Environmental Science & Technology

Critical Review

pubs.acs.org/est

Role of the Environment in the Transmission of Antimicrobial Resistance to Humans: A Review

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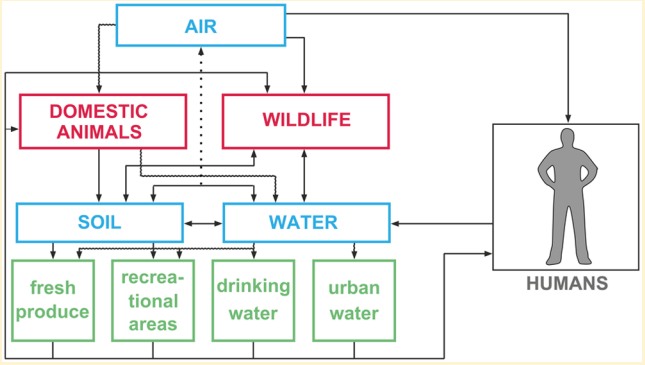
Supporting Information

ABSTRACT: To establish a possible role for the natural environment in the transmission of clinically relevant AMR bacteria to humans, a literature review was conducted to systematically collect and categorize evidence for human exposure to extended-spectrum β-lactamase-producing Enterobacteriaceae, methicillin-resistant *Staphylococcus aureus*, and vancomycin-resistant *Enterococcus* spp. in the environment. In total, 239 datasets adhered to inclusion criteria. AMR bacteria were detected at exposure-relevant sites (35/38), including recreational areas, drinking water, ambient air, and shellfish, and in fresh produce (8/16). More datasets were available for environmental compartments (139/157), including wildlife, water, soil, and air/dust. Quantitative data from exposure-relevant sites (6/35) and environmental compartments (11/139) were scarce. AMR bacteria were detected in the contamination sources (66/66) wastewater and manure, and molecular data supporting their transmission from wastewater to the environment (1/66) were found. The abundance of AMR bacteria at exposure-relevant sites suggests risk for human exposure. Of publications pertaining to both environmental and human isolates, however, only one compared isolates from samples that had a clear spatial and temporal relationship, and no direct evidence was found for transmission to humans through the environment. To what extent the environment, compared to the clinical and veterinary domains, contributes to human exposure needs to be quantified. AMR bacteria in the environment, including sites relevant for human exposure, originate from contamination sources. Intervention strategies targeted at these sources could therefore limit emission of AMR bacteria to the environment.



INTRODUCTION

The occurrence and spread of antimicrobial resistant (AMR) bacteria are pressing public health problems worldwide. Recently, in its first global report on surveillance of antimicrobial resistance, the World Health Organization (WHO) reported very high rates of resistance in bacteria (e.g., *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*) that cause common healthcare-associated and community-acquired infections in people in all WHO regions.[1] Infections caused by AMR bacteria are associated with excess mortality, prolonged hospital stays, and increased costs.[2] In order to formulate effective intervention strategies to combat intractable infections caused by AMR bacteria, it is important to discern which fraction of the total disease burden and costs are attributable to different sources, including animal reservoirs and vehicles such as foods or the environment.[3]

Enteric bacteria are introduced into the environment with human and animal feces, and people may be exposed to these bacteria through, e.g., recreation in contaminated surface water, consumption of contaminated drinking water, fresh produce, or (shell)fish, and inhalation of bioaerosols. Previous studies indicate that the risk of contracting *Salmonella* spp. or *Campylobacter* spp. in recreational waters is higher than or

Received: May 26, 2015
Revised: September 3, 2015
Accepted: September 10, 2015
Published: September 10, 2015

DOI: 10.1021/acs.est.5b02566
Environ. Sci. Technol. 2015, 49, 11993−12004

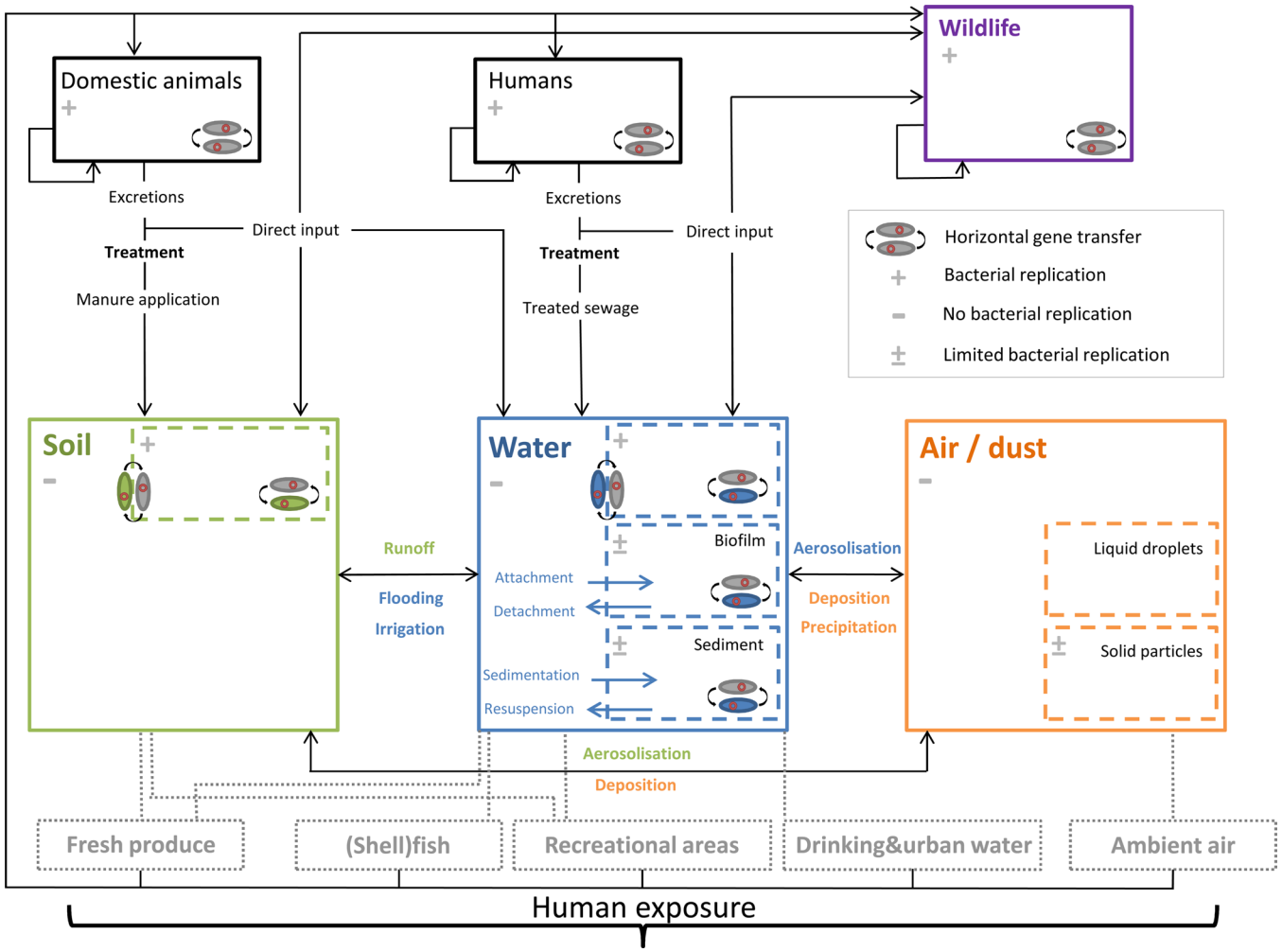


Figure 1. Schematic diagram of environmental compartments, contamination sources, exposure-relevant sites, and processes affecting survival and spread of bacteria.

equal to the risk of contracting the organisms through chicken consumption.[4−6] In the United States of America, 9% of all outbreaks with the pathogenic *E. coli* O157 are waterborne.[7] Based on global WHO risk assessments, it was estimated that there are over 120 million cases annually of gastrointestinal disease from exposure to coastal waters via recreation or by eating raw or lightly cooked shellfish.[8] Multiple studies have described outbreaks of infections with Enterobacteriaceae associated with consumption of fresh produce.[9] Examples include the 2011 outbreak of *E. coli* O104:H4 associated with sprouts in Europe and Northern America,[10] and the 2006 outbreak of *E. coli* O157:H7 associated with spinach in the United States.[11]

The natural environment has been identified as a pathway by which transmission of AMR bacteria to humans might occur.[12] The extent to which this occurs remains unknown, however. It is important to establish the relative role of the environment in the transmission of AMR bacteria to humans, compared with the spread of AMR bacteria through contact with animal carriers, consumption of food of animal origin, (international) travel, and their spread in healthcare and community settings. The aim of the current study was to establish a possible role for the natural environment, defined as soil, water, air/dust, and wildlife, in the transmission of clinically relevant AMR bacteria to humans by systematically reviewing the peer-reviewed literature. For this purpose, three AMR bacteria were selected: extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae (ESBL-Ent), methicillin-resistant *S. aureus* (MRSA), and vancomycin-resistant *Enterococcus* spp. (VRE). Additionally, the contribution of fecal contamination sources (human wastewater and animal manure) to the burden of the AMR bacteria in the environment was explored.

METHODS

Search Strategy. Two databases (Medline and Scopus) were searched on April 10, 2014, to identify publications describing one or more of three AMR bacteria in relation to the environment. The bacterial species were selected to represent Gram-negative and Gram-positive species, and species of fecal and non-fecal origin. Moreover, each of the bacterial species colonizes both animals and humans, has a clinically relevant type of resistance, and is sufficiently prevalent in humans and/or animals that its presence in the environment may be anticipated. Accordingly, ESBL-Ent (i.e., *E. coli*, *Enterobacter* spp., *K. pneumoniae*), MRSA, and VRE were selected, and carbapenemase-producing Enterobacteriaceae were not selected.

For the purpose of the present review, environment was defined as natural environment (or "outdoor" environment). Consequently, indoor environments such as hospitals or livestock housing were excluded. Four environmental compart-

DOI: 10.1021/acs.est.5b02566
Environ. Sci. Technol. 2015, 49, 11993−12004

ments were distinguished: soil, water, air/dust, and wildlife (Figure 1). Wildlife was categorized as an environmental compartment because these animals are not treated with antibiotics, and their carriage of AMR bacteria is most likely explained by uptake from the natural environment during foraging and drinking. Wildlife can therefore be considered as an extension of the specified environmental compartments, as well as a vehicle for multiplication and spread of these bacteria. Within the environmental compartments, sites relevant for human exposure were defined and included in the search strategy: recreational areas (i.e., beach sand and recreational water), irrigation water, drinking water, urban water (e.g., fountains), shellfish, and ambient air. Fresh produce, i.e., food of plant origin such as vegetables and fruits, may be contaminated with AMR bacteria during growth in contaminated soil and/or irrigation with contaminated water. Consequently, this was also included as a "site" relevant for human exposure. Finally, the main sources of fecal contamination of the environment, wastewater and manure, were included in the search strategy. For the purpose of the current study, manure was defined as animal feces that is intentionally (e.g., for use as fertilizer) or unintentionally (e.g., droppings of free-range animals) introduced into the natural environment, or that is stored, presumably for use as fertilizer. Consequently, only measurements of AMR bacteria in animal feces introduced in natural environments (e.g., not fecal swabs or droppings sampled in stables) were included.

Combinations of key search terms for AMR bacteria and defined environmental compartments, contamination sources, and exposure-relevant sites were used to interrogate the online databases (Table 1). Specifically, the titles, abstracts, and key words of publications included in the online databases were screened for these key search terms.

Selection Criteria. Two reviewers independently screened titles and abstracts, resulting from the automated database search, for exclusion criteria. Publications were excluded if they were in a language other than English, Dutch, or German, contained non-original research (e.g., reviews), or did not investigate the specified compartments or AMR bacteria (Figure 2). Publications solely describing resistance genes (i.e., genes encoding ESBL or resistance to methicillin or vancomycin), independently of bacterial hosts, were excluded. In case of conflict of opinion about inclusion of an article based on title or abstract, this was discussed by both reviewers until consensus was reached. Full texts were retrieved for included publications, and for publications where the decision for inclusion or exclusion could not be based on the contents of title and abstract, or where abstracts were not available. Full texts were further assessed for compliance with the inclusion and exclusion criteria (Figure 2A).

Data Extraction. Study characteristics and data from included publications were extracted and exported to a database using a custom-made form. Full details of the extraction fields are available in Table S1 (Supporting Information). In case multiple publications described the same or overlapping sets of samples or isolates, these publications were considered as one data source, i.e., one publication. Publications were categorized on the basis of the type of investigated compartment (contamination sources, environmental compartments, exposure-relevant sites) and the type of AMR bacteria. Individual publications could be included within more than one category.

To ensure the quality of the dataset, the method used to isolate AMR bacteria was assessed. First, it was determined whether selective culture methods were used to detect the AMR bacteria. Selective culture was defined as culture (pre-enrichment or direct plating) in the presence of relevant antibiotics: third-generation cephalosporin-supplemented medium or commercial screening medium for the isolation of ESBL-Ent; methicillin-, oxacillin-, or cefoxitin-supplemented medium or commercial screening medium for MRSA; and vancomycin-supplemented medium for VRE. The rationale for this assessment is the lack of sensitivity of non-selective culturing methods for the detection of AMR bacteria, and the resulting unreliability for drawing conclusions on the basis of lack of detection when non-selective culturing was used. Second, it was determined whether the antibiotic resistance was confirmed using phenotypic and/or molecular methods. Phenotypic confirmation tests deemed valid included growth of isolates on media containing concentrations equal to or above clinical breakpoints, established minimal inhibitory concentrations (MICs) equal to or above clinical breakpoints, and, in the case of disc diffusion assays, inhibition zones smaller than clinical breakpoints as described in the Clinical and Laboratory Standards Institute (CLSI) guidelines.[13] VanB-carrying VRE

Table 1. Combinations of Key Search Terms Used for the Identification of Literature on AMR Bacteria from Environmental Compartments, Contamination Sources, and Exposure-Relevant Sites

key subjects	Medline search terms
bacteria	(enterobacter* or *Escherichia coli*? or *E. coli*? or klebsiella? or enterococc* or *Staphylococcus aureus* or s aureus).af
	AND
resistance types	(extended spectrum beta lactamase? or esbl? or (methicillin adj2 resistan*) or mrsa? or (vancomycin adj2 resistan*) or vre?).af
	AND
environmental compartments	(water* or freshwater? or seawater or aquatic or coastal or beach* or lake? or river? or soil? or land? or pasture? or sediment? or air or airborne or dust or wildlife or wild animal? or bird? or mammal? or rodent?).mp
	OR
contamination sources	(manure or dropping? or slurry or sludge or lagoon? or compost or fertili?er? or sewage or effluent? or wastewater?).mp
	OR
exposure-relevant sites[a]	(crop? or vegetable? or fresh produce or sprout? or shellfish or fish).mp
key subjects	**Scopus search terms**
bacteria	(TITLE-ABS-KEY(enterobacter* OR escherichia-coli OR e-coli OR klebsiella OR enterococc* OR staphylococcus-aureus OR s-aureus))
	AND
resistance types	((TITLE-ABS-KEY(extended-spectrum-beta-lactamase OR esbl OR mrsa OR vre) OR TITLE-ABS-KEY(vancomycin W/1 resistan*) OR TITLE-ABS-KEY(methicillin W/1 resistan*)))
	AND
environmental compartments	(TITLE-ABS-KEY(*water OR water* OR aquatic OR coastal OR beach OR lake OR river OR soil OR land OR pasture OR sediment OR air OR airborne OR dust OR wildlife OR wild-animal OR bird OR mammal OR rodent))
	OR
contamination sources	(TITLE-ABS-KEY(manure OR dropping OR slurry OR sludge OR lagoon OR compost OR fertili?er OR sewage OR effluent))
	OR
exposure-relevant sites[a]	(TITLE-ABS-KEY(crop OR vegetable OR fresh-produce OR sprout OR shellfish OR fish))

[a]The exposure-relevant sites recreational water, drinking water, urban water, irrigation water, and ambient air are captured by search terms included under environmental compartments.

DOI: 10.1021/acs.est.5b02566
Environ. Sci. Technol. 2015, 49, 11993−12004

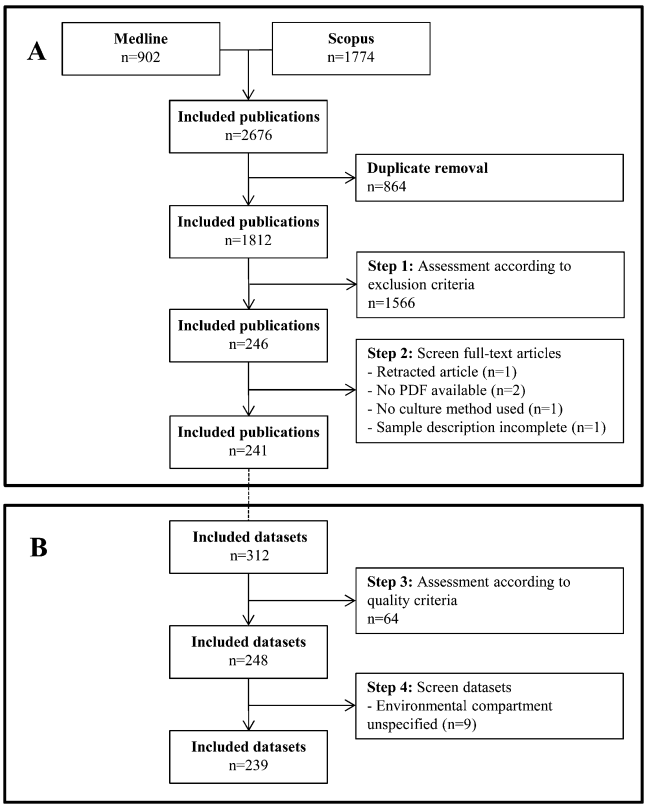


Figure 2. Flowchart of the (A) publication and (B) dataset selection process.

variants with MICs below the current CLSI breakpoint concentration (32 μg/mL) have been described;[14] therefore, growth at concentrations >16 μg/mL was also considered a valid phenotypic confirmation. Polymerase chain reactions (PCRs) detecting mecA and vanA or vanB genes were considered valid molecular tests for MRSA and VRE, respectively. For ESBL-Ent, PCRs detecting CTX-M genes, PCRs detecting TEM, SHV, and OXA genes in combination with sequencing to establish subtypes, or PCRs detecting other ESBL genes were considered valid molecular confirmation tests. Sequencing of TEM, SHV, and OXA genes is required since non-ESBL-encoding alleles exist of these genes. Studies in which AMR bacteria were investigated but not detected using only non-selective culture methods, or where resistance of isolates was not confirmed appropriately, were excluded from analysis.

For each publication, it was assessed whether AMR bacteria were enumerated, and whether environmental isolates were compared with isolates from fecal contamination sources or with human isolates for phenotypic or genetic relatedness. From the perspective of the current review, i.e., study of documentation on transmission from the environment to humans and on emission from contamination sources to the environment, only comparisons between isolates from samples with a spatiotemporal relationship were considered relevant. Tests frequently used for establishing relatedness of isolates included pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), antibiotic resistance profiling, *Staphylococcus* protein A gene (spa)-typing (specifically for MRSA), and PhenePlate (PhP) biochemical fingerprinting (specifically for VRE). Additional tests included those identifying resistance genes and their genetic environment, e.g., identification of ESBL-genotype and plasmid characterization for ESBL-Ent, and staphylococcal cassette chromosome SCC*mec*-typing for MRSA.

RESULTS

General. The selection process yielded 241 publications that met the inclusion criteria (Figure 2A), and from these publications data were extracted. Fifteen publications described the same or overlapping samples or isolates, and these were combined to represent seven publications. Some publications described multiple AMR bacteria, environmental compartments, contamination sources, and/or exposure-relevant sites. For further study, data from included publications were divided into datasets, with each dataset describing one type of AMR bacteria (i.e., ESBL-Ent, MRSA, VRE) in combination with one type of environmental compartment (i.e., soil, water, air/dust, wildlife), contamination source (i.e., wastewater, manure), or fresh produce. The 241 included publications resulted in 312 datasets (Figure 2B). After applying the defined quality criteria, 64 datasets were excluded. For 9 datasets, the results could not be interpreted due to lack of specification in which of the studied compartment(s) AMR bacteria were found. These had not been not excluded earlier in the selection process because the publications also contained other, valid datasets. This left 239 datasets for further study. Details on publications describing these datasets are available in Table S2 (Supporting Information). The majority of datasets described wildlife (n = 71), wastewater (mostly municipal or urban sewage) (n = 60), and water (n = 56), followed by soil (n = 25), fresh produce (n = 16), manure (n = 6), and air/dust (n = 5). ESBL-Ent (n = 102) and VRE (n = 88) were described more often compared to MRSA (n = 49).

All continents were represented; however, more than half of the datasets described isolates collected in Europe (n = 139), followed by North America (n = 39), Asia (n = 38), Africa (n = 9), South America (n = 8), Oceania (n = 3), and the Antarctic (n = 2). One dataset contained isolates that were collected from both Europe and Asia.

Exposure-Relevant Sites and Fresh Produce. Of 157 datasets concerning environmental compartments (Table 2), 24% (38/157) included sites that are relevant for human exposure (Table 3). Of these, almost two-thirds were about recreational water (n = 15) or beach sand (n = 12). The remaining datasets were about drinking water (n = 5), ambient air (n = 3), shellfish (n = 2), and irrigation water (n = 1). No datasets pertaining to urban water (e.g., fountains) were identified. Furthermore, 16 datasets concerned fresh produce such as vegetables, fruits, sprouts, and herbs (Table 3). Overall, AMR bacteria were detected in 92% (35/38) of datasets about exposure-relevant sites, and in 50% (8/16) of datasets about fresh produce.

Only six datasets concerning exposure-relevant sites included quantitative data on the AMR bacteria (Table 4). Five datasets concerned concentrations in recreational areas, ranging from $10^{0.1}$ to 10^{3} CFU/100 mL.[15−18] One dataset provided information on concentrations in blue mussels, which was <10 CFU/g.[19]

Genetic Relatedness between Environmental and Human Isolates. Of all publications where AMR bacteria were detected in environmental samples, 12 included information on the relationship between human and environmental isolates by PFGE, PhP biochemical fingerprinting, MLST, spa-typing, or SCC*mec*-typing (ESBL-Ent, n = 4; MRSA, n = 6; VRE, n = 2). Together these publications contained six datasets on

DOI: 10.1021/acs.est.5b02566
Environ. Sci. Technol. 2015, 49, 11993−12004

Table 2. Detection of AMR Bacteria in Environmental Compartments

AMR bacteria[a]	geographic regions investigated	detection[b]		description of samples	
		n/*N*	%	detected	not detected
Environmental Compartment: Soil					
ESBL-Ent	Europe, Asia, S. America	4/5	80	manure-amended soil, pasture soil, river bank sediment, farm-related soil	agricultural soil
MRSA	Europe, N. America, Asia	12/12	100	beach sand, manure-amended soil, soil unknown origin, soil around farm	–
VRE	Europe, N. America, Asia	7/8	88	beach sand, farmland without manure, (non)agricultural soils, freshwater/coastal sediment	soil receiving/not receiving pig slurry
subtotal		23/25	92		
Environmental Compartment: Water					
ESBL-Ent	Europe, Africa, N. and S. America, Asia	24/26	92	marine/fresh surface waters, well water, water bags, public tap water	fresh surface water, irrigation water
MRSA	N. America, Asia	10/11	91	marine surface waters	fresh surface waters
VRE	Europe, Africa, N. and S. America, Asia	17/19	89	marine/fresh surface waters, ground/well water wells	marine/fresh surface waters, well water
subtotal		51/56	91		
Environmental Compartment: Air/Dust					
ESBL-Ent	Europe	2/2	100	air around wastewater treatment plants	–
MRSA	Europe	3/3	100	air around livestock farms	–
VRE	–	–	–	–	–
subtotal		5/5	100		
Environmental Compartment: Wildlife					
ESBL-Ent	Europe, Asia, N. and S. America, Africa, Antarctic	29/34	85	multiple wild bird/mammal species, fish	multiple wild bird species
MRSA	Europe, N. America, Asia	14/17	82	multiple wild bird/mammal species	multiple wild mammal species
VRE	Europe, N. America	17/20	85	multiple wild bird/mammal species, shellfish, fish	multiple wild bird/mammal species, amphibians, reptiles
subtotal		60/71	85		
Total		**139/157**	**89**		

[a]Abbreviataions used: AMR bacteria, antimicrobial resistant bacteria; ESBL-Ent, extended-spectrum β-lactamase-producing Enterobacteriaceae; MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant *Enterococcus* spp. [b]*n*/*N*, number of datasets where at least one positive sample was detected divided by the total number of datasets; %, percentage of datasets where AMR bacteria were detected.

wildlife, six on surface water, and four on soil. Two of the publications on soil and surface water concerned exposure-relevant sites (i.e., recreational waters and beach sand). Of the publications pertaining to environmental and human isolates, however, only one compared isolates from samples that had a clear spatial and temporal relationship.[20] Human nasal cultures, beach sand samples, and marine water samples were collected on the same day, and stranded pilot whale (*Globicephala macrorhynchus*) samples were collected within two months at a marine mammal conservancy in the United States. MRSA isolates from whales, human volunteers attending these animals, and the beach sand and marine water associated with the marine mammal conservancy showed a high degree of genetic relatedness (≥95% similar PFGE patterns and same SCC*mec*-type).[20]

Environmental Compartments. AMR bacteria were detected in at least one of the samples in 89% (139/157) of the datasets concerning environmental compartments (Table 2). The majority of these datasets were about ESBL-Ent (n = 67), followed by VRE (n = 47) and MRSA (n = 43). High prevalences, defined as the percentage of datasets with at least one positive sample for the AMR bacteria, were observed in all environmental compartments and ranged from 85 to 100% (Table 2). All air/dust datasets concerned AMR hotspots (i.e., wastewater treatment plants and livestock farms). In the case of soil, the majority of datasets were obtained at recreational beaches and from agriculturally related soils. By contrast, datasets concerning wildlife and water were obtained from a variety of species and locations, not necessarily related to AMR hotspots.

Bacterial concentrations were described in 8% (11/139) of datasets where AMR bacteria were detected: three soil datasets from Germany, Denmark, and United States; five water datasets from The Netherlands, United States, and Denmark; two air datasets from Germany; and one wildlife dataset from Denmark (Table 4).

In three publications, birds and mammals were investigated in conjunction with at least one other environmental compartment, at the same time and geographic location, to establish the relation between isolates from different compartments.[20−22] Doljeská et al.[21] showed that ESBL-Ent were present in pond water and in black-headed gulls (*Larus ridibundus*) nesting on the same pond. However, based on AMR profiles, ESBL genes, and macro-restriction profiles, isolates from wildlife and surface water were not related. Hernandez et al.[22] isolated ESBL-Ent from surface water but not from penguin (*Pygoscelis papua*) feces. Hower et al.[20] found MRSA isolates with 99% similar PFGE profiles and the same SCC*mec*- and *spa*-type in short-finned pilot whales (*G. macrorhynchus*), water, and beach sand.

Contamination Sources. AMR bacteria were detected in at least one of the samples in all (60/60) datasets concerning wastewater (Table 5). The majority of these datasets concerned

DOI: 10.1021/acs.est.5b02566
Environ. Sci. Technol. 2015, 49, 11993−12004

Table 3. Detection of AMR Bacteria at Exposure-Relevant Sites and in Fresh Produce

AMR bacteria[a]	countries investigated	detection[b] n/N	%
	Exposure-Relevant Site: Beach Sand		
ESBL-Ent	Portugal	1/1	100
MRSA	United States	8/8	100
VRE	Malaysia, United States	3/3	100
subtotal		12/12	100
	Exposure-Relevant Site: Recreational Water		
ESBL-Ent	Algeria, Netherlands	2/2	100
MRSA	United States	9/10	90
VRE	Malaysia, United States	3/3	100
subtotal		14/15	93
	Exposure-Relevant Site: Drinking Water		
ESBL-Ent	Bangladesh, Democratic Republic of Congo, Nicaragua	3/3	100
MRSA	–	–	–
VRE	Germany, South Africa	1/2	50
subtotal		4/5	80
	Exposure-Relevant Site: Ambient Air		
ESBL-Ent	Poland	2/2	100
MRSA	Germany	1/1	100
VRE	–	–	–
subtotal		3/3	100
	Exposure-Relevant Site: Shellfish		
ESBL-Ent	–	–	–
MRSA	–	–	–
VRE	Denmark, United Kingdom	2/2	100
subtotal		2/2	100
	Exposure-Relevant Site: Irrigation Water		
ESBL-Ent	Netherlands	0/1	0
MRSA	–	–	–
VRE	–	–	–
subtotal		0/1	0
Total		**35/38**	**92**
	In Fresh Produce[c]		
ESBL-Ent	Japan, Netherlands, Portugal, Saudi Arabia, Spain	4/7	57
MRSA	Iran, South Korea	2/2	100
VRE	Denmark, Germany, Italy, Portugal, South Africa, Spain, Sweden, Turkey, United Kingdom	2/7	29
Total		**8/16**	**50**

[a]Abbreviations used: AMR bacteria, antimicrobial resistant bacteria; ESBL-Ent, extended-spectrum β-lactamase-producing Enterobacteriaceae; MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant *Enterococcus* spp. [b]*n*/*N*, number of datasets where at least one positive sample was detected divided by the total number of datasets; %, percentage of datasets where AMR bacteria were detected. [c]AMR bacteria were detected in vegetables, fruits, (imported) herbs, sprouts, mixed salad, and pre-packaged fruit juice. They were not detected in other vegetables, lettuce from a farm, mixed salad, sprouts, and crops from a farm.

VRE (n = 33), followed by ESBL-Ent (n = 23) and MRSA (n = 4). Three types of wastewater could be distinguished: (1) community wastewater, or water derived from sewer systems and wastewater treatment plants (WWTP); (2) hospital wastewater; and (3) industrial wastewater, or water derived from slaughterhouses, factories, and farms. AMR bacteria were detected in at least one of the samples in all (6/6) datasets concerning manure. These datasets most often concerned ESBL-Ent (n = 5), followed by VRE (n = 1). No datasets on MRSA in manure were identified in the current review (Table 5).

Eighteen percent (12/66) of datasets about contamination sources included quantitative data: 11 of the wastewater datasets and one of the manure datasets (Table 6). The effect of wastewater treatment on concentrations of ESBL-Ent and VRE was investigated in seven publications originating from Algeria,[23] Spain,[24] Denmark,[19,25] United States,[26,27] and Portugal.[28] The majority of these publications described only mechanical (e.g., sedimentation) and biological (e.g., activated sludge) treatment of wastewater. A reduction of 1−4 $\log_{10}$-units was observed during secondary treatment. In three publications,[26−28] concentrations of VRE in tertiary effluent were additionally described. Tertiary treatment in these publications entailed UV treatment, chlorination, and lagooning and sand filtration, respectively. A further reduction of 2−3 $\log_{10}$-units was observed relative to secondary treated effluents. UV treatment, chlorination, and lagooning brought VRE concentrations below the detection limit, but VRE could still be detected after sand filtration.[26−28] No information was available on the effect of manure treatment on AMR bacterial concentrations.

Three publications about wastewater included bacterial concentrations in community wastewater receiving wastewater from AMR hotspots as well as hotspot wastewater, i.e., from a hospital[28] and drug production plants.[19,25] Concentrations of AMR bacteria were highest in AMR hotspot wastewater, followed by WWTP influent and WWTP effluent. When investigated,[19,25] WWTP influents or sewage not containing AMR hotspot wastewater had lower concentrations of AMR bacteria compared to the counterparts receiving hotspot wastewater (Table 6).

Relation between Isolates from Environmental Compartments and Contamination Sources. Twenty publications included datasets describing both wastewater and environmental compartments. In only nine of these, however, was it specified that the investigated wastewater and environmental compartments were geographically connected and sampled at the same time.[15,19,24,29−34] All of these publications investigated surface water in conjunction with wastewater and were performed in multiple European countries. Two of the datasets additionally included air at a Polish WWTP in which ESBL-Ent were detected, and another included Danish wildlife (mussels) in which VRE were detected.[19,30,31] In eight of nine publications describing surface water receiving wastewater from WWTP (all except ref 31), ESBL-Ent and VRE were detected in both wastewater and surface water. In three of these eight publications, the relation between isolates from wastewater and environmental isolates was not investigated.[24,32,34] In three other publications, isolates were compared with respect to PFGE profiles or ESBL genotype.[19,30,31] Isolates from wastewater and surface water had different PFGE profiles in the Danish study,[19] while similar ESBL genotypes were seen in wastewater, river water, and air at the Polish WWTP.[30,31] In the two remaining studies, isolates were compared with respect to multiple characteristics. Novais et al.[33] showed that isolates from wastewater and surface water had

DOI: 10.1021/acs.est.5b02566
Environ. Sci. Technol. 2015, 49, 11993−12004

Table 4. Concentrations of AMR Bacteria in Environmental Compartments, Including Exposure-Relevant Sites

AMR bacteria	type (source)	concentration[d]	country	ref
	Environmental Compartment: Soil			
MRSA	agricultural[a] (around turkey and broiler farm)	2.3×10^3–2.7×10^5 CFU/pair bootswabs	Germany	55
	sand (beach)[b,c]	2.0–66.2 MPN/100 mL	United States	17
VRE	agricultural[a] (research station)	ND, <10 CFU/g[e]	Denmark	19
	non-agricultural[a] (research station)	ND, <10 CFU/g[e]		
	Environmental Compartment: Water			
ESBL-Ent	fresh/marine (river, lake, North Sea)[b]	0.15–15 CFU/100 mL	Netherlands	15
	fresh (not under influence of WWTP)	10 CFU/100 mL		
	fresh (river at discharge WWTP)	10^2–10^3 CFU/100 mL		
MRSA	marine (Pacific Ocean)[b,c]	2.0–66.2 MPN/100 mL	United States	17
	marine (Pacific Ocean)[b]	0.65 CFU/100 mL	United States	16
	marine (Atlantic Ocean, bather related)[b]	<2–780 CFU/100 mL	United States	18
	marine (Atlantic Ocean, ambient)[b]	<2–260 CFU/100 mL		
VRE	marine (at outlet WWTP)	$\leq 10^{-2}$ CFU/mL	Denmark	19
	Environmental Compartment: Air/Dust			
MRSA	50 m outside stable (turkey farm)[b]	7–93 CFU/m^3	Germany	55
	150 m outside stable (turkey farm)[b]	11–23 CFU/m^3		
	directly outside stable (pig farm)	3.2×10^1–4.0×10^1 CFU/m^3	Germany	56
	Environmental Compartment: Wildlife			
VRE	shellfish (at outlet WWTP)[b]	<10 CFU/g	Denmark	19

[a]"agricultural", soil exposed to animal manure; "non-agricultural", soil not exposed to animal manure. [b]Exposure-relevant site. [c]Marine water, freshwater, and sand samples from two marine beaches were pooled to give this result. [d]Abbreviations used: CFU, colony-forming units; MPN, most probable number; ND, not detected. [e]VRE were not detected by direct plating on Slanetz–Bartley agar with vancomycin.

Table 5. Detection of AMR Bacteria in Datasets Describing Contamination Sources

AMR bacteria[a]	geographic regions investigated	detection[b]		description of samples
		n/N	%	
	Contamination Source: Wastewater			
ESBL-Ent	Africa, Europe, S. America, Oceania	23/23	100	untreated, secondary effluent, sludge, other
MRSA	Europe, N. America, Oceania	4/4	100	untreated, secondary effluent, tertiary effluent, sludge, other
VRE	Asia, Europe, N. America	33/33	100	untreated, secondary effluent, tertiary effluent, sludge, other, unspecified
subtotal		60/60	100	
	Contamination Source: Manure			
ESBL-Ent	Europe, Asia	5/5	100	broiler, laying hen, pig, duck, dairy cow
MRSA	–	–	–	–
VRE	Europe	1/1	100	pig
subtotal		6/6	100	
Total		**66/66**	**100**	

[a]Abbreviations used: AMR bacteria, antimicrobial resistant bacteria; ESBL-Ent, extended-spectrum β-lactamase-producing Enterobacteriaceae; MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant *Enterococcus* spp. [b]*n/N*, number of datasets where at least one positive sample was detected divided by the total number of datasets; %, percentage of datasets where AMR bacteria were detected.

different PFGE profiles, while resistance genes, virulence traits, and AMR profiles were similar, suggesting horizontal gene transfer. Blaak et al.[15] conducted multiple analyses on spatiotemporally related ESBL-Ent isolates obtained from Dutch wastewater and downstream surface water (including recreational water), and demonstrated identical isolates with respect to sequence type, phylogenetic group, AMR profile, and ESBL genotype. A contribution of WWTP/sewage to the presence of AMR bacteria in surface water is further supported by quantitative data from two studies: Blaak et al.[15] showed similar ESBL-Ent concentrations in surface water at WWTP effluent discharge points and in effluents (10^2–10^3 CFU/100 mL), and Gómez et al.[24] demonstrated that VRE concentrations were higher closer to the WWTP (10^4 CFU/100 mL), compared to upstream or further downstream (10^2–$10^{2.5}$ CFU/100 mL).

In five publications, manure was investigated in relation to environmental compartments.[35−39] In two of these, direct deposition of feces in the environment was investigated. Hasan et al.[37] detected ESBL-*E. coli* in wild birds inhabiting the same lakeshore as poultry and ducks from surrounding households. Isolates were spatially related, but their temporal relationship was unclear.[37] Ma et al.[38] detected ESBL-*E. coli* in water from ponds on a duck farm. Isolates from the ponds and ducks were spatiotemporally related and had similar PFGE profiles, phylogenetic groups, and/or ESBL genes.[38] The three remaining publications concerned livestock manure in conjunction with application to agricultural land. Friese et al.[35] detected ESBL-*E. coli* in samples taken from previously fertilized fields (within the past 6 weeks) around (unspecified distance) pig and broiler houses, and in slurry samples. It was not indicated, however, whether pig slurry from the farms was used to fertilize the fields.[35] Hartmann et al.[36] detected ESBL-*E. coli* in cultivated soil amended one year before with liquid cow manure from one farm and in pasture soil on another farm. No information was provided, however, on the spatiotemporal relationship between these isolates and those from fresh and composted manure.[36]

DOI: 10.1021/acs.est.5b02566
Environ. Sci. Technol. 2015, 49, 11993–12004

Table 6. Concentrations of AMR Bacteria in Contamination Sources

AMR bacteria	type (source)[a]	concentration[b]	country	ref
		Contamination Source: Wastewater		
ESBL-Ent	influent (WWTP)	4.6×10^3–1.6×10^5 CFU/100 mL	Algeria	23
	effluent (WWTP)	5.1×10^2–1.3×10^3 CFU/100 mL		
	rinse water (poultry farm)	3.9×10^6–5.8×10^7 CFU/L	Netherlands	57
	effluent (WWTP)	10^2–10^3 CFU/100 mL	Netherlands	15
	hospital	10^4–10^5 CFU/mL	Poland	58
VRE	influent (WWTP)	2–140 CFU/100 mL	United Kingdom	59
	influent (WWTP)	10^3–10^4 CFU/100 mL	Spain	24
	effluent (WWTP)	10–10^2 CFU/100 mL		
	factory	0.9×10^5–5.1×10^6 CFU/mL	Denmark	25
	influent (WWTP, inlet 1[c])	1.5×10^2–5.8×10^3 CFU/mL		
	influent (WWTP, inlet 2[d])	6.9×10^1–6.4×10^2 CFU/mL		
	sludge (WWTP)	4.2×10^2–6.1×10^4 CFU/mL		
	effluent (WWTP)	3.6×10^{-1}–2.8×10^0 CFU/mL		
	sludge (factory)	10^9 CFU/mL	Denmark	19
	effluent (factory)	10^3 CFU/mL		
	sewage[d] (sewer upstream)	<10–10^3 CFU/mL		
	sewage[c] (sewer downstream)	10^2–10^4 CFU/mL		
	influent (WWTP[d], inlet 1[c])	10^3–10^4 CFU/mL		
	influent (WWTP[d], inlet 2[d])	10^2–10^4 CFU/mL		
	influent (WWTP[c])	10^3 CFU/mL		
	sludge (WWTP[d])	10^3–10^4 CFU/mL		
	sludge (WWTP[c])	10^3 CFU/mL		
	effluent (WWTP[d])	10^{-1}–10^0 CFU/mL		
	effluent (WWTP[c])	10^{-1} CFU/mL		
	influent (WWTP)	2.5×10^3–8.6×10^4 CFU/100 mL	United States	27
	secondary effluent (WWTP)	9.6×10^1–1.0×10^3 CFU/100 mL		
	tertiary effluent (WWTP)	ND to 3.3 CFU/100 mL[e]		
	other (WWTP)	ND to 1.9×10^5 CFU/100 mL		
	influent (WWTP)	ND (winter); 10^4–10^5 CFU/100 mL (spring, summer)	United States	26
	secondary effluent (WWTP)	ND (winter); 400–5200 CFU/100 mL (spring, summer)		
	tertiary effluent (WWTP)	ND		
	hospital	1.6×10^1–2.2×10^3 CFU/mL	Portugal	28
	influent[c] (WWTP)	6.7×10^0–4.1×10^2 CFU/mL		
	effluent (WWTP)	~10^0 CFU/mL		
		Contamination Source: Manure		
ESBL-Ent	dung heap (poultry farm)	≥0.1 CFU/g	Netherlands	57
	storage tank (poultry farm)	<0.1 CFU/g		
	free-range area (poultry farm)	3.1×10^3–9.3×10^3 CFU/g		

[a]WWTP, wastewater treatment plant. [b]CFU, colony-forming units; ND, not detected. [c]Receiving and [d]not receiving wastewater from the specified hotspot (e.g., hospital or factory wastewater). [e]Only detected when chlorination was not operational.

Manero et al.[39] investigated whether VRE were present in crops receiving pig slurry and soils receiving or not receiving pig slurry, but VRE were not detected.

DISCUSSION

Transmission of clinically relevant AMR bacteria to humans by exposure to the natural environment, e.g., recreational water and beach sands, drinking water, ambient air, and shellfish, is plausible. Quantitative data available in a small proportion of the included datasets describing environmental compartments support this. There were no publications, however, providing direct evidence for transmission of AMR bacteria to humans resulting from exposure to the environment. In the current review, the highest level of evidence for transmission was considered when genetic relatedness was shown between bacterial strains through molecular typing of spatiotemporally related human and environmental isolates collected at exposure-relevant sites. Although a number of studies performed molecular typing of human and environmental isolates, only one obtained this level of evidence.[20] In this study, the direction of transmission could not be determined (environment transmitting AMR bacteria to humans or vice versa), however, nor could transmission via a common source be excluded. Tools to further investigate transmission of clinically relevant AMR bacteria to humans by exposure to the natural environment include risk assessments, microbial source tracking, and epidemiological studies. Ideally, these tools should be combined to place environmental exposure in context with exposures in the clinical and veterinary/agricultural domains. Attribution of different sources and pathways that play a role in the transmission of AMR bacteria to humans can help to identify and prioritize intervention strategies.

Using Quantitative Microbial Risk Assessment (QMRA), the risk of human exposure to AMR bacteria in the environment can

DOI: 10.1021/acs.est.5b02566
Environ. Sci. Technol. 2015, 49, 11993–12004

be quantified.[40] This approach requires knowledge of the concentrations of clinically relevant AMR bacteria at exposure-relevant sites. Furthermore, it requires dose estimates following human consumption of fresh produce or shellfish, ingestion of surface water, and inhalation of bioaerosols, together with the frequency and duration of these events. Although scarce, datasets including quantitative data from exposure-relevant sites were identified in the current review. Where AMR bacteria were not enumerated at exposure-relevant sites, additional aspects to be considered for risk assessment include survival in, and transport of, AMR bacteria to sites of exposure, and changes in bacterial concentration between the measured site (i.e., environmental compartment, contamination source) and site of exposure. Horizontal gene transfer rates between clinically relevant AMR bacteria and environmental bacteria must also be addressed for more accurate estimates of exposure.[41] Another aspect to be considered for risk assessment is *in vivo* fitness of AMR bacteria in the human host following ingestion; however, quantitative data are lacking.

Microbial source tracking might be used to gain further understanding of how clinically relevant AMR bacteria emitted from contamination sources are transmitted to humans via the environment.[42] This method involves phenotypic and genotypic characterization of AMR bacterial isolates of human, animal, and environmental origin for the purpose of identifying differences between groups of bacteria that can be used to ascertain the source from which they were derived.[3] Phenotypic characterization methods include serotyping and antibiotic susceptibility profiling.[3] Among genotypic methods, pulsed-field gel electrophoresis (PFGE) is considered the "gold standard", but microarrays, multilocus sequence typing (MLST), multilocus variable-number tandem repeat analysis (MLVA), and whole genome sequencing (WGS) are becoming widespread.[43] The current systematic review did not include articles that investigated the prevalence of antibiotic resistance genes without relating them to a specific bacterial species. This was based on the assumption of a more direct risk associated with exposure to AMR bacteria that are capable of colonizing or infecting humans. However, taking into account the spread of whole bacteria alone might underestimate transmission of AMR, as horizontal gene transfer also plays an important role in the dissemination of antibiotic resistance.[44] Compared to culture-dependent techniques, metagenomic approaches and next-generation sequencing could provide more insight into the prevalence and diversity of antibiotic resistance determinants in the environment, while quantitative PCR might be helpful in collecting information about their distribution.[45] Microbial source tracking to investigate transmission of AMR bacteria to humans at exposure-relevant sites should preferably take into account transfer of both bacteria and their resistance determinants.

Epidemiological approaches can be used to identify possible exposure routes responsible for carriage of, or infection with, AMR bacteria. For example, Frank et al.[46] describe an outbreak of an infection with Shiga-toxin-producing *E. coli* O104:H4 harboring an ESBL gene (CTX-M-15) in Germany, where sprouts were identified as the most likely vehicle of infection.[47] In a study population comprising 100 cases and 190 controls, Søraas et al.[48] showed that recreational freshwater swimming was an independent risk factor for ESBL-positive urinary tract infections in people in Norway, along with travel to Asia, the Middle East, or Africa during the past 6 weeks to 24 months, recent use of fluoroquinolones and β-lactams, and diabetes mellitus. In a cross-sectional study by Huijbers et al.,[49] however, swimming in a river, lake, or pond was not identified as a risk factor for ESBL-Ent carriage in 1025 Dutch adults. Furthermore, Rosenberg Goldstein et al.[50] found no significant difference in the odds of methicillin-susceptible *S. aureus* (MSSA), multidrug resistant MSSA, and vancomycin-susceptible enterococci colonization among spray irrigation workers using reclaimed water (n = 19) and controls not routinely exposed to reclaimed water (n = 24). Also, none of the sampled individuals were positive for MRSA or VRE.[50] These particular studies were not included in the current review, as they did not actually investigate the presence of ESBL-Ent, MRSA, and/or VRE in the environment. This might have led to the exclusion of studies that support the role of the environment in transmission of clinically relevant AMR bacteria. A search for epidemiological studies reporting a relation between environmental exposure and infection with AMR bacteria showed that these were scarce, however (data not shown). It might also be possible to consider data on susceptible variants of selected bacterial species and assume that a proportion of these infections were caused by resistant variants. This approach does not take into account that the survival and spread of AMR bacteria might be different compared to those of susceptible strains both in the environment and upon entering the human body. Furthermore, for the purpose of risk assessment and attribution of different transmission routes, which provide targets for interventions, information about prevalence, concentration, and types of AMR bacteria in the environment is imperative.

Quantitative and molecular data provide evidence for dissemination of AMR bacteria from contamination sources to the environment, including to exposure-relevant sites. AMR bacteria were detected in all publications investigating wastewater. Moreover, where AMR bacteria were enumerated, high concentrations were observed, also in wastewater that is discharged onto surface water. This, together with molecular typing results, demonstrates the contribution of sewage and WWTP effluent to the presence of AMR bacteria in surface water. AMR bacteria were also detected in the six publications concerning manure in relation to the environment. There are currently no studies investigating the prevalence and concentration of AMR bacteria in manure or slurry prior to soil application. There are also no studies demonstrating the effect of manure or slurry application on the prevalence and concentration of AMR bacteria in soil and on fresh produce. Studies investigating the effect of manure application on resistance genes in soil have been conducted, however. For example, Fahrenfeld et al. (2014) showed significant increases in soil gene copy numbers of antibiotic resistance genes (*sul1*, *sul2*, and *ermF*) after manure application, and dissipation of these genes to background levels within 2 months.[51] The role of manure application on environmental contamination with clinically relevant AMR bacteria needs to be addressed further and placed into perspective relative to environmental contamination from human sources. An aspect not addressed by this review, but also important to consider here, is the dissemination of antibiotic residues to the environment through wastewater and manure.[40] For example, half-lives of 5 days for β-lactams to 100 days for tetracyclines and sulfonamides have been reported in manure,[52] suggesting that, when applied to land, they might act as selective agents to help propagate AMR bacteria or resistance genes.

It is clear that, in order to estimate exposures and risks associated with environmental pathways of AMR bacteria, further investigation is necessary. Management options exist, however, that can work synergistically with existing policies and goals, and could be put into effect immediately.[53] Dissemination

DOI: 10.1021/acs.est.5b02566
Environ. Sci. Technol. 2015, 49, 11993−12004

via wastewater to exposure-relevant sites is suspected; therefore, an effective target for intervention could be at wastewater collection points and wastewater treatment plants. It has been shown that concentrations of AMR bacteria were reduced by advanced wastewater treatment processes such as ozone, UV, ultrafiltration, and chlorination.[26,27] In addition, membrane bioreactor processes have been shown to be very effective in reducing bacterial numbers by over 6 $\log_{10}$,[54] and might prove useful in diminishing AMR bacteria. Another intervention measure could be treatment of manure; however, data on AMR bacteria in manure are scarce, and there are no studies investigating the effect of manure treatment on concentrations of AMR bacteria in the environment. The efficiency of reducing AMR bacteria by composting and other digestion processes should be evaluated.

In conclusion, the abundance of AMR bacteria at exposure-relevant sites suggests that risk of human exposure to AMR bacteria in the environment is plausible. To what extent the environment contributes to human exposure, also compared to the clinical and veterinary/agricultural domains, needs to be quantified. Important knowledge gaps have been identified that should be addressed in future studies. AMR bacteria in the environment, including sites relevant for human exposure, originate from wastewater and probably manure. Intervention strategies targeted at these sources could therefore limit emission of AMR bacteria to the environment.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b02566.

Data extraction form (Table S1) and list of all articles included in the systematic review (Table S2) (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was financially supported by The Netherlands Organization for Health Research and Development (ZonMw) and the Dutch Ministry of Health, Welfare and Sport (VWS). We thank drs. A. M. P. Kersten for her valuable assistance with the literature search, and Prof. Dr. J. W. M. van der Meer and Prof. Dr. T. J. M. Verheij for their helpful suggestions on the manuscript.

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DOI: 10.1021/acs.est.5b02566
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