consequently, Hendriksen et al. argued that metagenomics offers the advantage of detecting transmissible resistance genes from a variety of bacterial species (Hendriksen et al., 2019).

Our findings quantify AMR among the analyzed lakes. We specifically focused on the association of resistance genes to four antibiotics, namely tetracyclines, cephalosporins, (fluoro)quinolones, and sulfonamides, with agriculture (Annual report on antimicrobial agents intended for use in animals, 2018). However, none of these show a significant correlation with agriculture. The data suggest that low human impact on AMR can be observed in the European freshwater lakes, and our findings may serve as a reference for monitoring AMR development in European freshwater lakes in the future.

We selected the 39 lakes as a representative subset of the overall lakes analyzed based on their geographical distribution, thus limiting the country-specific findings. Nevertheless, our methodology and data will be valuable as a reference to track the temporal development of AMR in Europe, and comparisons for those studied from countries of other continents. Our standardized approach contrasts from studies that already identified significant accumulation of AMR from lakes (Chakraborty et al., 2020; Kong et al., 2021; Ram and Kumar, 2020; Wang et al., 2020) and could avoid conditions, for instance, present in China and India, where, albeit governmental actions have been already taken, the environment is highly suffering from a mis- and overuse of antibiotics (Kakk et al., 2017; Qu et al., 2019). One limitation in our current study is concerning the chromosomal and non-chromosomal elements such as plasmids, as the AMR genes are not necessarily vertically inherited, and the 16 s rRNA survey, therefore, most likely yields an incomplete list of AMR-related genera. Furthermore, characteristic mutations leading to resistance, e.g., in chromosomal genes gyrA and gyrB, were not considered in more detail nor correlated to phenotypic resistance of the bacteria. Moreover, Cox and Wright (2013) underpin the role of antibiotic-producing bacteria in soils (Cox and Wright, 2013) or species with chromosome-encoded elements, e.g., non-specific efflux pumps (Peterson and Kaur, 2018), which can be further disseminated by horizontal gene transfer (Cyclo et al., 2019). Thus, the exposure of intrinsically resistant bacteria to man-made environmental factors is not an explanation for their AMR and the natural resistome in European freshwater lakes.

In addition, we only considered agriculture, e.g., livestock farming, as an external impact on AMR levels in freshwater lakes. Hence our results might be biased towards agriculture (Collignon et al., 2018). Nevertheless, recent statistics about developments in agriculture in the European Union (EU) states that Romania, Italy, France, Poland, and Germany are among those countries with the most significant proportion of farming land (Agriculture, 2018). The findings by the EU coincides with the observations made by our study, i.e., our results verified not only indications for resistance against the four drug classes aforementioned but also specifically in these countries. Moreover, higher resistance against cephalosporins can be observed for lakes in France, Germany, or Romania, albeit their use is restricted in the EU (Fig. 4).

Our results support recent studies which reported increased levels of AMR resistance genes in various environments, e.g., in water samples, in groundwater (Balzer et al., 2016), in a further study which reported sewage as a source for AMR in the sediment of freshwater lakes (Czekalski et al., 2014), and in general, overuse of antibiotic agents in livestock farming (Hernando-Amado et al., 2019). We observed AMR in freshwater lakes, emphasizing AMR as a significant challenge for current and future healthcare systems. However, we could not rule out an overestimation of strain confidence completely, and the observed drug class resistances cannot be confidentially associated with present drug-resistant bacteria.

5. Conclusion

We comprehensively analyzed the resistome of freshwater lakes from European countries and focused explicitly on the antimicrobial resistance genes to four important classes of antibiotics, namely tetracyclines, cephalosporins, (fluoro)quinolones, and sulfonamides. Our findings provide a reference for the surveillance and monitoring of AMR development in European freshwater lakes and comparisons to those of other countries.

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Author contributions

SS and DH developed the concept and designed the experiments. JB designed the sampling campaign. JKN, DB, and JB collected and preprocessed the sequencing data. SS and LE performed the experiments and analyzed the data. SS, LE, MI, and DH interpreted the results. SS and DH wrote the manuscript. JB and DH supervised the study. All authors read and approved the final manuscript.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106821.

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