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Review article

Recent advances in environmental antibiotic resistance genes detection and research focus: From genes to ecosystems

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ABSTRACT

Antibiotic resistance genes (ARGs) persistence and potential harm have become more widely recognized in the environment due to its fast-paced research. However, the bibliometric review on the detection, research hotspot, and development trend of environmental ARGs has not been widely conducted. It is essential to provide a comprehensive overview of the last 30 years of research on environmental ARGs to clarify the changes in the research landscape and ascertain future prospects. This study presents a visualized analysis of data from the Web of Science to enhance our understanding of ARGs. The findings indicate that solid-phase extraction provides a reliable method for extracting ARG. Technological advancements in commercial kits and microfluidics have facilitated the efficacy of ARGs extraction with significantly reducing processing times. PCR and its derivatives, DNA sequencing, and multi-omics technology are the prevalent methodologies for ARGs detection, enabling the expansion of ARG research from individual strains to more intricate microbial communities in the environment. Furthermore, due to the development of combination, hybridization and mass spectrometer technologies, considerable advancements have been achieved in terms of sensitivity and accuracy as well as lowering the cost of ARGs detection. Currently, high-frequency terms such as "Antibiotic Resistance, Antibiotics, and Metagenomics" are the center of attention for study in this area. Prominent topics include the investigation of anthropogenic impacts on environmental resistance, as well as the dynamics of migration, dissemination, and adaptation of environmental ARGs, etc. The research on environmental ARGs has made significant advancements in the fields of "Microbiology" and "Biotechnology Applied Microbiology". Over the past decade, there has been a notable increase in the fields of "Environmental Sciences Ecology" and "Engineering" with a similar growth trend observed in "Water Resources". These three domains are expected to continue driving extensive study within the realm of environmental ARGs.

1. Introduction

Alexander Fleming's discovery of penicillin has profoundly revolutionized the efficacy of antibiotics in combating bacterial diseases, effectively eliminating human susceptibility to such infections. However, due to the extensive utilization of antibiotics in husbandry and aquaculture, there has been a notable rise in the prevalence of antibiotic-resistant strains in animals (Forsberg et al., 2012; Zhu et al., 2013). These strains, found in the guts of farm animals, are regarded as the primary contributors to the environmental antibiotic resistance genes (ARGs) (Crofts et al., 2017). Antibiotic-resistant bacteria (ARB) tend to spread in the water and soil ecosystems through surface runoff, promoting the probabilities of environmental bacteria acquiring resistance (Xu et al., 2022). ARGs found in strains, even in small amounts, facilitate the transmission and dissemination of ARGs by vertical gene transfer (VGT) and horizontal gene transfer (HGT), giving specific ARB a

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competitive edge (Forsberg et al., 2012; Zhu et al., 2013). Consequently, this significantly increases the probability of ARGs migration and propagation in different environmental media. Multiple ARGs (e.g., tetracycline, sulfonamides, beta-lactam, etc.) have been found in environmental sources such as wastewater, sediments, and soils (He et al., 2022; Luo et al., 2022; Zhang et al., 2021). Currently, the rise of ARGs and ARB has resulted in the diminished or even ineffective nature of antibiotics in combating bacterial infections, causing widespread concern among the broader population (Huang et al., 2018; Vila-Costa et al., 2017). Statistical calculations indicate that approximately 495 million deaths were linked to antimicrobial resistance (AMR) in 2019. It is estimated that up to 10 million individuals could die from AMR each year by 2050 (Church et al. 2021). The rise of AMR has become a paramount challenge, posing a grave threat to human health in the 21st century.

Although environmental bacteria have harbored ARGs for billions of years, their propagation has accelerated under elevated environmental selective pressure. Due to previous technical limitations, the understanding of ARB in the environment was insufficient, and the transfer of ARGs between environmental strains and human pathogens was underestimated. In the initial stage, DNA probes and PCR techniques offer novel perspectives for the observation and investigation of ARGs dissemination in natural habitats (Rosa et al., 1998; Sandt and Herson, 1991). By collecting diverse environmental samples and employing DNA probe or PCR screening, a plethora of varied sources of ARGs pollution have been identified in soil, water, and other common ecosystems (Riesenfeld et al., 2004; Smalla et al., 2000; Zhu, 2006). Due to breakthroughs and advancements in detection technologies, the investigation of environmental ARGs has undergone profound scientific scrutiny. Currently, we are experiencing an escalating prevalence of ARGs. This can be attributed to the use of antibiotics in both humans and animals, which spreads ARGs on mobile genetic elements (MGEs) and allows these MGEs to be exchanged across different bacteria, including pathogens (He et al., 2016; Zhu, 2006). To monitor the spread of environmental ARGs, various detection techniques and data resources have been developed to rapidly interpret information related to antibiotic resistance. These advanced technologies, such as high-throughput sequencing and multi-omics, enable rapid and accurate detection of the type and abundance of ARGs in environmental samples (Huang et al., 2012; Yang et al., 2017; Zheng et al., 2018). Additionally, scientists are actively engaged in conducting extensive data analysis and establishing comprehensive databases to better understand the evolution of ARGs in the environment and elucidate their potential threat to public health security (Kumar et al., 2022; Li et al., 2022). The objective of these endeavors is to create a reliable and all-encompassing repository for governments, healthcare institutions, and the agricultural sector, enabling them to make well-informed decisions regarding the mitigation of antibiotic resistance.

Recently, environmental ARGs research has exploded due to the rapid development of detection techniques. An extensive review of the literature on ARGs in the context of global environmental change over the past three decades is imperative. Gaining insights into its history is of immense significance for understanding the research status, research priorities, and future perspectives in this field. Bibliometrics combines statistical and mathematical techniques to track the development of a research area, enabling a thorough evaluation of the significance of research outcomes. In this study, we conducted a bibliometric analysis using VOSview to investigate the literature pertaining to ARGs in the environment (Fig. 1). Based on the published literature, a statistical analysis of publication categories, sources, and annual indicators was performed. Additionally, we provide a comprehensive examination of ARGs detection techniques in environmental sample data in the interim, covering sources as well as qualitative and quantitative approaches. Through keyword co-occurrence analysis, we elucidated the present trending subjects and predicted potential research focuses and future directions.

2. Data and methods

2.1. Data collection and sources

The data used in this study were retrieved from the Clarivate Analytics Web of Science (WOS), which is the online version of the Science Citation Index Expanded, including WOS Core Collection, MEDLINE®, Inspec®, Chinese Science Citation Database℠, KCI-Korean Journal Database, and SciELO Citation Index. We conducted searches in the topic field (title, abstract, keywords) using variations of the phrases “antibiotic resistance genes” or “antibiotics resistance genes” or “antibiotic resistance gene” or “antibiotic resistant genes” or “antibiotic resistant gene” or “antibiotics resistant genes” and “environment” or

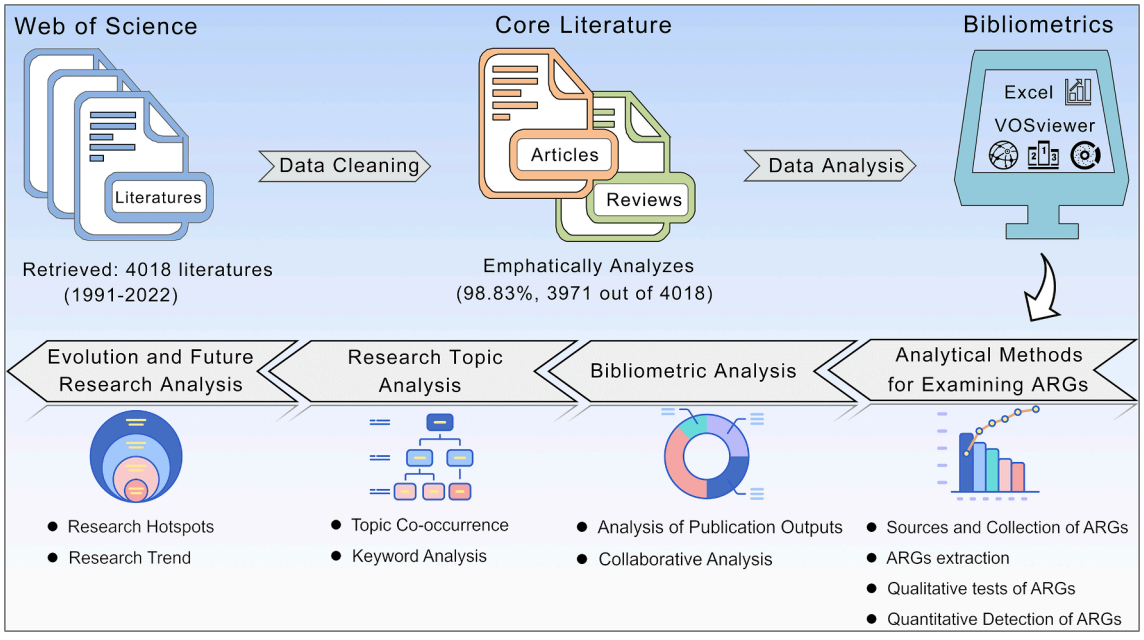


Fig. 1. Paper selection and flow chart of research framework.

"environmental" from January 1, 1991 up to December 31, 2022. To guarantee the accuracy of search outcomes, we meticulously screen documents by their title, abstract and keywords, exclude those that are irrelevant to the subject, and access their eligibility. The comprehensive publication data includes the abstract, keywords, source, reference, and other relevant information.

2.2. Data analyses

Initially, a rudimentary statistical analysis was conducted using the WOS database and Microsoft Excel to scrutinize fundamental publication metrics, including the total number of publications, annual publications, and national publications. The graphics were generated using the OriginPro 2021 software (the logistic model was employed to fit the R-values of the annual publication trends) and Figdraw online platforms. Bibliometric and visual analysis using VOSviewer (version 1.6.18, Leiden University) are employed to create a network map through co-occurrence and co-citation analysis, demonstrating (1) collaborations among different countries and (2) the co-occurrence of keywords and topic categories. The nodes in the network graph represent countries, keywords, and topics. The size of the nodes indicates the frequency or number of these items, while the lines between them indicate a connection or similarity. The node calculation in VOSviewer is conducted through the analysis of keywords within the text data and their co-occurrence associations. VOSviewer utilizes the "co-occurrence matrix method" to calculate the frequency of keyword co-occurrences in the text dataset. It processes and weighs this co-occurrence information to determine the similarity value between each pair of terms, which is then converted into the strength of the links. In addition to visualizing nodes and edge connections, VOSviewer offers a diverse range of features for conducting in-depth analysis of network structure. This involves employing clustering algorithms to categorize nodes based on their similar features or attributes and differentiate them using distinct colors or shapes.

3. Detection of ARGs in the environment

3.1. Sources and collection of environmental ARGs

Before humans began mass producing antibiotics to treat infectious diseases, numerous bacteria in the environment had already evolved and developed resistance to antibiotics. For instance, ARGs have been detected in samples collected from glaciers and permafrost, as well as in other habitats that are free from human contamination (Kashuba et al., 2017; Segawa et al., 2013). Due to the increased influence of human activities on environmental resistance, there has been a rapid evolution and dissemination of ARGs (Fig. 2). The wastewater (e.g., from medical facilities, livestock farms, and industries) discharged from human activities in aquatic environments contains a diverse range of hazardous substances and ARB (Hassoun-Kheir et al., 2020; Pereira et al., 2021; Zhu et al., 2021). ARB present in wastewater carry a diverse array of ARGs, which can be disseminated to the aquatic environment through various pathways. For example, it can be transferred directly to other microorganisms or released into water through MGEs or cell lysis (Pereira et al., 2021). In addition, the spread and transfer of ARGs vary across different types of water bodies, such as rivers, lakes, and oceans (Ren and Luo, 2022; Su et al., 2020). Due to the distinct features of various water bodies, such as their geographical location, climatic conditions, and pollution sources, these differences will directly affect the quantity and types of resistance strains they contain, thus further shaping the problem of drug resistance in the region. Applying manure that contains antibiotic residues to the soil, particularly on agricultural land, can result in soil contamination and facilitate the dissemination of ARGs (Zhu et al., 2013). Prolonged utilization of broad-spectrum antibiotics can readily induce bacterial resistance and facilitate the transmission of ARGs to other microorganisms in the soil (Xu et al., 2022). The risk of cross-border transmission of ARB and associated MGEs is increasing globally due to variations in manure treatment and regulatory standards at regional and national levels (Jacquiod et al., 2017).

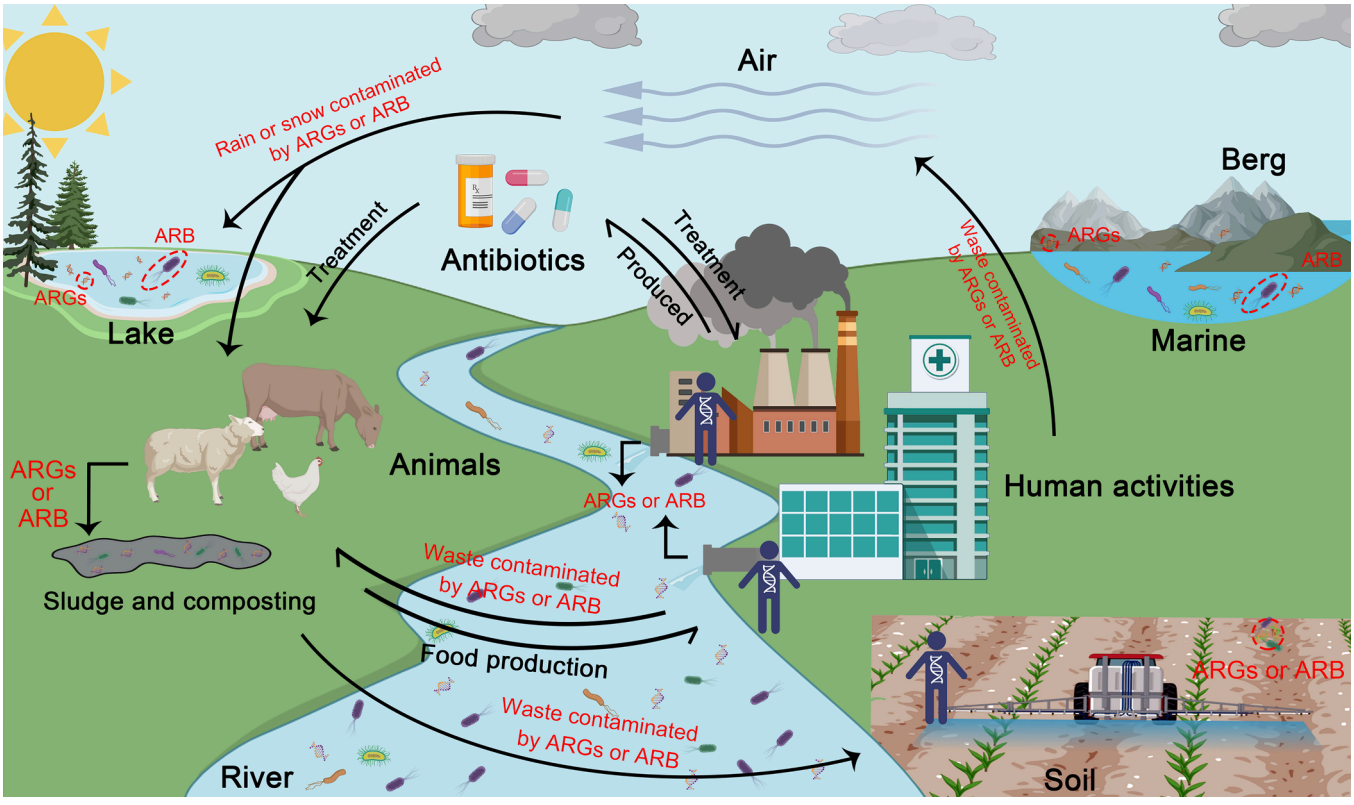


Fig. 2. Sources of ARGs in the environment.

This further exacerbates the worldwide problem of drug resistance and poses a challenge to public health security. Air pollution can also induce the development of bacterial resistance and facilitate the spread of ARGs in the atmosphere. Particulate matter or volatile organic compounds emitted from vehicle exhaust, factories, and waste incineration can act as carriers to introduce ARGs into the atmosphere and subsequently deposit them on the surface through rainfall (Ouyang et al., 2020). Furthermore, studies have shown that ARGs like *tet*(X) and *tet*(W) can be spread through the air, and have been detected in both indoor and outdoor settings, including animal habitats and human communities (Jin et al., 2022; Ling et al., 2013). These ARGs are carried by aerosols, including haze and dust, and are transmitted through inhalation or ingestion, such as penetration into the alveolar regions of humans or animals (Wang et al., 2022; Xie et al., 2018; Zhou et al., 2021).

To facilitate subsequent analysis, an optimal sample technique for ARGs should involve the collection of both intracellular and extracellular nucleic acids, while also guaranteeing the preservation of their integrity. The sampling time, locations, and methods for environmental ARGs are typically determined in advance. Regarding solid sample collection, most studies have documented the use of random or quarterly sampling methods using conventional or custom devices at designated study sites (e.g., soil, sludge, compost, and sediment) (Cao et al., 2020; Huang et al., 2018; Reichert et al., 2021). Water sample collection campaigns are typically designed based on the physicochemical properties of water. In particular, the ARGs in the water samples are isolated using a microfiltration membrane (e.g., 0.02 μm, 0.22 μm, or 0.45 μm pore size), then immediately collected in a sterile container for cryopreservation, and promptly transported to the laboratory for further analysis (Guo et al., 2018). Because gases can easily diffuse and do not have a stable state, most techniques for collecting ARGs from air and aerosols rely on the use of shock sampling methods or samplers. Bioaerosol particles aggregate via inertia on the surface of a solid or liquid medium. For instance, a wet concentrate sampler can be employed to collect aerosols into sterile phosphate buffers, while a high-volume total suspended particle sampler can gather air samples from urban, suburban, and rural areas (Gao et al., 2018; Ling et al., 2013; Tao et al., 2021). Furthermore, the microfiltration membrane is equally adept at capturing ARGs from gas samples. Specifically, as bioaerosol particles pass through different filter materials, the openings on the microfiltration membrane will either prevent or attract these particles based on electrical forces, capturing them on the filter. Prior to measuring the samples, it is necessary to perform a pre-separation and concentration process (Wang et al., 2021).

3.2. ARGs extraction

Nucleic acids primarily exist in both intracellular and extracellular states. Consequently, ARGs carried within nucleic acids also manifest as intracellular and extracellular entities, with the latter encompassing phage-borne ARGs and freely circulating ARGs (Barnes et al., 2014; Mao et al., 2014). Intracellular ARGs (iARGs) can be released into the extracellular environment either through cell lysis or active secretion by living cells, resulting in the presence of extracellular ARGs (eARGs). Similarly, eARGs present in the environment can also be converted into iARGs (Zou et al., 2022). The extraction of these ARGs involves four distinct approaches: Liquid–liquid extraction, solid-phase extraction, commercially available kits, and microfluidic technology (Fig. 3). Liquid-liquid extraction is a conventional method used to extract and separate compounds by disparity in solubility between two immiscible solutions (Liu et al., 2022). The procedure involves the utilization of diverse apparatus and equipment to facilitate the transfer of compounds between different liquid phases. An approach to isolating ARGs is by using CsCl gradient centrifugation, which is based on the previously described principle. Similarly, alkaline extraction, organic extraction, and salting-out methods all require cell disruption to generate lysates, followed by the precipitation of macromolecular compounds like cytoplasm and carbohydrates. Subsequently, ARGs are recovered through ethanol precipitation (Lu et al., 2022). The aforementioned extraction techniques are generally acknowledged and can be used to isolate ARGs from diverse environmental samples. Additionally, it should be noted that the CTAB extraction principle aligns with the previously discussed approach. However, it is imperative to freeze the sample using liquid nitrogen prior to grinding and subsequently suspending it in the CTAB buffer solution (Zhang et al., 2022a).

Liquid-liquid extraction has proven to be a robust technical foundation for extracting ARGs in the environmental. However, the utilization of liquid–liquid extraction necessitates a substantial quantity of reagents, making the entire process not only intricate and time-

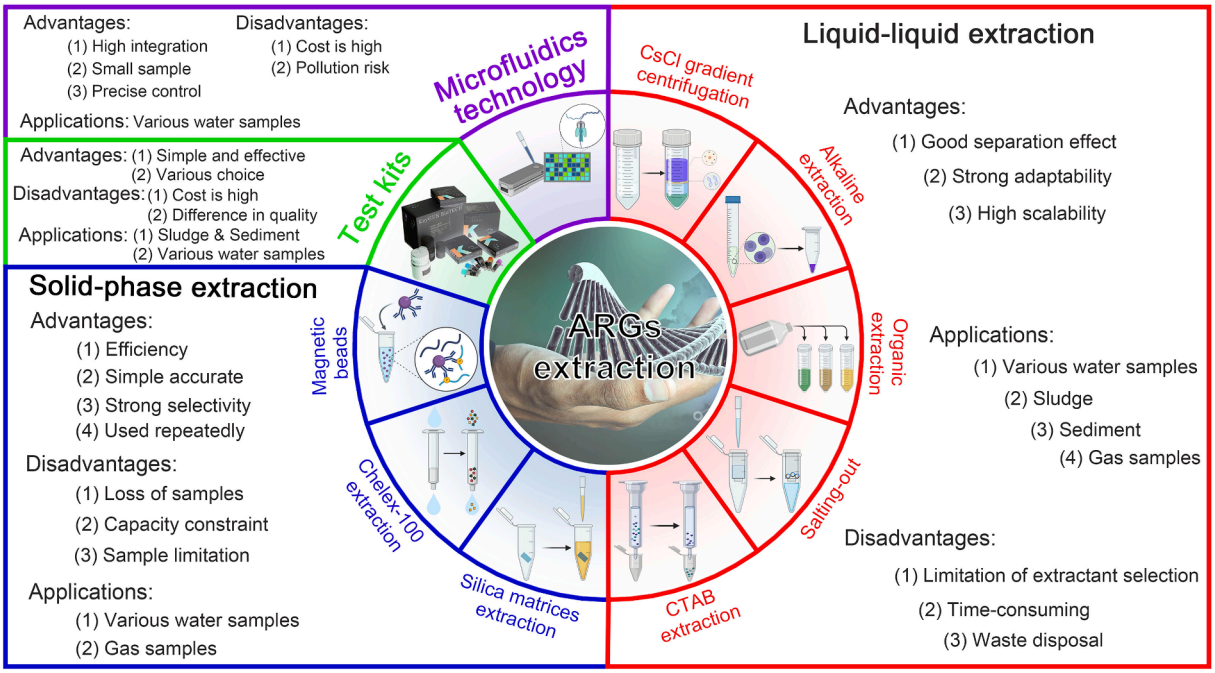
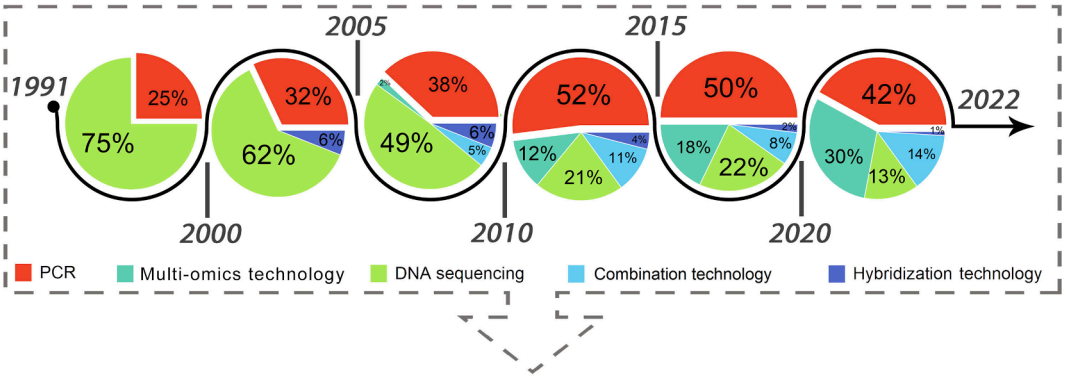
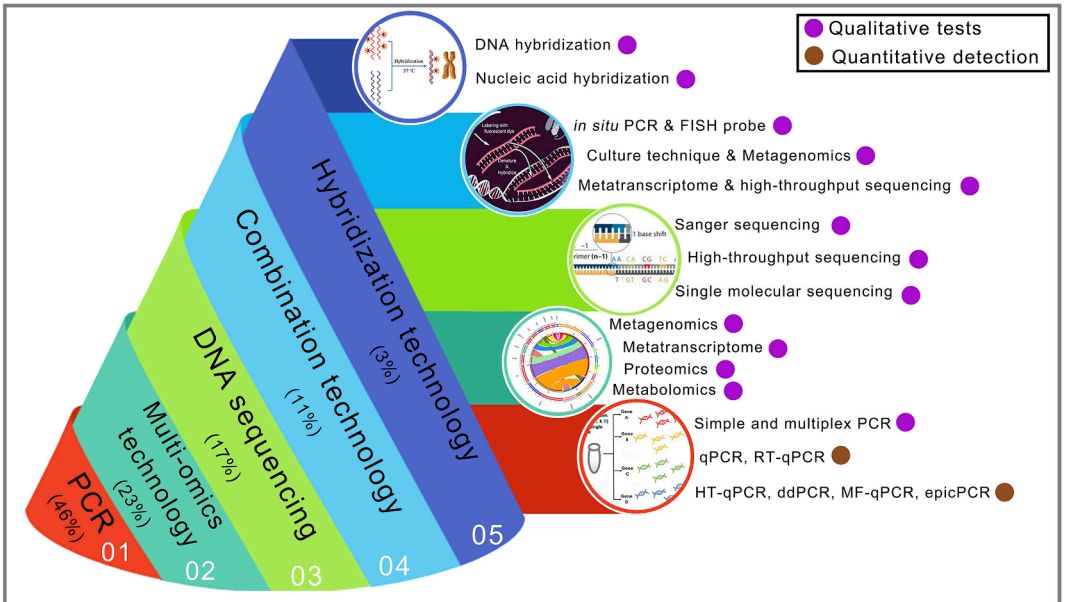


Fig. 3. The methodology for extracting ARGs.

consuming but also susceptible to potential environmental contamination. Consequently, solid-phase extraction has emerged as a viable alternative for liquid–liquid extraction, effectively addressing its inherent drawbacks. The principle of solid-phase extraction involves the adsorption of ARGs onto a stationary phase and subsequently extracting them from the mixture (Mohammadi et al., 2022). Silica matrices extraction is the most prevalent method, where silica acts as an adsorbent that binds to the ARGs in the lysate, thereby isolating them from other substances in the solution. The extraction efficiency of this method is reported to be 40 % of that achieved by traditional methods (Liu et al., 2020). Unlike extraction techniques using silica matrices, a cation exchange resin of Chelex-100 exhibits high bonding strength and facilitates the extraction of ARGs from various samples (Schwendener et al., 2020). This method not only has a minimal expense but also avoids the use of detrimental chemicals. Additionally, magnetic-based methodologies are employed for the extraction of ARGs (Fu et al., 2021). This approach utilizes functionalized magnetic beads or particles to form hydrogen bonds with nucleotides and then release them in a reversible





Technology	Advantage	Disadvantage
Simple PCR	Universal; Simple and effective	Unable to quantify
Multiplex PCR		
qPCR	High sensitivity and specificity; Rapid and efficient	Primer design difficult; High costs
RT-qPCR		Complex operation; Vulnerable to pollution
HT-qPCR	High sensitivity and specificity; Rapid and efficient; High automation	High costs; Vulnerable to pollution
ddPCR	High sensitivity and accuracy; Rapid and efficient; High reliability	High costs; Complex operation; Sample limitation
MF-qPCR	Selective and flexible; Small sample; High sensitivity and fast	High costs; Complex operation; High technical requirement
epicPCR	High sensitivity and specificity; Small sample; Rapid and efficient	Complex operation; Vulnerable to pollution; Primer design difficult
Metagenomics	Comprehensive; Diversity; High-throughput, High sensitivity; High resolution	Data processing complex; High costs; High technical requirement
Metatranscriptome		
Proteomics		
Metabolomics		
Sanger sequencing	High accuracy; Long read length	High costs; Low flux
High-throughput sequencing	High-throughput; Low costs; Rapid; Automation	Short read length
Single molecular sequencing	High-throughput; Rapid; Automation	High costs
Combination technology	High sensitivity and specificity; Rapid and efficient; Flexible; Low costs	Complex operation; High technical requirement
Hybridization technology		

Fig. 4. Analytical techniques for ARGs.

manner, thereby providing enhanced binding sites for ARGs. Consequently, this method offers a more streamlined and efficient means of extraction.

Advancements in technology have led to the development of various commercial kits that enhance the extraction of ARGs from environmental samples, resulting in a significant reduction in the time required for this process. These commercial kits are well-suited for high-throughput processing of numerous samples within a restricted timeframe (Miłobedzka et al., 2022). Similarly, the retrieval of ARGs using microfluidic technology has been successfully achieved, which is particularly noteworthy (Jin et al., 2020). This approach not only requires a minimal sample size but also effectively reduces expensive reagent consumption and processing time (Sun et al., 2021).

3.3. Qualitative analysis of ARGs

After reviewing the advancements in ARGs analytical techniques over the past 30 years, five categories are presented in Fig. 4. Conventional methods include PCR (simple and multiplex PCR), DNA sequencing, and multi-omics technologies (including metagenomics, metatranscriptome, proteomics, and metabolomics). Additionally, there are numerous approaches based on hybridization and combination technologies. Among these techniques, PCR is the predominant and most efficient method. A single PCR can detect known ARGs without the requirement of isolating and culturing viruses or bacteria. Multiplex PCR enables the simultaneous characterization of multiple gene targets and exhibits greater resilience compared to single PCR. PCR-based technologies are widely used to determine the occurrence or spread of well-recognized ARGs (tetracycline, fluoroquinolone, sulfadiazine, beta-lactam, quinolone, and erythromycin) in the environment. These genes can originate from specific sources, such as sewage treatment plants and pharmaceutical factories, or from more diffuse sources like livestock and contaminated soil/water (Ergie et al., 2019; Gunathilaka et al., 2017; Liu et al., 2019). This method has been broadly applied to monitor ARGs in sewage and manure in many countries, such as China, the United States, and Germany (Li et al., 2020; Opazo-Capurro et al., 2019; Parnanen et al., 2016).

DNA sequencing is essential for gene analysis. Over the past three decades, there has been significant development in DNA sequencing technology, enabling us to gain insights into the genetic composition of animals, plants, and microorganisms (Allwood et al., 2020; Krehenwinkel et al., 2019; Shen et al., 2021). Specifically, DNA sequencing has been employed to assess ARGs. For instance, Sanger sequencing (the first generation) has demonstrated its capability of detecting variations at the strain level and quantifying ARGs abundance (Li et al., 2013). However, despite its long read length and high accuracy, Sanger sequencing has shortcomings such as high cost and low flux, which have led to the application of high-throughput sequencing technology (the second generation). This technology is based on the development of PCR and gene chips for DNA sequencing, which can provide a fast, accurate, and cost-effective method of investigation (Zheng et al., 2018). Previous studies have demonstrated that high-throughput sequencing technology is useful for quickly and precisely analyzing ARGs in various environmental settings, particularly in wastewater systems encompassing hospitals, livestock farms, and residential buildings (Huang et al., 2012; Lu et al., 2010). The progress of technology has led to the development of DNA sequencing, which has evolved from the first and second generations to the emergence of third generation sequencing technology (single molecular sequencing). Third generation sequencing overcomes the restrictions of short read length while maintaining the speed and throughput advantages of the second generation. Recent studies have also shown that single molecular sequencing will be used for ARGs detection (An et al., 2022; Bratulic et al., 2015; Gomez-Simmonds et al., 2021).

PCR and DNA sequencing are proficient at identifying homologs of known ARGs, but they are incapable of detecting unknown or distantly related ARGs. Utilizing multi-omics technologies in such scenarios effectively bridges knowledge gaps and significantly enhances our comprehension of ARGs. Metagenomics employs both sequence-based and function-based approaches (Guo et al., 2017; Yang et al., 2017). Sequence-based metagenomics involves direct sequencing of all DNA isolated from environmental samples (Chi et al., 2020). Function-based metagenomics usually entails creating metagenomics libraries, and the gene libraries associated with ARGs have experienced significant expansion (Kumar et al., 2022; Li et al., 2022). Combined with machine learning classification, the application of metagenomics ARGs profiles to explicitly assign source contributions has been feasible. Through the identification of indicative ARGs and reconstruction of the host genome of ARGs, function-based metagenomics will enhance the reliability and accuracy of source tracking (Li et al., 2020). However, metagenomics is unable to differentiate between genomic DNA derived from living cells and the actual expression of predicted genes. As a high-throughput technique, the metatranscriptome has been developed for comprehensive analysis of gene expression, enabling the exploration of the entire transcriptome in an organism and its application in ARGs detection (Loh John et al., 2018; Sharma et al., 2010). Metatranscriptome allows for the simultaneous study of gene expression for a large number of genes in certain settings to explore ARGs. For instance, it is used to study the effects of short-term antibiotic exposure on microbial gene expression, physiology, and structure (Korry et al., 2020). Additionally, proteomics and metabolomics are crucial for the detection and study of ARGs. These technologies can assist in identifying protein biomarkers and metabolites linked to antibiotic resistance, thereby investigating the mechanisms of transfer and transmission of ARGs. Through the use of proteomics analysis, researchers can reveal the potential ecological risks of silver and silver nanoparticles in the environment to the spread of ARGs (Lu et al., 2020). Similarly, metabolomics analysis can provide much useful information for understanding the effects of extracellular polymeric substance production on the disinfection of biofilms in drinking water pipelines, especially for controlling ARGs (Wang et al., 2024). In summary, multi-omics technologies provide a crucial scientific basis for exploring the transmission, dissemination, and elimination of ARGs.

While multi-omics technologies have the advantage of not being confined to known gene sequences, the identification of specific ARGs may still be challenging due to their limited expression. Therefore, hybridization-based and combinatorial detection techniques have been developed in recent years, for instance, the widespread use of DNA hybridization and nucleic acid hybridization techniques (southern blot and northern blot) (Liang et al., 2019; Wan et al., 2023). Amirmozafari et al. used southern blot technology to elucidate the antibiotic resistance mechanisms of the *tox*A and *exo*S genes (Amirmozafari et al., 2016). Moreover, combination-based techniques can be flexibly and unrestrictedly combined, which overcomes the technical limitations of single detection methods and results in reduced detection costs and improved sensitivity, accuracy, and throughput. Various techniques have been published for detecting ARGs, such as *in situ* PCR & FISH probes, culture techniques & metagenomics, and metatranscriptome & high-throughput sequencing (Conwell et al., 2021; Jia et al., 2021; Zhang et al., 2022b).

3.4. Quantitative detection of ARGs

Conventional PCR methods, although sensitive and time-saving, are limited to qualitative detection and lack quantitative expression. Based on the application of extended PCR, quantitative PCR (qPCR), real-time qPCR (RT-qPCR), and high-throughput qPCR (HT-qPCR) are the most frequently used quantitative detection techniques for ARGs (Fig. 4). qPCR is a powerful molecular detection tool with higher sensitivity and specificity than conventional PCR. Studies have proved that qPCR can quantitatively analyze the target genes of major ARGs (e.g., *tet*A, *tet*B, *tet*M, *erm*B, *sul*1, *amp*C, and *qnr*S) in municipal sewage and quantify the abundance of ARGs in sediment samples (Al Salah et al., 2020; Nolvak

et al., 2016). RT-qPCR monitors the entire amplification process by introducing fluorescence-binding dyes or fluorescent-labeled probes into the PCR reaction system. It then quantitatively analyzes the unknown template by measuring the real-time accumulation of fluorescence signals (Hardwick et al., 2008; Navarro et al., 2015). For instance, RT-qPCR based on SYBR Green fluorochrome and TaqMan probes has been widely used for quantifying the absolute levels of ARGs in soil, sewage, and air (Borjesson et al., 2009; Burch et al., 2013; McKinney et al., 2018; Zhou et al., 2018). However, only a restricted number of ARGs can be analyzed by qPCR or RT-qPCR (Loftie-Eaton et al., 2014). This technique is significantly limited when analyzing a substantial quantity of samples. HT-qPCR provides a rapid and convenient method to concurrently detect hundreds of ARGs (Franklin et al., 2021; Liu et al., 2019).

Recently, several novel PCR techniques have emerged, including droplet digital PCR (ddPCR), microfluidic qPCR (MF-qPCR), and emulsion-paired separation-tandem PCR (epicPCR). The ddPCR method uses segmentation and Poisson statistics to generate thousands of individual droplets in a water–oil emulsion, enabling absolute quantification of nucleic acids without the need for external calibrators (Cave et al., 2016). ddPCR enables absolute quantification and tolerance to inhibitors, and it is considered the preferred method to overcome the limitations of RT-qPCR (Yao et al., 2022). It has been successfully used to quantitatively detect the distribution and spread of ARGs in soil, fertilizer, municipal waste, rivers, and aerosol environments (Cave et al., 2016; Tao et al., 2022; Wang et al., 2018). Furthermore, a high-density small-volume qPCR platform based on MF-qPCR has been developed (Ishii et al., 2014). Several investigations have demonstrated that MF-qPCR is both selective and flexible in its ability to identify ARGs (Bueno et al., 2019; Sandberg et al., 2018). Similarly, epicPCR is considered to break through the technical bottleneck in current research on ARGs (Jenniet al. 2018). epicPCR facilitates the connection of functional and phylogenetic genes within individual droplets, allowing for the simultaneous sequencing of millions of droplets, offering the advantages of high specificity and throughput (Sakowski et al., 2021).

4. Research status on environmental ARGs

Based on the WOS Core Collection, the primary sources of literature were derived from five databases, with the Science Citation Index Expanded ranking at the top (Fig. S1a). The predominant type was research articles (n = 3,406; 84.77 %), with reviews being the second most prevalent (n = 565; 14.06 %) (Fig. S1b). From 1991 to 2022, a total of 4,018 publications have been dedicated to studying environmental ARGs, and the annual publication volume increased exponentially (R^2 = 0.97) (Fig. 5a). Between 1991 and 2008, the cumulative number of publications in this field was little over 100, with an average of less than 10 per year. This indicates that scholars had a relatively low level of interest in the field throughout that period. Following 2009, there was a steady rise in growth, with a notable surge in 2017, when the number of publications exceeded 200 and the cumulative number of articles increased rapidly. Between 2018 and 2022, a total of 2,975 studies were published, accounting for approximately 74.0 % of the total research output, indicating that the discipline is experiencing rapid development. Furthermore, statistical results show that publications were contributed from 108 countries. We have selected the 20 most productive contributions, as shown in Table S1. China (n = 1732; 43.6 %) has the largest number of published papers, followed by the United States (n = 726; 18.3 %) and the United Kingdom (n = 206; 5.2 %). These three countries attach great importance to research in this field. It is worth noting that China, the United States, and the United Kingdom possess the greatest overall connection strength, with values of 652, 541 and 361, respectively (Fig. 5b and Table S2). By aggregating the number of publications in these three countries, it becomes evident that these countries have substantial global impact in the realm of environmental ARGs research and engage in frequent scholarly collaborations with other nations.

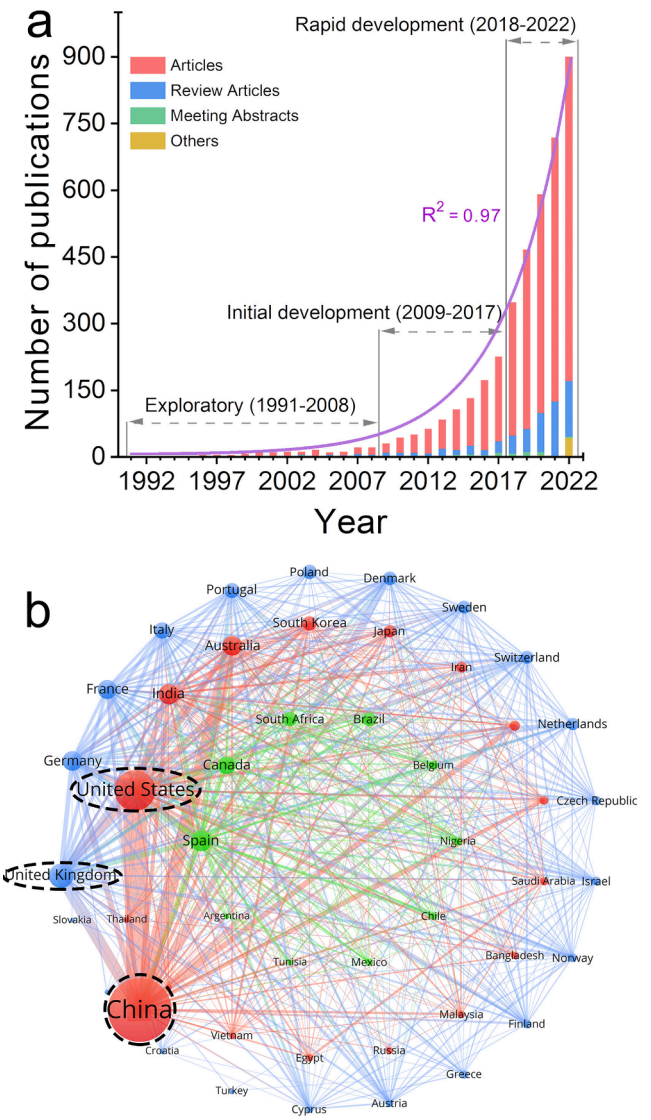


Fig. 5. Analysis of output publications on ARGs in the environment. (a) Annual trends in the number of publications; (b) Cooperation networks analysis of country.

Simultaneously, developed countries play a significant role in building a body of research knowledge in this field, as 8 out of the top 10 producing countries are developed countries.

5. Research focus of ARGs in the environment

An examination of keyword co-occurrence enables rapid identification of research hotspots within a certain topic. To further investigate the significant research pertaining to environmental ARGs, we utilized VOSview to conduct comprehensive statistical analysis of relevant keywords. Besides “ARGs and Environment”, “Antibiotic Resistance, Antibiotic, and Metagenome” are the three most frequently used terms (Fig. 6 & Table S3). Additional notable keywords consist of “Horizontal Gene Transfer, Removal Efficiency, Wastewater, Sewage Sludge, and qPCR”. These top-ranked keywords represent the research focuses of ARGs in the environment. The largest cluster (red): Studies within this cluster primarily involve the impact of human activities on environmental resistance, as well as the migration, dissemination, and adaptation of ARGs in the environment (Chowdhury et al., 2022; Forsberg et al., 2012; Wang et al., 2023). Cluster (purple): It focuses on antibiotics, ARB, ARGs in the environment and their impact on human and

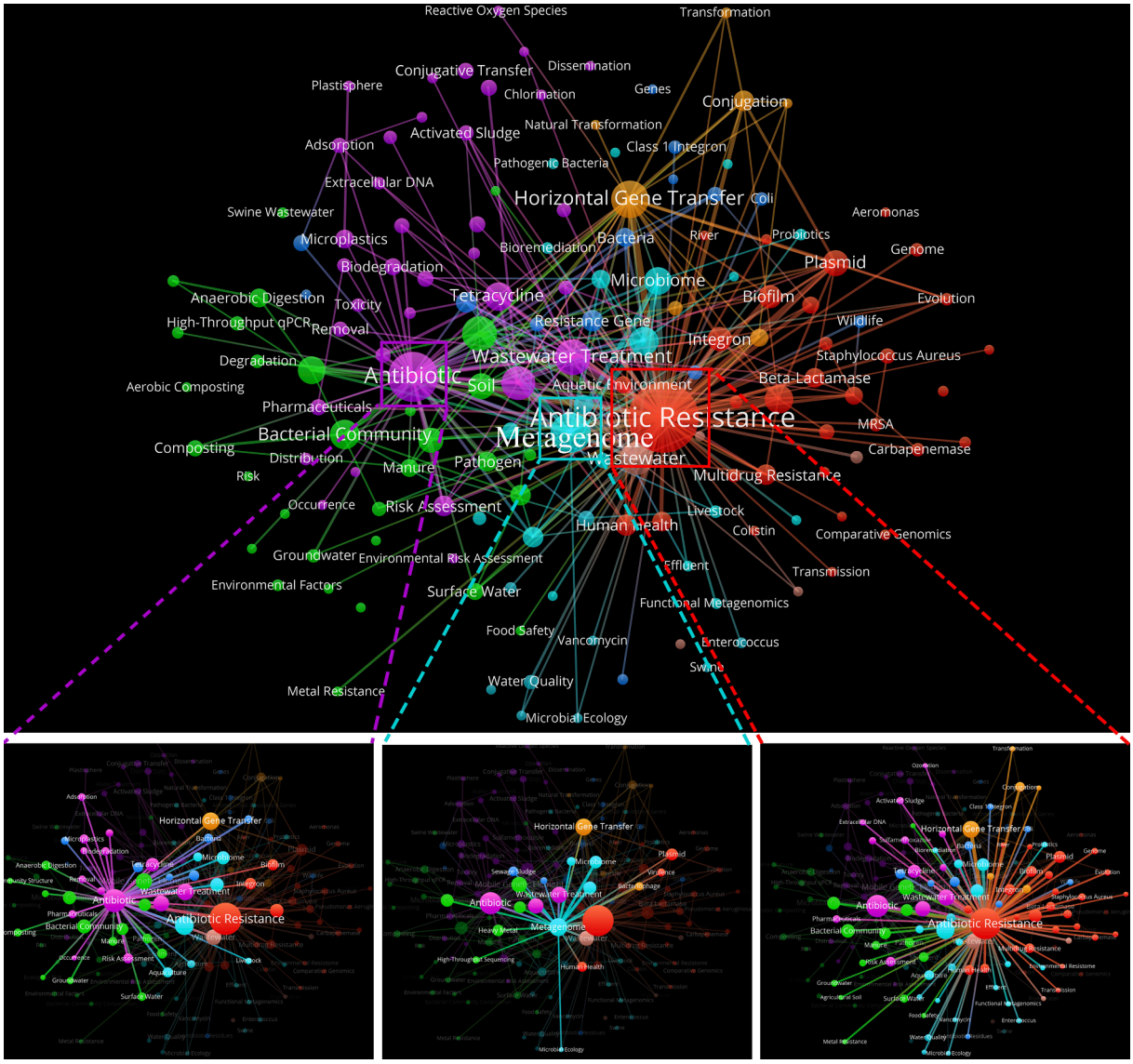


Fig. 6. Visual analysis of the co-occurrence network of keywords related to ARGs in the environment.

ecological health; the origin and dissemination of ARGs; the fate and dispersion mechanisms of ARGs across various soil types (Jin et al., 2022; Ling et al., 2013). Cluster (cyan): The research focuses on the impact of antibiotics on the structure and function of microbiota; the diversity, abundance, and persistence of ARGs in different types of compost; and the relationship between the abundance of ARGs and bacterial community diversity (Crofts et al., 2017; Xu et al., 2022; Zhang et al., 2021; Zhu et al., 2013). Additionally, other significant areas of interest include diverse events pertaining to ARGs transfer (VGT or HGT) and genetic diversity (Hu et al., 2022). This includes studying the occurrence and elimination of ARGs (e.g., MGEs) in wastewater treatment processes and agricultural land applications (Che et al., 2019; Wei et al., 2021; Zainab et al., 2020); molecular techniques such as specific PCR, multi-omics technologies, DNA sequencing, and other methods are used to detect environmental ARGs (Burch et al., 2013; Zhang et al., 2022b; Zhou et al., 2018).

The evolution of hot topics in environmental ARGs research remains unclear, even if these high-frequency keywords are labeled as the current focus. We concentrate on three crucial stages (Fig. 5a) in the development of environmental ARGs research and utilize VOSview for co-occurrence analysis. This sheds light on the evolution of keywords, hence disclosing shifts in hotspots for environmental ARGs research. During the exploration period, the high-frequency keywords “Antibiotic Resistance, Horizontal Gene Transfer, and Gene Transfer” emerged prominently (Fig. S2a). This implies that the transfer events and genetic diversity of ARGs have raised significant worries, along with the potential transmission risks from genetically modified crops or organisms to microorganisms (Beaber et al., 2004; Pruden et al., 2006; Zhu, 2006). During this period, DNA sequencing and PCR techniques emerged as pivotal tools for investigating environmental ARGs. DNA sequencing enables researchers to accurately analyze and interpret ARGs in diverse microorganisms, thereby unveiling their transmission pathways (Riesenfeld et al., 2004). PCR technology enables rapid identification of specific ARGs in microorganisms, facilitating a more comprehensive assessment of potential environmental risks (Chee-Sanford et al., 2001). During the developmental phase, the term “Antibiotic Resistance” was consistently the most often used keyword, along with “Antibiotics, Wastewater, and qPCR” (Fig. S2b). Multiple studies have demonstrated that the administration of antibiotics can facilitate the development of antibiotic resistance and the proliferation of ARGs, particularly within wastewater treatment systems (Hassoun-Kheir et al., 2020). Given the profound implications of antibiotic resistance on human and ecological health, a comprehensive understanding of the evolution of antibiotic resistance requires investigation of natural environments and clinical

ecosystems. Consequently, the rapid advancement of analytical techniques for ARGs, such as PCR and its derivatives, DNA sequencing, along with the widespread adoption of multi-omics technologies, has enhanced our knowledge of environmental ARGs (Bratulic et al., 2015; Liu et al., 2019; Lu et al., 2020). During the rapid development period, the keywords "Antibiotic Resistance, Antibiotics, and qPCR" have once again been the most frequently used. Furthermore, recently emerged terms such as "Antibiotic Resistant Bacteria" and "Microbiome" have become more prevalent (Fig. S2c). The emergence of new techniques for detecting ARGs has significantly increased and expanded public interest and research scope on ARGs in the environment. Research hotspots include the fate of ARGs in wastewater treatment plants worldwide; the diversity, abundance, and propagation mechanisms of ARGs in environmental media; the migration factors of ARGs between host bacteria and environmental media; the occurrence, distribution, and origin tracking of ARGs, etc. (Michael et al., 2013; Pruden et al., 2006; Qiao et al., 2018).

In summary, future research ought to be more in-depth and comprehensive in light of the aforementioned topics. Primarily, an efficient and accurate standardization scheme for the analysis of ARGs in various environmental media must be established, the protocol should include steps for sampling, purification, identification, and quantification. Therefore, it is imperative to update and evaluate methods for the detection of ARGs to establish an efficient monitoring system capable of

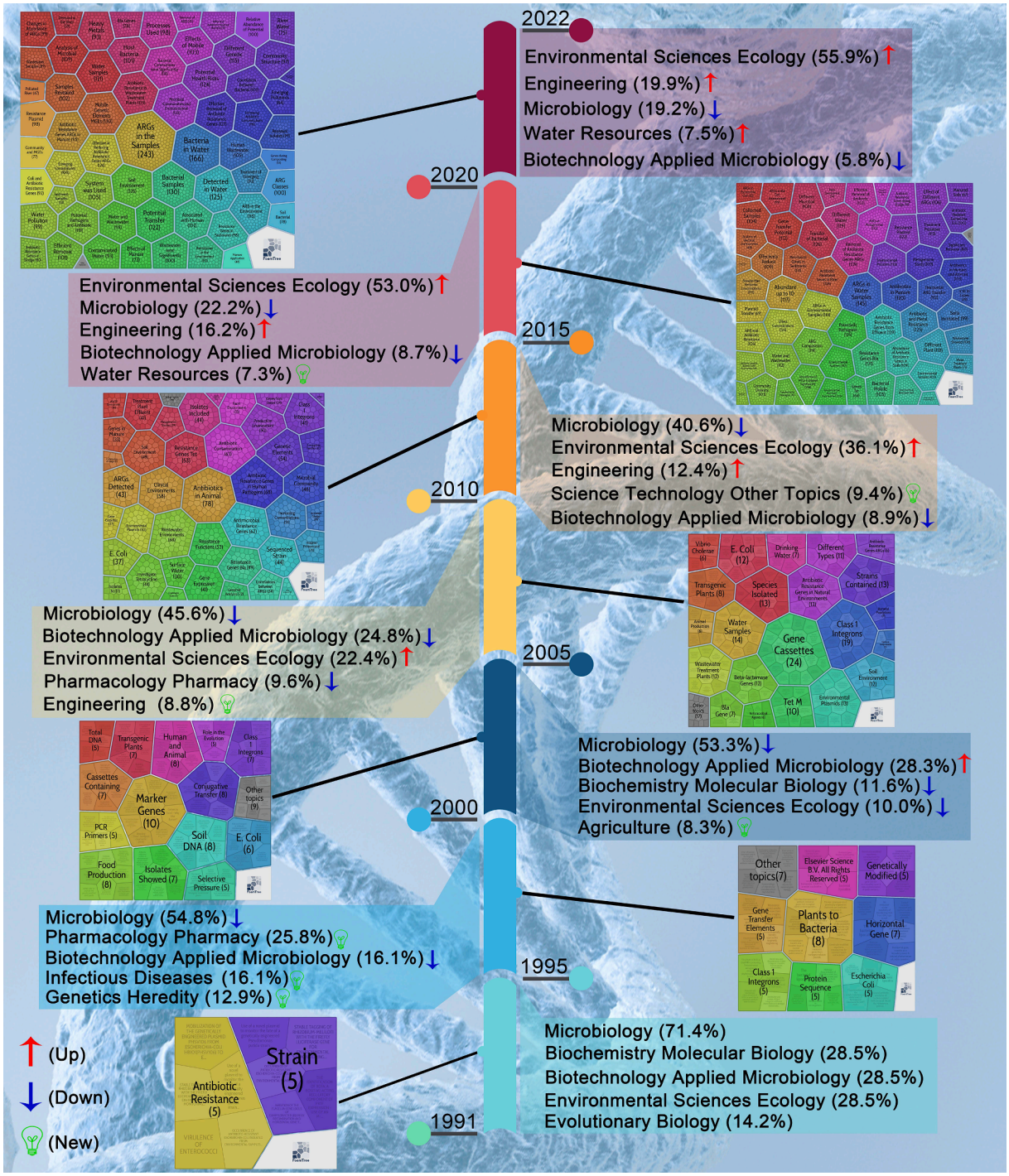


Fig. 7. Research area succession of ARGs in the environment.

assessing the distribution of ARGs. Simultaneously, it is also necessary to study the species differences and source characteristics of ARGs between different regions, pay attention to the transformation and migration rules of ARGs among various environmental media, and analyze the mechanism using modern molecular technology to provide a scientific basis for establishing an efficient monitoring system. Furthermore, in order to effectively control the dissemination of ARGs, safeguard human health, and preserve ecological equilibrium, it is crucial to expedite the development of novel and highly efficient technologies capable of eradicating ARGs from the environment while preemptively mitigating their potential for proliferation. Ultimately, addressing the issue of environmental ARGs necessitates international collaboration and enhanced information exchange. This can be achieved through the establishment of a platform for sharing experiences and fostering technological innovation, as well as the development of a unified global standard and policy framework.

6. Development trends of studied on environmental ARGs

Analyzing the evolution of environmental ARGs can help researchers systematically understand the trajectory and trends of studies. In this study, we present a comprehensive account of the evolutionary trajectory of research on environmental ARGs over the past three decades and systematically synthesize the research areas published every five years based on the "Analyze Results" provided by the WOS platform (Fig. 7).

From 1991 to 1995, research on environmental ARGs was mainly focused on "Microbiology", accounting for 71.4 % of all research areas. During this period, the academic community has shown minimal interest in this topic because of the delay in detection technologies. Untreated sewage has been identified as the primary reservoir of ARB through DNA sequencing. However, there has been a lack of inquiry into whether bacteria harboring ARGs pose a potential environmental risk (Baldini and Cabezali, 1991; Jett et al., 1994). Between 1995 and 2000, in addition to "Microbiology", several new domains such as "Pharmacology Pharmacy, Infectious Diseases, and Genetics Heredity" emerged. Evidence suggests that the demand for and utilization of antibiotics contribute to elevated antibiotic concentrations in natural ecosystems, thereby amplifying the resistance of pathogenic bacteria in the environment (Falkinham, 1996; Gouka et al., 1999; Iamtham and Day, 2000). The exploitation of PCR technology has provided further evidence to substantiate the dissemination of ARB in various environments, either within a single environment or across different ecosystems. Starting in 2000, the field of "Microbiology" continued to be the main focus, while "Agriculture" has emerged as a new area of environmental ARGs study. During this period, numerous studies have demonstrated that the use of antibiotics in animal husbandry facilitates the emergence and proliferation of antibiotic resistance in agricultural settings. Especially, feces have become a concerned reservoir for ARB and antibiotics. Consequently, their application in agricultural soils can significantly enhance the dissemination and migration of ARGs and ARB (Andersen et al., 2001; Roe and Pillai, 2003). From 2005 onwards, the percentage of research publications focused on "Microbiology" fell below 50 % for the first time, while that of "Environmental Sciences Ecology" increased significantly. Similarly, the emphasis on the "Microbiology" area experienced a continuous decline between 2010 and 2015; however, it still maintained its prominent position. This trend can be attributed to the emergence and advancement of other scientific areas. For instance, "Environmental Science Ecology" as well as "Engineering" have progressively garnered attention and emerged as research focal points. The emphasis on "Science Technology Other Topics" has also witnessed a surge, indicating a keen interest in diverse areas of study. During this period, advanced molecular biology techniques such as PCR, DNA sequencing, and multi-omics have provided efficient and reliable technical support for the research of ARGs. However, the field of "Microbiology" dropped to second place starting in 2015 and was replaced by "Environmental Science Ecology" as the new primary field. In addition to "Water Resources", areas such as "Engineering" and "Biotechnology Applied Microbiology" have also garnered widespread attention and shown rapid development. This change may reflect an increasing concern regarding the presence of ARGs in water resources and their integration into relevant research. After 2020, disciplines such as "Environmental Science Ecology, Engineering, and Water Resources" will thrive and emerge as cutting-edge areas. Each area will delve into novel subjects and conduct in-depth research.

Overall, in the past 30 years, research on environmental ARGs has evolved from a singular to a multifaceted aspect. With consistent progress in science and technology and ongoing innovation in research methods, our comprehension of ARGs in the environment is gradually expanding. The field of "Microbiology" has not vanished from our perspective, and it remains a fundamental and important part of the study of environmental ARGs. However, in recent decades, the representation of "Microbiology" within the broader research area has gradually diminished, primarily attributed to advancements in other interconnected areas. With growing public concern for environmental issues and advancements in engineering technology, the fields of "Environmental Sciences Ecology, Engineering, and Water Resources" have started to thrive. These fields are specifically focused on addressing intricate environmental issues caused by ARGs through incorporating expertise from natural science, social science, and engineering. In the future, these fields will continue to drive a more comprehensive and in-depth understanding of the spread of ARGs in nature and their impact on human health and ecosystems.

7. Conclusions

In this study, VOSviewer is used to visualized analysis the literature on environmental ARGs in the core collection of WOS. Based on this foundation, we present a comprehensive overview of the advancements in analysis technology for ARGs over the past three decades. Liquid-liquid extraction is an important method for extracting ARGs, but solid-phase extraction can reduce solvent consumption and the risk of environmental pollution. Simultaneously, the advancement of commercial kits and microfluidic technology has facilitated efficient extraction of ARGs while reducing processing time. The utilization of PCR (including extension techniques), DNA sequencing, multi-omics, combination and hybridization techniques has significantly facilitated the qualitative and quantitative detection of ARGs. The current research primarily focuses on investigating the impact of human activities on environmental resistance; migration, spread, and evolution of ARGs in the environment. Furthermore, research on environmental ARGs has evolved from a singular focus to encompass multiple areas such as "Microbiology, Biotechnology Applied Microbiology, and Science Technology Other Topics", etc. The main fields of study have shifted towards "Environmental Sciences Ecology" and "Engineering" in recent years, with increased attention being given to "Water Resources".

Although significant progress has been made in the study of environmental ARGs, further exploration is still required to address unknown issues. Through a bibliometric review, we propose potential research directions for the future:

(1) The source, abundance, species, and migration of resistance genes in soil, water, and atmosphere necessitate further investigation to provide scientific guidance for future ARGs management.
(2) Develop and promote a standardized protocol for the analysis of ARGs, encompassing sampling, purification, identification, and quantification across various environmental medias. Therefore, it is imperative to update and evaluate the analytical methodologies employed for ARGs detection in order to establish an efficient surveillance system.
(3) In order to effectively control and limit the spread of ARGs, safeguard human health, and maintain ecological balance, there

is an urgent need for novel and efficient technologies that can remove ARGs present in the environment.

8. Limitations

The insights gained in this study notwithstanding, there are still certain limitations pertaining to the methods employed for literature retrieval and analysis. First, the data collected were limited to the relevant keywords of "ARGs and environment" (including synonyms) used in the title, abstract, and keywords. Therefore, paper that do not contain these specific keywords are excluded from the dataset. Secondly, this paper solely utilizes the database of WOS while disregarding other databases. For instance, numerous Chinese researchers actively engage in the investigation of environmental ARGs and tend to publish their findings in domestic journals written in Chinese. The absence of these research results in the WOS database has impact on the bibliometric outcomes.

CRediT authorship contribution statement

Bowei Ouyang: Writing – original draft, Methodology, Conceptualization. **Cong Yang:** Methodology, Data curation. **Ziyue Lv:** Methodology, Data curation. **Baowei Chen:** Methodology, Conceptualization. **Lei Tong:** Supervision, Funding acquisition, Conceptualization. **Jianbo Shi:** Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2024.108989.

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