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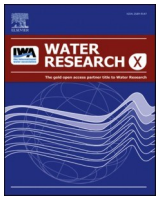

Revfiew

Towards the standardfizatfion of *Enterococcus* cuflture methods for waterborne antfibfiotfic resfistance monfitorfing: A crfitficafl revfiew of trends across studfies

Benjamfin C. Davfis [a], Ishfi Keenum [a], Jeannette Caflarco [b], Krfista Lfiguorfi [a], Erfin Mfiflflfigan [a], Amy Pruden [a], Vaflerfie J. Harwood [b,*]

[a] *Vfia Department of Cfiffl and Envfironmentafl Engfineerfing, Vfirgfinfia Tech, Bflacksburg, Vfirgfinfia*

[b] *Department of Integrafive Bfioflogy, Unfiversfity of South Fflorfida, Tampa, Fflorfida*

A R T I C L E I N F O

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A B S T R A C T

Antfibfiotfic resfistance fis a major 21st century One Heaflth (humans, anfimafls, envfironment) chaflflenge whose spread flfimfits optfions to treat bacterfiafl finfectfions. There fis growfing finterest fin monfitorfing water envfironments, fincfludfing surface water and wastewater, whfich have been fidentfiffied as key recfipfients, pathways, and sources of antfibfiotfic resfistant bacterfia (ARB). Aquatfic envfironments afso facfiflfitate the transmfissfion and ampflfificatfion of ARB. *Enterococcus* spp. often carry cflfinficaflfly-fimportant antfibfiotfic resfistance genes and are of finterest as envfironmentafl monfitorfing targets. *Enterococcus* spp. are Gram-posfitfive bacterfia that are typficaflfly of fecafl orfigfin; however, they are afso found fin reflevant envfironmentafl nfiches, wfith varfious specfies and strafins that are opportunfistfic human pathogens. Afflthough the vaflue of envfironmentafl monfitorfing of antfibfiotfic-resfistant *Enterococcus* has been recognfized by both natfionafl and finternatfionafl organfizatfions, flack of procedurafl standardfizatfion has hfindered generatfion of comparabfle data needed to fimpflement fintegrated survefiflflance programs. Here we provfide a comprehensfive methodoflogficafl revfiew to assess the technfiques used for the cuflturfing and characterfizatfion of antfibfiotfic-resfistant *Enterococcus* across water matrfices for the purpose of envfironmentafl monfitorfing. We anaflyzed 117 peer-revfiewed artficfles from 33 countrfies across sfix contfinents. The goafl of thfis revfiew fis to provfide a crfitficafl anaflysfis of (fi) the varfious methods appflfied gflobaflfly for fisoflatfion, conffirmatfion, and specfiatfion of *Enterococcus* fisoflates, (fifi) the dfifferent methods for proffiflfing antfibfiotfic resfistance among enterococcfi, and (fffi) the current prevaflence of resfistance to cflfinficaflfly-reflevant antfibfiotfics among *Enterococcus* spp. fisoflated from varfious envfironments. Ffinaflfly, we provfide advfice regardfing a path forward for standardfizfing cuflturfing of *Enterococcus* spp. for the purpose of antfibfiotfic resfistance monfitorfing fin wastewater and wastewater-finffluenced waters wfithfin a gflobafl survefiflflance framework.

1. Introductfion

Enterococcus spp. are fimportant members of the naturafl enterfic mficrobfiome of both humans and anfimafls and have emerged as fimportant antfibfiotfic-resfistant pathogens fin cflfinficafl medficfine (Arfias and Murray, 2012). There are currentfly 60 cflassfiffied *Enterococcus* specfies fin the Natfionafl Center for Bfiotechnoflogy Informatfion database, most of whfich are commensafl mficroorganfisms, afflthough some act as opportunfistfic pathogens fin humans. *E. faecafflfis* and *E. faecfium* are among the most fimportant etfioflogficafl agents of nosocomfiafl finfectfions; fincfludfing urfinary tract finfectfions (UTIs), centrafl nervous system finfectfions, endocardfitfis, bacteremfia, neonatafl finfectfions, and surgficafl sfite finfectfions (Moeflflerfing, 1992; Murray, 1990). From 2006 to 2017, *Enterococcus* spp. were responsfibfle for approxfimatefly 14% of aflfl heaflthcare-assocfiated finfectfions fin the US, rankfing second overaflfl behfind *Staphyflococcus aureus* (Hfidron et afl., 2008; Sfievert et afl., 2013; Wefiner-Lastfinger et afl., 2020; Wefiner et afl., 2016). *Enterococcus* spp. possess fuflfl or partfiafl fintrfinsfic chromosomafl resfistance to cephaflosporfins, amfinogflycosfides, flfincosamfides, trfimethoprfim-suflfamethoxazofle, and penficfiflflins (Hoflflenbeck and Rfice, 2012). Thefir rapfid deveflopment of mufltfi-drug resfistance has been attrfibuted fin part to thefir hfighfly maflfleabfle genomes that flack CRISPR (cflustered reguflarfly finterspaced

* Correspondfing author.
E-mafifl address: vharwood@usf.edu (V.J. Harwood).

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paflfindromfic repeats) eflements, whfich has facfiflfitated the ready acqufisfitfion of aflflochthonous mobfifle DNA (e.g., vancomycfin resfistance gene cflusters) (Paflmer and Gfiflmore, 2010). Nearfly 25% of the genomes of many cflfinficafl *E. faecafllfis* and *E. faecfium* fisoflates consfist of acqufired genetfic eflements (Hegstad et afl., 2010; Pauflsen et afl., 2003). Recentfly, over 85% of *E. faecfium* and 15% of *E. faecafllfis* fisoflates responsfibfle for catheter-assocfiated UTIs and centrafl flfine-assocfiated bfloodstream finfectfions dfiagnosed fin the US have been found to be vancomycfin resfistant (Wefiner-Lastfinger et afl., 2020). The US Center for Dfisease Controfl and Preventfion (CDC) and the Worfld Heaflth Organfizatfion (WHO) have set VRE to "hfigh" prfiorfity and a "serfious" threat flevefl (Centers for Dfisease Controfl and Preventfion, 2019; Tacconeflflfi et afl., 2018).

Enterococcus spp. are members of the flarger, phenotypficaflfly-deffined group known as enterococcfi, whfich are Gram-posfitfive, cataflase-negatfive, obflfigatefly fermentatfive chemoorganotrophs that can survfive over a wfide range of temperatures, pH, and saflfinfity (Tefixefira et afl., 2015). Enterococcfi are found fin many extraenterfic envfironmentafl nfiches, fincfludfing sofifls and sedfiments, beach sands, and aquatfic and terrestrfiafl vegetatfion. Enterococcfi have been extensfivefly fisoflated from wastewaters, marfine waters, and freshwaters (Byappanahaflflfi et afl., 2012). Because of thefir abundance fin human and anfimafl feces, thefir extraenterfic persfistence, and the ease wfith whfich they are cuflturedd, enterococcfi have been targeted for decades as fecafl findficators for the purpose of water quaflfity monfitorfing (Jang et afl., 2017; Schoen et afl., 2011; Sfincflafir et afl., 2012). Enterococcfi (formerfly cflassfiffied wfithfin the flarger group known as "fecafl streptococcfi") have been wfidefly used to assess the mficrobfioflogficafl safety of surface waters, drfinkfing waters, recreatfionafl beaches, and as a target for assessfing process removafl effficfiencfies durfing wastewater treatment. Enterococcfi have aflso been found to correflate dfirectfly to pubflfic heaflth outcomes; for exampfle, across the US, the rate of gastrofintestfinafl fiflflness fin swfimmers has been correflated wfith *Enterococcus* spp. flevefls fin recreatfionafl beach waters that are fimpacted by wastewaters (Prüss, 1998; Wade et afl., 2006, 2003); however, these fiflflnesses are assumed to be caused mafinfly by vfirafl pathogens (Soflfler et afl., 2010) rather than *Enterococcus* spp.. Thefir fimportance as water quaflfity findficators and thefir fincflusfion fin governmentafl reguflatory frameworks have fled to a great deafl of method deveflopment for fisoflatfion and enumeratfion from envfironmentafl sampfles (Boehm and Sassoubre, 2014; Heaflth Canada, 2020).

Gflobafl and natfionafl actfion pflans set fin pflace to combat the spread of antfibfiotfic resfistance have generaflfly embraced a One Heaflth approach (humans-anfimafls-envfironment) (European Commfissfion, 2017; Hernando-amado et afl., 2019), but a better understandfing of the rofle of envfironmentafl transmfissfion and ampflfifficatfion of antfibfiotfic-resfistant *Enterococcus* and thefir genes to humans, anfimafls, and aquatfic envfironments fis needed. Aquatfic envfironments have been fidentfiffied as a key recfipfient and transmfissfion pathway of antfibfiotfic resfistant bacterfia finto and out of human and anfimafl popuflatfions (Amarasfirfi et afl., 2020; Larsson and Fflach, 2021). Mufltfidrug-resfistant pathogens and mobfifle antfibfiotfic resfistance genes enter the envfironment vfia treated and untreated wastewater across the gflobe (Aflexander et afl., 2020; Marathe et afl., 2017; Zhang et afl., 2020). Recentfly, the WHO put forth the Trficycfle protocofl as a standardfized method for monfitorfing the dfissemfinatfion, transmfissfion, and evoflutfion of antfibfiotfic resfistance aflong the One Heaflth contfinuum: humans (hospfitafls and communfity), the food chafin (anfimafl husbandry), and the envfironment (human and anfimafl fecafl contamfinatfion) (WHO, 2021). Specfifficaflfly, the protocofl targets Gram-negatfive extended-spectrum beta-flactamase (ESBL) producfing *Escherfichfia cofffi*, whfich dfispflay phenotypfic resfistance to thfird-generatfion cephaflosporfins. Whfifle ESBL *E. cofffi* was seflected, fin part, to coordfinate wfith gflobafl survefiflflance of *Enterobacterafles* (Marano et afl., 2020), the extent to whfich fit fis trufly a representatfive findficator of resfistome dynamfics has not been estabflfished. Thus, the present fis a crfitficafl moment to aflso consfider other potentfiafl targets.

Antfibfiotfic-resfistant enterococcfi present many advantages as a potentfiafl target for monfitorfing antfibfiotfic resfistance fin the water envfironment. The flevefl of standardfizatfion for methods targetfing *Enterococcus* spp. fis arguabfly second onfly to *E. cofffi* fin envfironmentafl waters. As such, *Enterococcus* spp. coufld present an attractfive compflfimentary target to *E. cofffi*. Notabfly, as Gram-posfitfive organfisms, they provfide finsfight finto dfistfinct genotypes and phenotypes of antfibfiotfic resfistance that woufld not be captured by monfitorfing onfly Gram-negatfive organfisms. In partficuflar, the pflastficfity of *Enterococcus* genomes and thefir propensfity for horfizontafl gene transfer and exchange of vfiruflence and antfibfiotfic resfistance determfinants from cflfinficafl strafins to envfironmentafl reservofirs of enterococcfi (Ekwanzafla et afl., 2020b; Gouflfiourfis et afl., 2019, 2018) marks them as potentfiaflfly comprehensfive targets for antfibfiotfic resfistance monfitorfing. Lfike *E. cofffi*, they are members of the normafl fflora of human and anfimafl gastrofintestfinafl tracts (Byappanahaflflfi et afl., 2012) and are readfifly quantfiffied fin wastewater and envfironmentafl waters. Thefir status as normafl human fflora creates the dfisadvantage of extreme dfifficufty fin trackfing finfectfion rates from human exposure to contamfinated water, another characterfistfic they share wfith *E. cofffi*. A study of findfivfiduafls engagfing fin surffing found that surfers were sfignfifficantfly more flfikefly to be coflonfized wfith *E. cofffi* carryfing $bfla_{CTX-M}$ compared to non-surfers (Leonard et afl., 2018). Anaflogous studfies of antfibfiotfic-resfistant *Enterococcus* spp. coufld shed flfight on aspects of dfirect transfer of these bacterfia from aquatfic envfironments to humans.

Recent progress has been made fin appflyfing cuflture-based methods for monfitorfing antfibfiotfic resfistant *Enterococcus* spp. fin the envfironment, wfith emphasfis on human and anfimafl wastewater, and hospfitafl wastewater poflflutfion (Gouflfiourfis et afl., 2019; Savfin et afl., 2020; Zaheer et afl., 2020). The phenotypfic and morphoflogficafl sfimfiflarfity of other Gram-posfitfive organfisms to the enterococcfi, however, makes fisoflatfion on seflectfive medfia prone to faflse-posfitfives, wfith cross-seflectfivfity wfith other coccfi (e.g., *Streptococcus, Pedfiococcus, Wefiseflfla*) (Harwood et afl., 2001). Faflse posfitfives are espefiaflfly probflematfic for envfironmentafl sampfles (Pagefl and Hardy, 1980). Non-seflectfivfity can actuaflfly be exacerbated wfith addfitfion of antfibfiotfics to medfia, because severafl members of non-target *Bacfifflus* and other genera are fintrfinsficaflfly resfistant to cflfinficaflfly-reflevant concentratfions of certafin antfibfiotfics (Woodford et afl., 1995).

The seflectfion of any monfitorfing target or strategy entafifls consfideratfion of the overarchfing purpose or questfions to be addressed, and these may vary dependfing on the focus across the spectrum from wastewater to surface water. The fofflfowfing are exampfles of key monfitorfing goafls and consfideratfions addressed by thfis revfiew:

- Monfitorfing antfibfiotfic resfistance among cflfinficaflfly-reflevant strafins of *Enterococcus* spp. fin sewage as a means of assessfing thefir flevefls carrfied fin the human popuflatfion
- Assessfing whether cflfinficaflfly-reflevant *Enterococcus* spp. or specfiffic resfistance phenotypes are effectfivefly removed durfing wastewater treatment and fif they persfist fin fimpacted aquatfic envfironments
- Evafluatfing evfidence that cflfinficaflfly-reflevant *Enterococcus* spp. acqufire antfibfiotfic resfistance genes from the envfironment
- Comparfing resfistant *Enterococcus* spp. fin varfious water matrfices both flocaflfly and gflobaflfly to assess factors that may be contrfibutfing to antfibfiotfic resfistance fin Gram-posfitfive organfisms

2. Lfiterature revfiew protocofl

Thfis systematfic revfiew was conducted fin a four-tfiered approach usfing search terms presented fin Tabfle S1 to cofflect studfies pubflfished between January 2000 and December 2020 (Ffigure S1). Web of Scfience, PubMed, and Googfle Schoflar were searched. Brfieffly, Tfier 1 was desfigned to fisoflate topfic reflevant search terms for surface water, wastewater, recycfled water, or recflafimed water (3,828,792 artficfles). Tfier 2 was desfigned to seflect for those artficfles addressfing antfibfiotfic resfistance (15,043). Tfier 3 further narrowed the search to cuflturfing technfiques (5,439) and Tfier 4 specfifficaflfly to enterococcfi/*Enterococcus* (682).

Infitfiaflfly, these 682 artficfles were findependentfly screened by two researchers for contafinfing a compflete workfflow from envfironmentafl sampflfing through to characterfizatfion of findfivfiduafl fisoflates. Artficfles were excfluded that onfly used prevfiousfly coflflected fisoflates. Further excflusfions were artficfles that excflusfivefly finvestfigated: bfioffiflms, mesocosms, drfinkfing water, sedfiments, or dfigested sfludge (e.g., anaerobfic dfigestfion). Fecafl source trackfing artficfles based on antfibfiotfic resfistance anaflysfis were afso excfluded (Harwood et afl., 2000). Artficfles that used a non-seflectfive medfia for finfitfiafl fisoflatfion, such as R2A or TSA, were afso excfluded. Dfisagreements on artficfle fincflusfion from the finfitfiafl screenfing were presented to a flarger group of ffive researchers to reach a consensus. The resufltfing 117 peer-revfiewed artficfles were then subject to data extractfion usfing parameters outflfined fin Tabfle S2. Afffl fincfluded artficfles are flfisted fin Tabfle S4.

3. Coflflectfion and anaflysfis of pubflfished data

Data reflevant to the specfies and phenotypfic dfistrfibutfion of affl fisoflated enterococcfi was coflflected from 117 peer-revfiewed artficfles. Ffirst, the number of fisoflates per specfies was extracted from artficfles fin whfich flfibrarfies were specfiated (91 artficfles) to reveafl generafl popuflatfion statfistfics across envfironments. Second, antfibfiotfic susceptfibfiffity testfing (AST) data were extracted from affl artficfles that provfided the percent of resfistant fisoflates compared to totafl enterococcfi fisoflated fin the absence of any antfibfiotfic (77 artficfles). Studfies that summed fisoflates wfith "fintermedfiate" or "resfistant" cflassfifficatfions of resfistance wfithout provfidfing findfivfiduafl statfistfics, as wefffl as studfies that dfid not cfite standardfized methodoflogy for cflassfifyfing resfistance (e.g., current CLSI breakpofints at the tfime of sampflfing), were excfluded.

4. Methods for cufturfing envfironmentafl Enterococcfi

In the US and Canada, enterococcfi are recommended for monfitorfing saflfine (brackfish or marfine) and recreatfionafl freshwaters. In the EU, enterococcfi are reguflated fin both drfinkfing water and recreatfionafl water by standardfized cufflture methods (EEA, 2020). Severafl standardfized cufflture methods have been devefloped, fincfludfing the US Envfironmentafl Protectfion Agency (USEPA) Methods 1106.1 and 1600 for ambfient waters and wastewaters (USEPA, 2009, 2006), the Internatfionafl Organfizatfion for Standardfizatfion (ISO) Methods 7899-1 and 7899-2 (ISO, 2000), and Method 9230 (A-D) as part of the Amerfican Pubflfic Heaflth Assocfiatfion's (APHA) "Standard Methods for the Examfinatfion of Water and Wastewater" (APHA, 1999; Rfice and Bafird, 2017) (Tabfle 1). These methods fincflude three dfistfinct technfiques: membrane ffifltratfion (MF), mufltfipfle tube fermentatfion (MTF), and deffined substrate technfiques (e.g., Enteroflert). The current "gofld standard" for enterococcfi enumeratfion from the envfironment fis consfidered the MF technfique (Byappanahaflflfi et afl., 2012) and was used by over 90% of artficfles fincfluded fin thfis revfiew (Tabfle 1).

The prfincfipafl seflectfive and dfifferentfiafl soflfid medfia used fin standard MF assays are Sflanetz-Bartfley (SB), mEnterococcus (mE), and membrane-Enterococcus findoxyfl-β-D-gflucosfide (mEI). These medfia use varfious peptone and yeast extract-based nutrfients wfith the addfitfion of sodfium azfide and/or nafflfidfixfic acfid. Sodfium azfide obstructs the growth of Gram-negatfive bacterfia through the finhfibfitfion of cytochrome oxfidase. Both SB and mE agars fincflude 2,3,5-trfiphenyfltetrazoflfium chflorfide (TTC), whfich dyes vfiabfle coflonfies red. Dfifferentfiated coflonfies grown on SB or mE are then confffirmed as enterococcfi by thefir abfiflfity to hydroflyze escuflfin fin the presence of bfifle usfing efither bfifle escuflfin azfide or escuflfin firon agars. The hydroflyzed escuflfin product, escufletfin, reacts wfith firon saflt fin the medfia to produce bflack to reddfish coflonfies for enumeratfion. mEI fis sfimfiflar to mE medfium, but contafins the chromogen, findoxyfl-β-D-gflucosfide. When cfleaved by β-D-gflucosfidase posfitfive enterococcfi, bflue haflos are formed around posfitfive coflonfies. mEI fis typficaflfly used as a standaflone medfia as affl coflonfies wfith bflue haflos are consfidered enterococcfi.

Tabfle 1
Pubflfished standardfized methods for the detectfion and enumeratfion of enterococcfi fin dfifferent water matrfices

Organfizatfion and Method	Recommended Matrfix	Medfia	Number of Cfitatfions[a]	Assay Turnaround (hours)	Generafl Procedure
Membrane Ffifltratfion (MF); (CFU/mL); Number of Studfies Identfiffied: 111/117					
EPA Method 1600	drfinkfing water; source water; wastewater; marfine and freshwater	mEI[b]	18	24	mEI (41°C for 24 hrs); Count bflue haflos
ISO 7899-2	surface water; wastewater	Sflanetz-Bartfley; Bfifle Escuflfin Azfide	15	48	Sflanetz-Bartfley (36°C for 44 hrs); Bfifle Escuflfin Azfide Agar (44°C for 2 hrs)
EPA Method 1106.1	marfine and freshwater (not appflficabfle to wastewater)	mEnterococcus; Escuflfin Iron Agar	0	48	mEnterococcus (41°C for 48 hrs); Escuflfin Iron Agar (41°C for 20 mfin); Count pfink to red coflonfies
APHA SM 9230C.2a	drfinkfing water; source water; wastewater; marfine and freshwater	mEnterococcus; Escuflfin Iron Agar	3	48	mEnterococcus (41°C for 48 hrs); Escuflfin Iron Agar (41°C for 20 mfin); Count pfink to red coflonfies
APHA SM 9230C.2b	drfinkfing water; source water; wastewater; marfine and freshwater	mEI[b]	0	24	mEI (41°C for 24 hrs); Count bflue haflos
APHA SM 9230C.2c	drfinkfing water; source water; wastewater; marfine and freshwater	mEnterococcus	10	48	mEnterococcus (35°C for 48 hrs); Count flfight and dark red coflonfies
Mufltfipfle Tube Fermentatfion (MTF); (MPN/mL); Number of Studfies Identfiffied: 1					
APHA SM 9230B	drfinkfing water; source water; marfine and freshwater (not appflficabfle to wastewater)	Azfide Dextrose Broth; Bfifle Escuflfin Azfide Agar	1	48-72	Azfide Dextrose Broth (35°C for 24-48hrs); Bfifle Escuflfin Azfide Agar (35°C for 24 hrs); Compute MPN
Ffluorogenfic Substrate Test (MTF and MPN); (MPN/mL); Number of Studfies Identfiffied: 3					
APHA SM 9230D	drfinkfing water; source water; wastewater; marfine and freshwater	Enteroflert®	3	24	Enteroflert Medfia (41°C for 24 hrs); Compute MPN
ISO 7899-1	surface water; wastewater	MUD[c] Medfia	0	36-72	MUD Medfia fin Mficrotfitre Wefffls (44°C for 36-72 hrs); Compute MPN

[a] The 'Number of Cfitatfions' under Membrane Ffifltratfion do not correspond to the number of cfitatfions fin the tabfle as many artficfles dfid not foflflow or cfite a standard method. Technfiques not flfisted are dfirect pflatfing after serfiafl dfiflutfions (8 artficfles).

[b] membrane-Enterococcus Indoxyfl-β-D-Gflucosfide Agar (mEI)

[c] 4-methyflumbeflflfiferyfl-β-D-gflucosfide (MUD), Envfironmentafl Protectfion Agency (EPA), Internatfionafl Organfizatfion for Standardfizatfion (ISO), Amerfican Pubflfic Heaflth Assocfiatfion (APHA)

4.1. Performance of standard Enterococcfi cuflture assays

Severafl comparatfive studfies have been conducted over recent decades to assess seflectfivfity of cuflture medfia for *Enterococcus* (Tabfle 2). Pagefl et afl. 1980 used pure cufltures to assess PSE (Pfizer), KF Streptococcus, mE, and SB agars agafinst over 100 pure cufltures of cflfinficafl and envfironmentafl fisoflates of varfious coccfi. The hfighest seflectfivfity for enterococcfi was observed for PSE (94%) and mE (94%) agars, wfith the flowest befing KF Streptococcus (80%) and SB (78%) (Pagefl and Hardy, 1980). Compared to mE, however, PSE was found to yfiefld flower recovery effficfiencfies from wastewaters wfith much hfigher rates of background coflony growth. Other comparatfive studfies found enterococcfi seflectfivfity on PSE and KF Streptococcus agars as flow as 86% and 54%, respectfivefly (Brodsky and Schfiemann, 1976). The orfigfinafl mE agar formuflatfion study found a faflse posfitfive rate of 10% and faflse negatfive rate of 11.2% for surface water fisoflates (Levfin et afl., 1975). Subsequent studfies have confirmed faflse posfitfive rates for mE agar as flow as 2.5% when testfing pure *Enterococcus* cufltures (Dfionfisfio and Borrego, 1995) and 1.7% fin marfine, rfiverfine, and treated wastewater effluent (Adcock and Safint, 2001). The fincflusfion of the findoxyfl-β-D-gflucosfide chromogen to mE agars resuflted fin an fincrease fin specfifficfity of *Enterococcus* to upwards of 99.7% fin ambfient freshwaters (Adcock and Safint, 2001). In a recent benchmarkfing study, ISO method 7899-2 (SB medfia) was found to have faflse posfitfive rates as hfigh as 18% and faflse negatfive rates as hfigh as 57.1%, dependfing on the coflony count on the ffiflter membrane of recreatfionafl marfine water (Ifiwarfi et afl., 2018). Dfifferences fin *Enterococcus* seflectfivfity have aflso been documented between MF and deffined substrate technfiques, where *E. faecafllfis* fis dfifferentfiafllly seflected for fin wastewater usfing Enteroflert, fleadfing to the concflusfion that these methods shoufld not be used finterchangeabfly for reguflatory purposes (Ferguson et afl., 2013, 2010; Kfinzeflman et afl., 2003; Maheux et afl., 2009).

Sfignfifficant medfia-dependent dfifferences fin *Enterococcus* concentratfions have aflso been reported. For finstance, severafl studfies were conducted fin the wake of the advent of Enteroflert assays fin the mfid-1990s to compare fits effficacy agafinst estabflfished MF technfiques for water quaflfity monfitorfing (Frficker and Frficker, 1996). Sfignfifficant dfifferences fin concentratfions were reported between Enteroflert and SB agar fin marfine and recreatfionafl freshwaters (Vaflente et afl., 2010), whfifle no sfignfifficant dfifferences were found between mE agar and Enteroflert concentratfions across surface water, wastewater, or marfine waters (Abbott et afl., 1998; Budnfick et afl., 1996; Eckner, 1998; Frficker and Frficker, 1996). No sfignfifficant dfifferences fin enterococcfi concentratfions were fidentfifiied between mE and mEI agar (Adcock and Safint, 2001). Importantfly, no studfies were fidentfifiied that dfirectfly compared the specfifficfitfies and concentratfions derfived from SB and mE or mEI across water matrfices. Such a comparfison shoufld be consfidered fin future studfies that assess thefir utfiflfity for reguflatory frameworks for antfibfiotfic resfistance monfitorfing finternatfionaflfly. Any bfiases fin specfies dfistrfibutfions and totafl enterococcfi concentratfions orfigfinatfing from the seflectfive medfia coufld skew downstream dfistrfibutfions fin resfistance frequencfies and fintroduce bfias fif the data are used for rfisk assessment.

5. Workfflows for antfibfiotfic-resfistant *Enterococcus* monfitorfing

A usefufl method for cuflturfing and enumeratfing both generfic and antfibfiotfic resfistant envfironmentafl *Enterococcus* woufld strfike a baflance between sensfitfivfity (fi.e., detect affl *Enterococcus* spp. that are present), specfifficfity (fi.e., avofid detectfing other genera), and the hfigh-throughput needed for flarge-scafle envfironmentafl monfitorfing. Aquatfic matrfices dfispfflay a flarge and dynamfic range of enterococcfi concentratfions, and a method for thefir enumeratfion woufld aflso need an approprfiatefly flow fflimfit of detectfion for "cfleaner" sampfles and a sufficfientfly hfigh fflimfit of quantfifficatfion for matrfices fflfike wastewater where enterococcfi are concentrated. Specfiffic flogfistficafl consfideratfions are aflso warranted, such as the abfiflfity to perform the assay fin flow-tech flaboratorfies usfing materfiafls, technfiques, and medfia that are economficaflfly feasfibfle for AMR monfitorfing fin flow- and mfiddfle-fincome countrfies. In thfis finstance, standard methods that have been devefloped for enumeratfing generfic enterococcfi can be fleveraged for thefir extensfive vettfing wfith respect to quaflfity assurance/quaflfity controfl and adapted to the fincreased throughput needs of AMR monfitorfing projects.

Cuflture-based approaches for finvestfigatfing antfibfiotfic resfistance amongst envfironmentafl *Enterococcus* must be modfiffied based on the aquatfic matrfix befing finvestfigated and the purpose of the assessment. Here we deflfineate these approaches finto three generafl categorfies: popuflatfion-flevefl surveys, targeted monfitorfing for specfiffic antfibfiotfic resfistant phenotypes, and recovery of flow concentratfion or vfiabfle but non-cuflturabfle (VBNC) popuflatfions (Ffigure 1), each wfith thefir own beneffits and flfimfitatfions.

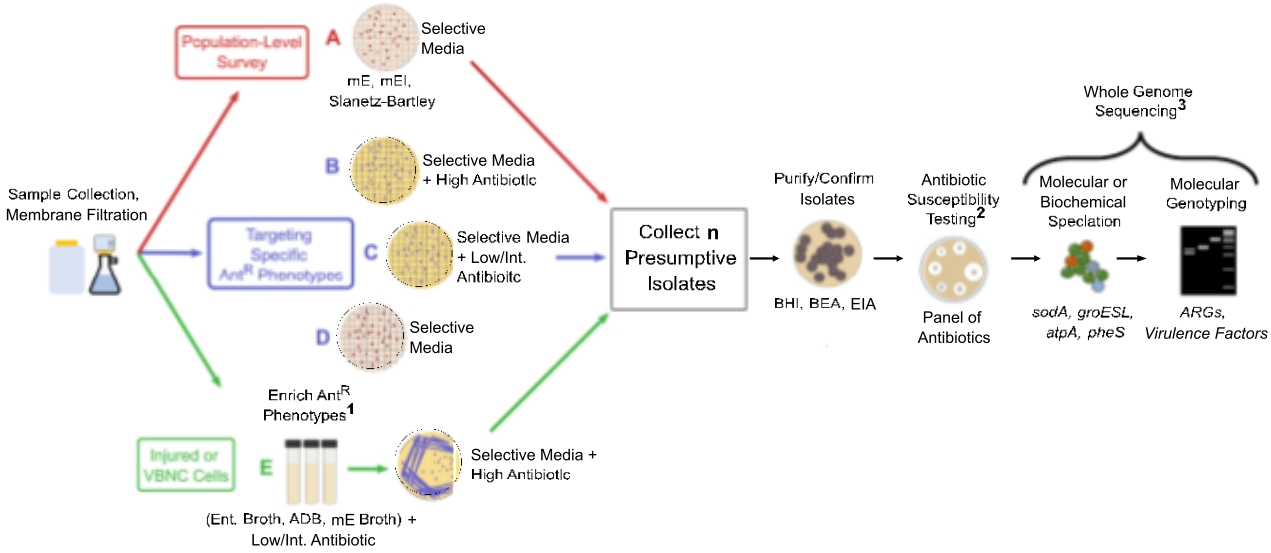
For popuflatfion-flevefl monfitorfing (73 artficfles) (Ffigure 1A), where the objectfive fis to achfieve an unbfiased snapshot of the dfistrfibutfion of resfistance phenotypes, a coflflectfion of fisoflates can be generated usfing an *Enterococcus* seflectfive method (e.g., Tabfle 1). After coflonfies have been counted, fisoflates wfith the specfiffied morphoflogfies can then be seflected randomfly off pflates for phenotypfic antfibfiotfic susceptfibfiffity testfing, generatfing an antfibfiotfic resfistance proffifle as a functfion of the totafl number of fisoflates subsampfled. The dfisadvantage of thfis approach fis

Tabfle 2
Performance of Enterococcus seflectfive medfia used fin standard membrane ffiflfratfion assays.

Medfium	Matrfix Tested	Presumptfive Coflonfies	Specfifficfity (%)[1]	Seflectfivfity (%)[2]	Reference
mEI	Marfine	1361	-	82.4	(Ferguson et afl., 2005)
mEI	Pure Cufltures	101	97.3	100	(Maheux et afl., 2009)
mEI	Surface	54	-	100	(Nfishfiyama et afl., 2015)
mEI	Surface; Wastewater; Marfine	1279	-	94.9	(Ferguson et afl., 2013)
mEI	Surface; Wastewater; Marfine	641	-	94.5	(Ferguson et afl., 2010)
mEI	Surface; Wastewater; Marfine	361	-	93.9	(Messer and Dufour, 1998)
mEnterococcus	Marfine	80	-	97.5	(Dfionfisfio and Borrego, 1995)
mEnterococcus	Marfine	624	-	94.2	(de Offivefira and Watanabe Pfinhata, 2008)
mEnterococcus	Pure Cufltures	93	91.0	88.2	(Pagefl and Hardy, 1980)
mEnterococcus	Surface	2231	-	88.5	(Levfin et afl., 1975)
mEnterococcus	Surface; Wastewater; Marfine	1043	-	90.2	(Adcock and Safint, 2001)
Sflanetz-Bartfley	Marfine	97	-	93.8	(Audficana et afl., 1995)
Sflanetz-Bartfley	Marfine	234	-	92.7	(Ifiwarfi et afl., 2018)
Sflanetz-Bartfley	Pure Cufltures	82	78	74.4	(Pagefl and Hardy, 1980)
Sflanetz-Bartfley	Surface	321	-	95.3	(Łuczkfiewficz et afl., 2010)
Sflanetz-Bartfley	Surface; Wastewater	385	-	93.8	(Frficker and Frficker, 1996)

[1] . Specfifficfity = (True Negatfives)/(True Negatfives + Faflse Posfitfives)

[2] . Seflectfivfity = True Posfitfives/(True Posfitfives + Faflse Posfitfives) or (Coflonfies Confirmed to Enterococcus Genus)/(Totafl Presumptfive Enterococcus Coflonfies fin Coflflectfion)



Ffig. 1. Workfflows for monfitorfing antfibfiotfic-resfistant Enterococcus fin the envfironment. A) Unbfiased ecoflogficafl survey of ant^R phenotypes. B) Interest fin onfly hfigh-flevefl resfistance (fi.e., cflinficafl reflevance). C) Capture broad range of ant^R phenotypes. D) Add seflectfive medfia wfithout antfibfiotfic to workfflow B or C fif finterested fin proportfion of ant^R fin totafl enterococcfi popuflatfion (WHO Trficycfle program). E) Interest fin flow concentratfion/finjured ceflfls (e.g., dfisfinfected water). [1]Note that pre-enrfichment for resfistance phenotypes (finjured or VBNC ceflfls) prevents thefir quantfifficatfion. [2]Antfibfiotfic susceptfibfiflfity testfing of subsampfled coflonfies often fincfludes orfigfinafl seflectfive antfibfiotfic to confirm fuflfl "resfistant" cflassfificatfion. [3]Whofle genome sequencfing fis recommended for the most accurate specfiatfion and comprehensfive genotypfing for gflobafl fisoflate comparfisons. Ant^R = antfibfiotfic resfistance, VBNC = vfiabfle but non-cufturabfle, ADB = azfide dextrose broth, BEA=bfifle escuflfin azfide, EIA=escuflfin firon agar, ARGs = antfibfiotfic resfistance genes

that most coflonfies screened may not be antfibfiotfic resfistant and ffindfing coflonfies wfith the resfistant phenotypes of finterest, and to achfieve requfired statfistficafl power, can be akfin to "searchfing for a needfle fin a haystack". The advantage to thfis approach fis that fit provfides a denomfinator for totafl *Enterococcus* fin the sampfle and an unbfiased dfistrfibutfion of both enterococcafl specfies and thefir genotypes and phenotypes, resufltfing fin an ecoflogficaflfly-reflevant anaflysfis (Cho et afl., 2019).

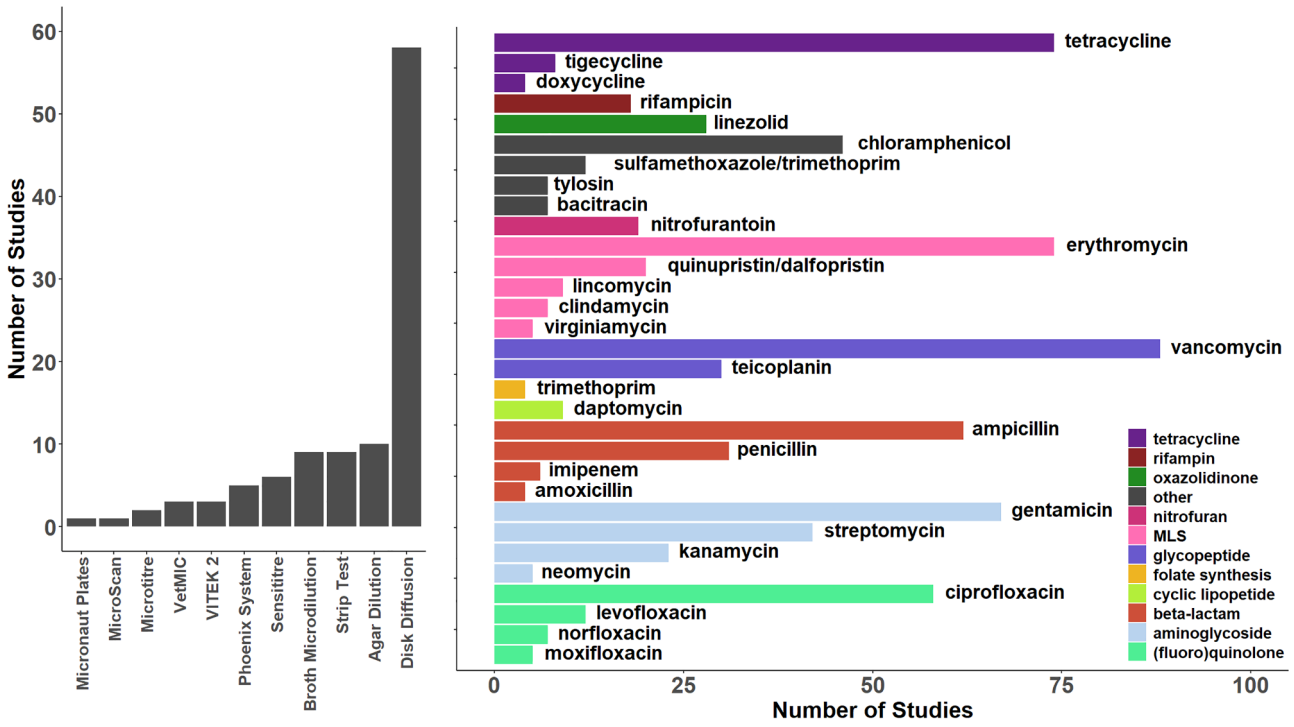
If the phenotype of finterest fis afready known (e.g., hfigh-flevefl VRE), targeted monfitorfing approaches may be more effficfient for fin-depth characterfizatfions of sub-popuflatfions of *Enterococcus*. Such approaches use an antfibfiotfic at cflinficaflfly-reflevant breakpofints to seflect for specfiffic resfistance phenotypes (Ffigure 1B). The use of flow/fintermedfiate breakpofints of antfibfiotfics may be usefufl for capturfing a broad range of phenotypes fin the envfironment but wfffl frequentfly capture cflinficaflfly-finreflevant organfisms, especfiaflfly fin the case of gflycopeptfide resfistance (Ffigure 1C). The sampfle can afso be pflated fin tandem on the seflectfive medfia wfithout the antfibfiotfic (Ffigure 1D), thus afflowfing the quantfifficatfion of the resfistant popuflatfion as a fractfion of the totafl enterococcfi measured fin CFU/unfit voflume, a unfiversafflfly comparabfle monfitorfing vaflue (e.g., see WHO Trficycfle Program recommendatfions (WHO, 2021)). Studfies utfiflfizfing targeted approaches often screen the fidentfified resfistant coflonfies agafinst a panefl of antfibfiotfics, whfich can fincflude the orfigfinafl seflectfive antfibfiotfic to confffirm cflinficaflfly-reflevant flevefls of resfistance. Thfis approach requfires the chofice of finfitfiafl seflectfive antfibfiotfic and therefore wfffl excflude strafins that are not resfistant to the prfimary seflectfive antfibfiotfic. Usfing a seflectfive antfibfiotfic wfffl afso skew the dfistrfibutfion of *Enterococcus* spp. away from the true dfistrfibutfion, often seflectfing for cflosefly reflated genera that share the same resfistance phenotype, such as *Lactobacfiflflus, Leuconostoc, Wefisseflfla*, and *Pedfiococcus* fin the case of hfigh flevefls of gflycopeptfide resfistance (Harwood et afl., 2001; Nfishfiyama et afl., 2017, 2015).

In some scenarfios, the recovery of very dfiflute phenotypes (rare targets) or stressed ceflfls fis desfirabfle, for finstance fin advanced treated wastewater fintended for reuse or other dfisfinfected waters. Pre-enrfichment of sampfles fin concentrated seflectfive broth (e.g., Enterococcosefl or Azfide Dextrose Broth) amended wfith the seflected antfibfiotfic at flow/fintermedfiate concentratfions can greatfly fincrease the detectfion fflimfit of rare phenotypes by heflpfing to recover VBNC coflonfies (Bflanch et afl., 2003; Vfiflanova and Bflanch, 2006). These recovered, resfistant coflonfies can then be streaked on hfigh-flevefls of the antfibfiotfic to recover cflinficaflfly-reflevant phenotypes of finterest. However, any protocofl empfloyfing a target enrfichment step wfffl precflude the abfiflfity to quantfify the resfistant *Enterococcus* popuflatfion or normaflfize to the totafl popuflatfion, a necessfity for unfiversaflfly comparabfle datatypes (Ffigure 1E).

After the coflflectfion of fisoflates fis generated, purfifficatfion of fisoflates can be performed on the fisoflatfion medfium or on other seflectfive-dfifferentfiafl medfia such as bfifle escuflfin azfide (BEA), or escuflfin firon agar (EIA). Ideaflfly, afll fisoflates shoufld be confffirmed to genus by a method such as qPCR for the 23S rRNA gene (US Envfironmentafl Pro-tectfion Agency, 2012). It fis crucfiafl that at fleast a sub-set be confffirmed to understand the faflse-posfitfive rate of the fisoflatfion method, and so that faflse-posfitfive fisoflates can be removed from the dataset. Kfirby-Bauer dfisc dfiffusfion testfing can be used to confffirm the flevefl of resfistance to the prfimary fisoflatfion antfibfiotfic and mufltfi-drug resfistance. Further fisoflate characterfizatfion may be desfirabfle, fincfludfing specfiatfion and genotypfing for ARGs and vfiruflence factors. Whofle genome sequencfing (WGS) fis recommended when resources afflfow, as fit fis the most accurate and comprehensfive method for specfiatfion and genotypfing and enabfles gflobafl fisoflate comparfisons. A comprehensfive evafluatfion of the sufite of methods used for fisoflate characterfizatfion fis presented fin the foflflowfing sectfions.

5.1. *Antfibfiotfic susceptfibfiflfity testfing methods*

Severafl technfiques and automated pflatforms exfist for generafflfized antfibfiotfic susceptfibfiflfity testfing (AST) or the determfinatfion of mfinfimum finhfibfitory concentratfions (MICs) of fisoflate ffibrarfies, fincfludfing Kfirby-Bauer dfisk dfiffusfion assays (~ 47% of artficfles), varfious commercfiafl automated systems (24%), manuafl broth or agar dfiflutfions (20%), or strfip test methods (9%) (Ffigure 2A). Over 90% of antfimficrobfiafl sensfi-tfivfity tests (AST) were performed on efither Mueflfler-Hfinton agar or fin Mueflfler-Hfinton broth. Concentratfions of antfibfiotfics chosen for AST were predomfinantfly determfined by referencfing the Cflinficafl and Research Standards Instfitute (CLSI; 70%) breakpofints for *Enterococcus*, accordfing



Ffig. 2. Summary of antfibfiotfic susceptfibfiflfity testfing (AST) methods appflfied across the revfiewed artficfles. A) Dfistrfibutfion of assays and commercfiaflfized pflatforms used for AST. B) Dfistrfibutfion of antfibfiotfics used for screenfing enterococcfi fisoflate coflflectfions.

to the most currentfly avafiflabfle gufideflfines. Other standardfized breakpofint concentratfions were specfiffic to a partficuflar natfion or governmentafl body, e.g., fincfludfing the European Commfittee on Antfimficrobfiafl Susceptfibfiflity Testfing (EUCAST; 7%), the Natfionafl Antfimficrobfiafl Resfistance Monfitorfing System (NARMS; USA; 2%), and the Canadfian Integrated Program for Antfimficrobfiafl Resfistance Surveffiflflance (CIPARS; 1%). Approxfimatefly 15% of studfies utfiflfizfing AST that afflso dfifferentfiated degrees of resfistance (fi.e., "susceptfibfle", "fintermedfiate", or "resfistant") dfid not cfite a justffificatfion for antfibfiotfic concentratfions nor a standardfized method for determfinfing the flevefl of resfistance observed.

The panefl of antfibfiotfics used to screen fisoflates varfied across studfies, but overarchfing trends were apparent. VRE was mentfioned fin the tfitfle of ~35% of artficfles fidentffiffied, and vancomycfin was fincfluded fin the screenfing panefl fin over 75% of the artficfles (Ffigure 2B). The number of observed phenotypes among cflfinficafl and envfironmentafl fisoflates of *Enterococcus* are wfide-rangfing, whfich warrants a dfiverse range of antfibfiotfics fincfluded fin the panefls. The antfibfiotfics tested were further categorfized finto twefflve dfistfinct cflasses by actfivfity. The most promfinent cflasses across affl studfies were gflycopeptfides (76%), macroflfideflfincosamfide-streptogramfin (MLS; 72%), tetracycflfines (72%), betaflactams (especfiaflfly penficfiflflfins) (70%), and amfinogflycosfides (64%). Interestfingfly, antfibfiotfics that are efither approved by the FDA to treat VRE finfectfions or are commonfly used to treat VRE (Arfias and Murray, 2012) were fless commonfly fincfluded fin panefls. These compounds fincflude flfinezoflfid (27%), qufinuprfistfin/daflfoprfistfin (21%), daptomycfin (8%), and the synthetfic gflycopeptfide, teficopflanfin (28%). Hfigh-flevefl amfinogflycosfide resfistance (e.g., gentamficfin, streptomycfin, and kanamycfin) fin enterococcfi fisoflated from the envfironment was the focus of smaflfl subset of studfies.

5.2. *Mufltfidrug resfistance proffiflfing*

Because enterococcfi are fintrfinsficaflfly resfistant to severafl antfibfiotfics; fincfludfing cephaflosporfins, penficfiflflfins, cflfindamycfin, and amfinogflycosfides, resfistant phenotypes are commonfly found fin envfironmentafl sampfles. Pflasmfid- and transposon-medfiated resfistance to tetracycflfines, erythromycfin, chfloramphenficofl, trfimethoprfim, vancomycfin, and cflfindamycfin; however, have further affflowed the genus to become a fleadfing cause of mufltfidrug resfistant nosocomfiafl finfectfions, partficuflarfly fin the US (Murray, 1998). Modern nosocomfiafl *E. faecfium* fisoflates, for exampfle, are commonfly resfistant to ampficfiflflfin, vancomycfin, and hfigh flevefls of amfinogflycosfides (Mfiflfler et afl., 2014). Recentfly, the emergence of mufltfidrug-resfistant VRE to newer, flast-resort antfibfiotfics; fincfludfing oxazoflfidfinone-flfinezoflfid, daptomycfin, qufinuprfistfin/daflfoprfistfin, and tfigecycflfine, have caused frequent treatment fafiflures and are of gflobafl concern (Ahmed and Baptfiste, 2018). Thus, screenfing for mufltfidrug resfistance amongst fisoflated envfironmentafl enterococcfi fis essentfiafl for monfitorfing the evoflutfion of the genus over tfime, specfifficaflfly as a functfion of anthropogenfic poflflutfion, as weflfl as assessfing the reflatfive hazard posed by the fisoflate. The chofice of antfibfiotfics to fincflude fin screenfing panefls fis crucfiafl, as most are not usefufl from a rfisk-based monfitorfing framework, aflthough some may hfighflfight ecoflogficafl reflevance. For finstance, screenfing for phenotypes that are fintrfinsfic to the genus may not have cflfinficafl reflevance but may be usefufl fin determfinfing the dfistrfibutfion of endemfic phenotypes to contextuaflfize the reflatfive frequency of VRE detectfion.

5.3. *Bfiochemficafl and moflecuflar confffirmatfion and specfiatfion*

Because the vfiruflence and resfistance characterfistfics across dfifferent *Enterococcus* spp. vary substantfiaflfly, specfiatfion of resfistant *Enterococcus* fisoflates fis desfirabfle. A sufite of bfiochemficafl tests has been devefloped by cflfinficafl mficrobfioflogfists to confffirm *Enterococcus* to the genus flevefl. These tests fincflude Gram stafinfing, cataflase testfing, thermafl growth range/thermotoflerance (growth at 10 and 45 °C), haflotoflerance (6.5% NaCfl), growth at pH 9.7, pyrroflfidonyflaryflamfidase actfivfity, and the abfiflfity to hydroflyze escuflfin fin the presence of bfifle saflts (e.g. growth on bfifle escuflfin agar) (Fackflam and Coflflfins, 1989; Tefixefira et afl., 2015). These tests were common features of nearfly three quarters of artficfles and served as a prerequfisfite for fincflusfion fin downstream characterfizatfion, fincfludfing further specfiatfion (Ffigure S2). Studfies that dfid not confffirm fisoflates to the genus flevefl efither reflfied on chromogenfic agar (e.g., mEI,

CHROMagar VRE) to seflect presumptfive enterococcfi or specfiated thefir flfibrary wfithout screenfing for characterfistfic metaboflfisms or morphoflogfies. Genus-specfiffic prfimers based on the 16S rRNA gene (Deasy et afl., 2000), 23S rRNA gene (EPA Method 1611) (EPA, 2012), or the eflongatfion factor EF-Tu (*tuf*) (Ke et afl., 1999) gene have afso been used for rapfid fidentfiffcatfion of the genus *Enterococcus*. However, 16S rRNA prfimer sets are known to fafifl to capture afifl *Enterococcus* spp. (Botfina and Sukhodoflets, 2006), whfifle EPA Method 1611 has proven to be very reflfiabfle fin our experfience.

Specfiatfion of enterococcfi flfibrarfies was common and performed fin 91/117 artficfles, the most common approach of whfich was PCR. There are severafl conserved protefins and correspondfing genes that are targeted fin these assays. The sfimufltaneous detectfion of enterococcafl specfies and gflycopeptfide resfistance was the ffirst moflecuflar approach to fimprove dfiagnostfic speeds for cflfinficafl enterococcfi and was based on the detectfion of genes encodfing D-aflanfine:D-aflanfine (*ddfl*) fligases and other gflycopeptfide resfistance determfinants. A reduced afffinfity for gflycopeptfides fin VanA- and VanB-type resfistance fin enterococcfi are due to the fintegratfion of D-aflanyfl:D-flacate finto peptfidogflycan precursors by the chromosomaflfly-encoded *ddfl* fligases (Dutka-Maflen et afl., 1995a). The *ddfl* enzymes fin *E. faecfium* ($ddfl_{E.\ faecfium}$) and *E. faecaflfis* ($ddfl_{E.\ faecafl fis}$) are conserved, and fin resfistant strafins, these enzymes are present fin addfitfion to *vanA* or *vanB*. Sfimfiflarfly conserved flfigases, *vanC1* and *vanC2-3* are hfighfly specfiffic for *E. gafffffinarum* (Dutka-Maflen et afl., 1992) and *E. casseflfifflavus* (Navarro and Courvaflfin, 1994), respectfivefly. The prfimers pubflfished by Dutka-Maflen et afl. (1995) for $ddfl_{E.\ faecfium}$, $ddfl_{E.\ faecafl fis}$, $vanC1_{E.\ gafflflfinarum}$, and $vanC2\text{-}3_{E.\ casseflfifflavus}$ have been the most wfidefly used for the specfiatfion of envfironmentafl enterococcfi, as many researchers are specfifficafllfly concerned wfith the fidentfiffcatfion of these four most common and cflfinficaflfly-reflevant specfies (Tabfle S3). (Karfiyama et afl., 2000) and (Depardfieu et afl., 2004) provfided addfitfionafl mufltfipflex PCR assays for more hfigh-throughput approaches to VRE surveffiflflance. A mufltfipflex PCR assay based on specfies-specfiffic superoxfide dfismutase (*sodA*) genes devefloped by Jackson et afl. (2004) fincfludes prfimers for 23 dfifferent enterococcafl specfies (Jackson et afl., 2004).

The PCR prfimers descrfibed above were fin part devefloped due to a flack of consensus between commercfiaflfly-avafiflabfle systems and kfits, such as the Anaflytficafl Proffifle Index (API; bfioMerfieux), PhenePflate (PhPflate Mficropflate Technfiques AB), Phoenfix Mficrobfioflogy Systems (BD Phoenfix), VITEK (bfioMerfieux), Mficronaut-Strep2 (MERLIN), MficroScan Waflk Away (Beckman Coufflter), and BBL Crystafl (MG Scfientfiffic) manuafl or automated rapfid fidentfiffcatfion systems. The prfincfipfles behfind these hfigher-throughput systems are derfived from conventfionafl bfiochemficafl phenotypfing of enterococcfi whfich finvofive dfifferentfiatfing carbohydrate fermentatfion of mannfitofl, sorbfitofl, sorbose, finuflfin, arabfinose, meflfibfiose, sucrose, rafffinose, trehaflose, flactose, gflycerofl, safflficfin, and mafltose, among others (Fackflam and Cofflflins, 1989; Tefixefira et afl., 2015). The commercfiafl methods empfloy a panefl of bfiochemficafl tests fin paraflflefl to reduce the flabor costs of manuafl phenotypfing. However, fif atypficafl specfies are present, these systems wfffl struggfle to fidentfify the organfism wfith acceptabfle flevefls of certafinty (Castfiflflo-Rojas et afl., 2013). Thfis fissue fis especfiaflfly probflematfic fin matrfices outsfide of the cflfinficafl settfing, as these systems were devefloped and vaflfidated targetfing common cflfinficafl strafins and reference cufltures wfith dfistfingufishabfle bfiochemficafl characterfistfics and not the wfide phenotypfic dfiversfity of envfironmentafl sampfles. A comprehensfive survey of these systems has been revfiewed prevfiousfly (Emery et afl., 2016). An emergfing technoflogy for the rapfid fidentfiffcatfion of mficroorgan-fisms fis matrfix assfisted flaser desorptfion fionfizatfion-tfime of fflfight mass spectrometry (MALDI-TOF MS) (Sfinghafl et afl., 2015). Thfis method uses flasers to generate sfingfly protonated fions from anaflytes fin the sampfle. In the process of fidentfifyfing unknown mficrobes, these anaflytes are prfimarfifly housekeepfing and rfibosomafl protefins, gfiven that they constfitute a flarge portfion of the dry wefight of mficrobfiafl ceflfls. The fionfized protefins are then separated by thefir mass-to-charge ratfio and dependfing on thefir tfime of fflfight through a channefl, a peptfide mass ffingerprfint fis generated that can be compared to openfly sourced databases. MALDI-TOF MS for the purpose of enterococcfi specfiatfion emerged fin the flfiterature fin 2017, as thfis fis an emergfing technfique fin envfironmentafl studfies. MALDI-TOF MS systems are consfiderabfly more expensfive than most moflecuflar or phenotypfic methods, but the throughput fis generaflfly flarger (thousands of fisoflates) and can better accommodate the demand fin cflfinficafl flaboratorfies.

16S rRNA gene sequencfing was fless frequentfly used than other methods due fits non-specfifficfity. Other genes may be sequenced, fincfludfing the *sodA, rpoA*, and *pheS* genes, whfich have shown to be more dfiscrfimfinatory than the 16S rRNA gene for cflosefly-reflated specfies, such as *E. casseflfifflavus* and *E. fflavescens* (Naser et afl., 2005; Poyart et afl., 2000). In a recent comparatfive study of varfious flocfi to dfifferentfiate cflosefly-reflated *Enterococcus* spp., fit was determfined that the aflpha subunfits of ATP synthase (*atpA*), chaperonfins (*groESL*), and phenyflaflnyfl-tRNA synthase aflpha subunfits (*pheS*) performed equaflfly weflfl or better than 16S rRNA gene sequencfing agafinst 308 enterococcfi fisoflates from untreated urban wastewater (Sanderson et afl., 2019). The rate of faflse fidentfiffcatfion of consensus reference strafins based on flocfi sequencfing was approxfimatefly 2%, much flower than the paraflflefflfized carbohydrate phenotypfing systems dfiscussed above, such as RapID STR, whfich had error rates of 15.9% for *E. faecafl fis*, 21.5% for *E. faecfium*, and 56.9% for *E. casseflfifflavus/ gafflflfinarum*. The gofld standard for specfiatfing enterococcfi, and any organfism for that matter, fis WGS (Sanderson et afl., 2019).

5.4. Genotypfing resfistant Enterococcfi

Numerous genetfic determfinants confer antfibfiotfic resfistance across the genus *Enterococcus*. Co-occurrence of resfistance genes and vfiruflence factors fis of partficuflar concern from a cflfinficafl standpofint and fis common among nosocomfiafl strafins (Guzman Prfieto et afl., 2016; Pontfinen et afl., 2021). Most genetfic determfinants of antfibfiotfic resfistance fin *Enterococcus* spp. are fintfinsficaflfly encoded, fi.e., they exfist on the chromosome wfithfin the core genome of the genus. Typficaflfly, acqufired resfistance, fi.e., a product of horfizontafl gene transfer, fis of greater finterest for monfitorfing, where the purpose fis to examfine trends fin resfistance patterns and fff they are changfing fin tfime and space. Acqufired resfistance genes are of prfimary concern as drfivers of fafifure of antfibfiotfic treatment fin cflfinficafl finfectfions. Canonficafl mobfifle resfistance determfinants wfithfin enterococcfi fincflude those that confer resfistance to gflycopeptfides (*van* gene cflusters), amfinogflycosfides (aac(6′)-Ie-aph(2″)-Ia and aph(3′)-IIIa), MLS (*ermB*), and tetracycflfines (*tetM* and *tetL*) (Ffigure S3).

Just over a thfird of anaflyzed artficfles (41/117) genotyped coflonfies for antfibfiotfic resfistance determfinants after they had been fisoflated on antfibfiotfic-contafinfing medfia, of whfich 37 used PCR and 4 used WGS. The *van* operon was commonfly targeted, wfith an emphasfis on *vanA* and *vanB* wfithfin VRE fisoflates themseflves (Ffigure S3). There are nfine dfistfinct gene cflusters conferrfing gflycopeptfide resfistance fin enterococcfi (VanA, B, C, D, E, G, L, M, N) (Hancock et afl., 2014; Tefixefira et afl., 2015) and these determfinants dfiffer both genetficaflfly and phenotypficaflfly based on thefir physficafl flocatfion (encoded on mobfifle genetfic eflements or chromosomafl), whether resfistance fis finducfibfle or constfitutfive, the type of peptfidogflycan precursor that fis produced, and ufltfimatefly the flevefl of resfistance conferred. VanA gene cflusters are the most common fin cflfinficafl fisoflates and are typficaflfly found on Tn*1546*-flfike transposons, are frequentfly fintegrated finto a wfide range of pflasmfids, and produce cflfinficafl flevefls of resfistance to vancomycfin (MIC 64-1,000 μg/mL) and tefficopflanfin (MIC 16-512 μg/mL) (Tefixefira et afl., 2015). Sfimfiflar to VanA, VanB gene cflusters are afso typficaflfly found fin cflfinficafl fisoflates and are present on transposons (Tn*1547* or Tn*1549* to Tn*5382*), but dfiffer from *van*A due thefir fiinabfiflfity to recognfize teficopflanfin, aflflowfing strafins wfith the VanB phenotype to remafin susceptfibfle (Mfiflfler et afl., 2014). These two gene cflusters are the most sfignfifficant genetfic determfinants fin cflfinficaflfly-resfistant enterococcfi and severafl PCR assays have been devefloped for thefir detectfion (Dutka-Maflen et afl., 1995b; Karfiyama

et afl., 2000; Nam et afl., 2013; Rathnayake et afl., 2011). Enterococcfi dfispfayfing susceptfibfle to fintermedfiate resfistance are typficaflfly attrfibuted to chromosomaflfly encoded *van* cflusters, flfike *vanC1* fin *E. gaflfflfinarum* and *vanC2/3* fin *E. casseflfifflavus*, whfich are commonfly detected fin envfironmentafl sampfles. The much more rare *vanD-N* genotypes were not detected fin any artficfles that screened for them (Kotzamanfidfis et afl., 2009; Taučer-Kaptefijn et afl., 2016; Zdragas et afl., 2008).

5.5. Vfiruflence factors and pathogenesfis

The pathogenesfis of finfectfions caused by enterococcfi fis sfffifl poorfly understood (Tefixefira et afl., 2015). However, severafl PCR assays have been devefloped for the detectfion of vfiruflence factors common to *Enterococcus*, fincfludfing: surface adhesfion protefins (*esp*), aggregatfion substances (*agg*), cytoflysfin (*cyfl*) and hemoflysfin (*hyfl*) secretfion operons, coflflagen adhesfion (*ace*), and geflatfinase secretfion protefins that are predomfinantfly found fin endocardfitfis fisoflates (*geflE*) (Eaton and Gasson, 2001; Mannu et afl., 2003; Vankerckhoven et afl., 2004). These vfiruflence factors were prfimarfifly screened for fin the studfied artficfles due to thefir fimpflficatfions fin pathogenesfis routes from envfironmentafl reservofirs back finto humans and anfimafls. Onfly 18 of the studfies fidentfified screened for vfiruflence factors, the most common befing *esp* (aflfl 18 artficfles), *cyfl*, and *geflE* (Ffigure S3). The ffirst vancomycfin-resfistant *E. faecaflfis* strafin documented fin the US was reveafled to carry a flarge, transmfissfibfle pathogenficfity fisfland contafinfing both *esp* and a compflete *cyfl* operon, and severafl other functfions that are non-essentfiafl to commensafl behavfior of the organfism (Shankar et afl., 2002). The *E. faecaflfis* pathogenficfity fisfland fis an fintegratfive conjugatfive eflement that can be mobfifflfized between pflasmfids and chromosomes fin *E. faecaflfis* and *E. faecfium*, transferrfing vfiruflence factors and antfibfiotfic resfistance determfinants (Laverde Gomez et afl., 2011; Manson et afl., 2010). The co-occurrence of vfiruflence wfith antfibfiotfic resfistance fis a key consfideratfion when screenfing the envfironment for the emergence of potentfiaflfly hyper-vfiruflent strafins. Rathnayake et afl. (2012) found sfignfifficant correflatfions between the presence of vfiruflence factors and phenotypfic antfibfiotfic resfistance among both *E. faecfium* and *E. faecaflfis* fisoflates fin surface waters and regfionafl cflfinficafl fisoflates fin Austraflfia (Rathnayake et afl., 2012). Sfimfifarfly, Lata et afl. (2016) documented wfidespread co-occurrence of *vanA* and *vanB* genotypes wfith *geflE, ace, efaA,* and *esp* vfiruflence factors fin both *E. faecaflfis* and *E. faecfium* fin fimpacted surface waters fin northern Indfia (Lata et afl., 2016). Such studfies demonstrate the vaflue of monfitorfing both antfibfiotfic resfistance and vfiruflence, partficuflarfly for gafinfing finsfight finto ecoflogficafl factors at pflay fin observed resfistance patterns.

5.6. Mufltfifocus sequence typfing

Mufltfifocus sequence typfing (MLST) fis a technfique by whfich mufltfipfle flocfi, or specfiffic fintemafl DNA fragments wfithfin an organfism's genome, are ampfififfied by PCR, sequenced, and then compared across mufltfipfle fisoflates of that specfies. The flocfi are typficaflfly housekeepfing genes common to the genus, and the afflefffic composfitfion of the set of targets determfines the "sequence type", aflfowfing for the determfinatfion of cflonafl compflexes (CCs; fisoflates wfith dfifferences of no more than 2 flocfi) and potentfiafl source attrfibutfion of fisoflates. Sequence types can then be compared to pubflficfly avafiflabfle and curated databases, such as PubMLST (Joflfley et afl., 2018), where gflobafl fisoflate comparfisons can be made. Puflse-ffiefld gefl eflectrophoresfis fis a sfimfiflar technfique to MLST and was prevfiousfly known as the "gofld standard" for source attrfibutfion and epfidemfioflogficafl flfinkages of bacterfiafl fisoflates, but flow finterflaboratory reproducfibfiflfity and finabfiflfity to perform phyflogenetfic or popuflatfion structure studfies makes fit unsufitabfle for gflobafl, flong-term epfidemfioflogficafl studfies (Nemoy et afl., 2005). MLST proffifles of *Enterococcus* have onfly been devefloped for *faecfium* (Homan et afl., 2002) and *faecaflfis* (Rufiz-Garbajosa et afl., 2006) and are therefore the onfly two specfies present fin the PubMLST database. The two proffifles are derfived from a mfixed set of 11 housekeepfing genes: *gdh* (gflucose-6-phosphate dehydrogenase), *purK* (phosphorfibosyflamfinofimfidazofl carboxyflase ATPase subunfit), *pstS* (phosphate ATP-bfindfing cassette transporter), *atpA* (ATP synthase, aflpha subunfit), *gyd* (gflycerafldehyde-3-phosphate dehydrogenase), *adk* (adenyflate kfinase), *ddfl, gkfi* (gflucokfinase), *aroE* (shfikfimate 5-dehydrogenase), *xpt* (shfikfimate 5-dehydrogenase), and *yqfiL* (acetyfl-coenzyme A acetyfltransferase), whfich were chosen for thefir flow ratfios of nonsynonymous to synonymous mutatfions and thefir dfispersed flocatfions on the chromosomes. MLST fis usefufl when expflorfing potentfiafl cflonafl reflatfionshfips between *Enterococcus* spp. fisoflated from the envfironment and those from crfitficafl AMR monfitorfing pofints such as hospfitafl wastewaters and pharmaceutficafl productfion waste. The cflonafl compflex 17 (CC17) of *Enterococcus faecfium*, for exampfle, fis a nosocomfiafl strafin assocfiated wfith outbreaks worfldwfide and fis generaflfly ampficfiflflin and qufinoflone resfistant and contafins the *esp* surface adhesfion protefin (Top et afl., 2008). CC17 has been detected fin severafl envfironmentafl sampfles and fis an findficatfion of the finterconnectedness of the envfironment and cflfinficafl wastewater streams (Capflfin et afl., 2008).

5.7. Whofle genome sequencfing

Despfite the deveflopment of severafl sophfistficated moflecuflar bfioflogficafl assays for the compflete characterfizatfion of enterococcafl fisoflates over the flast few decades, the advent and proflfiferatfion of next-generatfion sequencfing technfiques has afflowed for comprehensfive and hfigh-throughput functfionaflfity of aflfl the prevfious assays fin a sfinguflar method (Ffigure 1). WGS of fisoflates aflflows for the sfimufltaneous detectfion of ARGs, vfiruflence factors, pflasmfids, bacterfiophages, finsertfion sequences, transposons, and the sequence type and cfladafl reflatedness of fisoflates that can be comparted wfith enterococcafl flfibrarfies gflobaflfly. Onfly four artficfles revfiewed here performed WGS (Ekwanzafla et afl., 2020a; Gouflfiourfis et afl., 2019, 2018; Zaheer et afl., 2020), fincficatfing that comprehensfive epfidemfioflogficafl anaflysfis of antfibfiotfic resfistant, vfiruflent, hospfitafl-adapted enterococcafl cflades fis flargefy absent from the envfironmentafl flfiterature. In WGS workfflows, resfistant enterococcfi are finfifiaflfly screened for on seflectfive medfia suppflemented wfith antfibfiotfics (fin thfis case ampficfiflflin or vancomycfin) and then fisoflated and specfiated before they are subject to sequencfing. Genomfic surveffiflflance of *E. faecfium* fisoflates from retafifl meat, patfients wfith bfloodstream finfectfions, and wastewater treatment pflants reveafled dfistfinct cflades wfith flfimfited sharfing of ARGs between flfivestock and humans fin the UK (Gouflfiourfis et afl., 2018). There was, however, extensfive overflap between fisoflates from bfloodstream finfectfions and those from the finffluents and efffluents of 17 dfifferent wastewater treatment pflants (WWTPs) fin the regfion, whfich coufld findficate the emergence of new flfineages of *E. faecfium* that are both hospfitafl-adapted and persfist fin the envfironment (Gouflfiourfis et afl., 2019). Sfimfiflarfly, a South Afrfican study finvestfigated the prevaflence of VRE fin hospfitafl wastewater, munficfipafl wastewater, and the recefivfing surface water (Ekwanzafla et afl., 2020a).Thfirty-ffive percent of the enterococcfi exfifting the wastewater treatment pflant were vancomycfin resfistant, fleadfing to the greatest VRE floadfings fin the downstream sedfiment. Subsequent comparatfive genomfics found that ST40, a human pathogenfic *E. faecaflfis* sequence type, and CC17 of *E. faecfium* were found persfistfing fin downstream sedfiments, posfing a rfisk to human heaflth, and demonstratfing the need for more advanced wastewater treatment fin thfis scenarfio. Afllthough WGS fis more expensfive and dfiffficuflt to perform than PCR-based genotypfing, fits hfigh-throughput and robust anaflysfis fis qufickfly becomfing commonpflace as sequencfing costs contfinue to faflfl. Aflso, the storage and sharfing of sequenced genomes to pubflfic databases aflflows for flongfitudfinafl, phyflogenetfic trackfing of probflematfic cflones as they are transmfitted gflobaflfly (van Hafl et afl., 2021).

6. Trends fin totafl and antfibfiotfic-resfistant *Enterococcus* found fin water envfironments

A comprehensfive data extractfion and anaflysfis was performed to

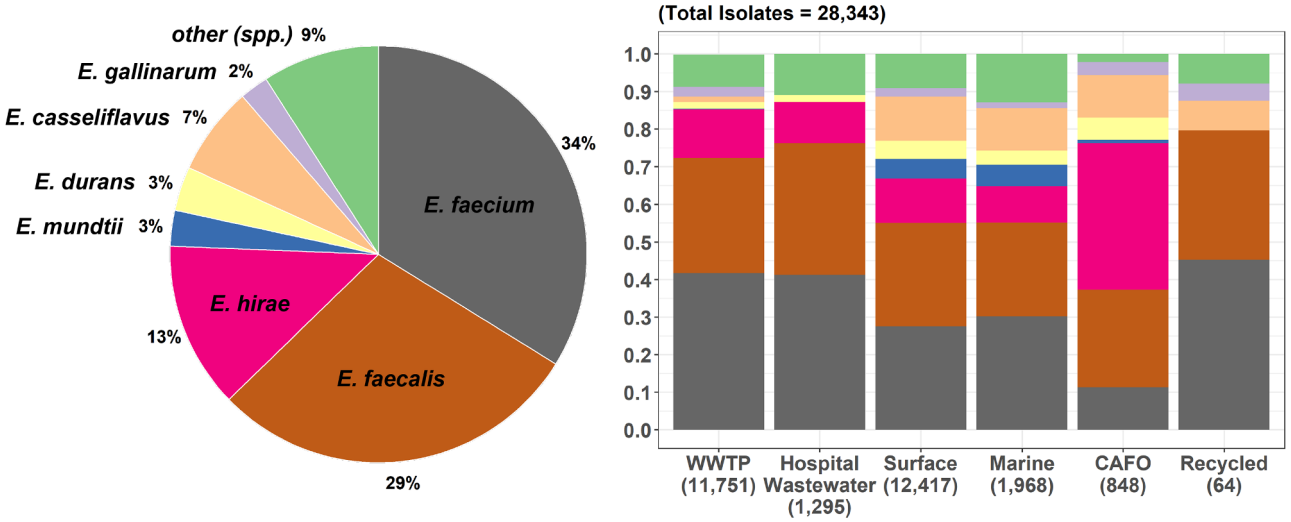
fidentfify overarchfing trends fin the specfies dfistrfibutfion of generfic and antfibfiotfic-resfistant *Enterococcus* spp. to fiflflumfinate generafl trends and finform sampflfing prfiorfitfies, extractfing data from aflfl artficfles that reported resfistance as a percentage of the totafl number of fisoflates fin a coflflectfion. Together, thfis coflflectfion consfisted of 42,459 fisoflates extracted from 95/117 artficfles. To reduce the amount of bfias fintroduced by sampflfing and enrfichment procedures, onfly *Enterococcus* AST data that was generated fin the absence of an finfitfafl seflectfive antfibfiotfic were used. Thfis reduced the coflflectfion sfize for AST data to 18,729 fisoflates extracted from 77 artficfles but aflflowed for an estfimatfion of the "true" phenotypfic dfiversfity of envfironmentafl antfibfiotfic-resfistant *Enterococcus*. Thfis approach aflso aflflows for the empfirficafl prfiorfitfizatfion of monfitorfing targets for *Enterococcus* resfistant to crfitficafl antfibfiotfics as weflfl as a baseflfine for further studfies across dfifferent water matrfices wfithout a bfias towards resfistant popuflatfions.

6.1. Totafl Enterococcfi

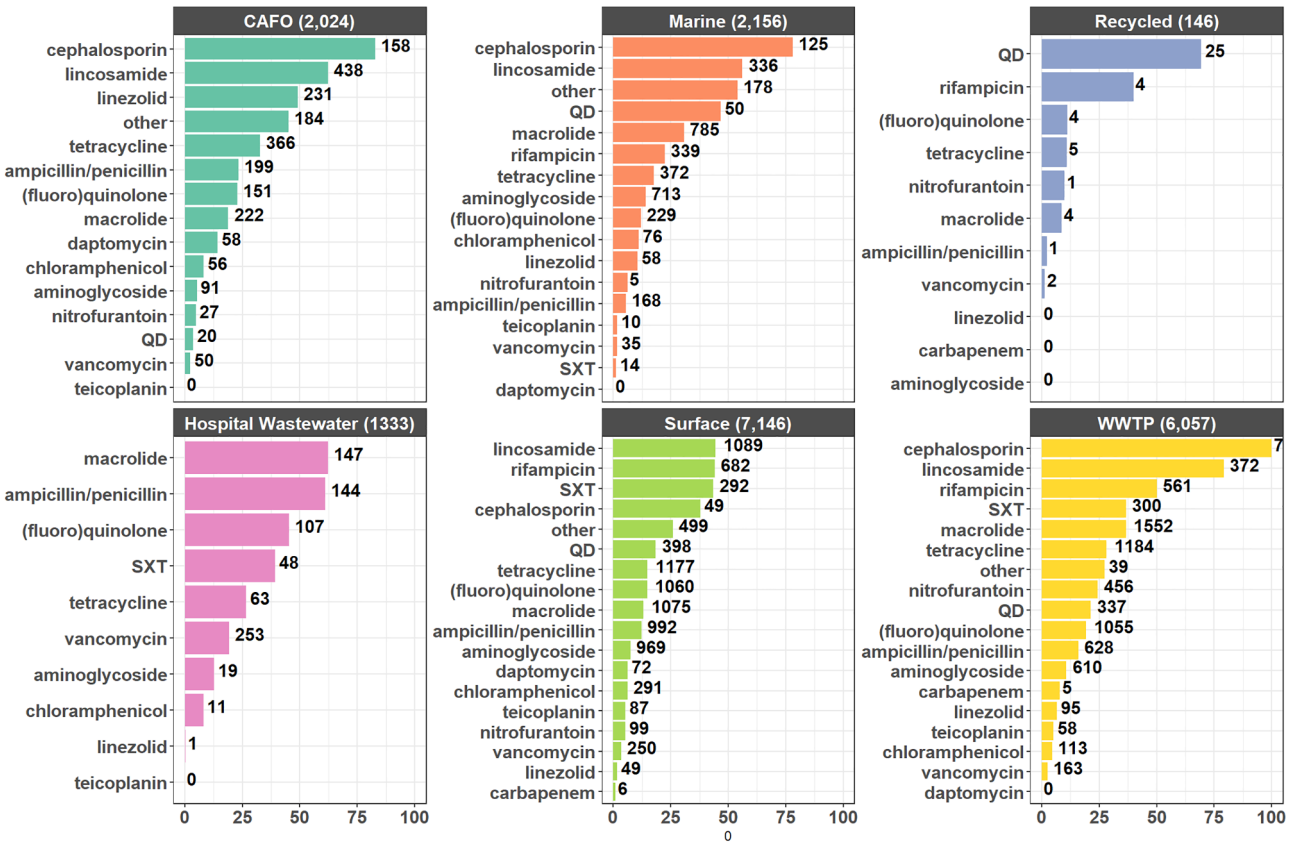
Onfly 62 of the 91 artficfles fin whfich flfibrarfies were specfiated were the data provfided fin a format that coufld be extracted unambfiguousfly (e.g., fin tabuflar format) or detafifled popuflatfion statfistfics reported, resufltfing fin a coflflectfive of 28,343 specfiated fisoflates for anaflysfis (Ffigure 3). The three most common *Enterococcus* spp. fidentfifiied across the 62 artficfles were *E. faecfium* (34.0%), *E. faecaflfis* (29.0%), and *E. hfirae* (13.0%). Mfinor specfies such as *E. rafffinosus, E. avfium,* and *E. pseudoavfium* (Tabfle S3) each represented fless than 2% of the totafl fisoflates, aflthough thfis fis flfikefly finffluenced by underrepresentatfion of these specfies fin common PCR conffirmatfion assays. In fact, fin many artficfles, onfly *E. faecaflfis, E. faecfium, E. gafllflfinarum,* and *E. casseflfifflavus* were screened, as these specfies represent the most encountered resfistant cflfinficafl fisoflates. Despfite the uneven representatfion of the number fisoflates from each water matrfix, there were cflear trends among enterococcafl popuflatfions. In WWTP and hospfitafl wastewater sampfles, the combfined proportfions of *E. faecfium, E. faecaflfis,* and *E. hfirae* were nearfly fidentficafl, makfing up approxfimatefly 90% of aflfl fisoflates (Ffigure 3). These proportfions are consfistent wfith the naturafl dfistrfibutfions of *Enterococcus* spp. fin the gastrofintestfinafl tract of heafflthy human aduflts and anfimafls (Sfiflva et afl., 2012). The proportfions of non-fecafl reflated or undeffined *Enterococcus* spp. were greater fin sur-face water and coastafl water sampfles, consfistent wfith dfiflutfion of fecafl contamfinatfion wfith envfironmentafl strafins. A flarge proportfion *E. hfirae*, the domfinant specfies excreted by cattfle (Jackson et afl., 2011), was found fin cattfle feedflot drafins (Zaheer et afl., 2020). The even proportfions of specfies across comparabfle water matrfices (e.g., WWTPs and hospfitafl wastewater) observed, despfite extensfive heterogenefity fin methodoflogy, suggests that there fis a generafl flack of systematfic errors or bfiases fin efither medfia seflectfivfity or specfiatfion techn fiques.

6.2. Surface water

The majorfity of fisoflates obtafined across studfies were derfived from surface water (fresh water), whfich fincfluded both urban and rurafl watersheds and rfiverfine sfites dfirectfly fimpacted by munficfipafl or hospfitafl wastewater dfischarge (7,146 fisoflates) (Ffigure 4). Compared to the other envfironments examfined, surface waters dfispflayed some of the flowest percentages of resfistant fisoflates to aflfl 18 antfibfiotfics and antfibfiotfic cflasses. It shoufld be noted that, despfite dfiflutfion effects and envfironmentafl attenuatfion, 3.5% of 7,146 *Enterococcus* spp. fisoflates were fuflfly resfistant to vancomycfin. The vast majorfity of these fisoflates were efither *E. faecfium* or *E. faecaflfis*. By contrast, fuflfl vancomycfin resfistance was vfirtuaflfly undetected fin *E. casseflfifflavus, E. gafllflfinarum* and the other fless domfinant specfies (Ffigure 4; Tabfle S3). Despfite fintrfinsfic resfistance of *E. casseflfifflavus* and *E. gafllflfinarum* vfia *vanC* genes, thefir phenotypes rarefly exceeded CLSI breakpofints for fuflfl cflfinficafl resfistance. Łuczkfiewficz et afl. (2010) examfined *Enterococcus* resfistance to 13 dfifferent antfibfiotfics fin an urban rfiver system fin Pofland fin the absence of wastewater treatment pflant dfischarge and found that resfistance to erythromycfin, cfiproffloxacfin, and tetracycflfine was common among aflfl fisoflates (Łuczkfiewficz et afl., 2010). They aflso found mufltfidrug resfistance (some to aflfl 13 antfibfiotfics tested), fincfludfing vancomycfin and hfigh-flevefl amfinogflycosfide resfistance, among *E. faecaflfis* and *E. faecfium* fisoflates fin the two mafin trfibutarfies feedfing the coastafl waters. The authors suggested that rfiverfine enterococcfi shoufld be consfidered as a potentfiafl rfisk for downstream recreatfionafl bathers, even fin the absence of pofint-source wastewater poflflutfion. In contrast, studfies of rurafl watersheds (< 1 % urban) fin Ontarfio (Canada) and Georgfia (US) found that the dfiversfity and dfistrfibutfion of antfibfiotfic resfistance among *Enterococcus* were strfikfingfly dfifferent than fin more anthropogenficaflfly-fimpacted water bodfies (Cho et afl., 2019; Lanthfier et afl., 2011). These two studfies, together comprfisfing 2,195 fisoflates, findficate that the *Enterococcus* specfies and thefir phenotypes were stochastficaflfly dfistrfibuted and sparse, wfith few mufltfidrug (< 6 antfibfiotfics) resfistant strafins and no fisoflates reachfing the CLSI breakpofints for vancomycfin, teficopflanfin, or flfinezoflfid. They



Ffig. 3. Dfistrfibutfion of Enterococcus spp. fisoflates across aflfl studfies. A) Specfies dfistrfibutfion of the totafl specfiated meta-flfibrary across aflfl water types (27,464 fisoflates). "other (spp.)" fis any specfies that was not detected at >2% of the totafl meta-flfibrary abundance or was reported onfly as Enterococcus spp. B) The dfistrfibutfion of generfic enterococcafl fisoflates by water matrfix. The totafl number of fisoflates representfing each matrfix fis findficated parenthetficaflfly. Concentrated anfimafl feedfing operatfion (CAFO) sampfles encompass wastewater flagoons, feedflot drafins, and any on-sfite treatment systems.



Ffig. 4. Dfistrfibutfion of percent of Enterococcus fisoflates resfistant to each antfibfiotfic by sampfle matrfix. Isoflates were onfly fincfluded fif they were finfitfiafllfly fisoflated fin the absence of a seflectfive antfibfiotfic. The number of unfique fisoflates representfing each matrfix fis fin parentheses fin the headers but note that not every fisoflate was tested for every antfibfiotfic. The number of resfistant fisoflates per antfibfiotfic tested fis represented adjacent to each bar fin each panefl. CAFO = concentrated anfimafl feedfing operatfion, WWTP = wastewater treatment pflant. QD = qufinuprfistfin/daflfoprfistfin; SXT = suflfamethoxazofle/trfimethoprfim

attrfibuted the dfispersfion of the resfistant fecafl findficators to domestficated anfimafl and wfifldlfife fecafl poflflutfion and thefir potentfiafl for dfissemfinatfion to be flfimfited. The hfighest rates of resfistance among *Enterococcus* fisoflates from surface water studfies came from the North West provfince of South Afrfica where 86/124 *Enterococcus* spp. were phenotypficaflfly resfistant to vancomycfin. These were fisoflated fin the absence of a seflectfive antfibfiotfic and dfispflayed mufltfidrug resfistance to ampficifllflfin, amoxficifllflfin, penficifllflfin, cfiproffloxacfin, erythromycfin, and tetracycflfine. Interestfingfly, a sfingfle fisoflate of *E. suflfureus* was found to be resfistant to ampficifllflfin, amoxficifllflfin, penficifllflfin, streptomycfin, vancomycfin, chfloramphenficofl, cfiproffloxacfin, erythromycfin, and tetracycflfine (Moflafle and Bezufidenhout, 2016).

Upstream and downstream sampflfing of munficfipafl wastewater dfischarge was carrfied out finseverafl studfies. The detectfion of enterococcfi wfith resfistance to cflfinficaflfly-reflevant antfibfiotfics downstream of munficfipafl wastewater dfischarge was regfionaflfly dependent and flfinked to the degree of treatment empfloyed by the WWTP. A Tunfisfian study found no ampficifllflfin-resfistant *Enterococcus* (ARE) or VRE fin the finffluent, efffluent, or recefivfing water, suggestfing the regfionafl varfiatfion fin thefir dfistrfibutfion (Ben Safid et afl., 2015). Another study (Bessa et afl., 2014) found *vanA* posfitfive *E. faecfium* mostfly upstream of WWTP dfischarge fin Portugafl, a regfion where VRE fis prevaflent fin the popuflatfion.

6.4. *Munficfipafl and hospfitafl wastewater*

Munficfipafl and hospfitafl wastewater were commonfly sampfled across the fidentfiffied artficfles. Together they accounted for 7,400 fisoflates fin the coflflectfion and dfispflayed hfigh percentages of cflfinficaflfly-reflevant antfibfiotfic resfistant phenotypes (Ffigure 4). *Enterococcus* spp. fisoflates from untreated hospfitafl wastewater dfispflayed the hfighest percentages of resfistance to ffluoroqufinoflones, ampficifllflfin/penficifllflfin, macroflfides, and vancomycfin.

WWTPs, whfich encompassed raw finffluent, actfivated sfludge, secondary efffluents, and varfiabfly treated ffinafl efffluents across 15 dfifferent countrfies, yfiefdded *Enterococcus* spp. fisoflates wfith the hfighest percentages of resfistance to cephaflosporfins, flfincosamfides, nfitrofurantofin, and teficopflanfin (Ffigure 4). Wfithfin the "WWTP" category, onfly 2.7% of 6,057fisoflates were resfistant to vancomycfin fin the coflflectfion. The hfighest percentages of fuflfl vancomycfin resfistance across studfies of WWTP finffluents came from Germany (19%; 16/84 fisoflates) (Gaflflert et afl., 2005), Portugafl (3.4%; 17/499 fisoflates) (Araújo et afl., 2010), Iran (3.3%; 19/577) (Taflebfi et afl., 2007), and Pofland (3.2%; 6/185 fisoflates) (Luczkfiewficz et afl., 2013). The 2005 paper (Gaflflert et afl., 2005), fis the ofldest of the group, and reveafls the chaflflenges of accuratefly assessfing antfibfiotfic-resfistant *Enterococcus,* partficuflarfly VRE, whose phenotypfic characterfistfics are mfimficked by genera such as *Leuconostoc, Pedfiococcus* and *Wefiseflfla* (Harwood et afl., 2001). In thfis case bacterfia were fisoflated on reflatfivefly non-seflectfive bfifle escuflfin agar, and were confffirmed onfly by abfiflfity to grow at 6.5% NaCfl and by mficroscopy for ocflfl morphoflogy. Furthermore, the method for assessfing antfibfiotfic resfistance reflfied on a set zone of finhfibfitfion of 2 mm for aflfl antfibfiotfics, rather than standardfized zone dfiameters.

Afllthough they represented a smafflfer fractfion of fisoflates, hospfitafl wastewater envfironments showed the most deffinfitfive contrfibutfions of phenotypfic resfistance to recefivfing waters. For exampfle, Novafis et afl. (2005) found statfistficaflfly sfignfifficant fincreases fin phenotypfic resfistance exceedfing CLSI breakpofints among *E. faecfium* and *E. faecaflfis* for vancomycfin, teficopflanfin, cfiproffloxacfin, and ampficifllflfin fin urban sewers recefivfing hospfitafl wastewater dfischarge fin Portugafl (Novafis et afl., 2005). Cflonafl anaflysfis, Tn*1546* typfing, and vfiruflence factor assays were afllso consfistent wfith flocafl cflfinfics befing the source of downstream surface

water fisoflates. Sfimfiflar observatfions were made aflong a medficafl center-retfirement home-wastewater treatment pflant-rfiver contfinuum fin France fin 2013, where the hospfitafl-adapted cflonafl compflex, CC17 *E. faecfium*, was cuflturabfle aflong the contfinuum and finto recefivfing wa-ters (Lecflercq et afl., 2013). The CC17 concentratfion, though, was attenuated by the WWTP and the proportfion of CC17 became out-wefighed by envfironmentafl strafins. Further, epfidemfioflogficafl source trackfing usfing WGS of VRE fisoflated from 20 WWTPs fin the UK fin 2019 determfined that there was wfidespread dfissemfinatfion of hospfitafl-adapted *E. faecfium* fin WWTP efffluents across eastern Engfland (Gouflfiourfis et afl., 2019). They found that WWTPs recefivfing hospfitafl wastewater had sfignfifficantfly hfigher VRE and ARE concentratfions than non-hospfitafl assocfiated treatment pflants and found hfighfly sfimfiflar fisoflates shared between the flocafl teachfing hospfitafl and those emfitted from surroundfing WWTPs. Together, these studfies provfide strong support of *Enterococcus* spp. as both a cflfinficaflfly- and envfironmentaflfly-reflevant target for waterborne monfitorfing. Hospfitafl wastewaters, the munficfipafl wastewater finfrastructure responsfibfle for treatment, and recefivfing wa-ters are key monfitorfing pofints for trackfing thefir dfissemfinatfion.

6.5. Pharmaceutficafl wastewater

Pharmaceutficafl wastewater fis a crfitficafl monfitorfing pofint fin the dfissemfinatfion of resfistant mficroorganfisms and fis currentfly understudfied fin the ffiefld. Onfly two such artficfles focused on resfistant *Enterococcus* were encountered fin thfis revfiew. Guardabassfi et afl. (2002) documented fin-termedfiate resfistance of presumptfive *Enterococcus* to 20 ug/mL vanco-mycfin and evfidence of *vanA* and *vanD* fisoflates fin the waste bfiomass from the fermentatfion tanks used fin the productfion of vancomycfin (Guarda-bassfi et afl., 2002). The WWTP treatfing the waste was aflso enrfiched wfith presumptfive, fintermedfiate-flevefl VRE, and quantfiffiabfle CFUs were routfinefly emfitted from the pflant after secondary cflarfiffficatfion wfithout dfisfinfectfion. PFGE anaflysfis found fidentficafl VRE patterns between van-comycfin productfion fermentatfion waste and the ffinafl efffluents of the WWTP, suggestfing that pharmaceutficafl productfion waste can be a dfirect contrfibutor to the dfissemfinatfion of VRE finto the envfironment. Further, Guardabassfi et afl. (2004) finvestfigated the reflatfionshfip of Tn*1546*-flfike eflements fin *Enterococcus* spp. fisoflated from munficfipafl sewage, actfivated sfludge, vancomycfin productfion waste, human feces, mussefls, and sofifl usfing flong PCR-restrfictfion fragment flength poflymorphfism and found findfistfinguishabfle eflements shared across the ecoflogficaflfly dfistfinct flocatfions and between enterococcafl specfies, suggestfing ready transferabfiflfity of the *vanA* genotype between cflfinficafl and envfironmentafl strafins (Guardabassfi and Daflsgaard, 2004).

6.6. Recycfled water

Due to the fincreased pressures on freshwater around the worfld, wastewaters are fincreasfingfly befing treated and refintroduced finto water and food cycfles as the recycfled water fis used for crop fimfigatfion, groundwater recharge, and even dfirect potabfle reuse. Onfly three studfies fin the current revfiew examfined resfistant *Enterococcus* fin wastewaters fintended for reuse. Goflds tefin et afl. (2014) sampfled two WWTPs fin the Mfid-Atflantfic and two from the Mfidwest regfions of the US that reuse thefir treated efffluents and detected VRE fin 27% of wastewater sampfles, wfith hfigher rates fin the Mfid-Atflantfic pflants. VRE were onfly detected fin ffinafl treated efffluents when there was flack of chflorfinatfion (Goflds tefin et afl., 2014). Subsequent studfies from the same WWTPs found that VRE are detectabfle at flow concentratfions at the pofint of use after recovery from UV dfisfinfectfion, aflthough other phenotypes are more prevaflent (Carey et afl., 2016). Both WWTPs studfied recefive hospfitafl wastewater and thefir efffluents were used for spray fimfigatfion (Goflds tefin et afl., 2012).

6.7. Marfine waters and recreatfionafl beaches

Freshwater and marfine water envfironments used for recreatfionafl bathfing are at the dfirect finterface between envfironmentafl fecafl poflflu-tfion and human exposure and are therefore fimportant monfitorfing pofints. Studfies of enterococcafl popuflatfions of marfine and freshwater beaches from Spafin, Puerto Rfico, Pofland, Greece, Maflaysfia, Brazfifl, Itafly, and Mfichfigan confffirmed that many drug resfistant strafins are readfifly cuflturabfle fin recreatfionafl marfine water and sand (Aflm et afl., 2014; Arvanfitfidou et afl., 2001; Dada et afl., 2013; de Oflfivefira and Watanabe Pfinhata, 2008; Montficeflflfi et afl., 2019; Sadowy and Luczkfiewficz, 2014; Santfiago-Rodrfiguez et afl., 2013; Tejedor Junco et afl., 2001). Hfigh phenotypfic and phyflogenetfic dfiversfity was observed across aflfl studfies, and many envfironmentafl-assocfiated enterococcfi domfinated flocafl pop-uflatfions, fincfludfing *E. casseflfifflavus* (Montficeflflfi et afl., 2019), *E. hfirae* (Sadowy and Luczkfiewficz, 2014), and *E. avfium* and *E. rafffinosus* (Arva-nfitfidou et afl., 2001). Beach sands are of partficuflar finterest as they represent a nfiche envfironment for enterococcfi where horfizontafl gene transfer can occur at hfigher frequencfies than among pflanktonfic bacterfia, accefleratfing the rate at whfich fecafl mficrobfiota exchange genes wfith pathogens and facfiflfitatfing human-pathogen finteractfions (Aflm et afl., 2014; Oravcova et afl., 2017).

6.8. CAFOs and fimfigatfion water

Studfies of the effects of cattfle and swfine concentrated anfimafl feedfing operatfion (CAFO) wastewater on downstream envfironments were aflso prevaflent fin the flfiterature. The enterococcafl fisoflates from CAFO studfies fin the coflflectfion showed the hfighest resfistance prevaflence to tetracy-cflfines, whfich fis not surprfisfing gfiven that tetracycflfines make up nearfly 40% of afffl antfimficrobfiafls used fin anfimafl husbandry fin the US. (CDC, 2013). As many CAFOs contafin and treat thefir wastewater on-sfite, thefir dfirect fimpact on groundwater or downstream surface water (e.g., due to runoff or unfintentfionafl dfischarge) was a concern. The anfimafl products themseflves aflso represent a potentfiafl dfirect flfine of exposure from anfi-mafls to humans through the food chafin.

Sapkota et afl. (2007) sampfled upstream and downstream of a hfigh-densfity swfine operatfion fin the Mfid-Atflantfic regfion of the US and found hfigher MICs for cflfindamycfin and tetracycflfine amongst the *Enterococcus* spp. fin both downstream groundwater and surface water (Sapkota et afl., 2007). Sfimfiflarfly, Stfine et afl. (2007) sampfled the waste flagoons, surface waters, and weflfl water of a swfine CAFO fin the US. that had been admfinfisterfing tetracycflfine-contafinfing feed for over 20 years and found that 68% of aflfl enterococcfi were resfistant to tetracycflfines, and a totafl of 60 dfifferent specfies dfispflayed phenotypfic resfistance to tetra-cycflfines across the sfites (Stfine et afl., 2007). Further evfidence for the dfirect dfissemfinatfion of cflfinficaflfly-reflevant enterococcfi finto ambfient surface waters by CAFOs was documented by Jahne et afl. (2015) (Jahne et afl., 2015). They documented a cattfle CAFO and fits on-sfite wastewater treatment system comprfisfing of an finffifltratfion basfin wfith subsequent sequestratfion by a constructed wetfland. Enterococcfi that dfispflayed co-resfistance to vancomycfin, flfinezofldfid, and daptomycfin were common fin the wastewater and, durfing rafin events, the fincreased hydrauflfic floadfing on the finffifltratfion basfin and constructed wetfland resuflted fin the dfirect emfissfion of these organfisms finto downstream surface waters.

7. Concflusfion and recommendatfions

In thfis revfiew we recounted the flast 20 years of research assessfing antfibfiotfic-resfistant enterococcfi fin varfious water envfironments. Because *Enterococcus* has been shown to be a refffiabfle findficator of fecafl contam-finatfion of water bodfies, severafl natfionaflfly and finternatfionaflfly recog-nfized standard cuflture methods have been devefloped for thefir enumeratfion. Varfious *Enterococcus* spp. are both cflfinficaflfly-reflevant and survfive and persfist fin the envfironment. The studfies surveyed here flay the groundwork for consfiderfing *Enterococcus* spp. as a standardfized target for waterborne monfitorfing of antfibfiotfic resfistance.

Recentfly, the WHO put forward a standardfized, comprehensfive surveffiflflance program for One Heaflth-finspfired monfitorfing, fi.e., the

Trficycfle protocofl (WHO, 2021), whfich targets the Gram-negatfive ESBL *E. cofffi*. Here, *Enterococcus* spp., as Gram-posfitfive organfisms, represent a compeflflfing target to consfider as a compflement to such monfitorfing programs. *Enterococcus* spp. dfispflay resfistance to crfitficaflfly-fimportant antfibfiotfics that woufld not be captured by an *E. cofffi*-targeted monfitorfing program aflone. As observed fin thfis crfitficafl revfiew, *Enterococcus* spp. aflso dfispflay sensfitfive responses to anthropogenfic poflflutfion, fincfludfing hospfitafls and CAFOs, that are apparent fin thefir dfistfinct geographficafl occurrence patterns.

The recommended path forward for standardfizfing envfironmentafl antfibfiotfic-resfistant *Enterococcus* monfitorfing shoufld ensure the comparabfiflfity of monfitorfing pofints, methods empfloyed, and reportfing metrfics. Accessfibfiflfity and ease of appflficatfion are aflso fimportant consfideratfions. Ideaflfly, *Enterococcus* spp. monfitorfing for the purpose of antfibfiotfic resfistance survefiflflance coufld be fincorporated finto exfistfing monfitorfing programs, especfiaflfly consfiderfing the hfigh flevefl of exfistfing standardfizatfion and reguflatory requfirements. The exfistence of natfionaflfly and fintematfionaflfly recognfized standard cuflture methods fis of great vaflue fin ensurfing comparabfiflfity of the data gathered fin space and tfime, however, further standardfizatfion fis needed for the purpose of antfibfiotfic resfistance survefiflflance specfifficaflfly. The concflusfions and recommendatfions based on thfis crfitficafl revfiew are as foflflows:

- mEI fis a prfime candfidate for a standard seflectfive medfia, gfiven that fit yfiefds the hfighest seflectfivfity over mEnterococcus and Sflanetz-Bartfley and fis fintegrated finto exfistfing reguflatory recommendatfions fin the US. Stfiflf, the ffindfings here were encouragfing that studfies are generaflfly consfistent, even fif dfifferent medfia were empfloyed, but confffirmatfion of the genus fis crfitficafl because no *Enterococcus* medfia fis 100% seflectfive. The addfitfion of a seflectfive antfibfiotfic to the medfia can decrease specfifficfity by seflectfing for fintrfinsficaflfly-resfistant, non-target genera.
- In flfine wfith recommendatfions made fin the WHO Trficycfle protocofl, pflatfing envfironmentafl sampfles on the seflectfive medfia wfith and wfithout a prfimary seflectfive antfibfiotfic produces both a percent resfistance of the enterococcfi popuflatfion and a CFU/unfit voflume measurement (e.g., CFU/mfl). A CFU/mL measurement represents a unfiversaflfly comparabfle magnfitude of antfibfiotfic-resfistant bacterfia.
- Dependfing on the research questfion, vancomycfin and other antfibfiotfics used for prfimary seflectfion may be added to medfia at the breakpofint for fuflfl, fintermedfiate, or flow resfistance. The fuflfl resfistance breakpofint fis the most usefufl for cflfinficafl reflevance, but fin some envfironments, one may be finterested fin fintermedfiate resfistance. Where one expects bacterfia to be compromfised (finjured), use of flow-flevefl antfibfiotfic fin enrfichment cufltures may be usefufl for flowerfing detectfion flfimfits. The use of flow/fintermedfiate breakpofints may compflficate human-heaflth rfisk assessments due to the fisoflatfion of fintrfinsficaflfly-resfistant specfies that are common fin the envfironment unfless further testfing fis done.
- A deffined set of key antfibfiotfics afimed at treatfing VRE finfectfions coufld aflso be empfloyed for monfitorfing emergfing phenotypes and mufltfidrug resfistance. These fincflude ampficfiflflin, teficopflanfin, oxazoflidfinone-flfinezofid, daptomycfin, qufinuprfistfin/daflfoprfistfin, and tfigecycflfine. The emergence of mufltfidrug resfistant VRE that are co-resfistant to these 6 antfibfiotfics are of great concern.
- Antfimficrobfiafl sensfitfivfity testfing usfing dfisk dfiffusfion on Mueflfler-Hfinton agar or agar dfiflutfions fin Mueflfler-Hfinton broth shoufld be foflflowed accordfing to the most up to date CLSI gufideflfines.
- Specfiatfion and vfiruflence typfing of resfistant enterococcfi fis of finterest for rfisk assessment and flongfitudfinaflfly trackfing changfing genotypes and phenotypes across the genus. PCR-based approaches are appropfriate for flow-tech flabs, specfiffic flocfi sequencfing, and emergfing technoflogfies such as MALDI-TOF MS wffffl fincrease throughput wfithout sacrfifficfing accuracy.
- Key monfitorfing pofints to consfider for the dfissemfinatfion of resfistant enterococcfi are the hospfitafl-munficfipafl wastewater contfinuum and thefir recefivfing water bodfies, especfiaflfly where they fimpact recreatfionafl waters. Pharmaceutficafl wastewater and recycfled water are crfitficaflfly understudfied for resfistant *Enterococcus*. The surroundfing areas of CAFOs are of partficuflar concern after storm events.
- Whenever possfibfle, fisoflatfion of antfibfiotfic-resfistant coflonfies for WGS wffffl afid fin determfinfing the sequence type, vfiruflence genotype, pflasmfid type, acqufired AMR genes, and chromosomafl pofint mutatfions. Pubflfic sharfing of WGS data wffffl heflp to advance understandfing of the ecoflogy, epfidemfioflogy, and gflobafl transmfissfion of thfis fimportant pathogen.

Decflaratfion of Competfing Interest

The authors decflare the foflflowfing ffinancfiafl finterests/personafl reflatfionshfips whfich may be consfidered as potentfiafl competfing finterests: Amy Pruden and Vaflerfie J. Harwood reports ffinancfiafl support was provfided by Water Research Foundatfion.

Data avafiflabfiflfity

Data wffffl be made avafiflabfle on request.

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Suppflementary materfiafls

Suppflementary materfiafl assocfiated wfith thfis artficfle can be found, fin the onflfine versfion, at dofi:10.1016/j.wroa.2022.100161.

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