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Systematic Review

Impact of point sources on antibiotic resistance genes in the natural environment: a systematic review of the evidence

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Abstract

There is a growing concern about the role of the environment in the dissemination of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG). In this systematic review, we summarize evidence for increases of ARG in the natural environment associated with potential sources of ARB and ARG such as agricultural facilities and wastewater treatment plants. A total of 5247 citations were identified, including studies that ascertained both ARG and ARB outcomes. All studies were screened for relevance to the question and methodology. This paper summarizes the evidence only for those studies with ARG outcomes ($n = 24$). Sixteen studies were at high ($n = 3$) or at unclear ($n = 13$) risk of bias in the estimation of source effects due to lack of information or failure to control for confounders. Statistical methods were used in nine studies; three studies assessed the effect of multiple sources using modeling approaches, and none reported effect measures. Most studies reported higher ARG concentration downstream/near the source, but heterogeneous findings hindered making any sound conclusions. To quantify increases of ARG in the environment due to specific point sources, there is a need for studies that emphasize analytic or design control of confounding, and that provide effect measure estimates.

Keywords: antibiotic resistance genes, effect measure, point source, systematic review.

Introduction

Antimicrobial resistance (AMR) is a serious global public health challenge. Antibiotic resistance in human pathogens can cause

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treatment failure, prolong the duration of illnesses and increase mortality rates, exacting high human and economic costs to society (Friedman *et al.*, 2016). The wide and increasing use of antibiotics and other antimicrobial agents in human medicine, veterinary medicine, animal husbandry, horticulture and around the household have enhanced the selection and spread of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Baker-Austin *et al.*, 2006; Meek *et al.*, 2015; O'Neill, 2015).

The possible role of the natural environment, and surface water in particular, in transmission pathways of ARB and associated ARG has been the subject of much recent discussion (Wooldridge, 2012; Woolhouse *et al.*, 2015). A range of human activities, including activities of daily living, medical care and agriculture, generate waste that contains varying levels of antibiotics (and metabolites), ARB, and ARG. This waste is ultimately released into environmental media. Point sources, defined as 'any single identifiable source of pollution from which pollutants are discharged' (Armon and Starosvetsky, 2015), represent an important and definable contribution to this effluent stream.

Once in the environment, these ARB and ARGs pose potential health risks to humans and animals (Ashbolt *et al.*, 2013). They can persist in the environment, spread over land and water, and be transmitted via free-ranging wildlife (Baquero *et al.*, 2008; Berendonk *et al.*, 2015; Vittecoq *et al.*, 2016). Within environmental niches, ARGs can increase clonally when a bacterial cell hosting an ARG divides, or be transferred between bacterial cells through horizontal gene transfer (HGT) (Allen *et al.*, 2010; Ashbolt *et al.*, 2013; Berglund, 2015).

Despite an increase in the number of studies reporting AMR in diverse natural environmental media, including water, soil, sediment and wildlife, the relative contribution of specific anthropogenic sources to the quantity of ARB and ARGs in the environment is an area of debate (Wooldridge, 2012; Woolhouse *et al.*, 2015; Williams-Nguyen *et al.*, 2016b). Therefore, in this study we sought to systematically identify and summarize evidence in the existing scientific literature pertaining to an association between effluent point sources and the quantity of ARGs in adjacent environmental media. In particular, we looked for measures of impact (i.e. effect measures), which quantify the magnitude or strength of the effect between a point source(s) and the frequency or concentration of resistance elements in the surrounding environment. The specific review question was: *Is the prevalence or concentration of antibiotic resistance genes in soil, water, air or free-living wildlife higher in close proximity to, downstream from or downwind from, known or suspected sources compared to areas more distant, upstream, or upwind from these sources?*

Because the majority of bacteria cannot be cultured, many researchers have begun to measure bacterial genes, including ARGs, in environmental media using culture-independent methods (Luby *et al.*, 2016). These approaches, such as quantitative real-time polymerase chain reaction (q-PCR) and metagenomics (Henriques *et al.*, 2011), are able to provide insight into the environmental resistome in a way not possible using other technologies that rely on culture-dependent methods. Here we report systematic review results pertaining to ARG outcomes (ascertained via culture-independent methods).

Methods

The steps of the systematic review process are summarized in Fig. 1. A systematic review of the literature was conducted following a previously published protocol (Williams-Nguyen *et al.*, 2016a) using the population, exposure, comparator, outcome, study design (PECOS) framework. The population of interest refers to environmental samples; soil, water, air, or free-living wild animal samples as such were considered. Non-wild animals were not considered as environmental samples because they are not a naturally occurring component of environmental systems free of humans.

The systematic review team was composed of six people, which included expertise on AMR, epidemiology, and systematic review methodology. PubMed©, Commonwealth Agricultural Bureaux (CAB Abstracts©), and Scopus© were searched on 14 October 2014 from inception date using specific search strategies. The search was updated on 19 April 2016 using identical search terms. The PubMed© controlled-vocabulary search string was as follows:

"drug resistance, microbial"[Mesh] AND ("water pollutants"[Mesh] OR "environment"[MeSH Terms] OR "soil"[MeSH Terms] OR "water"[MeSH Terms] OR "water pollution"[MeSH

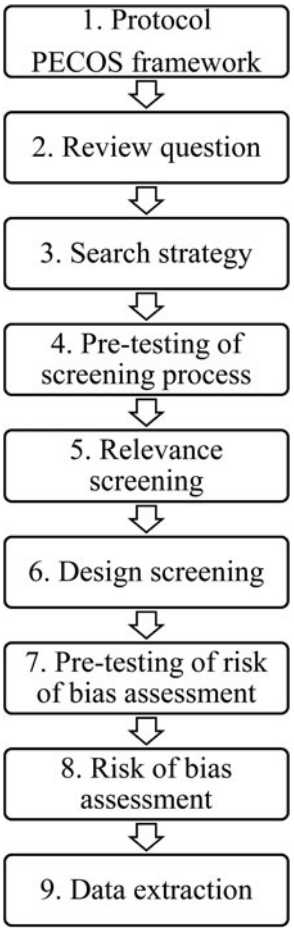

Fig. 1. Diagram summarizing the steps of the systematic review process.

Terms] OR "air pollution"[MeSH Terms] OR "air pollutants" [MeSH Terms] OR "animals, wild"[MeSH Terms]) AND ("Animals"[MeSH Terms] OR "humans"[MeSH Terms] OR "animal feed"[MeSH Terms] OR "manure"[MeSH Terms] OR "aquaculture"[MeSH Terms] OR "waste water"[MeSH Terms] OR "sewage"[MeSH Terms] OR "hospitals"[MeSH Terms] OR "hospitals, animal"[MeSH Terms] OR "cities"[MeSH Terms]) NOT "therapeutics"[MeSH Terms] NOT "drug discovery" [MeSH Terms] NOT "aids"[All Fields] NOT "hiv"[All Fields] NOT "influenza"[All Fields].

The search string for CAB Abstracts© was:

("Drug Resistance".mp. and ("environment$" or "soil" or "water" or "water pollution" or "air pollut$" or "wild animals").hw. and ("animals" or "man" or "feeds" or "manures" or "aquaculture" or "wastewater$" or "sewage" or "hospitals" or "animal hospitals" or "urban areas").hw.) not "Therapeutics".af. not "Drug discovery".af. not "aids".af. not "hiv".af. not "influenza".af.

The search string for Scopus© was:

TITLE-ABS-KEY ((antibiotic OR antimicrob*) AND resistan*) AND KEY ("environment*" OR "soil" OR "water" OR "water pollution" OR "air pollut*" OR "wild animals") AND KEY ("animals" OR "man" OR "feeds" OR "manures" OR "aquaculture" OR "wastewater*" OR "sewage" OR "hospitals" OR "animal hospitals" OR "urban areas") AND NOT TITLE-ABS-KEY ("Therapeutics") AND NOT TITLE-ABS-KEY ("Drug discovery") AND NOT TITLE-ABS-KEY ("aids") AND NOT TITLE-ABS-KEY ("hiv") AND NOT TITLE-ABS-KEY ("influenza")

The same protocol was used for both culture-independent (ARG) and culture-dependent (ARB) outcomes, and thus studies with both outcome types were assessed as a whole up to the data extraction process, at which point ARG and ARB outcomes were independently evaluated. Therefore, although the focus of this publication is ARGs, the initial results include publication numbers relevant to ARB and ARGs.

There were no language or geographical limits on the search. All citations were imported into the EndNote reference management software package (Thomson Reuters, Philadelphia, PA), and duplicate records were removed.

Titles and abstracts of all citations were then screened to include only those relevant to the question. Specifically, studies were included if they: (a) were primary research; (b) collected environmental samples (soil, water, sediment, air, biological samples from wildlife); and (c) reported prevalence or concentration of ARG. An additional exclusion criterion – not stated in the original protocol (Williams-Nguyen *et al.*, 2016a) – was added that asked: 'Does the study use microbial source tracking techniques?' Microbial source tracking techniques compare characteristics of fecal bacteria isolated from environmental sources with characteristics of fecal bacteria from known sources in an effort to identify the source of environmental isolates. Because these types of studies often fail to compare sites based on physical distance or direction from the source (e.g. (Edge and Hill, 2005; Dickerson *et al.*, 2007; Mthembu *et al.*, 2010; Murugan *et al.*, 2012)), such studies do not provide evidence for this systematic review question. Any study that did not meet all these criteria was excluded. Those studies where it could not be ascertained from the title and abstract if they met all criteria were considered 'unclear' and passed through to the following screening phase for further clarification.

Full-text of remaining articles was retrieved, and the methods section only was reviewed. It was then determined whether the methodology used for each study was adequate to answer the systematic review question using the following inclusion criteria. Studies were included if they: (a) reported proximity to, or direction from a potential point source; and (b) had a comparison group (i.e. samples taken a fixed distance from or upstream from the source) or compared across a range of distances (i.e. samples taken at different distances from the source). Those studies that did not meet both criteria were excluded. An additional question not stated in the protocol (Williams-Nguyen *et al.*, 2016a) *a priori* was added at this screening stage as follows: 'Does the study implicitly or explicitly define a point source with reference to which a comparison was defined?'. During this screening phase, articles not written in English were identified, and an effort was made to translate the full text as review team resources allowed.

Pre-testing of the screening process was done by reviewing a sample of articles among all the citations from the database. Specifically, four articles that featured comparison groups based on information in the title or abstract were chosen. Papers of this type were selected to ensure testing of the second screening level (design screening). Two independent reviewers evaluated this phase, and improvements to the screening process and data entry were made based upon the reviewer's feedback. Final screening decisions were entered into a spreadsheet designed for this systematic review (Microsoft Office Excel 2013® Microsoft Corporation, Redmond, WA, USA).

For both screening phases (title/abstract and methods section of the article's full-text), two reviewers independently assessed each record. Consensus was required, and conflicts were resolved through phone conferences and e-mail.

Following the application of the inclusion/exclusion criteria, the full-text of each included study was evaluated for potential threats to internal validity (risk of bias assessment) by two independent reviewers per article. A customized relational database (Microsoft Access 2013®) was used for data entry on the risk of bias assessment. First, a qualitative rubric (explained below) was pre-tested by reviewing a sample from the included full-text articles after the two screening stages by three independent reviewers. A total of three articles were evaluated for this purpose. The pre-testing improved the consistency of the risk of bias assessment across reviewers, as well as the design of the data entry tool.

Articles were divided equally between each participating reviewer. A qualitative rubric of low, high, and unclear was assigned to each study for the potential risk of bias in the reported effect measure or other outcome variable due to selection bias, information bias, and confounding (Williams-Nguyen *et al.*, 2016a). The risk of bias assessment was conducted at the

study level and not at the outcome level due to the large number of possible outcomes per study. Selection bias was defined as systematic differences between the comparison groups with respect to how samples were collected in the study (methods used across sites). Information bias was defined as systematic differences in the methods for ascertaining ARG between comparison groups (i.e. use of different laboratory methods for the samples in the comparison groups). Confounding was evaluated with respect to the presence of point or non-point sources other than the source of interest that could have affected the study outcomes. It was assumed that a study that assessed the impact of a point source using sampling locations within a large spatial scale (e.g. 100 km distance between sampling locations) was at higher risk of confounding than a study where the spatial scale was smaller (e.g. a 10 km scale) due to the possible influence on the outcome of a larger number of alternative point and non-point sources, unless adequate confounding control measures were described. For all three types of biases, strategies to control or minimize the impact of these biases on the internal validity of the study were factored into the decision to classify them as low, unclear or high.

A final qualitative (low, high, and unclear) overall bias rubric was assigned to each study by considering the risk of bias from each domain after consensus was reached between the reviewers. In general, if a study had at least one out of the three domains classified as high risk, the overall result was considered high risk of bias, and the same applied for unclear risk of bias. However, there were exceptions, and the overall decision was made on a case-by-case basis relying on the judgment of the three reviewers involved in the risk of bias assessment.

Data from all studies, including the ones that were deemed to be at high risk of bias, were extracted and synthesized. Data consisted of characteristics of the study (geographic location, publication year, spatial scale, sampling design, type of laboratory detection method used), the exposure (point source), and the outcome: ARG prevalence or ARG concentration (either relative gene abundance, defined as ARG copies normalized to 16S copies, (2) absolute gene abundance or (3) gene concentration, defined as ARG copies divided by a measurement of volume), as reported by the authors, without further manipulation of that data. Any available information on statistical methods or modeling approaches used, and effect measures (and variability) reported for the comparison of interest were recorded. Data were entered into the same custom relational database, albeit in a different table from the one used for the risk of bias assessment. Additionally, a summary of the most relevant findings for the comparison of interest from each individual study was conducted and is presented in Tables 2 and 3.

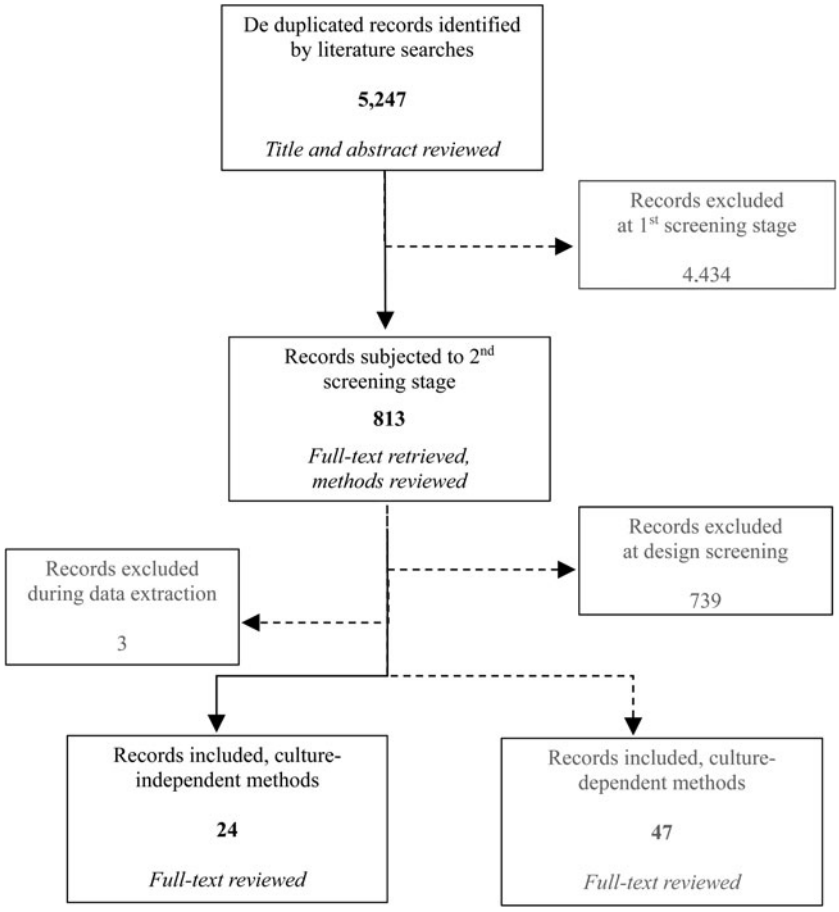

Fig. 2. Flowchart summarizing the selection process for the studies (the shaded boxes depict the articles excluded from the process and the records for the ARB outcome, not assessed in this paper).

Table 1. Descriptive information for each one of the 24 studies included in this systematic review

Citation	Country(s)	Spatial scale	Source type	Environmental media/system
Amos *et al.* (2015)	UK	50 km	Human waste (WWTP)	Sediment cores/river
Berglund *et al.* (2015)	Sweden	3.5 km	Human waste (WWTP)	Sediment/river
Czekalski *et al.* (2012)	Switzerland	3.2 km	Human waste (WWTP)	Surface water, Sediment/lake
Czekalski *et al.* (2014)	Switzerland	4 km	Human waste (WWTP)	Sediment/lake
Harnisz *et al.* (2015)	Poland	400 m	Aquaculture (fish farm)	Surface water/river
Hong *et al.* (2013)	USA	15 m	Terrestrial agriculture (swine farm)	Ground water/river
Jia *et al.* (2014)	China	10 km	Terrestrial agriculture (swine farm)	Surface water/river
Khan *et al.* (2013)	Pakistan	20 km	Human waste (urban area)	Sediment/river
Kristiansson *et al.* (2011)	India, Sweden	20 km	Human/industrial waste (WWTP receiving pharmaceutical manufacturing waste)	Sediment/river
Lapara *et al.* (2011)	USA	8 km	Human waste (WWTP)	Surface water, Sediment/river, lake
Lapara *et al.* (2015)	USA	>960 km	Human waste (WWTP)	Surface water/river
Makowska *et al.* (2016)	Poland	Not reported	Human waste (WWTP)	Surface water/river
Marti *et al.* (2013)	Spain	200 m	Human waste (WWTP)	Sediment, biofilm/river
Mceachran *et al.* (2015)	USA	10–20 m	Terrestrial agriculture (Beef feedlot)	Air
Pei *et al.* (2006)	USA	50 km	Human waste (urban area)	Sediment/river
Proia *et al.* (2016)	Spain	1.1 km	Human waste (WWTP)	Biofilm/river
Pruden *et al.* (2012)	USA	>100 km	Human waste, CAFOs	Surface water/river
Rodriguez-Mozaz *et al.* (2015)	Spain	0.5 km	Human waste (WWTP)	Surface water/river
Sidrach-Cardona *et al.* (2014)	Spain	1.5 km	Human/industrial waste (WWTP, antibiotic-production plant)	Sediment, surface water/river
Stalder *et al.* (2014)	Not reported	5 km	Human waste (WWTP)	Surface water/river
Tamminen *et al.* (2011)	Finland, Sweden	1 km	Aquaculture (fish farm)	Sediment/sea
Uyaguari *et al.* (2011)	USA	100 km	Human waste (WWTP)	Surface water, sediment/sea
Xu *et al.* (2015)	China	Not reported	Human waste (WWTP)	Surface water/river
Zhang *et al.* (2013)	China	50 km	Human waste (urban area)	Surface water/river

In contrast to the original protocol (Williams-Nguyen *et al.*, 2016a), the risk of bias assessment was conducted prior to the data extraction. To minimize introduction of bias by conducting these steps in reverse order, the reviewers who assessed studies during the risk of bias stage did not review the same studies during the data extraction, and were blinded to the risk of bias assessment decisions. Afterwards, a review team member uninvolved in either risk of bias assessment or data extraction validated all extracted data.

Results

The total number of records (including both culture-dependent to ascertain ARB and culture-independent methods for ARGs) returned by search strings totaled 5247 after de-duplication. The number of articles remaining after each screening step was 813 and 75, respectively. In total, 27 of the 75 included articles used culture-independent methods to ascertain ARGs. At the point of data extraction, three studies were identified wherein data were presented as aggregated and no qualitative or quantitative comparison of ARG prevalence or ARG concentration by distance or direction from the source was available. Therefore, these studies were excluded as providing no information about this systematic review question (Auerbach *et al.*, 2007; Bajaj *et al.*, 2015; Xi *et al.*, 2015). Hence, the final number of studies assessed in this review was 24 (Fig. 2).

For the overall risk of bias assessment, three studies were categorized as high risk of bias, 13 were at an unclear risk of bias, and eight were deemed to be at low risk for bias. The rubric for the risk of bias levels was previously published (Williams-Nguyen *et al.*, 2016a).

An example of a study considered at high risk of bias was Zhang *et al.* (2013). This study involved collection of samples at a spatial scale of about 50 km and did not adjust for potential confounders in the analysis, such as other point or non-point sources in the 50 km study area. An example of a low risk of bias study was Pruden *et al.* (2012). Despite a spatial scale of more than 100 km, this study controlled for potential confounders from many other sources of anthropogenic effluent by using linear regression modeling to account for distance to different source types. An example of a study with unclear risk of bias was Lapara *et al.* (2011). In this study, description on the selection of samples at different locations was lacking. Additionally, there were possible confounders such as effluent from non-point sources from agricultural and recreational

Table 2. Findings for the studies included in this systematic review that assessed human waste (WWTP, industrial, urban areas) as a point source, organized by risk of bias (from low to high) ($n = 19$).

Citation	Relevant comparison	Overall risk of bias	Gene(s)	Relevant findings
Amos *et al.* (2015)	Log-log regression model (Model 2) explaining relative abundance of *intI1* in Thames River sediment at sites across a range of WWTP impacts (defined as a function of type of, size of, and river course distance from WWTPs), adjusting for land cover, season and rainfall. Relative gene abundance was calculated as: number of *intI1*/number of 16S rRNA genes	Low (despite large spatial scale, they adjust for potential confounders)	*intI1*	Assuming all variables included in Model 2 are independent predictors of *intI1* relative abundance, the model indicated that a 10% increase in the total WWTP impact at a given site is associated with a 3.2% increase in *intI1* relative abundance adjusting for land cover, season and rainfall ($\beta = 0.3207 \pm 0.0723$, $P < 0.001$). The model predicted the impact of a large activated sludge-treatment plant on a clean site in the river to be a 200-fold (0.01–2.44%) increase in *intI1* relative abundance immediately downstream and 65-fold 10 km downstream. This model explained 83% of the variation in log *intI1* relative abundance at a single point in a river at any season within the sample used to construct the model (adjusted $R^2 = 0.83$) and 78% of the variation in a sample of four independent sites from elsewhere on the River Thames (out of sample validation)
Berglund *et al.* (2015)	Relative gene abundance in river water between sites immediately (R3), 1 km (R4) and 2.5 km (R5) downstream compared with sites immediately (R2) and 1 km (R1) upstream of a WWTP. Relative gene abundance was calculated as: ARG copies/16S rDNA copies	Low (small spatial scale-unlikely influence from other point or non-point sources)	*dfr1* *ermB* *intI1* *sul1* *tetA* *tetB* *vanB*	Overall, relative gene abundances were higher at downstream (R3) compared with upstream (R1 and R2) sites. ***dfr1***: above detection limit downstream vs. below detection limit upstream ***ermB***: relative abundance higher downstream vs. upstream ($P < 0.01$) ***intI1***: relative abundance approximately 10 times higher downstream vs. upstream ($P < 0.001$) ***sul1***: relative abundance 10 times higher downstream vs. upstream ($P < 0.01$) ***tetA***: relative abundance 10 times higher downstream vs. upstream ($P < 0.01$) ***tetB***: above detection limit at 2 of 3 downstream sites (R3 and R4) vs. below detection limit upstream ***vanB***: no statistical difference between sites; not detected at R2
Proia *et al.* (2016)	On 4 rivers (designated ARB, BRE, GUA, SMP) each featuring a WWTP, relative gene abundance in biofilm from sites 50–100 m (DW) and 1 km (DW1) downstream of WWTP compared with a site 100 m upstream (UP) accounting for variation between rivers. Relative gene abundance was calculated as: ARG copies/16S rRNA gene copies	Low (small spatial scale for each comparison, and lack of other activities that may influence the outcome is mentioned)	$bla_{CTX\text{-}M}$ *ermB* *qnrS* *sul1*	Overall, despite variation between rivers, relative gene abundance was significantly higher at downstream sites, particularly site DW, compared with upstream. ANOVA results indicated that the magnitude of this effect differed between rivers. No effect estimates were provided. Alpha cut-off for statistical inference was $\alpha = 0.05$

Table 2. (*Cont.*)

Citation	Relevant comparison	Overall risk of bias	Gene(s)	Relevant findings
Pruden *et al.* (2012)	General linear regression models (Model 9) explaining log relative gene abundance averaged across river sediment type (bed and suspended) and across season in a river basin as function of upstream capacities of animal feeding operations and WWTPs weighted for inverse distance along surface water pathways. Samples were collected at 10 sites along the river basin representing a range of exposure types. Relative gene abundance was calculated as: ARG copies/16S rRNA gene copies	Low (despite a large spatial scale, potential confounding was controlled for)	*sul1* *tetW*	The association between average log relative ***sul1*** abundance and the combined impact of inverse-distance-weighted upstream WWTP and animal feeding operation capacities was statistically significant (Model 9_{sul1}, $F = 40.2$, $P < 0.0001$, DF = 7, $R^2 = 0.92$). Average log relative ***tetW*** abundance was not found to be associated with upstream WWTPs and animal feeding operations (Model 9_{tetW}, $F = 0.2$, $P = 0.8391$, DF = 7, $R^2 = 0.06$). Individual effect estimates for WWTPs and animal feeding operations, respectively, and accompanying statistical inference were not provided in a fully adjusted model for either gene
Sidrach-Cardona *et al.* (2014)	Relative gene abundance in river water and sediment samples at a site downstream (site 2) compared with upstream (site 1) of an antibiotic production plant (APP). Relative gene abundance was calculated as: log (ARG copies/16S rRNA gene copies)	Low (unlike introduction of any type of bias)	$bla_{CTX\text{-}M}$ bla_{SHV} bla_{TEM}	In surface water or sediment, graphical inspection of relative abundance means and standard errors compared between sites 2 and 1 did not support an effect of the APP on downstream gene abundance for any gene investigated. ***bla*^{SHV}** was not detected at either site in sediment. Effect estimates and statistical inference were not provided
Sidrach-Cardona *et al.* (2014)	Relative gene abundance in river water and sediment samples at sites downstream (sites 4–6) compared with upstream (site 3) of a WWTP. Sites 3–6 were located 10 km downstream of an antibiotic production plant. Relative gene abundance was calculated as: log (ARG copies/16S rRNA gene copies)	Low (unlike introduction of any type of bias)	$bla_{CTX\text{-}M}$ bla_{SHV} bla_{TEM}	Evidence was mixed for an effect of the WWTP on downstream relative gene abundance. Graphical inspection of relative abundance means and standard errors at site 3 compared with sites 4–6 suggest that abundance of ***bla*$^{CTX\text{-}M}$** and ***bla*^{SHV}** in river water and ***bla*^{SHV}** in sediment may have been significantly higher downstream of the WWTP compared with upstream. But similar findings were not evident for other comparisons. Effect estimates and statistical inference were not provided
Stalder *et al.* (2014)	In river water at sites 3 km downstream compared with 2 km upstream of WWTP discharge point. Relative gene abundance was calculated as: ARG copies/(16S rRNA gene copies)	Low (unlike influence of other sources)	*intl1* *intl2* *intl3*	No difference in relative gene abundance was observed between upstream and downstream sites ($P > 0.05$) for any gene tested
Czekalski *et al.* (2012)	Relative gene abundance in lake water near WWTP outfall site (STEP) compared with site 3.2 km away (DP). Relative gene abundance was calculated as: ARG copies/16S rRNA copies	Unclear (insufficient information about possible confounding due to lake depth)	*sul1* *sul2*	Qualitatively, no difference in relative abundance of ***sul1, sul2*** between STEP and DP not reported (both less than 1% in relative abundance at both sites)

Czekalski *et al.* (2014)	Relative gene abundance in lake sediment compared across a range of distances (0 to ~6 km) from WWTP outfall (STEP). Relative gene abundance was calculated as: ARG copies/16S rRNA copies	Unclear (insufficient information about possible confounding due to lake depth)	*sul1* *sul2* *tetB* *tetM* *tetW* *qnrA*	Graphical regression analysis supported exponential decay of ***sul1***, ***sul2***, ***tetB***, ***tetM***, and ***tetW*** relative abundance at increasing distance from STEP, and interpolation analysis suggested directionality of impact. Relative ***qnrA*** abundance was below the detection limit at all sites. Statistical inference for these differences was not provided
Khan *et al.* (2013)	Relative gene abundance in river sediment sampled at downstream sites near (R2) and 19 km (R3) from Lahore city center compared with a site 6 km upstream (R1) from the city. Relative gene abundance was calculated as: ARG copies/10^6 × 16S rRNA gene copies	Unclear (not enough information about other potential sources)	*dfrA1* *ermB* *intI1* *sul1* *tetA* *tetB*	Evidence suggested an effect of distance to Lahore city center on the relative abundance of all target genes. Although quantitative effect measures were not provided, a significant increasing trend from R1 to R3 was reported ($P < 0.01$)
Kristiansson *et al.* (2011)	Relative gene abundance in river sediment at sites 0.05 km (R4), 2.3 km (R2), 2.7 km (R3), and 17.5 km (R1) downstream from WWTP compared with sites located 1.9 km (R5) and 2.2 km (R6) upstream (India). Relative gene abundance was calculated in relation to the total number of identified bacterial cells	Unclear (not enough information provided about sampling sites to determine if other sources would influence the outcome)	*qnrS* *sul2* *strA* *strB*	Relative abundance of ***sul2*** (66 times), ***strA*** (22 times), and ***strB*** (54 times) was higher downstream compared with upstream. Relative abundance of ***qnrS*** was lower downstream compared with upstream. Effect estimates (computed differences) and accompanying statistical inference was not provided
Kristiansson *et al.* (2011)	Relative gene abundance in river sediment a site 25–230 m (N) downstream from WWTP compared with a site located 5–100 m (U) upstream (Sweden). Relative gene abundance was calculated in relation to the total number of identified bacterial cells	Unclear (not enough information provided about sampling sites to determine if other sources would influence the outcome	*qnrS* *sul2* *strA* *strB*	***sul2***, ***strA*** and ***strB*** were not detected at any site. Relative abundance of qnrS was slightly higher downstream compared with upstream. Effect estimates (computed differences) and accompanying statistical inference was not provided
Lapara *et al.* (2011)	Gene concentration in surface water at sites approximately 1.5–24 km downstream/ distant from WWTP compared with site approximately 1.6–9 km miles upstream; and gene concentration in sediment at sites near WWTP outfall compared with sites approximately 5 km and 16 km distant. Gene concentration was calculated as: ARG copies per mL water	Unclear (not enough information about selection of sampling sites or influence of other potential sources)	*intI1* *tetA* *tetX* *tetW*	In water, concentrations of ***intI1***, ***tetA***, ***tetX***, and ***tetW*** were higher immediately at the WWTP outfall, but there was little or no apparent difference between upstream and downstream/distant sites. In sediment samples, concentrations of ***intI1***, ***tetA***, ***tetX***, and ***tetW*** were higher at a site near the WTTP (DH1) compared to the more distant sites (DH3 and LS1). Effect estimates (computed differences) and accompanying statistical inference was not provided
Makowska *et al.* (2016)	Mean relative gene abundance in river water at sites downstream of WWTP compared to sites upstream of WWTP. The distances from WWTP were not reported. The relative abundance was calculated: gene copies/16S rRNA gene × average number of 16S rRNA gene copies per bacterial cell × 100	Unclear (not enough information about other potential sources)	*intI1* *sul1* *sul2* *tetA* *tetB* *tetM*	Mean relative gene abundances were higher at downstream sites compared to upstream sites for all genes tested, however none of these differences were found to be statistically significant. Effect estimates (computed differences) and p-values not reported ***intI1***: 0.65 (downstream) vs. 0.21 (upstream) ***sul1***: 0.49 (downstream) vs. 0.07 (upstream) ***sul2***: 0.40 (downstream) vs. 0.17 (upstream) ***tetA***: 0.053 (downstream) vs. 0.004 (upstream) ***tetB***: 0.053 (downstream) vs. 0.014 (upstream) ***tetM***: 0.016 (downstream) vs. 0.009 (upstream)

Table 2. (*Cont.*)

Citation	Relevant comparison	Overall risk of bias	Gene(s)	Relevant findings
Marti *et al.* (2013)	Relative gene abundance in sediment and biofilm samples from a site 100 m downstream compared with a site 100 m upstream of the WWTP. Relative gene abundance was calculated as: ARG copies/16S rRNA gene copies	Unclear (not enough information about other potential sources affecting the upstream site)	$bla_{CTX\text{-}M}$ bla_{SHV} bla_{TEM} *ermB* *qnrA* *qnrB* *qnrS* *sul1* *sul2* *tetO* *tetW*	Overall, there was evidence that the WWTP impacted the relative abundance of resistance genes in sediment and biofilm samples. Effect estimates were not provided In sediment samples, relative abundance of ***ermB*** was significantly higher downstream compared with upstream. Relative abundance was also slightly higher downstream than upstream for most other genes (***bla$^{CTX\text{-}M}$, bla^{SHV},bla^{TEM} , qnrS, sul1, sul2, tetO, tetW***), but these difference were not significant. The remaining gene examined, ***qnrA***, was not detected at either river site In biofilm samples, relative abundance was significantly higher downstream compared to upstream for most genes examined (***bla^{TEM}, bla^{SHV}, ermB, qnrB, qnrS, sul1, sul2, tetO***, and ***tetW***) at $\alpha = 0.05$. And while relative gene abundance was also higher downstream compared with upstream for ***bla$^{CTX\text{-}M}$***, this difference was not significant. For ***qnrA***, relative gene abundance was lower (not detected) downstream compared with upstream, but the difference was not significant Alpha cut-off for comparison was $\alpha = 0.05$
Pei *et al.* (2006)	Relative gene abundance in river sediment sampled at a site downstream from a city and point of discharge of a wastewater reclamation facility (site 4) compared to a site upstream from the city and discharge point (site 2). Sampling was conducted during high-flow and low-flow conditions, yielding a comparison for each sampling condition. Relative gene abundance was calculated as: ARG copies/16S rRNA gene copies	Unclear (not enough information provided about the potential influence of other point sources)	*sul1* *sul2* *tetO* *tetW*	Evidence supporting an effect of the city and discharge point on gene abundance was mixed. Qualitative comparison indicated higher mean relative abundance of ***sul1*** (during high-and low-flow conditions) and ***tetO*** (during high-flow conditions) when comparing downstream (site 4) with upstream (site 2) sites. However, there was no difference or a lower abundance downstream for ***sul1***, ***tetW*** and ***tetO*** (during high-flow conditions). Effect measures and accompanying statistical inference were not provided
Rodriguez-Mozaz *et al.* (2015)	Absolute gene concentration in river water at a site 250 m downstream compared with a site 250 m upstream from a WWTP. Gene concentration was calculated as: log (ARG copies per mL water)	Unclear (not enough information provided about selection of samples, other potential sources, or about inclusion of other covariates in the analysis)	bla_{TEM} *ermB* *qnrS* *sul1* *tetW*	Mean concentration of ***ermB***, ***qnrS***, and ***sul1*** was significantly higher downstream compared with upstream ($P < 0.05$). Mean concentration of ***bla^{TEM}***, ***tetW*** was not significantly different at upstream and downstream sites ($P > 0.05$)

Uyaguari *et al.* (2011)	Gene concentration (copies per g sediment) and relative gene abundance (copies per ng DNA) in sediment and river water samples from sites near (site 2) and downstream of (site 4) WWTP outfall compared with site upstream (site 3)	Unclear (different type of water, river and ocean, could affect the outcome; Not enough information about potential influence of other sources)	$bla_{M\text{-}1}$	Evidence did not support an increase in gene concentration or relative abundance associated with the WWTP. Both concentration and relative abundance were significantly lower near the WWTP outfall and downstream compared with upstream ($P < 0.05$)
Xu *et al.* (2015)	Relative gene abundance in river water at a site downstream (T2) compared to upstream (T1) of a WWTP. Relative gene abundance was calculated as: ARG copies/16S rRNA gene copies	Unclear (not enough information about sampling locations or other potential sources upstream or around the source)	*gyrA* *parC* *qnrC* *qnrD* *sul1* *sul2* *sul3* *tetA* *tetB* *tete* *tetM* *tetW* *tetZ*	Qualitative evidence was conflicting for an effect of the WWTP on relative gene abundance generally. Mean relative abundances of ***parC, qnrC, qnrD, sul1, tetA, tetE, tetZ*** were higher downstream compared with upstream, while the differences in relative abundance of ***gyrA, sul2, sul3, tetB, tetW*** were not significantly different between downstream and upstream sites. Relative abundance of ***tetM*** was significantly lower downstream compared with upstream. Effect estimates and accompanying statistical inference were not provided
Lapara *et al.* (2015)	Fluid kinetics (plug-flow) model explaining relative abundance of genes in the upper Mississippi River (>960 km reach) as a function of river flow rates, downstream distance from WWTPs, volume of fluid inputs, and modeling assumptions. Relative gene abundance was calculated as: ARG copies/16S rRNA copies	High (large spatial scale assessing WWTP but not considering influence of agricultural and other sources)	*bla* *ermB* *intl1* IncA/C plasmid *qnrA* *sul1* *tetA* *tetW* *tetX*	Overall, qualitative comparison of model predictions with measurements from 12 sampling locations along the river reach did not show good fit to the data for ***intl1***, ***ermB***, ***sul1***, ***tetA***, ***tetW***, and ***tetX***. **IncA/C plasmids** and a synthetic beta-lactamase (***bla***) gene were not detected in river water. Model results for ***qnrA*** were not reported. Summary effect estimates were not available due to the nonlinearity of the model
Zhang *et al.* (2013)	Relative gene abundance in river water at a site downstream (N6) compared with upstream (N5) from a town. Relative abundance was calculated as: log (ARG copies/16S rRNA gene copies)	High (many potential sources besides the one of interest, and large spatial scale)	*aacC1* bla_{TEM} bla_{OXA1} *cmlA5* *dfrA1* *ermB* *sul2* *tetA* *tetG* *strA* *vanA*	There was little support for an effect of the town on relative gene abundance in river water. Differences in the mean relative abundance were not qualitatively apparent for any gene tested. Effect measures (difference in means) and accompanying statistical inference were not provided for this comparison

In the Relevant findings column, specific genes from each study are emphasized in bold. WWTP: wastewater treatment plant.

Table 3. Findings for the studies included in the systematic review that had animal agriculture (both terrestrial and aquaculture) as a point source, presented by risk of bias (from low to high) ($n = 5$)

Citation	Relevant comparison	Overall risk of bias	Gene (s)	Relevant findings
Harnisz *et al.* (2015)	Relative gene abundance at a site approximately 200 m downstream from the discharge point of a freshwater fish farm (DRW) compared with a site approximately 200 m upstream (URW) from that point. Relative gene abundance was calculated as: log (ARG copies/16S rRNA gene copies)	Low (small spatial scale, hence unlike influence from other sources)	*tetA* *tetC* *tetL* *tetO*	Relative gene abundance was higher downstream compared with upstream for some genes for some sampling periods, but these differences were not statistically significant ($P > 0.13$ for all comparisons)
Mceachran *et al.* (2015)	Relative gene abundance in airborne particulate matter collected at sites 10–20 m downwind from beef cattle feedlots compared with sites 10–20 m upwind. Relative gene abundance was calculated as: ARG copies/16S copies	Low (confounding is addressed by weather and other feed yards by restriction, plus small spatial scale)	*tetB* *tetL* *tetM* *tetO* *tetQ* *tetW*	Evidence supports an effect of the cattle feedlot on the *tet* gene abundance in airborne particulate matter. Genes ranged from 100 to over 1000-fold more abundant in downwind samples compared to upwind samples ($P < 0.002$). The greatest relative increase was observed for ***tetM***. Statistical test used for inference was not described
Hong *et al.* (2013)	Gene abundance in groundwater at sites down-gradient from a swine farm (E4, E6, E7) compared with sites up-gradient from the farm (E1, Facility well). Gene abundance was reported as log copies per ng DNA	Unclear (not enough information provided about other potential sources)	*tetZ* *tetQ* *intI1* *intI2*	Qualitative comparison of down-gradient vs. up-gradient sites does not support an effect of the farm on gene abundance. Genes were detected in qualitatively similar abundance at the facility well and down-gradient sites. Genes were not detected at site E1. No effect measures or accompanying statistical inference were provided
Tamminen *et al.* (2011)	Relative gene abundance in marine sediment collected at varying distances (200, 400, 600, 800, and 1000 m) from a fish farm boundary on several sampling occasions over a 2 year period. Relative gene abundance was calculated as: gene copies/16S rRNA gene copies	Unclear (Not enough information provided about the sampling locations)	*tetA* *tetC* *tetH* *tetM*	There was no evidence of an effect of distance to the fish farm on relative abundance of the targeted *tet* genes in sediment. Abundances were below the limit of detection at all sites outside the fish farm boundary
Jia *et al.* (2014)	Relative gene abundance in surface water sampled along water course at varying distance from a swine farm. Sites S2 and S3 were along the drainage gutter at approximately 0.5 and 1 km downstream, respectively. Sites S4–S8 were along the receiving river system at approximately 2–6 km downstream. Relative gene abundance was calculated as ARG copies/16S rRNA copies	High (no information provided about other sources with an overall study spatial scale of 10 km)	*tetC* *tetM* *tetO* *tetQ* *tetW* *tetX*	Qualitatively, evidence suggested a sigmoidal decay of gene abundance at increasing distance from the swine farm supporting a possible effect of the farm on gene abundance in the waterway. In particular, relative abundance was higher in the drainage gutter compared with the river. However, effect estimates and statistical inference were not provided

water use that were not mentioned. Given the lack of information, it was not possible to determine if the risk of bias of estimates of the relationship between the source of interest (WWTP) and ARG concentration (*intI1*, *tetA*, *tetX*, and *tetW*) in river and lake surface waters and sediments was high or low in this study, and thus it was classified as unclear.

While all included studies were written in English, one study written in Chinese (Liu *et al.*, 2012) was deemed relevant to the review question based on the title and abstract that were available in English; however, full-text translation was not feasible, hence it is uncertain if it would have been finally included. No other non-English articles met eligibility criteria based on English title or abstract (or translations of the title/abstract).

The geographic location of the studies ($n = 24$) was diverse: China ($n = 3$), Finland ($n = 1$), India ($n = 1$), Pakistan ($n = 1$), Poland ($n = 2$), Spain ($n = 4$), Sweden ($n = 3$), Switzerland ($n = 2$), UK ($n = 1$), USA ($n = 7$). There was one study (Stalder *et al.*, 2014) in which the location could not be ascertained after reviewing the full-text, and two studies involved two different countries (Kristiansson *et al.*, 2011; Tamminen *et al.*, 2011).

Date of publication ranged from 2006 to 2016, with the highest number of publications in 2015 ($n = 7$). The spatial scale for the sampling frame ranged from 10–20 m (McEachran *et al.*, 2015) to more than 900 km (Lapara *et al.*, 2015).

The majority of studies investigated not only point sources of human waste, especially wastewater treatment plants ($n = 16$), but also urban areas ($n = 3$). Terrestrial animal agriculture was examined in three studies: two studies examined swine farms and one study examined a beef cattle feedlot. Aquaculture (fish farms) was assessed in two studies.

Surface water was the most common type of environmental media sampled ($n = 13$), followed by sediment ($n = 12$), biofilm ($n = 2$), air ($n = 1$), and groundwater ($n = 1$). None of the included studies sampled wildlife. Five of the studies collected more than one sample type. For a summary of the sampling information, see Table 1.

Overall, the most common target gene outcomes were *sul1* ($n = 12$), *tetW* ($n = 11$), *tetA* ($n = 9$), and *sul2* ($n = 8$). The number of genes per study ranged from 1 to 13, with the majority of studies evaluating four different genes ($n = 7$). Most studies used q-PCR to ascertain ARGs ($n = 23$), and one study used shotgun metagenomics (Kristiansson *et al.*, 2011).

Regarding the outcome data type, 20 studies compared relative gene abundance only, three compared absolute gene concentration only, and one study compared both relative gene abundance and absolute gene concentration. None of the studies used prevalence as their outcome type.

With reference to statistical methods and modeling approaches, nine out of the 24 studies conducted statistical analysis to compare ARG outcomes upstream vs. downstream (or near vs. far sites) with reference to a single point source, and three out of the 24 studies used modeling approaches to describe the effect of multiple sources. However, no effect measures were described in any study. Specifically, one study used a *t*-test to compare relative gene abundance of each one of the target ARG between upstream and downstream sites from a WWTP (Berglund *et al.*, 2015). Eight studies compared the relative gene abundance (Khan *et al.*, 2013; Marti *et al.*, 2013; Stalder *et al.*, 2014; Harnisz *et al.*, 2015; Makowska *et al.*, 2016; Proia *et al.*, 2016) or the absolute gene concentration (Uyaguari *et al.*, 2011; Rodriguez-Mozaz *et al.*, 2015) across sites using either ANOVA or a non-parametric method for comparison of means such as Kruskal–Wallis, Friedman, or Mann–Whitney tests at the 0.05 significance level. One study compared the relative gene abundance of ARGs across sites based on distance from the source using graphical regression and interpolation (Czekalski *et al.*, 2014). Of the nine studies that reported statistical inference, six found a significant relationship for the majority of the target ARG (Uyaguari *et al.*, 2011; Khan *et al.*, 2013; Marti *et al.*, 2013; Berglund *et al.*, 2015; McEachran *et al.*, 2015; Proia *et al.*, 2016), and three did not (Stalder *et al.*, 2014; Harnisz *et al.*, 2015; Makowska *et al.*, 2016). Of the three studies that conducted modeling approaches, Amos *et al.* (2015) used a log–log regression model to explain the relative abundance of *intI1* in river sediment samples at sites across a range of WWTP outputs, adjusting for other variables; Lapara *et al.* (2015) used a fluid-kinetics (plug-flow) model to explain the relative abundance of ARG in a river as a function of several variables, including distance from the multiple WWTP; and Pruden *et al.* (2012) conducted general linear regression models to explain the log relative gene abundance along a river with an exposure gradient as a function of several variables.

In the section that follows, results are summarized for each group of point source investigated (human waste and animal agriculture) by the type of comparison made (upstream vs. downstream in rivers or based on distance from the source) and by type of outcome reported (relative gene abundance or absolute gene concentration).

Human waste (n = 19)

From the 19 studies, 16 assessed WWTP and/or industrial waste, and three studies urban areas. Among the 16 that evaluated WWTP and/or industrial waste, 13 compared ARG outcomes in unidirectional systems ($n = 10$) or based on distance ($n = 3$) with reference to a single point source, while three studies described the effect of multiple point sources using modeling approaches.

Among the 10 studies that assessed the impact of WWTP and/or industrial waste in unidirectional systems (i.e. rivers), eight reported relative gene abundance only, one reported absolute gene concentration only, and one reported both. Among the first group, four studies showed a higher relative gene abundance at downstream sites from the source compared with upstream sites (Kristiansson *et al.*, 2011; Berglund *et al.*, 2015; Makowska *et al.*, 2016; Proia *et al.*, 2016). One study reported no difference in relative gene abundance downstream compared with upstream (Stalder *et al.*, 2014). The remaining three studies presented conflicting evidence for the effect of WWTP/industrial waste on the relative gene abundance (Marti *et al.*, 2013; Sidrach-Cardona *et al.*, 2014; Xu *et al.*, 2015). The only study that evaluated absolute gene concentration presented conflicting evidence (Rodriguez-Mozaz *et al.*, 2015). Finally, Uyaguari *et al.*

(2011) reported both a lower gene concentration and a lower relative gene abundance downstream.

Three studies assessed the impact of WWTP across a range of distances. Two of them reported relative gene abundance and one study reported gene concentration. Among the former group, one study found higher relative gene abundance at sites closer to the source compared with distant sites (Czekalski *et al.*, 2014) and the other study found no difference in relative gene abundance between near and far sites (Czekalski *et al.*, 2012). The study reporting gene concentration found higher gene concentration at sites closer to the source compared with distant sites (Lapara *et al.*, 2011). The remaining three studies assessing the impact of WWTP conducted modeling approaches and they all reported relative gene abundance. The model conducted by Amos *et al.* (2015) indicated that a 10% increase in the total WWTP impact (defined as a function of type of, size of, and river course distance from upstream WWTPs) at a given site was associated with a 3.2% increase in the relative abundance of *intI1*, adjusting for land cover, season, and rainfall. The fluid kinetics model predictions by Lapara *et al.* (2015) for the Mississippi river did not show a good fit for the target genes, and the general linear regression models in Pruden *et al.* (2012) in a river system in Colorado showed an association between average log relative *sul1* abundance and the impact of inverse-distance weighted upstream WWTP and animal feeding operation capacities; however, they did not find such an association for the other target gene (*tetW*).

The three studies that assessed the impact of urban areas as the source of human waste reported relative gene abundance in river systems. Khan *et al.* (2013) found a higher relative gene abundance downstream compared with upstream sites; Zhang *et al.* (2013) found no difference between upstream and downstream sites from a city; and Pei *et al.* (2006) reported mixed evidence depending on the sampling season (high vs. low water flow) and on the target genes.

Animal agriculture (n = 5)

Of these five studies, three assessed terrestrial agriculture and two aquaculture. Among the three studies that assessed the impact of terrestrial animal agriculture, two were conducted in unidirectional systems (i.e. rivers), of which one reported relative gene abundance, and one absolute gene abundance. Specifically, McEachran *et al.* (2015) reported a higher relative gene abundance downwind compared with upwind sites from beef cattle feedlots; and Hong *et al.* (2013) did not find a difference in absolute gene abundance in groundwater samples between up-gradient and down-gradient sites from a swine farm. The third study examining terrestrial animal agriculture made comparisons based on distance from a swine farm, reporting higher relative gene abundance near the farm compared with sites farther away (Jia *et al.*, 2014).

The two studies assessing aquaculture as the point source made comparisons of relative gene abundance in a river system (Harnisz *et al.*, 2015) and based on distance from the fish farm (Tamminen *et al.*, 2011). Harnisz *et al.* (2015) found a higher relative abundance of some target genes downstream compared with upstream sites from a fish farm depending on the sampling season, while Tamminen *et al.* (2011) did not find an apparent impact of the fish farm across a range of distances on the relative gene abundance. For more details on the results for individual studies refer to Tables 2 and 3.

Discussion

This systematic review aimed to identify and summarize the available evidence on the impact of anthropogenic point sources on the increase of ARGs in the environment. Based on the authors' prior knowledge of the literature on this subject, the assumption was made that etiologic research on this review question would be uncommon, and that a narrowly focused question would not provide sufficient evidence to be meaningfully summarized. Thus, the review question was broadly formulated, permitting evaluation of a larger pool of evidence but also increasing the heterogeneity among the studies.

Most studies were considered to be unclear for risk of bias. The common reason for this was lack of information about potential confounders that might bias the observed relationship between proximity to a point source and levels of ARG in environmental media. The predominant confounder of concern to the review team was the introduction of antibiotics, ARB, or ARGs from other sources that could differentially affect the exposed and comparator sites. Many studies did not provide details about other possible contributors to resistance in the system or did not explain the location of other contributors and sampling sites. Studies with moderate to large spatial scales and no information about potential confounders were common. Most of these were considered to be an unclear or high risk of confounding bias.

We note that risk of bias assessment was conducted before the data extraction, which is a deviation from the original protocol. Though non-standard, this is unlikely to have introduced additional biases into the review findings because different reviewers evaluated the same study at the two different stages, and we extracted data from all studies including those considered at high risk of bias. The risk of bias assessment was conducted using subjective judgment, and despite reviewer consensus, this is a limitation of this review process.

As we noted, the most commonly evaluated point source was WWTPs, which has been recognized to contain a large diversity of ARB and ARGs (Rizzo *et al.*, 2013). Human waste, which can include antibiotics, bacteria, and potentially ARB and ARGs, is treated at WWTPs. However, ARGs are still found after the treatment process, at the WWTP discharge, or at sites downstream from the WWTP (Rizzo *et al.*, 2013); most studies reported the highest levels of ARGs (relative gene abundance or concentration) in river sites downstream from the point source (the WWTP) as compared with upstream sites or near the WWTP (compared with sites far from it). Only five studies assessing the impact of animal agriculture (three terrestrial representing swine farms and a beef feedlot, and two in aquaculture representing fish farms) were included in the final pool of studies to review and reported mixed findings. The small number of

studies, the heterogeneity among the animal systems and other gathered evidence revealed insufficient scientific evidence about the impact of animal agriculture on a measurable increase of antibiotic resistance in the surrounding environment. One potential reason for this knowledge gap is that agricultural farms are more challenging point sources to assess compared with wastewater treatment plants, highlighting the need for additional studies of animal systems.

Overall, there was consistency in the results for the outcome data types (relative or absolute gene abundance or gene concentration) with most studies reporting a higher relative gene abundance and /or gene concentration downstream from the source (in unidirectional studies) or near the source (for those studies based on distance) across all source types.

Across all studies in the review, *sul1*, *sul2*, *tetA*, and *intl1* were the most frequently studied and detected ARGs. *Sul1* and *sul2*, mainly found in Gram-negative bacteria (Sköld, 2000), confer resistance to sulfonamide antibiotics, which are used in both animal and human practice, by modifying the dihydropteroate synthase related to protein synthesis. Among the large group of *tet* genes, *tetA* confers resistance to tetracycline via efflux pumps (Roberts, 2005). In the case of *intl1*, it codes for the integrase enzyme associated with many drug resistant bacteria (Mazel, 2006). However, *intl1*, *intl2*, *intl3* (integrons) are not always associated with AMR. Other ARG commonly detected were bla_{TEM}, bla_{SHV}, and *ermB*. The first two confer resistance to β-lactam antibiotics (e.g. penicillins, cephalosporins, carbapenems) by encoding for β-lactamase enzymes, and *ermB* confers resistance to macrolide antibiotics through the modification of 23S by rRNA methylation (Szczepanowski *et al.*, 2009). None of the studies reported detecting *floR* (perhaps because they did not search for it specifically), which has a wide global distribution and has been found associated with both agriculture and aquaculture (Cloeckaert *et al.*, 2000; Fernandez-Alarcon *et al.*, 2010).

Among those studies that conducted a statistical analysis, ANOVA or an equivalent non-parametric method was the most common approach. Such methods for comparison of means (unlike regression methods) cannot produce a quantitative summary effect measure when used to evaluate complex systems with a large number of relevant comparison groups or covariates. A combination of regression methods such as the ones proposed by Pruden *et al.* (2012) and Amos *et al.* (2015) together with spatial analysis, as used in the study by Czekalski *et al.* (2014) can provide a good framework to address some of the challenges related to bias, and quantification of the impact of point sources on the prevalence or concentration of ARG in the environment.

In light of this review process, the protocol (Williams-Nguyen *et al.*, 2016a) would have benefited from a few modifications (besides the ones we made *a posteriori*) to minimize the limitations and challenges encountered throughout the review. For instance, it would have been valuable to have an available tool to address the quality of the methodology and evidence provided by the studies for our specific review question; a possible solution to this would have been to include only those studies that explicitly defined a comparison of relevance to the review question or that conducted a statistical analysis for such a comparison.

Potential publication bias could not be assessed for this body of evidence. Publication bias is the exaggeration of treatment effect sizes caused by the propensity for journals to preferentially publish research showing statistically significant results (Song *et al.*, 2013). Such bias can cause a meta-analysis to give a misleading picture of the effect size in question, such that the average effect size appears to exist when none is truly present or to exaggerate the magnitude of a significant effect size. Quantitative assessment of the presence of publications bias is possible when the distribution of sufficiently homogeneous effect measures can be examined via funnel plots and other methods (Duval and Tweedie, 2000). This review did not identify such a pool of quantitative results, thus publication bias could not be evaluated. Additionally, some existing evidence may not have been identified by our search. Although some of the databases searched do index grey literature, our search strategy did not identify any. Furthermore, a meta-analysis was not conducted in this review for the same reason (lack of quantifiable homogeneous outcomes).

We identified a number of important considerations for future studies seeking to estimate the effect of a specific point source on environmental levels of ARGs. Our review highlighted the need for epidemiological and/or ecological observational studies that control for selection bias, information bias, and confounding to the extent possible. Such studies will need to describe and adjust for confounders (especially due to other sources of antibiotics or resistant bacteria and/or genes). A good example of such an approach is the study by Pruden *et al.* (2012). Additionally, there remains a need for studies where the data analysis provides effect measures such as odds ratios or risk ratios (for studies with ARG prevalence as the outcome data type) or mean differences (for studies with ARG concentration as the outcome data type) to quantify the magnitude or strength of the effect of the exposure (i.e. the point source) on the outcome (i.e. the prevalence or concentration of ARG in the surrounding environment), accompanied by measures of variability. Pruden *et al.* (2012), despite creating relevant generalized linear regression models of the relationship of interest, did not provide parameter estimates from these models which would be needed to quantify the effect WWTPs had on ARGs after accounting for other sources such as animal feeding operations, and conversely, the effect that animal feeding operations on ARGs after accounting for WWTPs.

Similarly, researchers should use statistical methods to infer the significance of the study findings, and report these along with study results. The most appropriate statistical model(s) will depend on specifics of the study design and on the outcome of interest. Enhanced collaborative work between microbiologists, ecologists, and other scientists to provide expertise where needed will aid in successful efforts to conduct etiologic research.

There is no doubt that the increase of ARB is a global health crisis, and that there is a need to understand and to intervene with the dissemination pathways. The role of the natural environment in the dynamics of antibiotic resistance is an area of

great interest and concern (Singer *et al.*, 2006; Allen *et al.*, 2010). Research on the issue must use methodology able to contend with the inherent complexity of environmental systems subject to flux, as well as the necessarily observational nature of most scientific evidence.

This systematic review provides a strong imperative to improve research methods in order to provide interpretable, quantitative information about the effect of point sources on resistance in the environment. Such information will ultimately be vital for developing effective interventions that will address resistance in the environment and benefit human and animal health.

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