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Needles in haystacks: monitoring the potential escape of bioaerosolised antimicrobial resistance genes from wastewater treatment plants with air and phyllosphere sampling

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Abstract

Wastewater treatment plants are well known point sources of emissions of antimicrobial resistance genes (ARGs) into the environment. Although most work to date has focused on ARG dispersal via effluent, aerial dispersal in bioaerosols is a poorly understood, but likely important vector for ARG dispersal. Recent evidence suggests that ARG profiles of the conifer needle phyllosphere could be used to measure bioaerosol dispersal from anthropogenic sources. Here we assessed airborne dispersal of ARGs from wastewater treatment plants in Wales, UK and Quebec, Canada,

using conifer needles as passive bioaerosol monitors. ARG profiles of wastewater were compared to those of conifer phyllosphere using high-throughput qPCR. ARG richness was significantly lower in conifer phyllosphere samples than wastewater samples, though no differences were observed across the dispersal gradients. Mean copy number of ARGs followed a similar trend. ARG profiles showed limited, but consistent patterns with increasing distance from wastewater treatment plants, but these did not align with those of wastewater samples. For example, proportional abundance of aminoglycosides decreased over the dispersal gradient in Wales, whereas mobile genetic elements showed the inverse relationship. In summary, while distinct ARG profiles exist along dispersal gradients, links to those of wastewater were not apparent.

Les usines de traitement des eaux usées sont de potentielles sources d'émission de gènes de résistance aux antibiotiques (ARGs) dans l'environnement. La majorité des travaux publiés à ce jour se concentrent principalement sur la dispersion des ARGs dans les effluents, mais la dispersion des ARGs peut également se produire dans l'air sous forme de bioaérosols. L'air comme mode de transport des ARGs est encore relativement peu étudié et mal compris. De récentes études ont démontré que la phyllosphère des aiguilles de conifères pouvait être utilisée pour détecter et identifier le profil d'ARGs retrouvé dans l'air à proximité de sources anthropiques. La présente étude se concentrait sur l'évaluation de la dispersion des ARGs près d'usines de traitement des eaux usées situées au Pays de Galles (Royaume-Uni) et au Québec (Canada), en utilisant des aiguilles de conifères comme échantillonneurs passifs de bioaérosols. La qPCR à haut débit a été utilisée pour comparer le profil d'ARGs des eaux usées avec celui de la phyllosphère des aiguilles de conifères. La richesse en ARGs était significativement plus faible dans les échantillons de phyllosphère des aiguilles que dans les échantillons d'eaux usées, bien qu'aucune différence n'ait

été observée parmi les différents gradients de dispersion. Le nombre moyen de copies d'ARGs était relativement similaire. Les profils d'ARGs sur les aiguilles de conifères tendent à changer plus la distance de la source augmente. Par exemple, la proportion de gènes de résistance aux aminoglycosides tend à diminuer en s'éloignant de l'usine du Pays de Galles tandis que les éléments génétiques mobiles (MGEs) suivaient une tendance contraire. En somme, bien que des profils d'ARGs spécifiques peuvent être observés selon les gradients de dispersion, aucun lien clair ne peut être établi entre les profils des aiguilles et ceux des eaux usées. D'autres recherches devront être menées pour évaluer à plus long terme les changements dans les profils d'ARGs et la biodiversité bactérienne pour déterminer de potentiels contaminants aéroportés.

Keywords: Conifers; Biomonitoring; Environmental AMR; High-throughput qPCR; Bioaerosols

Introduction

In 2019, an estimated 4.95 million deaths were caused by infections by antimicrobial resistant (AMR) bacteria globally (Murray et al., 2022). Unfortunately, the consequences of increasing AMR extend further than simple mortality. As of 2019, AMR is estimated to cost the European Union €1.5 billion annually in lost economic productivity and healthcare spending (Anderson et al., 2019). In Canada, the present costs of AMR are similar (1.4 billion CAD), but are expected to increase to 8 billion CAD annually by 2050 (Council of Canadian Academics, 2019). Yet despite increasing efforts to monitor AMR bacterial infections in human and animal health, there is a clear lack of monitoring capacity to track the spread of antibacterial resistance genes (ARGs) into the environment (Pruden et al., 2021). Improving environmental monitoring capacity for AMR would adhere to the One Health ethos, which calls for multidisciplinary and

intersectoral approaches to protect human, animal, and environmental health (Pokharel et al., 2020; Robinson et al., 2016). Monitoring wastewater effluent using molecular methods can effectively quantify ARG loads and track their dispersal through the environment (Pruden et al., 2021). In turn, this could provide early detection of risks associated with AMR bacteria helping to prevent outbreaks or promote prescribing-based interventions.

Indeed, the dispersal of AMR in the wider environment from anthropogenic sources has received much attention, particularly the contamination of soils and waterways (Singer et al., 2016; Wu et al., 2023). Wastewater treatment plants (WWTPs) in particular, are well-known point-sources for the discharge of ARM bacteria and associated mobile genetic elements (MGEs) carrying ARGs (Singer et al., 2016; Shi et al., 2023; de Nies et al., 2022). Several studies have demonstrated that sites near WWTPs harbour greater quantities of ARGs, thus posing a risk for primary human exposure and secondary exposure through contamination of the food chain (Czekalski et al., 2014; Marti et al., 2013; Gaviria-Figueroa et al., 2019). Further, the totality of ARGs and MGEs of wastewater effluent is often enhanced during the treatment process (Ju et al., 2019; Corno et al., 2019; Raza et al., 2022; Shi et al., 2023; Thornton, 2020). As such, resilient AMR bacteria can be recovered in the downstream water column (Amos et al., 2014; Munir et al., 2011) and can propagate ARGs in the wider environment through horizontal gene transfer (Jacquiod et al., 2017). Given the fact that the release of AMR bacteria from WWTPs can exceed 10^{10} CFU day^{-1} (Manaia et al., 2010; Novo and Manaia, 2010), it is vital that these point sources of AMR pollutants be monitored.

There is growing evidence that aerial dispersal of AMR bacteria may represent an important vector for ARGs to enter the environment (Bai et al., 2022; Gaviria-Figueroa et al., 2019). During the wastewater treatment process, microorganisms become aerosolised through

93 aeration, mechanical mixing, and bubble bursting (Kataki et al., 2022), with the airborne bacterial
94 biomass ranging between 10^6-10^8 genomic units (based on 16S rRNA copy numbers) per m^3 of air
95 (Bélanger Cayouette et al., 2022; Blais-Lecours et al., 2014; Mbareche et al., 2022). Many families
96 of ARGs have been detected in WWTP bioaerosols using metagenomics (Gaviria-Figueroa et al.,
97 2019; Yang et al., 2018) and quantitative PCR (qPCR) approaches (e.g. Bélanger Cayouette et al.,
98 2022; Ouradou et al., 2023; Wang et al., 2019). Though information on long-distance bioaerosol
99 dispersal is limited, Gaviria-Figueroa et al. (2019) used a modelling approach to estimate that over
100 a 24 h period more than 220,000 individual ARGs could be transported across a 10 km radius
101 surrounding an American WWTP capable of processing 15 million L of waste per day, with a
102 further 10,000 ARG copies transported up to 120 km away. This suggests that WWTP bioaerosols
103 have the potential impact microbial communities over an incredibly large area. More work is
104 therefore needed to monitor the long-distance dispersal of WWTPs bioaerosols and their potential
105 roles as environmental contaminants.

106 In comparison to waterborne vectors, the extent to which bioaerosols contribute to the
107 propagation of AMR in the wider environment remains unclear. Further, new modelling
108 approaches are needed to quantify the risk posed by airborne ARGs. Recent work has shown that
109 vegetation can be used as passive collectors when sampling bioaerosols (Theofel et al., 2020;
110 George et al., 2022). This mirrors the use of conifer trees as barriers around point sources of
111 pollution to limit emissions of fine particulate matter, ammonia, and odours (Adrizal et al., 2008;
112 Lin et al., 2006). For example, Galès et al. (2014) delineated the dispersal patterns of
113 *Saccharopolyspora rectivirgula* emissions from a composting plant along a gradient of pine trees.
114 Assessments of the conifer phyllosphere have also revealed different ARG profiles in agricultural
115 and forest regions of Quebec, Canada (George et al., 2022a). Harnessing the conifer phyllosphere

as a monitoring tool could allow for long-term monitoring of ARGs in bioaerosols, contributing to the assessment of anthropogenic influences on AMR bacteria and ARG dispersal (Manibusan and Mainelis, 2022). Indeed, this method draws inspiration from the use of biofilms as environmental markers of ARG dispersal from wastewater effluent (Engemann et al., 2008; Marti et al., 2013). It is assumed that ARGs will be transferred via colonisation and plasmid exchange on conifer needles in the same way as ARGs are accumulated in biofilms as AMR bacteria settle out of the contaminated water column (Engemann et al., 2008).

Here, the ARGs profiles of wastewater, air, and the conifer phyllosphere were assessed along a dispersal gradient at two contrasting WWTPs in Quebec, Canada and Wales, UK. Our two main objectives were to: *i*) assess whether the conifer phyllosphere could delineate ARG dispersal from WWTPs; *ii*) assess differences in ARG profiles could be detected using a common panel of ARGs. We hypothesised that the ARG profile of conifer needles would resemble those of WWTPs but decrease with distance away from the WWTP. Further, it was hypothesised that our common ARG panel was diverse enough to be applicable to both sampling regions.

Materials and methods

Study sites

The study focused on two exemplar sites, one located in Quebec, Canada (Site 1) and one located in Bangor, North Wales, UK (Site 2). Both WWTPs are open air treatment systems with primary settling and secondary aeration ponds where bioaerosols can directly enter the environment.

Site 1: The WWTP in Quebec was located at Sainte-Brigitte-de-Laval (N47°00’00”, W71°12’00”). The site serves a population of 8,500 and has a daily flow of 1041.2 l day^{-1}. In

September 2022, wastewater samples were collected from one of the settling lagoons over the course of 3 h. Air samples were collected from 3 points across the WWTP simultaneously with wastewater samples in a 200 m diagonal transect within the WWTP. Conifer needles were collected from trees surrounding the WWTP in a ~150 m radius. Due to the geography of the area surrounding Sainte-Brigitte-de-Laval WWTP, it was not possible to follow the downwind dispersal of bioaerosols in this region. Air and conifer needles were also collected from an upwind control site (Lac-Beauport, QC, Canada) located ~10.5 km from the Sainte-Brigitte-de-Laval WWTP, to serve as reference samples for the environment surrounding the WWTP (Fig. 1).

Site 2: The WWTP in North Wales was located at Treborth, Gwynedd, (N53°12’34”, W04°10’59”). The WWTP serves a population of 40,000 and has a daily average flow of 285 l s^{-1}. In July 2022, air and conifer needle samples were collected from sites across a ~16 km transect in North Wales, UK. Conifer needle samples were classified as upwind (Y Felinheli) (within a 3.8 km radius), in proximity to the WWTP (within a < 1 km radius), in Bangor city centre (~4.2 km from WWTP), or distant (Beaumaris and Abergwyngregyn sites, ~7.5 km and ~11 km from WWTP site, respectively; Fig. 1). A control sample of conifer needles was taken 65 km away from the WWTP at Lake Vyrnwy, Powys, in Mid Wales (Fig. 1).

Wastewater sampling

At both WWTPs, three wastewater samples were collected in polypropylene bottles over the course of 1 h, with 100 ml of wastewater collected every 10 mins to produce one composite 700 ml sample. Samples were stored at 4 °C until analysis. Prior to DNA extraction, 100 ml of wastewater per composite sample was centrifuged for 10 min at 10,000 x g at a temperature of 4°C. Supernatants were discarded and the pellet resuspended in PowerSoil Pro lysis buffer C1. DNA extraction was performed immediately following processing, as described below.

162 Briefly, wastewater pH and electrical conductivity were determined using standard
163 electrodes. Ammonium (NH_4^+) and orthophosphate (PO_4^{3-}) were determined colorimetrically as
164 described in Mulvaney (1996) and Murphy and Riley (1962), respectively. Turbidity was
165 measured with an Orion™ AQ4500 Turbidimeter (ThermoFisher Scientific, UK). Chemical
166 properties of wastewater samples are presented in Table 1.

167 *Air sampling*

168 Simultaneous to wastewater sampling, air samples were collected over the course of 1 h
169 using a SASS 3100 Dry Air Sampler (Research International, Monroe, WA, USA) with charged
170 electret filters. At the WWTPs, each sample was collected from a different location across a 50 m
171 transect, with data from all 3 grouped together to reduce within-site variation. Reference air
172 samples were collected from upwind control sites (Y Felinheli and Lac-Beauport) and at
173 downwind sites in Wales, again each over the course of 1 h. Field blanks were collected at upwind
174 sites and WWTPs by exposing a clean filter to the environment but not running the sampling
175 apparatus. Filters were stored in sealed bags at room temperature until DNA extraction.

176 To facilitate DNA extraction, a protocol developed by the *Institut de recherche Robert-*
177 *Sauvé en santé et en sécurité du travail* was followed. Briefly, filters were immersed in 10 ml of
178 SASS extraction buffer (0.1 M sodium phosphate, pH 7.4) and vortexed for 10 min at 2,500 rev
179 min^{-1}. This solution was then centrifuged at 17,500 rev min^{-1} for 10 min to create a pellet for DNA
180 extraction.

181 *Conifer needle sampling*

182 Conifer needles were collected following the methods of George et al. (2022) and Galès et
183 al. (2014). Briefly, 2 branch ends from each tree ~20 cm in length were collected from 1.5–2.5 m
184 height and stored in sterile plastic bags and kept at 4 °C until processing. A total of 15 g of conifer

185 needles were removed and homogenised in a 50 ml tube of 0.05 % Tween20 saline buffer with a
186 paddle mixer for 5 min. Debris were removed from this solution by differential centrifugation at
187 250 rev min^{-1} for 3 min. The remaining supernatant was centrifuged at 17,500 rev min^{-1} for 30 min
188 to create pellets from which DNA was extracted. In Wales, 38 needle samples were collected, of
189 which 10 came from proximity of the Treborth WWTP, 11 came from Bangor city centre, 10 came
190 from distant sites, and 7 came from the Lake Vyrnwy control site. In Quebec, 12 needles samples
191 were collected from the perimeter of the WWTP, and 5 samples were collected at the Lac-Beauport
192 control site. Due to geographic and access limitations, we could not replicate the dispersal transect
193 as in Wales.

194 *DNA extraction & qPCR analyses*

195 DNA extraction was performed for all sample types using a DNeasy PowerSoil Pro Kits
196 (Qiagen, Montreal, Canada and Hilden, Germany) following manufacturer's instructions. Total
197 bacterial biomass was estimated using qPCR analyses of the 16S rRNA gene and FAM
198 fluorescence probes following Bach et al. (2002), on a Bio-Rad CFX-384 Touch™ Real-Time
199 PCR Detection System (Bio-Rad, Montreal, CA). The thermoprotocol was 95 °C for 3 min; then
200 95 °C for 20 s; and 62 °C for 1 min for 40 cycles. Results were deemed valid if accompanying
201 standard curves had efficiency values of 90-110%. This qPCR data was used to generate 16S rRNA
202 copy numbers per g of conifer needles or per ml of air or water using the following formulae:

203 $$16S\ rRNA\ copy\ number\ per\ g\ needles = \frac{\left(\frac{(Sq * 50)}{7.5}\right) * 50}{W}$$

204 $$16S\ rRNA\ copy\ number\ per\ ml(air\ or\ water) = \frac{(Sq * 50)}{V}$$

where Sq is the starting quantity estimate from qPCR analyses from 2 µl of DNA of a 100 µl extraction, 7.5 (ml) is the volume of supernatant used to make aliquots, 50 (ml) is the total volume of starting solution, *W* is the weight of needles (g), and *V* is the volume of air or water (ml).

The presence of crAssphage, a viral marker of human faecal contamination, was also assessed using qPCR to assess the spread of faecal matter in the environments surrounding WWTPs (Farkas et al., 2019). This was accomplished using the CPQ_056 primer pairs (Table S1) and a thermocycler protocol of 98 °C for 5 min, then 95 °C for 15 s, and finally 60 °C for 1 min for 40 cycles (Stachler et al., 2017).

A Takara SmartChip high-throughput qPCR (HT-qPCR) system (TakaraBio USA, San Jose, CA, USA) was used to assess the abundance and diversity of ARGs and MGEs. A total of 39 genes were assessed including aminoglycosides (3), beta-lactams (10), colistin (1), macrolides (4), MGEs (3), quinolones (2), sulfonamides (2), tetracyclines (10), and vancomycins (4) as explained in George et al. (2022). These genes were selected based on their frequent use in the literature (Stedfelt et al., 2018) and at the suggestion of colleagues, as in the case of mcr-1 and certain carbapenemase genes, as part of an effort to monitor AMR across sectors in Canada (George et al., 2022). Information on mode of action was retrieved from the Comprehensive Antibiotic Resistance Database (https://card.mcmaster.ca). A full list of genes and primer sequences can be found in Table S1. These primers used SYBR Green dye fluorescence except for those of the *mcr1* and *blaCTX-M-1* genes, which used a FAM probe for fluorescence. Analyses on the Takara SmartChip HT-qPCR system used a shared a thermocycler protocol of an initial step of 95 °C for 3 min; then 95 °C for 10 s; 60 °C for 30 s; and 55 °C for 31 s; with a melt curve of 55 °C for 5 s + 0.5 °C/cycle for a total of 45 cycles. Copy numbers of ARGs/MGEs were calculated following the comparative CT method (Schmittgen and Livak, 2008):

228 $$Relative\ gene\ copy\ number = 2^{-(Ct(ARG) - Ct(16S))}$$

229 Relative abundance of ARGs/MGEs was normalised by multiplying the ARG/MGE copy
230 number by 16S rRNA gene copy number.
231
232 *Statistical analyses*
233 Relative copy numbers were $\log_{10}$-transformed for all subsequent statistical analyses. The
234 presence/absence and proportional abundance of target genes is presented in Table S2. Total 16S
235 biomass, ARG/MGE richness linear models with either sample type or location as categorical
236 factors. Differences in the composition of ARGs/MGEs across samples were assessed by non-
237 metric dimensional scaling (NMDS) with Bray-Curtis distances using the vegan package (Oksanen
238 et al., 2022). Differences between groups were assessed by PERMANOVA (adonis2). All
239 statistical analyses were performed using R v. 4.2.2 (R Core Team, 2022).
240
241 **Results**
242 Quantifiable levels of bacterial DNA were recovered from all samples, except for the Y
243 Felinheli upwind control sample. Total bacterial biomass based on 16S rRNA gene copies
244 followed identical trends in both areas (Fig. 2). Biomass was lower in air samples than in
245 wastewater and needle samples in both Wales ($F_{2,47} = 108.4$; $p < 0.001$) and Quebec ($F_{2,20} = 91.19$;
246 $p < 0.001$).
247 Richness of ARGs was significantly different between sample types in both sample
248 locations (Wales: $F_{2,46} = 35.36$; $p < 0.001$; Quebec: $F_{2,20} = 26.62$; $p < 0.001$). Richness was highest
249 in wastewater samples and lowest in air samples (Fig. 3A). Although no significant differences
250 were observed along distance gradients radiating from WWTPs, ARG richness was higher in

wastewater samples than in phyllosphere samples in both Wales and Quebec ($F_{7,54} = 18.37$; $p < 0.001$). Richness of ARGs was however higher in wastewater than needles (Fig. 3B). CrAssphage was only quantifiable in a small number of samples besides those of wastewater, namely the Treborth WWTP air and one needles sample from Bangor itself (Fig. S1).

Quinolone genes were overwhelmingly abundant in Quebec samples (Fig. S2) and they overshadow the subtleties. When they were removed from the data file, more detailed trends became evident. Wastewater profiles in both sites were dominated by MGEs. Aminoglycosides and sulfonamides were more prevalent in Quebec whereas tetracycline and macrolide resistance genes were more present in Welsh wastewater (Fig. 4A). This translated into a greater proportion of transposases/integrases in Welsh wastewater and a greater proportion of antibiotic inactivation genes in Quebec wastewater (Fig. 4B). Quebec phyllosphere samples appear to have a more even distribution of ARGs when quinolones were removed compared to Welsh needle samples, which had high proportions of aminoglycosides (Fig. 4A). These genes were primarily associated with antibiotic inactivation (Fig. 4B). Air samples from Wales had a high proportion of tetracycline resistance genes. In Quebec, aminoglycoside and vancomycin resistance genes were important contributors (Fig. 4A).

There were significant differences ($F_{7,54} = 4.56$; $p < 0.001$) in ARG copy numbers along dispersal gradients from WWTPs based on PERMANOVA results. When $\log_{10} +1$ transformed abundances were visualised via NMDS (stress = 0.15) wastewater samples were clustered together. There was also evidence that Quebec phyllosphere samples clustered. However, phyllosphere samples collected along the dispersal gradient in Wales were so widely distributed that they overshadowed other trends (Fig. 5A). Copy numbers of ARGs decreased over distance from the WWTPs. Yet there was a high abundance of ARGs from the Lake Vyrnwy control site (Fig. 5B).

This was likely influenced by the proportion of number of beta-lactamase genes in these samples (Fig. 5C).

Proportional abundances of ARGs in needle samples were vastly different from wastewater. Welsh wastewater samples were dominated by MGEs. Oddly, their abundance was lowest in proximity to the WWTP and grew with distance. They had an inverse relationship with aminoglycoside resistance genes. Beta-lactam resistance genes were especially prevalent in the Lake Vyrnwy control site (Fig. 5C). In Quebec, there was a decline in aminoglycoside resistance genes from the wastewater to the control site. Needles collected from around the WWTP had a much different ARG profile to the wastewater. Macrolides were strongly present. The control area at Lac-Beauport had a more even distribution of ARGs (Fig. 5C).

Discussion

Monitoring ARG emissions via bioaerosols is an increasingly important aspect of addressing the AMR crisis (George et al., 2022b). Yet tracking emissions across landscapes remains challenging. Assessing changes in ARG profiles of phyllosphere communities represents a potential low-cost tool to monitor ARG pollution in bioaerosol emissions. Previous work has shown that AMR profiles within the phyllosphere can be differentiated with distance from anthropogenic ARG sources (George et al., 2022a; Huang et al., 2023; Xiang et al., 2020). In past studies, forest phyllospheres commonly exhibited fewer ARGs when compared to urban or agricultural areas (George et al., 2022a; Xiang et al., 2020). However, this phenomenon was not exhibited in the two forested sites used in the present study. Rather, ARG richness was not significantly different across phyllosphere samples.

High-throughput qPCR revealed distinct trends in the proportional abundances of ARG across the dispersal gradient. The similarity between wastewater ARG profiles between British

and Canadian sites reflects the clustering of Western nations observed in recent global analyses (Prieto Riquelme et al., 2022). Yet these trends do not extend to the ARG profiles of phyllosphere samples. In Wales, the increasing prevalence of aminoglycoside resistance genes across the dispersal gradient may reflect the impacts of other anthropogenic activities. For example, high-throughput qPCR data has shown aminoglycoside resistance genes are important components of animal husbandry facilities (Yang et al., 2018). Indeed, aminoglycosides are the third most commonly used antibiotics in British sheep farms (Davies et al., 2017). This could indicate bioaerosols from agricultural sources are more important contaminants in this area. Given the prevalence of sheep raising in the surrounding areas, this may indicate that animal waste and management are more important sources of bioaerosols than the WWTP.

This work was predicated on the hypothesis that WWTP would be the most prominent ARG emission source. However, the dispersal patterns of bioaerosols from point sources like WWTPs are difficult to determine. Although there are some estimates for ARG dispersal over long distances (Bai et al., 2022; Gaviria-Figueroa et al., 2019), so far no study has conclusively tracked such emissions. Such local dispersal dynamics are likely important in Wales, where precipitation was higher during the sampling period. Realised dispersal ranges are likely to be much smaller than mathematical predictions due to variation in particle composition or changes in air pressure or windspeed, for instance (Brunet et al, 2018). Recent work has demonstrated that ARGs are present in clouds, however, how their deposition in precipitation may influence terrestrial communities is unknown (Rossi et al., 2023). Indeed, soils are important reservoirs of both anthropogenic and naturally occurring ARGs (Cytryn, 2013; Singer et al., 2016). Comparisons with the ARG profiles of soil were not made in the present study. Shared ARG profiles between the phyllosphere and soil may indicate deposition of local bioaerosols, such as those generated by

rainfall (Joung et al., 2017). It is likely that agricultural bioaerosols and run-off are important vectors for ARG transport in soils along the dispersal gradient (Singer et al., 2016).

To our knowledge the present work represents the first time phyllosphere ARG profiles have been directly compared to source material. However, more intensive studies are needed to validate the potential for monitoring airborne ARGs with conifer needles sampling. This must include longer sampling campaigns with multiple sampling events, which are beyond the scope of this study. Further, greater efforts must be made to account for confounding aerosol sources. Although complementary air samples were taken, the equipment needed is expensive and logistically difficult to run in long-term field experiments. Passive air samplers could be installed in forested areas so that the aerial component of phyllosphere samples over a set time period can be observed in isolation. This could be done using the Rutgers Electrostatic Passive Sampler (REPS) system, for example. The REPS is compact, affordable, and validated for collecting viable bacteria outdoors (Grogan and Mainelis, 2022). Further, soil samples should be periodically collected from below selected conifer trees so that confounding effects of local bioaerosol emissions can be eliminated. Incorporating these additional measures will better validate future tests of this monitoring method.

Conclusion

Mitigating ARG emissions via bioaerosols will be a vital aspect of future efforts to combat the AMR crisis. To achieve this, effective monitoring programmes must be developed. Other monitoring programmes suggest that the conifer needle phyllosphere may be an effective monitoring tool. However, our results indicate that further refinement is needed for this to be effective. Although distinct trends in ARG profiles were observed across a dispersal gradient from WWTPs, these findings were incongruous with those of wastewater. Future work should include

343 repeat sampling at multiple time points to account for seasonal variation in emissions and
344 phyllosphere community composition. Further, molecular approaches such as amplicon or shotgun
345 metagenomic sequencing should be employed to better identify bioindicators of contamination.
346 These lessons will inform future attempts to create ARG monitoring networks.

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356 **Data availability**

357 All data are presented in the manuscript. Files can be provided upon reasonable request via the
358 corresponding author.

359 **Conflict of interest**

360 The authors have no financial or personal conflicts of interest in the work presented.

361 **Author contributions**

362 PBLG, DLJ, and CD conceived the project which was further refined by ECC and LH. ECC, LH,
363 PBLG, SL, and JL collected and processed samples in both Quebec and Wales. PBLG led
364 statistical analyses with help from LH. LH and PBLG led the writing of the first draft with
365 contributions from ECC, SL, and JL. All authors edited and revised subsequent drafts.

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Figure captions

Fig. 1. Sampling locations visited in Quebec and Wales. Points represent the centre of cities/villages sampled. Molecular analyses were conducted at Université Laval in Quebec City (marked in blue).

Fig. 2. Box plots of $\log_{10}$-transformed bacterial biomass across sample types (air, phyllosphere, wastewater) in Wales and Quebec. Boxes denote the first and third quartiles. Horizontal lines mark medians. Whiskers represent 1.5X the interquartile range. Black dots represent outliers. Individual data points are displayed next to boxes.

Fig. 3. Box plot of ARG richness across **A**) sample types and **B**) from conifer phyllosphere samples along distance gradients from WWTPs. Boxes denote the first and third quartiles. Horizontal lines mark medians. Whiskers represent 1.5X the interquartile range. Black dots represent outliers.

Individual data points are displayed next to boxes. SBL stands for Saint-Brigitte-de-Laval. Control sites are labelled in grey; all other sites are labelled in black.

Fig 4. Proportionate abundances of **A**) ARG type and **B**) method of action for sample types (air, phyllosphere, and wastewater) at two WWTPs located in Wales and Quebec. Note that quinolones have been removed from this figure demonstrate trends in other genes. SBL stands for Saint-Brigitte-de-Laval.

Fig. 5. ARGs profiles of wastewater and conifer phyllosphere samples. Shown are **A**) NMDS ordination of phyllosphere and wastewater samples; **B**) the mean and standard error of ARG copy numbers. Control sites are labelled in grey; all other sites are labelled in black; and **C**) proportional abundances of ARGs. SBL stands for Saint-Brigitte-de-Laval.

Table 1. Chemical properties of the three wastewater samples collected at both the Treborth and Sainte-Brigitte-de-Laval WWTPs at the time of aerosol sampling. Values represent means (± SE).

Property	**Treborth**	**Sainte-Brigitte-de-Laval**
pH	7.60 ± 0.02	6.98 ± 0.03
Electrical conductivity (µS/cm)	1018 ± 17	652 ± 2
Turbidity (NTU)	210.3 ± 12.8	145.2 ± 10.7
NH_4^+ (mg N/l)	30.3 ± 0.6	20.0 ± 0.3
PO_4^{3-} (mg P/l)	8.1 ± 0.62	19.4 ± 0.71