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Raman biosensor and molecular tools for integrated monitoring of pathogens and antimicrobial resistance in wastewater

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ABSTRACT

Pathogens and antimicrobial resistance (AMR) are emerging as major global threats to public health. Wastewater, as the unique interface between environments and humans receiving and spreading pathogens and AMR, is playing a more important role than ever before for monitoring of public health. Here, by pinpointing pathogens and AMR, we reviewed the most recent technological advancements in Raman biosensors (single-cell Raman, Raman-stable isotope probing, surface-enhanced Raman, statistical analysis) and molecular methods (polymerase chain reaction, metagenomics and single-cell genomics) for phenotypic and genotypic surveillance, respectively. In particular, the importance of integrating phenotypic and genotypic analysis via targeted single-cell sorting for a complementary and holistic surveillance and understanding of health risk was highlighted. We further suggest technological requirements to enhance wastewater surveillance and better inform tackling strategy against pathogens and AMR.

1. Introduction

1.1. Pathogens and antimicrobial resistance in wastewater and health impact

Wastewater is a unique interface between environments and humans. Human activities generate a huge amount of wastewater, which is a collection of both biological and chemical loads from an entire community. In light of the outbreak of COVID-19 pandemic, biological loads especially the rising prevalence of microbial pathogens (virus, bacteria, fungi, protozoa) and antimicrobial resistance (AMR), are gaining unprecedented attentions [1,2]. They have been posing a burgeoning threat to public health globally and imparting a significant economic burden to global healthcare systems [3,4]. On the top ten threats to global health listed by World Health Organization (WHO) in 2019, four of them were directly related to pathogens (pandemic influenza, Ebola, HIV) and AMR [5]. Wastewater is a sink and hotspot for capturing, accumulating, and spreading of pathogens and AMR [6–8]. The entire pathogen load with certain degrees of disease burden and AMR will be discharged from the local catchment of different origins, including human feces, hospital, industry, and rainfall runoff finally conveyed and concentrated into a single waste stream for treatment (Fig. 1). This fact makes wastewater an ideal place for surveillance of pathogens and AMR of the entire community, and leads to development of a new powerful tool named wastewater-based epidemiology (WBE) for tracking the outbreak and transmission of diseases [2,9].

An extensive range of pathogens have been detected in wastewater worldwide, including nearly all clinical bacterial pathogens and viruses incurring the recent major outbreak of epidemic or pandemic such as COVID-19 (2019–2020), Zika virus (2015–2016), Ebola (2014–2016), SARS (2002–2003) [7,10,11]. In addition to pathogens, AMR-the ability of bacteria, fungi, and virus to resist drugs, is posing a more profound threat to the global health by compromising the treatment of microbial infections [1,3,5,12]. More and more pathogens have developed resistance to one or multiple drugs [13]. Superbugs that can resist nearly all antimicrobial drugs have been frequently reported in both developed and developing countries [14,15]. Even now, infections with antimicrobial resistant pathogens cause about 700,000 deaths per year around the world, and this number is forecast to increase to 10 million people a year by 2050 [1,3]. WHO strongly urged to combat AMR by stating “no action today, no cure tomorrow” in 2011 [12], then approved a global action plan to cope with AMR and established Global Antimicrobial

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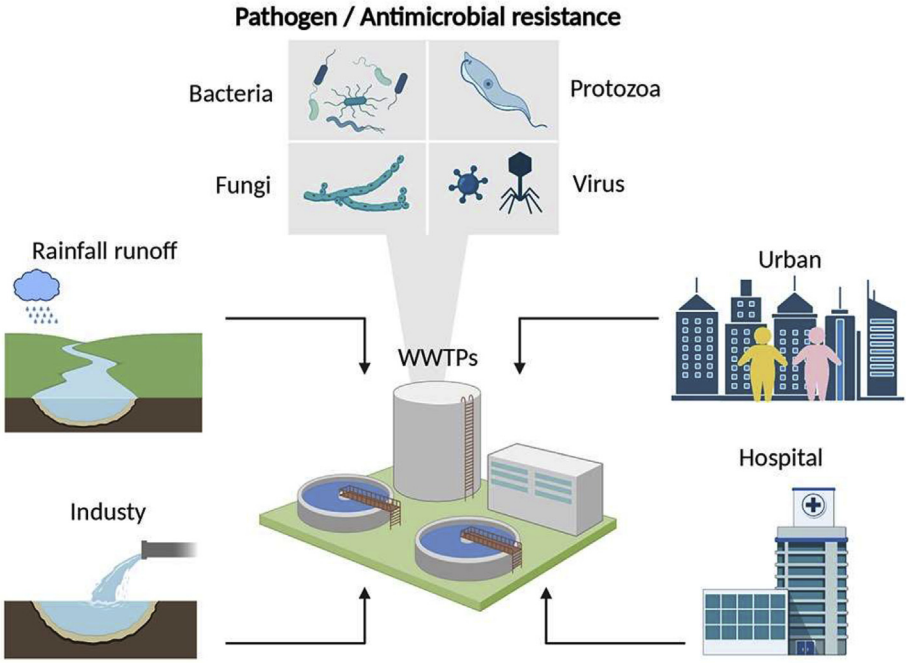

Fig. 1. An overview of the role of wastewater as a reservoir of pathogens and antimicrobial resistance.

Resistance Surveillance System (GLASS) in 2015 [16,17], and further published its first list of global antibiotic-resistant priority pathogens in 2017 to guide research and development of new antibiotics [13]. Antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are widespread in wastewater, and ARGs conferring resistance to antibiotics of nearly all mechanisms have been detected in wastewater [18–21]. Moreover, wastewater treatment plants (WWTPs) are an enormous biological reactor with a purpose of degrading pollutants. Such concentrated environments create an ideal place for pathogens and AMR to multiply, transfer and mutate efficiently under diverse selective agents such as antibiotics, heavy metals, pesticides [22–26]. Some work found that most ARGs detected in WWTPs were carried by mobile genetic elements (MGEs) such as plasmids, further facilitating the spread of ARGs [20].

Wastewater treatment plants cannot effectively remove pathogens and AMR due to the current technology [19]. As such, the effluent acts as a point source and causes further contamination in downstream environments such as soil and freshwater [27–30]. It is estimated that around 80% of wastewater without sufficient treatment is discharged to the environments [31]. In addition, more than one hundred types of ARGs were detected in park soils irrigated with wastewater effluent [30], and wastewater sewage sludge even after composting still contained a high abundance and diversity of ARGs [32]. These facts illustrate the great importance to surveil pathogens and AMR in wastewater.

In the context of global efforts to eliminate pathogens and AMR, sampling from wastewater represents a large and important part of the environmental surveillance programs. Systematic and targeted monitoring of pathogens and AMR in wastewater is essential to address the increasing public health challenges. Microbial threats might be always in the world, and infectious pathogens spreading from non-human species to humans appear to be increasingly risky. In these regards, technique advancements capable of enhancing surveillance capacity in terms of early warning and outbreak tracking become very important. The acquired data can better inform proactive prevention, survey the effectiveness of interventions, and even prevent disease from spreading around the world. Therefore, new advancements in monitoring techniques for such purposes merit strong attentions and a review.

1.2. Needs for an integrated genotypic and phenotypic surveillance

Both genotypic and phenotypic analysis have their own advantages and drawbacks for pathogen and AMR surveillance (Fig. 2). For genotypic surveillance, molecular methods such as metagenome sequencing, amplicon sequencing, and polymerase chain reaction (PCR) have produced extensive and immense DNA sequence-based information on pathogens (species, virulence factor) and AMR (ARGs, MGEs). Such large information enables the construction of local, regional, and even global-scale distribution of pathogens and AMR. The high resolution also allows quantitative and comparative studies of the correlation among human, animal, and environmental samples, thereby providing an approach to surveil pathogens and AMR under the One Health framework [33,34]. It is also possible to illustrate the microbial hosts, genetic locations and context of ARGs and virulence factors [20]. More importantly, molecular methods allow reconstruction of putative transmission networks to determine the source and evolution of pathogens/AMR that lead to diseases outbreaks [35].

However, molecular methods are not the choice under all circumstances. Gene sequences only provide the potential or inferred pathogenicity and AMR instead of phenotypic features. In addition, genotypic analysis cannot distinguish the origins of genes from extracellular DNA, dead, nonactive and active cells which risks are highly different [36]. In contrast, phenotypic analysis indicates practical microbial infections or disease outbreaks (Fig. 2). It can also be used to quantify the level of phenotypic AMR, so it is actionable to guide the clinical treatments of bacterial infections, and promote research and development of new drugs to combat the growing global AMR [35,37]. Phenotypic characterization is also an indispensable way to reveal the function of unknown or novel ARGs and virulence factors. However, compared with the rapid development of genotypic characterization, methods for phenotypic studies largely lag behind. Conventional culture-based phenotypic methods

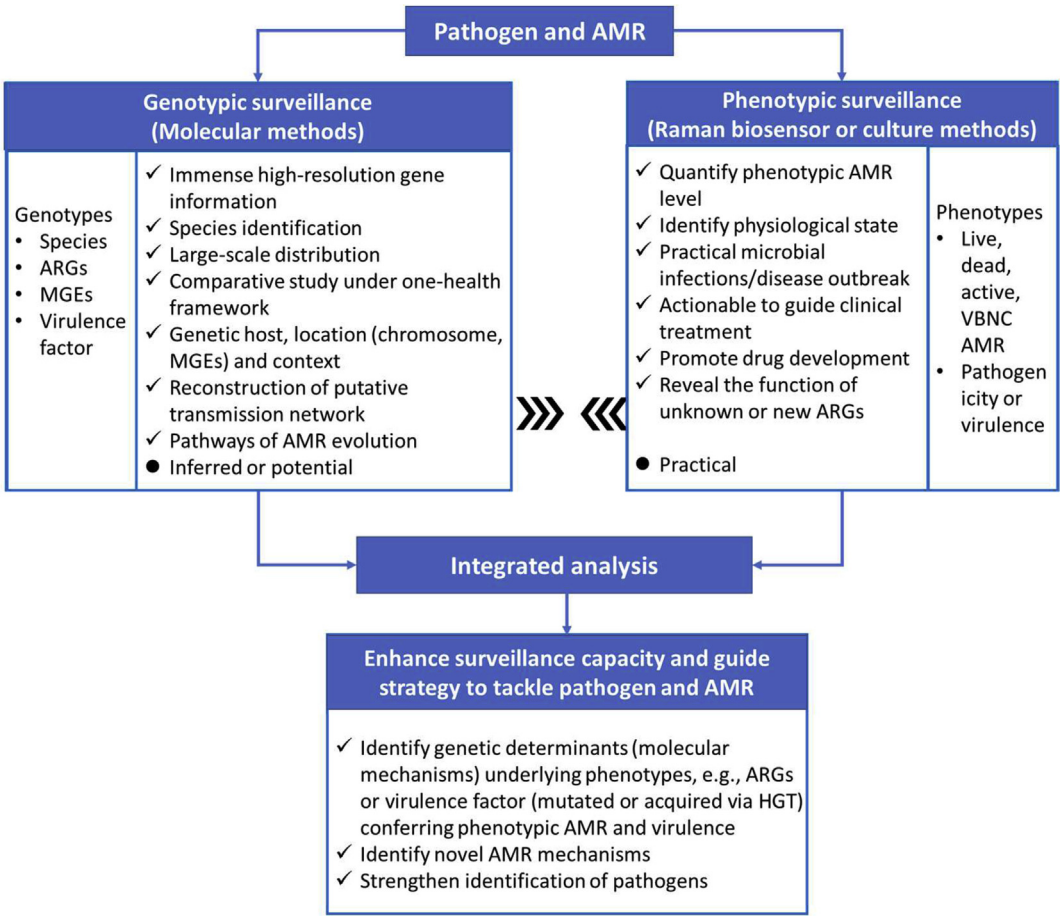

Fig. 2. Integration of genotypic and phenotypic analysis for a holistic and enhanced surveillance of pathogens and antimicrobial resistance.

have obvious drawbacks in implementation, i.e., they are time and labor-consuming, low throughput, provide limited information to only cultivable bacteria, but preclude studies on uncultured cells which represent a large fraction in diverse microbial communities (including wastewater) and may play a great role in the occurrence and spread of pathogens and AMR. New phenotypic methods are highly demanded.

Presently, there are still discrepancy and knowledge gap between genotypic and phenotypic analysis [38]. For example, there are cases where ARGs are present but resistant phenotypes are not found because ARGs are not always expressed. There are also cases where no known ARGs are present but resistant phenotypes are observed due to the presence of novel ARGs or mechanisms. It is also possible that the same genotypic determinants confer varied phenotypic profiles. To bridge the gap and achieve a comprehensive understanding of the threat of pathogens and AMR to public health, there is a great need to integrate genotypic and phenotypic analysis for a complementary surveillance (Fig. 2). When phenotypes are determined, genotypic analysis will add valuable information on genomic determinants underlying the pathogens with specific phenotypes. For example, genomic data can clarify ARGs conferring phenotypic antibiotic resistance, determine genomic mutations or acquired genes accounting for increased expression of phenotypic resistance or pathogenicity, and strengthen the identification of infection-causing pathogens more accurately [35]. Therefore, an integrated approach will be invaluable for enhancing surveillance capacity and guiding strategy to tackle pathogens and AMR.

In this review, for phenotypic analysis, the newly developed Raman spectroscopy-based tools showing great potentials for pathogen identification and phenotypic characterization of AMR will be reviewed in detail. For genotypic analysis, molecular methods including PCR, metagenomic sequencing, single-cell genomic sequencing and their recent applications in pathogens and AMR surveillance will be reviewed. Finally, new advancements integrating phenotypic and genotypic surveillance of pathogens and AMR bridged via targeted single-cell sorting and sequencing will be introduced.

2. Raman biosensor for pathogen and phenotypic antimicrobial resistance analysis

2.1. Principle and advantages of Raman spectroscopy

2.1.1. What is Raman spectroscopy?

Raman spectroscopy is a member of vibrational spectroscopic techniques. It detects photons that are scattered to frequencies different from that of incident photons due to the energy exchange between incident monochromic photons and a vibrating molecule. It was first discovered by C. V. Raman in 1928 [39,40]. When monochromatic light interacts with molecules, most photons are scattered at the same frequency as that of the incident photons, an effect called elastic scattering or Rayleigh scattering, while about 1 out of 10^6-10^8 photons are scattered at a frequency shifted from that of the incident photons. This effect is called inelastic scattering or Raman scattering. The energy difference is equal to the vibrational energy of chemical bonds, constituting the basis for Raman detection. Microbial samples are composed of different biomolecules each with some characteristic chemical bonds. Hence,

Raman spectroscopy can take a chemical image pinpointing the chemical compositions of microorganisms, which represent different cellular phenotypes such as cell types, metabolic states, and stress responses to environmental stimuli.

Normal Raman scattering is inherently weak in signal intensities due to the low quantum efficiency. However, if the excitation energy of incident photons matches the electronic transition of molecules, resonance Raman (RR) can be excited, and the resulting RR signal can be selectively enhanced by 4–6 orders of magnitude. Some pigment molecules such as carotenoids and cytochrome *c* (Cyt *c*) in bacteria can be selectively enhanced via RR [41,42]. Surface-enhanced Raman spectroscopy (SERS) provides another way for signal enhancement by 4–11 orders of magnitude [43]. This is achieved by means of the strong electromagnetic enhancement generated by the excitation of surface plasmon resonance of nanometals, typically Au, Ag, Cu nanometals. Because SERS enhancement is highly localized, only the Raman signal of molecules adsorbed or close to the metallic nanostructures can be selectively enhanced. This feature confers SERS with additional abilities of surface characterization and fluorescence quenching due to the energy transfer between molecules and nanometals. Moreover, by conjugating nanometals with elements for recognition (antibodies, aptamer), detection (molecules with strong SERS signals), separation or enrichment (magnetic particles) of microbes, the constructed versatile SERS biosensors can be used for multifunctional microbial studies such as capturing, detecting, and even inactivating of microbial pathogens in environments.

2.1.2. Raman - stable isotope probing theory

Stable isotope probing (SIP) is an attractive technology that has developed rapidly and evolved to a state-of-art approach capable of elucidating the function, activity, and metabolic flux of microbes in complex microbial communities [44]. Many elements essential for microbial growth or activity have their corresponding stable isotopes, such as ^{13}C, ^{15}N, ^{2}D, ^{18}O, ^{34}S. The use of SIP approach is based on the principle of “you are what you eat” [45], that is, when microbes uptake and metabolize isotope-labeled substrates, the isotope will appear in some newly synthesized cellular components such as nucleic acids, protein, lipid, pigments. Detection of isotope-labeled biomolecules provides a means to target microbes with interested metabolic features.

Raman spectroscopy, including normal Raman, RR and SERS, all characterizes the vibrational frequency of chemical bonds, so when an atom is replaced by its heavier isotope in molecules, although the chemical structure remains unchanged, the vibrational frequencies of the involved chemical bonds decrease, as can be detected as distinct Raman red shifts. To facilitate understanding, two atoms connected by a massless spring are used to describe the vibration of chemical bond. Here, the two atoms are considered as mathematical points and the spring represents the chemical bonds between the atoms. The wavenumber of the bond vibration is described by Equation (1):

$$\tilde{\nu} = \frac{1}{2\pi c}\sqrt{\frac{k}{\mu}} \tag{1}$$

where $\tilde{\nu}$ is the wavenumber of the bond vibration (cm^{-1}), c is the speed of light in vacuum (m s^{-1}), k is the force constant of a diatomic bond (N m^{-1}), and μ is the reduced mass (g) calculated by Equation (2):

$$\mu = \frac{m_1 m_2}{m_1 + m_2} \tag{2}$$

where m_i is the mass of the atom (g). For instance, when a hydrogen atom is replaced by deuterium in a C–H bond, the ratio of vibrational wavenumber before and after D replacement can be calculated by Equation (3), resulting in a ratio being around 0.734.

$$\frac{\tilde{\nu}_2}{\tilde{\nu}_1} = \sqrt{\frac{\mu_1}{\mu_2}} \approx 0.734 \tag{3}$$

where $\tilde{\nu}_i$ is the wavenumber of C–H or C-D bond, μ_i is the reduced mass of C–H or C-D bond. Generally, the C–H vibration is located between 2800 and 3100 cm^{-1}, while the C-D vibration usually appears in the silence region of 2040–2300 cm^{-1}. Since there are no spectral interferences, the C-D vibration is of great advantage for the investigation of metabolic activity of microbial cells. To date, ^{2}D, ^{13}C, and ^{15}N were the most used isotopes for Raman-SIP applications in microbial studies [37,41,42,44,46–55]. Although other isotopes (e.g., ^{18}O and ^{34}S) could also be potentially applied to Raman-SIP, they have been rarely illustrated for microbial studies [56].

2.1.3. Advantages of Raman spectroscopy

A rapid and reliable identification of pathogens and phenotypic characterization of AMR is of critical importance for surveillance. In this regard, Raman spectroscopy-based biosensor is the top and hot candidate technology for surveillance purpose, because it allows a rapid, phenotypic, noninvasive characterization of microbes at single-cell level that could complement genotypic and microbiological methods. The advantages of Raman spectroscopy are detailed as below. Firstly, Raman spectroscopy provides intrinsic fingerprint information of microorganisms representing a nearly complete biomolecule profile, including proteins, nucleic acids, lipids, pigments (Cyt *c* and carotenoids), storage compounds (polyhydroxybutyrate (PHB), polyphosphate (Poly-P), magnetosome, and sulfur), and metabolites (Fig. 3A) [41,42,44,57–62]. Moreover, Raman profiles of microbes vary sensitively with the change of chemical, physiological or metabolic states of microorganisms, so Raman spectroscopy is a phenotypic method. When combined with statistical analysis, Raman allows differentiation of pathogens at different taxonomic levels from genera, species, to even strains such as methicillin-resistant and sensitive *Staphylococcus aureus* (MRSA and MSSA) [63]. Moreover, Raman can be well combined with SIP for sensing functional and active cells, especially for phenotypic characterization of AMR, based on the characteristic Raman shifts induced by the incorporation of isotope-labeled substrates by microbes (Fig. 3B–D). Secondly, although MALDI-TOF mass spectroscopy and infrared spectroscopy are also applicable for identification of microbes after standard cultivation, Raman spectroscopy outperforms them in its cultivation independence owing to the ability of single-cell level detection. This is achieved by combing Raman with optical microscopy to offer a high spatial resolution down to 1 μm, just the size of a single microbial cell [64]. With this culture-free ability, Raman spectroscopy provides a rapid means to identify pathogens without necessity of lengthy cultivation. This is beneficial for directly detecting yet uncultured microbes or those in a viable but nonculturable state (VBNC) in complex environmental matrices. Thirdly, Raman is a noninvasive technique and thus allows downstream sorting of cells of interest for single-cell genomic analysis, providing a great way to integrate microbial phenotypes with genotypes [65]. Such integration is very important for an in-depth and comprehensive surveillance of pathogens and AMR in natural environments. Fourthly, Raman-derived biosensors, such as SERS, can be readily integrated with versatile platforms for pathogen capture, signal enhancement, identification and even inactivation.

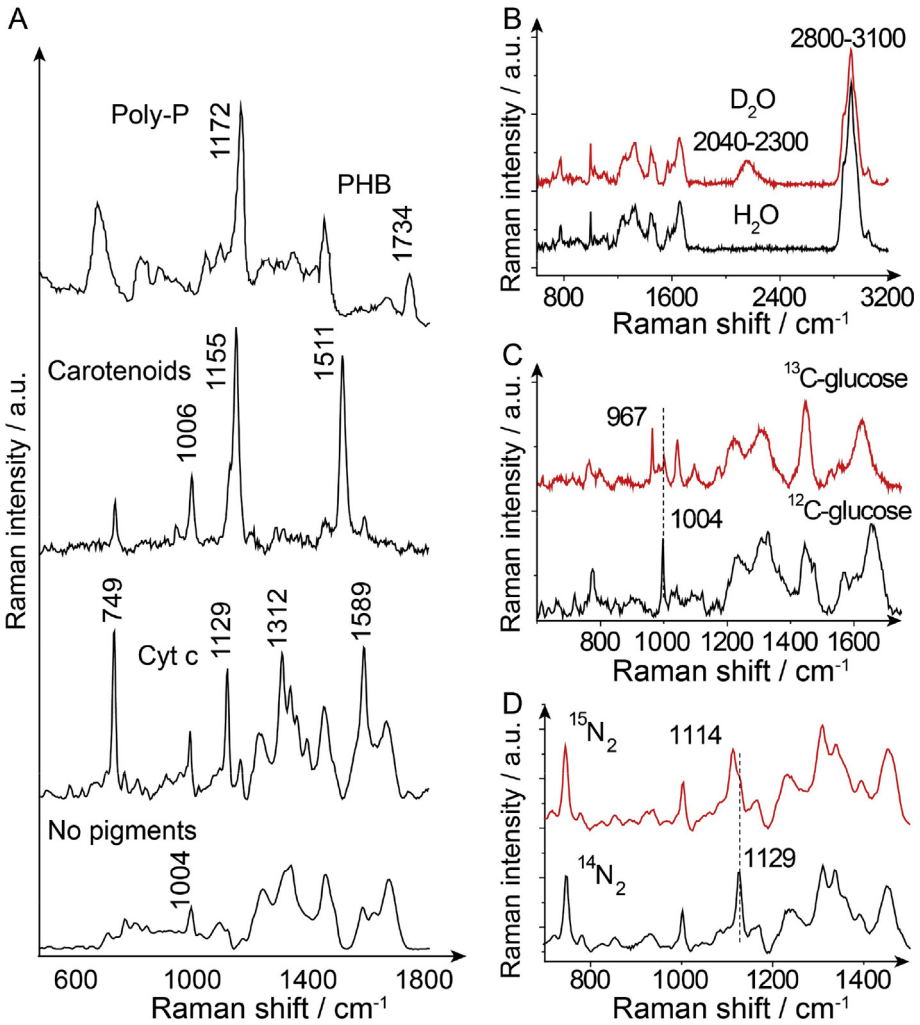

Fig. 3. (A) Exemplary Raman spectra of microbial cells that contained different storage compounds and pigments, such as Poly-P, carotenoids, Cyt *c*. Stable isotope-induced band shifts in Raman spectra of *E. coli* labeled with D_2O (B), ^{13}C-glucose (C), and N_2-fixing *Azotobacter* sp. labeled with $^{15}N_2$ (D). Reproduced with permission from Refs. [42,44,50,52,66].

2.2. Raman biosensor for pathogen identification

2.2.1. Single-cell Raman for a rapid and culture-free pathogen identification

Based on the cellular spectral fingerprints unique to the chemical composition of different microbial species, Raman spectroscopy has been employed for a rapid pathogen identification (Table 1). Schuster et al. first introduced Raman spectroscopy for microbial investigations at the single-cell level [67,68]. Huang et al. further demonstrated that single-cell Raman spectroscopy (SCRS) coupled with multivariate methods allowed taxa discrimination of bacteria [61]. Recently, pathogens from food and water, such as *Legionella* spp. [69], *Bacillus* spp. [70], *Pseudomonas* spp. [71], and *Burkholderia* spp [72]. can be identified at species or even strain level by SCRS with chemometric analysis. For example, Dragana et al. combined SCRS with tree-based multiclass support vector machines (MC-SVM) to discriminate *Legionella* at the species level with 87.3% accuracy [69]. They further demonstrated that environmental factors (pH, mineral content) and growth phases can influence the differentiation of *Pseudomonas* spp. in bottled natural mineral water. By combining SCRS with SVM, the identification accuracies were up to 85% for *P. aeruginosa* at strain level from independent samples [71]. Recently, To capture and detect bacteria from real-world samples, several Raman compatible isolation strategies have been developed, such as optical tweezers and dielectrophoresis (DEP), which can trap bacteria by optical gradient force [73] and electrokinetic pressure [74], respectively. Xie et al. combined optical tweezers with SCRS for pathogen identification in aqueous solutions [75]. Six bacterial pathogens were well discriminated at species level based on their spectra and chemometric analysis. Schröder et al. developed an integrated setup of DEP and Raman for discrimination of pathogens from urine samples [76]. In a proof-of-principle study, spiked pathogens of either *E. coli* or *E. faecalis* can be rapidly identified with linear discriminant analysis (LDA) from urine samples without time-consuming cultivation.

It is worth noting that physiological states of microbes and environmental factors can easily induce variations of microbial Raman spectral features. Huang et al. reported that growth phases and nutrient conditions caused variations of Raman spectra due to potential changes in the microbial composition [61,77], and resulted in an obscure boundary for species differentiation. Therefore, for a reliable and robust Raman identification of pathogens, it is recommended that growth conditions (e.g., growth media and growth phase) [61,78], environmental factors (e.g., temperature, pH, and moisture etc.) [77,79], and sample processing [80] should be all taken into account to build Raman database and classification model.

Table 1
Summary of classification algorithms used for rapid Raman-based pathogen identification.

Sample type	Target pathogens	Raman techniques	Algorithms[a]	Identification accuracies (%)	Ref.
Pure culture	Bacteria	Raman	ANN	80	[86]
Pure culture	Bacteria	Raman	SVM	92	[88]
Pure culture	Bacteria	Raman	SVM	83.6	[89]
Pure culture	Bacteria	Raman	SVM	87.3	[69]
Meat	Bacteria	Raman	SVM	90.6–99.5	[95]
Water	Bacteria	Raman	SVM	85	[71]
Pure culture	Bacteria	Raman	CNN	82	[63]
Pure culture	Bacteria and fungi	Raman	CNN	95.64	[93]
Pure culture	Bacteria	Raman	CNN	95	[96]
Pure culture	Bacteria	Raman	GAN	100	[94]
Pure culture	Virus	SERS	PCA-HCA	87.75	[97]
Pure culture	Virus	SERS	SIMCA	95	[98]
Clinical	Virus	SERS	LR	90	[99]
Water	Virus	SERS	PCA-LDA	93.33	[100]
Pure culture	Fungal spores	Raman	PCA-LDA	89.4	[101]
Clinical	Fungi	SERS	PCA	98	[102]
Pure culture	Fungi	SERS	FPCA-LDA	97.8	[103]

[a] ANN, Artificial neural networks; SVM, support vector machine; CNN, Convolutional neural networks; GAN, generative adversarial network; PCA, principal component analysis; HCA, hierarchical cluster analysis; SIMCA, soft independent modeling of class analogy; LR, logistic regression; LDA, linear discriminate analysis; FPCA, fuzzy principal component analysis.

2.2.2. Classification algorithms for Raman-based pathogen identification

Raman spectra usually contain a superposition of all biomolecules within the laser focus of a cell. The intrinsic complexity requires advanced data processing to mine useful information from spectral data. Moreover, the spectral profile of two different microbial species may differ slightly because of subtle variations in the essential components. To recognize these small spectral variations with high accuracy and sensitivity, chemometric approaches have become indispensable for Raman-based classification (Table 1). Before chemometric analysis, raw spectral data should be preprocessed including cosmic spikes removal, baseline correction, and spectral normalization to promote its quality and reliability. Subsequently, unsupervised algorithms such as principal component analysis (PCA) [61,75,81–83] and hierarchical cluster analysis (HCA) [61,75,83–86] are used to reduce the dimensionality of spectra, extract the distinct features, and cluster unlabeled spectra according to their similarities in the dataset. After that, classification model can be built with chemometric algorithms such as LDA [82,83], discriminant functional analysis (DFA) [61,84,86], partial least squares discriminant analysis (PLS-DA) [87], SVM [69,72,88,89], and random forests [90,91]. Presently, these traditional chemometrics are increasingly challenged by the complexity of microbial spectral database, especially for microbial spectra with low signal-to-noise ratios (SNR). To address these challenges, deep learning algorithms, such as convolutional neural network (CNN) [63,86,92,93] and generative adversarial network (GAN) [94], are becoming a promising solution and recently gain momentum in pathogen identification (Table 1). For example, Ho et al. combined Raman spectroscopy with CNN algorithm for a rapid identification of pathogenic bacteria at strain level. The average identification accuracies of 30 isolated bacterial strains exceed 82% even on low SNR spectra, which outperforms the most traditional algorithms such as logistic regression (75.7%) and SVM (74.9%) (Fig. 4A) [63]. Moreover, this approach can distinguish MRSA and MSSA with 89.1% recognition accuracy, allowing for targeted treatment of bacterial infections. To use these algorithms for pathogen identification, the first step is to establish and train a classification model using Raman spectroscopic database of known bacterial species. The trained model should then be validated and even tuned using a biologically or technically independent dataset. Finally, the obtained classification model is used to identify unknow bacterial species from clinical or environment settings.

2.2.3. SERS for a versatile pathogen capture and identification

Compared with normal Raman, SERS provides strongly enhanced Raman signals, enabling the detection of pathogens even using portable Raman devices with a low detection sensitivity [104]. Generally, there are two strategies for SERS-based biosensing of pathogens including label-free and label-based methods [105]. In label-free methods, bare metal nanoparticles (NPs) are directly used for SERS detection based on the intrinsic biological information of pathogens. In label-based methods, metallic NPs are modified with different molecules to construct SERS biosensors for multiple function. To capture pathogens, SERS biosensors can be conjugated with specific pathogen recognition elements such as antibodies [106,107], aptamers [108], and vancomycin [109]. In addition, Raman reporter molecules with very strong SERS signals are normally introduced into SERS biosensors to improve the detection sensitivity of pathogens, such as 4-mercaptobenzoic acid (4-MPBA) [110], Rhodamine 6G [111], 4-aminothiophenol [107], 4-mercaptopyridine, and 5,5-dithiobis-2-nitrobenzoic acid [112]. Label-based SERS biosensors have enabled a highly sensitive and multiplex detection of pathogens in complex matrices such as clinical blood [109,110], food, and drink [106,108]. For instance, He et al. developed an approach capable of simultaneous capture, SERS detection, and inactivation of bacteria from blood using MBPA-modified Ag NPs (Fig. 4B). Moreover, SERS biosensors can combine with magnetic beads and microfluidic chip to isolate and enrich the captured pathogens for identification.

SERS biosensors also provides an ultrasensitive tool for virus detection and identification. Recently, Yeh et al. developed a VIRRION (virus capture with rapid Raman spectroscopy detection and identification) biosensor for rapid virus capture and optical identification without labelling from clinical samples (Fig. 4C) [99]. In this study, the viruses were enriched based on their size using carbon nanotube herringbone patterns decorated with Au NPs. These viruses were then identified using SERS in conjunction with machine learning algorithm. This nondestructive approach provides an innovative system for virus isolation and identification from clinical samples. In addition, Scully et al. combined tip-enhanced Raman scattering (TERS) with coherent anti-Stokes

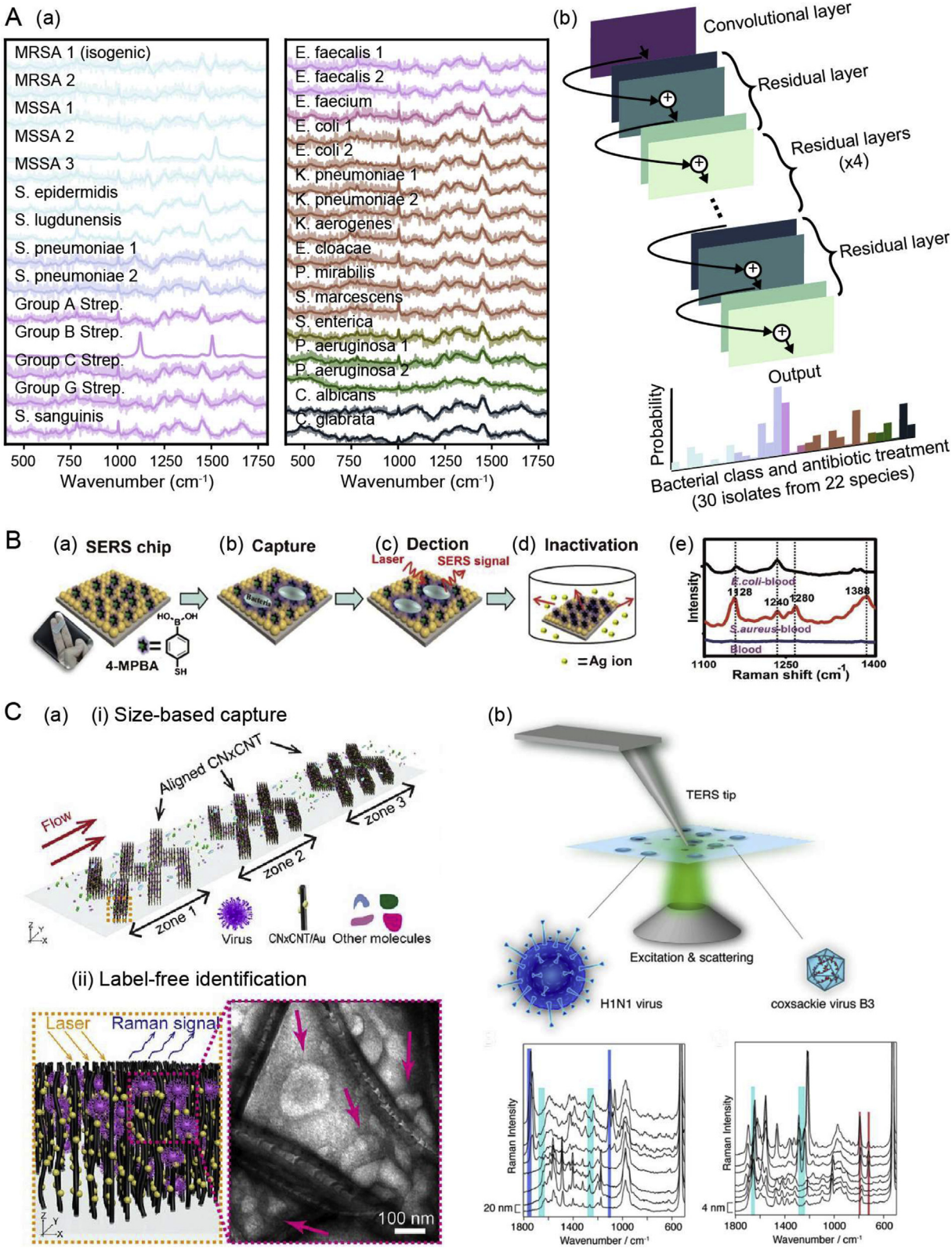

Fig. 4. Rapid identification of pathogens by normal Raman and SERS. (A) Raman spectroscopy combined with deep learning for rapid identification of pathogenic bacteria. (a) Average Raman spectra of 30 pathogens. (b) CNN architecture used for pathogen identification from low-signal Raman spectra. (B) Schematic of multifunctional SERS chip of Ag NPs modified with bacteria-binding 4-MPBA for bacterial capture, detection, and inactivation. (C) Raman spectroscopy used for virus identification. (a) Illustration of (i) size-based capture and (ii) label-free SERS for viruses identification. (b) Schematics of the transmission TERS setup used for viruses detection. Reproduced with permission from Refs. [63,99,110,113].

Raman spectroscopy to map the virion surface structure of a single virus particle (Fig. 4C), providing a way with nanometer resolution and chemical specificity for viral studies [113].

2.3. Raman biosensor for phenotypic analysis of antimicrobial resistance

2.3.1. Single-cell Raman-SIP for a rapid phenotypic AMR analysis

The emergence and spread of antibiotic resistance in pathogenic bacteria pose a significant threat to public health worldwide. Traditional growth-based methods such as broth dilution assay and disk diffusion test for AMR surveillance are very time-consuming. It typically takes days to weeks for pathogen isolation and antibiotic susceptibility testing (AST) [114]. By comparison, SCRS combined with deuterium substrates (e.g., D_2O and deuterated glucoses) provides a rapid means to circumvent the lengthy preculture and detect ARB with phenotypic resistance in their natural habitat. As described in section 2.1.2, when incubated with deuterium-labeled substrates, metabolically active microbes can incorporate ^2D into *de novo* synthesized biomass, generating a new carbon-deuterium (C-D) Raman band (Fig. 3B) [47,115]. Since this process is governed by the general metabolic activity of bacterial cells, C-D Raman shifts may serve as an indicator to monitor microbial metabolic activity [115]. Under antibiotics treatment, antibiotic resistant and susceptible strains display totally different metabolic activities and can thus be simply discriminated based on the intensity of C-D bands.

Recently, Yang et al. developed single-cell Raman combined with D_2O labelling (Raman-D_2O) for rapidly sensing phenotypic AST of pathogens in clinical urine samples (Fig. 5A) [47]. In this study, infectious pathogens were recovered directly from clinical urine samples and incubated in media containing D_2O and antibiotics. After single-cell Raman detection, antibiotic susceptibility readouts were reported according to the relative C-D ratio of pathogens. The total assay time was shortened to only 2.5 h from receiving urine samples to AST results. Yi et al. further extended the applicability of Raman-D_2O for AST of pathogens in more complex blood samples [116]. Stimulated Raman scattering provides another rapid way for AST by Raman imaging of multiple individual bacterial cells in one field of view [117,118]. In addition to AST of clinical pathogens, Raman-D_2O based biosensor has also been applied to detect ARB in various environments, such as plastic biofilms [37], river water [37,119], and human gut [53].

These results demonstrated the great potential of single-cell Raman-D_2O for phenotypically surveilling ARB in wastewater because it is cost-effective and easily applicable. In the future, with the development of devices integrating automated sample preparation, Raman detection and intelligent data processing, Raman-D_2O will find a way for routine ARB surveillance in wastewater.

2.3.2. Single-cell Raman-SIP for phenotypic tracking of AMR spread

Environments such as wastewater is a huge reservoir of antibiotic resistance. The widespread of antibiotic resistance in both clinical settings and environments threatens human health [120]. Horizontal gene transfer (HGT) allows ARGs exchanging across various bacterial strains and even concentrating into one cell, driving the evolution of superbugs resistant to all types of antibiotics [121,122]. Bacteria receiving ARGs and displaying phenotypic resistance are more dangerous than those without expression. In this regard, Raman-SIP-based biosensor provided an attractive way for HGT tracking because of its high detection sensitivity,

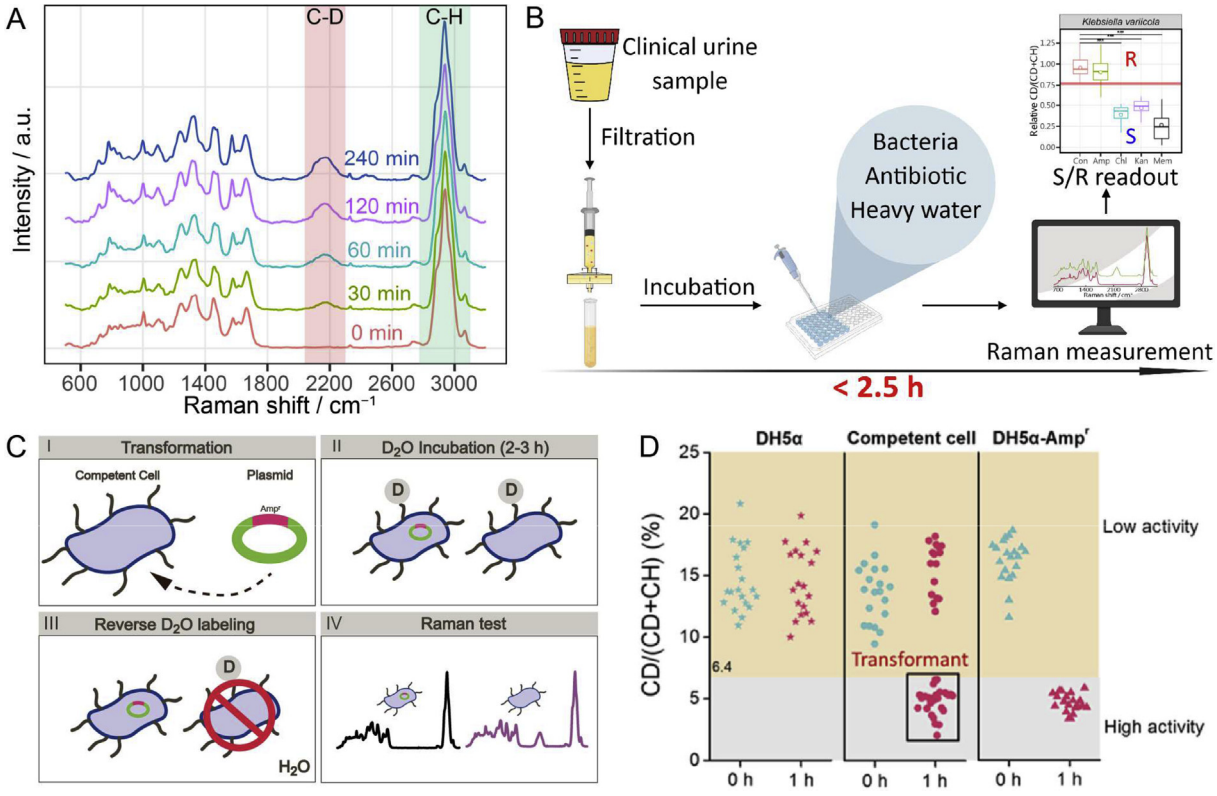

Fig. 5. Single-cell Raman-D_2O for phenotypic analysis of antibiotic resistance. (A) Single-cell Raman spectra of *E. coli* incubated with culture medium amended with D_2O for different time. (B) Schematic of rapid AST for clinical samples via single-cell Raman-D_2O. (C) Workflow for phenotypic tracking of antibiotic resistance spread via transformation by single-cell Raman-rD_2O. (D) Identification of antibiotic resistant transformants of *E. coli* DH5α receiving *bla* plasmids based on the lower C-D ratios after 1 h of incubation under ampicillin treatment. Reproduced with permission from Refs. [46,47].

phenotypic characterization, rapidness, and culture-independence compared with conventional plate culture and reporter gene technology. Based on the different metabolic activities of antibiotic resistant and sensitive bacteria in uptaking H to replace pre-labeled D under antibiotic treatment, Li et al. developed Raman with reverse D_2O labeling (Raman-rD_2O) as a more sensitive tool than forwarding labelling in distinguishing phenotypically resistant and susceptible bacteria (Fig. 5B) [46]. The rapid silence of C-D band was established as a sensitive indicator capable of discriminating phenotypically resistant transformants from a large pool of recipient bacteria. By using single cell Raman-rD_2O biosensor, the spread efficiency of multiple plasmid-borne ARGs extracted from soils against five clinically relevant antibiotics to *E.coli* was revealed. This approach is readily applicable to evaluate the spread risk of resistome in wastewater via HGT to clinically relevant pathogens, providing a means for target control of resistance spread.

3. Molecular tools for genotypic pathogen and antimicrobial resistance analysis

3.1. Principle and advantages of molecular tools

Cultivation is a classical method for identifying pathogens (e.g., bacteria, viruses, fungi, and protozoa) and their antibiotic resistance. Pure culture of microorganisms has always been considered as the basis of research on infectious disease. However, it is a lengthy and laborious process, due to the necessity of enriching and isolating pathogens on selective media from complex environmental microbial communities [123]. Moreover, low-abundance pathogens in the environment increase the difficulties to enrich and separate, although they may still pose a high risk of spread and infection. Pathogens can be also in a VBNC state after exposure to a hostile environment for a long time [124]. Therefore, cultivation technique has many limitations for monitoring of pathogens and their antibiotic resistance in environments. Recently, more and more culture-independent molecular tools, such as PCR, loop-mediated isothermal amplification (LAMP), high-throughput qPCR, metagenomic sequencing, and single-cell genomic sequencing, have been applied to detect pathogens and their antibiotic resistance in environments (Fig. 6) [125–127]. These culture-independent molecular tools are efficient at detecting genes of microorganisms that cannot grow in the lab, multiply very slowly or are in a VBNC state in environments. They are also operated in a high-throughput manner allowing capturing many different types of genetic information from taxonomy to functional genes such as ARGs, MGEs, virulence factor. Molecular methods have been recruited as a standard approach in the GLASS launched by WHO [17], and are thus of great potential for wastewater surveillance to interpret pathogen and AMR profiles and understand their prevalence. The advantages and disadvantages of different molecular tools for the surveillance of pathogens and AMR are summarized in Fig. 6.

3.2. PCR-based molecular tools for pathogens and antimicrobial resistance analysis

PCR, as a gold standard technique of gene amplification, is the most commonly used molecular tool for detecting the specific segments of pathogen genomes (DNA or RNA). Multiplex PCR assays with many different primers can be designed to detect multiple pathogens and/or resistance genes at the same time. Presently, the traditional agarose gel-based detection of PCR amplification has no longer been able to meet the high demand of accurate quantification and rapid detection of target genes. Real-time quantitative PCR (qPCR) makes up for this deficiency and can rapidly detect target genes without electrophoresis and monitor DNA amplification in real time [128,129]. SYBR Green and TaqMan are the two commonly used methods in qPCR to quantify the concentration of

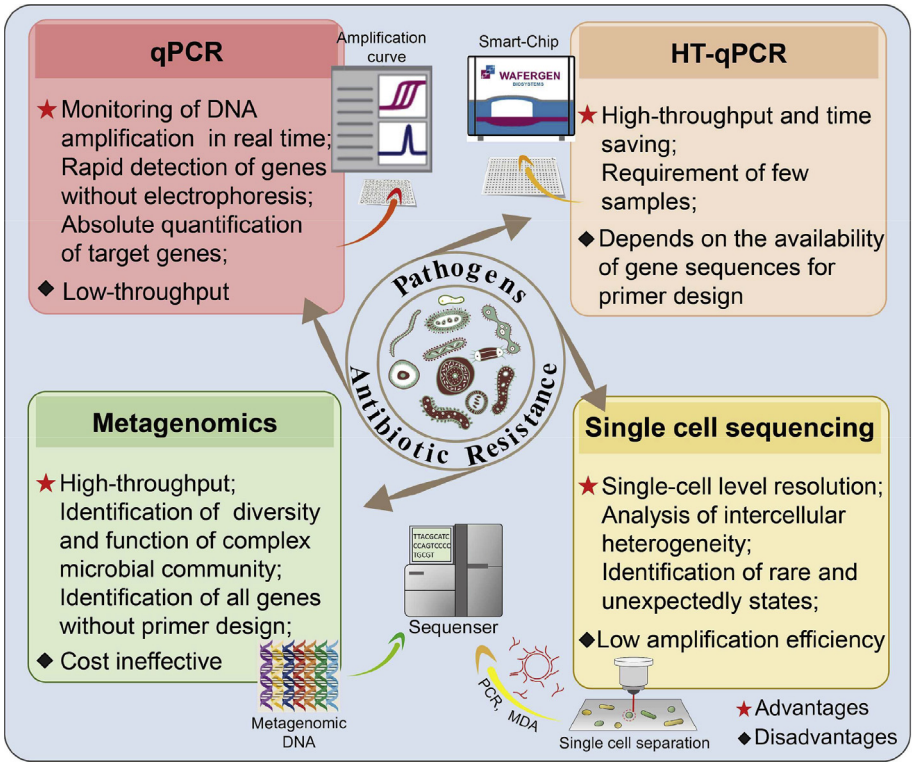

Fig. 6. Advantages and disadvantages of different molecular tools for surveillance of pathogens and antibiotic resistance.

target genes by monitoring the real-time changes of fluorescence during thermal cycling [130]. TaqMan probe has a higher specificity than SYBR Green but is more costly. The high specificity of TaqMan probe is more effective for analyzing diverse pathogens and AMR-associated genes from complex environmental matrices [131]. In the past years, qPCR has been widely employed to detect different types of common pathogens in wastewater, such as *Cryptosporidium* and *Giardia*, which are the major cause of diarrheal disease in humans worldwide [132]. Shannon et al. detected thirteen diverse pathogens during five stages of wastewater treatment (i.e., raw wastewater, primary effluents, mixed liquor, waste activated sludge and final effluents) [131]. Surprisingly, some of pathogens including *K. pneumoniae, E. coli, E. faecalis*, and *C. perfringens* were detected throughout the wastewater treatment process, confirming the risk of exposure to these stubborn pathogens. In addition, Calderón-Franco et al. investigated the ARGs harbored in intracellular and extracellular DNA in wastewater environments using qPCR, raising concerns about the spread of ARGs via extracellular DNA during the wastewater treatment progress [128].

Reverse-transcriptase quantitative PCR (RT-qPCR) is an extension of qPCR technology. It has been demonstrated as an excellent method for detecting RNA viruses (e.g., SARS-CoV-2 and norovirus) pollution in wastewater [133,134]. During the Covid-19 pandemic, RT-qPCR has been widely used to detect SARS-CoV-2 in both human and environmental samples such as wastewater [135,136]. Medema et al. found that the RNA signals of SARS-CoV-2 detected by RT-qPCR in wastewater correlated significantly with the prevalence of COVID-19 [135]. The surveillance of SARS-CoV-2 RNA concentrations at the inlet and outlet of WWTPs can serve to early warn virus circulation in the population. Due to the low concentration of viruses in the environment, concentration steps such as ultrafiltration, polyethylene glycol precipitation, and electronegative membrane adsorption, are normally needed prior to direct RNA extraction. Droplet digital PCR (ddPCR) is another new sensitive method for nucleic acid detection based on Poisson distribution statistics by fractionating DNA/RNA samples into tens of thousands of nanodroplets for PCR amplification within each individual droplet. Compared to traditional qPCR, the benefits of ddPCR include absolute quantification of target DNA copies without the requirement of running standard curves, reduced PCR bias by removing the amplification efficiency reliance of qPCR, increased signal-to-noise ratio allowing for sensitive and precise detection of rare targets due to the highly diluted nucleic acid templates and effective amplification in droplet [137]. ddPCR is a sensitive tool for target DNA measurement, microbial quantification, and viral load analysis. It has been applied to detect and quantify different pathogens in wastewater, such as norovirus, adenovirus, Shiga toxin-producing *E. coli* [126,138], and the abundance of different types of ARGs encoding resistance to tetracycline, sulfonamide, and macrolides [139]. The high sensitivity and precision of ddPCR enabled the quantification of viruses with low abundance in graywater that is hard for traditional qPCR.

In addition to PCR-based DNA measurement technology, LAMP is also a simple, rapid, specific, and cost-effective nucleic acid measurement technology. Different from PCR, LAMP can complete amplification at a constant temperature (60–65 °C) without thermal cycler [140]. This advantage endows it great ability for field testing by using a paper-based device or microfluidic chip [127,141]. LAMP has been widely used for the rapid detection of bacterial DNA of foodborne, airborne, and waterborne pathogens, such as *Pseudomonas aeruginosa*, *Legionella pneumohila*, *Vibrio parahemolyticus*, and *Vibrio cholerae* [127,142,143]. Recently, LAMP was used for the rapid and on-site detection of RNA viruses (e.g., Avian Influenza Virus) by combining with reverse transcription [144]. Furthermore, LAMP can rapidly detect ARGs [145,146], and the results showed a 98.2% agreement with those detected by qPCR assays. Moreover, DNA-based nanosensor has also been used to specifically detect pathogens like *V. cholera*, *E. coli*, MRSA, *Aspergillus*, *Candida*, and *Bacillus subtilis* [147]. Specific genes of pathogens could interact with bioreceptor and induce changes in the DNA molecules of bioreceptor. Such changes can be converted to appropriate signals by transducer and readily detected. There are many advantages of this technology, such as excellent stability, low detection limit, user friendly, and measurement in real time. However, the toxicity of nanomaterials and difficulty in sensor regeneration limit the wide application of DNA-based nanosensors [148]. Hybridization-based method is another systematic technique for the detection of target genes (e.g., resistance genes). It can be subdivided into those based on arrays, line probe assays and fluorescent in situ hybridization (FISH) [149]. The hybridization-based method has been used for the detection of pathogens and their carried ARGs in wastewater. Santiago et al. developed a FISH method with SA23 probe and used it to detect the prevalence of *Salmonella* and antibiotic resistance in reclaimed wastewater even after UV disinfection, a great matter of public health concern [150].

3.3. High-throughput qPCR for pathogens and antimicrobial resistance analysis

With the rapid development of DNA sequencing, the number of available genomic information on pathogens and AMR has been growing rapidly. However, a comprehensive surveillance of the great variety of pathogens and ARGs in environments by conventional qPCR is quite time consuming, cost ineffective and burdensome [151,152]. Thus, many previous works using conventional qPCR only targeted a small fraction of pathogens and ARGs in environmental matrices [153,154], rendering the results not broadly representative. It is essential to simultaneously quantify the abundance of a large number of pathogens and ARGs for a comprehensive evaluation of the contamination level and health risk.

The recently developed high-throughput qPCR (HT-qPCR), which is capable of detecting PCR reactions of hundreds of different primers targeting almost all major classes of ARGs and pathogens in one chip, is becoming more and more popular for environmental surveillance [151,155]. HT-qPCR has been used to quantify the antibiotic resistome of a wide variety of environmental matrices, including soil, sediment, wastewater, drinking water, freshwater, air, snow, plant, food, etc [20,156–160]. Wastewater plays an important role in accumulating, exchanging, and disseminating pathogens and AMR in the environments [153]. By using HT-qPCR, previous studies have found that the abundance of ARGs in wastewater cannot be completely removed after WWTPs treatment, instead, some of them may persist or even increase in effluent [161–163]. Mao et al. found that the absolute abundance of ARGs reduced by 89.0%–99.8% from the raw influent to the effluent, but considerable ARG levels from $(1.0 \pm 0.2) \times 10^3$ to $(9.5 \pm 1.8) \times 10^5$ copies/mL can still be detected in the effluents of WWTPs [161]. An et al. further evaluated ARGs dynamics using HT-qPCR during wastewater treatment process from a broad range of 11 WWTPs in China [19]. They found that the abundance and diversity of ARGs were significantly reduced after wastewater treatment process, while seven ARGs mainly conferring resistance to aminoglycosides and beta-lactams persisted in all WWTPs after treatments. Yu et al. detected 261 ARGs in wastewater using HT-qPCR, including those conferring resistance to multidrug, beta-lactams, aminoglycoside, tetracycline, marcrolide-lincosamide-streptogramin B (MLSB) [164], and found that extracellular ARGs as an important source were transferred from WWTPs to the receiving environment.

Based on the idea of HT-qPCR detection of antibiotic resistome, researchers further designed primers specifically targeting virulence factor of pathogens and fecal markers to achieve a HT-qPCR detection. An et al. firstly established and employed HT-qPCR to simultaneously quantify 68 marker genes of 33 human pathogens and 23 fecal markers of 10 hosts in marine recreational water samples in Xiamen, China [151]. The established HT-qPCR assay covered many common pathogens, including *S. aureus*, *Salmonella*, *Klebsiella pneumoniae*, *Legionella, Helicobacter pylori*, *V. cholera*, and *P. aeruginosa*, many of which have been listed as priority pathogens by WHO [165]. Before the advent of HT-qPCR, identification of multiple pathogens in the environment is commonly made using metagenomic sequencing. Although the sequencing technology has the high-throughput ability for pathogens surveillance, quantitative information of target pathogens is difficult to obtain. In addition, 16S rRNA gene-based sequencing cannot recognize the genes encoding the pathogenicity of microorganisms [166]. By comparison, HT-qPCR can more accurately identify pathogens based on the genes encoding virulence factor. Moreover, the source of pathogens in environments can be identified by monitoring the marker genes specific for mammal hosts from different samples. Therefore, HT-qPCR has a great potential to be employed for an extensive surveillance of pathogens, antibiotic resistance and even their origins in wastewater.

Although HT-qPCR is useful for the detection of a large numbers of ARGs and pathogens, there are also some limitations. Firstly, HT-qPCR depends on the known genes for primer design, thus the unknown ARGs and virulence factors in environments cannot be detected by this method [167]. The target gene pool still needs to be expanded and improved constantly; Secondly, the uniform amplification conditions of HT-qPCR throughout the assay may not be the optimal conditions for each gene [168]; Thirdly, like other molecular methods, it is unable to differentiate viable cells, dead cells and extracellular genes of the detected pathogens, thus the actual health risk to humans is unknown [155]. Advancements in metagenomic/single-cell genomic sequencing and their integration with phenotypic characterization may provide a good solution.

3.4. Metagenomic sequencing of pathogens and antimicrobial resistance

Genomic sequencing, especially metagenomic sequencing, is being increasingly used for pathogens and AMR surveillance. Different from PCR that only targets selected genes, metagenomic sequencing offers a vast information and highest resolution of all known genes by sequencing the whole genome of all microbes inhabiting in environments [169–171]. It thus can not only reveal microbial community structure but also its functional genes such as ARGs, MGEs, virulence factor and their microbial hosts in complex environments, such as wastewater [125,172].

With more and more people living in urban areas, an increasing proportion of population is connected to sewer systems [173]. Metagenomic sequencing of urban sewage has been employed for global monitoring of antibiotic resistance in 60 countries across 6 continents including Europe, North-America, Oceania, Africa, Asia, South-America [174]. The diversity and abundance of ARGs were found to vary greatly by continents. This work also proposed the use of metagenomic analysis of sewage as an economically affordable and ethically acceptable option for a continuous surveillance of AMR, including resource-poor countries [174]. It was also employed to study urban sewage samples across 32 WWTPs within 17 Chinese cities. Extensively shared resistome was observed in Chinese urban sewage samples, and the shared resistome was strongly associated with human gut microbiota, demonstrating the potentially strong interaction between human health and urban health [18]. Particularly, hospital wastewater represents a collection of fecal matter of patients receiving antibiotic treatment, thus contributing more to AMR and posing a great exposure risk to people than other sources [175]. A study in Israel using shotgun metagenomics revealed diverse ARGs conferring resistance to antibiotics of high clinical relevance in the hospital wastewater [176]. In another working using Illumina HiSeq-based shotgun metagenomics, novel ARGs encoding carbapenemases not yet found in clinical strains were identified, indicating that hospital wastewater served as a reservoir of novel ARGs [177].

The problem of antibiotic-resistant infections is exacerbated by the dissemination of ARGs via HGT. WWTPs are recognized as hotspots for HGT of ARGs. Metagenomics revealed that ARGs carried by MGEs such as class 1 integrons and plasmids were widely found in wastewater [20,177]. The co-occurrence of pathogens and resistance genes further increased the opportunities of unwanted HGT events [177]. It was also employed to study ARGs in bacteriophages. A higher prevalence of ARGs on phage DNA than bacterial DNA was revealed in hospital wastewater, indicating the role of bacteriophages as potential vehicles for horizontal transfer of ARGs [178]. In addition, dissemination of ARGs between humans and the environments they inhabit is a public health priority. By using metagenomic and 16S sequencing, Pehrsson et al. investigated the resistance exchange network and microbial community between interconnected human fecal and environmental microbiota from two low-income human habitats in Latin America. They identified MGEs-associated key ARGs across habitats which were determined to be generally structured by bacterial phylogeny [179]. This work lays the foundation for surveillance of resistance gene dissemination across interconnected habitats [179].

WWTPs are fundamental infrastructures to reduce chemical, physical, and microbial contaminants in wastewater and improve the water quality of effluent [6], and are thus called the city's gut. However, the treated sewage and sludge still bear potential risks of spreading antibiotic resistance and pathogenicity [180]. Metagenomic analysis was employed to evaluate the treatment efficiency of WWTPs in removing ARGs. For example, Christgen et al. revealed that anaerobic–aerobic sequence bioreactor was effective in reducing the abundance of aminoglycoside, tetracycline, and β-lactam ARGs [181]. However, the abundance of sulfonamide and chloramphenicol ARGs were largely unaffected by treatment. Ju et al. compared the removal versus enrichment of ARGs and pathogens in municipal sewage sludge digesters. The results showed that ARGs and pathogens could not be removed by anaerobic digestion, and ARGs conferring multidrug resistance tended to co-occur with pathogens [182].

In addition to the distribution and composition of ARGs in wastewater, it is also important to elucidate the genetic location (e.g., MGEs or chromosome) and microbial hosts for an in-depth understanding of the dynamic spread of ARGs and ARB in wastewater. However, the short reads from Illumina-based metagenomic sequencing and the presence of numerous insertion sequences and repetitive elements in MGEs hamper the effective assembly of short reads and understanding of the genetic context of ARGs. This problem can be resolved by using long read sequencing that spans most insertion and repetitive sequences [183]. Che et al. combined long read Oxford Nanopore and metagenomic sequencing to uncover the resistome context and track the host of ARGs in WWTPs. Most of ARGs in WWTPs were found to be carried by plasmids, and a higher prevalence of transposons and integrons were detected on plasmids than chromosome, indicating a potentially important role of these MGEs in promoting the spread of ARGs in wastewater [20].

Metagenomic sequencing was also used to explore the metagenome of viral pathogens. Over 200 known viruses and 51 novel viruses were identified in untreated wastewater [184]. Reclaimed

water, the end-product of wastewater treatment and an important alternative urban water supply [185], was found to contain 1000-fold more viruses than drinking water [186]. Viral metagenomics was applied to sewage sludge from 5 large WWTPs in U.S. each serving a population between 100,000 and 1,000,000. A total of 43 different types of human viruses were identified in sewage sludge, including newly emerging viruses (Klassevirus, Coronavirus HKU1 and Cosavirus) associated with respiration and Enteroviruses [187]. Viral metagenomics was also used to obtain RNA virosphere in the wastewater from Santiago in South America. The results showed that 88% of sequences from RNA virosphere were unknown, while known viral sequences were mainly from viruses infecting bacteria, invertebrates and humans [125]. Obviously, sewage viral metagenomics is useful for public health surveillance.

Metagenomic analysis can be also combined with metatranscriptomic analysis to comprehensively reveal the prevalence and expression of ARGs. A total of 330 ARGs conferring resistance to 21 antibiotic classes were detected in the gut microbiome of human and animal, but only around half of them were transcriptionally active [188]. By applying metagenomic and metatranscriptomic analysis in activated sludge from three WWTPs in Taiwan, most of ARGs were found to be actively transcribed, and their transcript abundance was correlated with ARGs abundance. Meanwhile, plasmid-associated ARGs were found to display a higher transcription probability than ARGs in chromosomes [189].

In addition to the above sequence-based metagenomics, functional metagenomics, which screens expressed ARGs from metagenomic clones in microbial hosts on media with target antibiotics [190], provides a way to identify completely novel ARGs. By using this technology, a novel tetracycline resistance gene was identified in pig manure [191]. Functional screening of plasmid and small-insert libraries identified 80 ARGs, one of which was discovered as a novel clade of chloramphenicol acetyltransferases. By combining functional metagenomic with long read sequencing technology, a broad resource for understanding the dispersal of ARGs was provided in agriculture and clinical settings [192].

3.5. Single-cell genomic sequencing of pathogens and antimicrobial resistance

Single-cell sequencing can provide insights into genomics by deciphering the contribution of individual cells. It advances rapidly and produces some new discoveries that bulk metagenomics fails to achieve in recent years. For instances, single-cell sequencing is a high-resolution approach enabling study of rare but important species (bacteria, virus, protist) or event (HGT or mutation) that may present a lower copy number than what metagenomic sequencing can resolve. It may also distinguish high homology sequencing derived from divergent species that metagenomics fail to do [193], or identify cell-by-cell genomic variation to enable evolutionary analysis of population. It also provides a potentially unambiguous way to link ARGs, MGEs, virulence factor with microbial hosts. When combined with targeted sorting of bacteria with phenotypic resistance, single-cell sequencing can potentially identify novel ARGs encoding known or unknown resistance mechanisms from the large majority of uncultivated ARB in environment.

To obtain single-cell genomes, efficient isolation and sorting of individual cells is the first critical step. The presently developed sorting techniques include gradient dilution based on Poisson distribution [194], optical tweezers [65,195], laser ejection and also high-throughput methods such as flow cytometry and microfluidic devices capable of droplet generation [196–199] When combining these sorting techniques with fluorescence or Raman spectroscopy, targeted sorting of cells of interested based on the specific fluorescence or Raman signals can be achieved. Another critical step is multiple displacement amplification (MDA) which is now a gold standard for amplification of single genomes to generate enough genomic template for direct sequencing or a further PCR amplification [200,201].

Presently, single-cell genome sequencing has been employed in bacteria, virus, and protist. Some new insights, especially into novel or rare taxa and genes that lacked representation in the known sequence databases, were provided. The feasibility of single-cell genomic sequencing of low-abundance microorganisms in complex microbial communities has been validated [202]. Liu et al. developed a novel microfluidic-based microdroplet platform to isolate ARB and detected novel single-nucleotide polymorphisms of *fusA* in a mutant [203]. In addition, an agarose droplet–based microfluidic emulsion PCR was developed to detect rare pathogenic *E. coli* O157:H7 from 100,000 of normal cells [198]. Recently, Lan et al. developed an ultrahigh-throughput droplet microfluidics approach to capture single cells and barcode individual genomes to enable a sequencing pool of thousands of tagged genomes. This approach has been applied in marine samples to link ARGs to their host genera [199]. Single-cell genomic sequencing has also been applied in eukaryotes studies. Single-celled protists from Tara Oceans were obtained by flow cytometric sorting. The results indicated that the genotype of uncultured stramenopiles showed a specific spatial distribution and heterotrophic marine protists performed diverse functions in ocean [204].

Single-cell genomics was further applied in viruses. Viruses are the most diverse biological entities in earth, however, traditional methods in virosome depend on cultivable virus-host systems. Using *E. coli* phage as a model, viral particles were sorted through flow cytometry. The sorted viruses were amplified through MDA and the amplified products were then used for sequencing [205]. By applying single-cell genomics and metagenomics to a hot spring mat, host–virus interactions based on DNA sequence were predicted. It was found that 26% of single cells contained at least one viral contigs, and the copy number of viral genomes was lower compared to their hosts, indicating that lysogeny is the predominant viral lifestyle in low mobility environments [197].

Single-cell RNA sequencing (scRNA-seq) is the future of genome biology [206]. Transcriptome is a crucial constituent for the maintenance of bacteria identity which is highly heterogeneous even in isogenic populations [207]. ScRNA-seq for eukaryotic cells has been well developed and can provide a comprehensive gene expression heterogeneity profile for tens of thousands of cells. For example, using a high throughput scRNA-seq, cells in Arabidopsis were found to be highly heterogeneous during root cell differentiation [208]. However, adapting scRNA-seq to microbes is highly challenging because bacteria have extremely lower mRNA content than eukaryotic cells. Recently, split-pool ligation-based transcriptome sequencing has enabled a high-throughput characterization of bacterial gene expression. Using this method, cell states at different growth phases were discriminated, and a rare cell subpopulation with prophage induction was revealed [209,210]. It is anticipated that scRNA-seq contributes to reveal the underlying gene expression associated with antibiotic resistance.

Overall, there are still technical challenges associated with single-cell genomic sequencing. DNA contamination is a challenging unresolved problem when sequencing and assembling genomes after MDA. Methods have been reported for removing irrelevant DNA from the isolated single cells by repetitive cell sorting [211]. Setting a strict control treatment was also an effective way to remove contamination induced by MDA reagents [212]. In addition, because amplification bias such as over-representation in one MDA reaction often occurs [213], reducing the reaction volume of MDA has been adopted to reduce the amplification bias [214].

Melting and limited re-annealing of MDA products was also used to reduce the representation of abundant sequences [211].

4. Integration of phenotypic and genotypic analysis

Despite extensive sequencing-based characterization of microbiomes, there remains a dramatic lack of understanding of microbial functions, because most of functional genes and whether they can be expressed are unknow. For example, only 30−60% of genes in human microbiome gene catalog can be annotated [215]. The lack of understanding of bacterial functions and the underlying genetic mechanisms calls for the integration of phenotypic with genotypic analysis. SIP is a powerful tool for exploiting the ecophysiology and functions of microbiomes based on the assimilation of isotopes (C, N, O, H, S) into newly synthesized biomass [44,216,217]. It not only allows in situ study of specific physiological activities of microorganisms without prior knowledge of genetic basis [218], but also provides a way to distinguish functional bacteria incorporating isotopes without knowing the bacterial metabolic process [217].

DNA-SIP is a bulk approach that can identify bacterial population assimilating stable isotopes by separating SIP-labeled nucleic acids based on their different densities. The selected fractions can be analyzed through gene sequencing to reveal the phylogenetic and functional gene composition of the associated microbial population. Ouyang et al. applied DNA-SIP to investigate sulfamethoxazole-degrading bacteria in soil microcosms, and *Actinobacteria* was determined as a crucial player in yet-uncultivated indigenous bacteria for antibiotics degradation [219]. Although DNA-SIP approach has been frequently performed, there are few studies of combining SIP with metabolomics, so this is an exciting area for future research [220].

Recently, SIP approach is also developed to combine with single-cell Raman-activated cell sorting to achieve a high-resolution linking of phenotypic and genotypic analysis. The workflow is shown in Fig. 7. As Raman measurement is nondestructive, bacteria of interest are allowed for subsequent single-cell sorting and molecular analysis [115]. For example, by incubating colonic microbiota with different mucosal O-glycan monosaccharides in the presence of D_2O, members of the underexplored family Muribaculaceae, Lachnospiraceae, Rikenellaceae and Bacteroidaceae were revealed as the major mucin monosaccharide foragers by sequencing of sorted cells. Based on this information, a five-member consortium using the above bacteria were built to impede the growth of intestinal pathogen *Clostridioides difficile* [221]. Under antibiotics treatment, single-cell Raman-D_2O can be used to discriminate phenotypically antibiotic resistant and susceptible bacteria based on their metabolic activity. This approach has been applied to identify ARB in river, and Raman activated cell ejection was applied to isolate single ARB for the first time. Potential human pathogens were identified in the sorted cells by 16s RNA sequencing [119]. Wang et al. used Raman-D_2O to detect ARB in human gut [53]. To establish the link between phenotypes and genotypes of the ARB, tens of antibiotic active cells were sorted and the mini-metagenomic DNA of the sorted bacteria was amplified and sequenced. The sorted ARB and their associated ARGs were identified. This study revealed that bacterial genotype did not necessarily lead to resistance phenotype due to the lack of gene expression [53]. Recently, Raman-activated gravity-driven single-cell encapsulation and sequencing (RAGE-Seq) was developed to provide a high coverage of genome assemblies. RAGE-Seq has successfully unraveled genome-wide antibiotic resistance mutation map of single bacteria from urine [222]. Hence, it is important

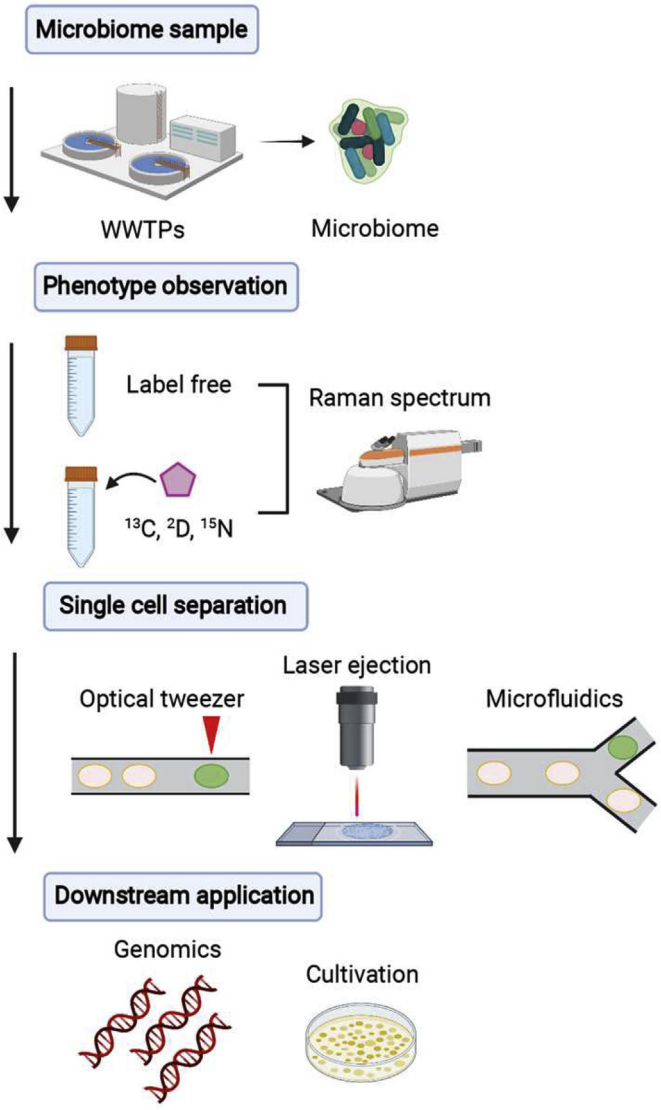

Fig. 7. Workflow for integration of phenotypic (Raman) and genotypic (molecular methods) analysis to study microorganisms. Microbial samples obtained from WWTPs were subjected to Raman detection (label free or SIP) for phenotypic characterization. After identifying a cell of interest, the target cells were sorted by optical tweezers, laser ejection or microfluidics. The sorted cells were then committed to downstream studies such as genome sequencing and cultivation.

to study both genotype and phenotype to uncover the functionality of ARB. These examples highlighted how key microorganisms with specific metabolisms of interest can be identified without prior knowledge of genetic basis underlying the functional ability. Therefore, single-cell Raman SIP-activated soring opens a new window to study ARB, especially the underexplored large majority of unculured ARB in environments.

Moreover, Raman spectroscopy has the potential to identify the species and antibiotic resistance of bacteria without SIP. Different bacterial phenotypes can be characterized by the intrinsic molecular compositions of individual cell, leading to subtle difference among bacterial Raman spectra. By training a classification model via machine learning such as convolutional neural network, Ho et al. distinguished the methicillin-resistant and -susceptible isolates of *S. aureus*. Antibiotic resistance and susceptibility diagnosis

of clinical isolates was further achieved at accuracies of 65% in clinical samples [63]. Raman-based bacterial classification was also used to identify pathogens in water and food in spiked culture systems [69,95], so it is a potentially powerful way to integrate Raman classification and genotypic sequencing for a further in-depth understanding of pathogens and AMR.

5. Conclusions and future outlook

With population and urbanization expanding, pathogens and AMR are emerging as the major threat to public health, which is true during the current pandemic. Wastewater, as the collector and spreader of pathogen and AMR, is being increasingly discharged with the intensifying human activities. It can be anticipated that wastewater and WBE will play a more important role than ever before for monitoring and early warning of public health. Here, by pinpointing the current two major global health threats of pathogens (especially bacteria and virus) and AMR, we reviewed the most recent technological advancements in Raman biosensor and molecular methods for phenotypically and genotypically surveilling of pathogens and AMR respectively, and also their integration for a more holistic understanding of health risk.

In the future, technological breakthrough will determine the depth and width that WBS can achieve, especially those previously unachievable new insights into public health. Moreover, methods standardization and surveillance range are also critical for indicating the regional and global-scale public health. Here we put forward some perspectives on technological development aiming to enhance wastewater surveillance capacity to early and better inform tackling strategy.

1) **Multiplex phenotypic and genotypic surveillance**

Important health risk-associated microbial phenotypes can be antibiotic resistance, physiological states (dead, dominant, VBNC, persistent, active), and pathogenicity. The genotypes can be genome, resistome, mobilome, transcriptome and proteome. There is a great necessity for multiplex phenotypic and genotypic surveillance. As such, not only health risks but also the spread risks and the underlying mechanisms can be illustrated. The resulting comprehensive information is highly valuable for a more realistic and in-depth risk evaluation and predication. For example, some bacteria harboring multiple ARGs may not be a superbug, but these ARGs, especially those located on MGEs (mobilome), still hold great potentials to disseminate to other species which may become a superbug phenotypically resistant to nearly all antibiotics. In this case, it is important to know the mechanism accounting for the species with expression of ARGs (transcriptome).

To integrate phenome and genome profiling, single cell-based techniques such as single-cell Raman activated sorting and sequencing techniques are highly promising. Presently, linkage of microbial phenotypes detected by Raman with genotypes of single (single-cell amplified genome) or multiple targeted cells (mini-metagenome) has been developed and applied in some environmental communities. However, this technique has not been developed to a point that it can be most effective. The successful applications were still very limited. Technique advances to obtain high-quality DNA amplified from single targeted cells and improve the throughput and automatic level are urgently needed for its future robust applications. In comparison with single-cell DNA sequencing (genome), single-cell RNA (transcriptome) and protein (proteome) sequencing are more challenging due to the much lower concentration of mRNA or protein than DNA. Integration of single-cell Raman with multiomics is currently unachievable, but worth great efforts to solve technical hurdles toward their application in the complex wastewater matrix.

2) **Rapid and automated online surveillance system**

We have to respond faster. This is an urgent need because bacteria/virus and AMR are disseminating at an unprecedented speed and scale with the increasing waste disposal, more frequent global travel and trades. Rapid and real time monitoring is particularly important to signal the occurrence for early warning and track the dissemination trend to guide timely intervention strategy. Despite the advantages of Raman spectroscopy for a rapid cultivation-free identification of pathogens and AMR, combination with automatic sampling, detection, advanced data processing and output such as deep learning, are all needed to achieve real time monitoring. For molecular methods, laboratory-based assays are not enough for rapid and online surveillance, instead, miniaturized and portable devices, such as nanopore sequencing and loop-mediated isothermal amplification-based paper biosensors, show great promise for rapid, on-site and sensitive monitoring of pathogen and AMR. Moreover, they are low-cost and easily operated, endowing them great potentials to be popularized in more countries including low and middle-income countries.

3) **Standardization of surveillance methods**

Different laboratories are using their own methods (protocols, bioinformatics algorithms) and database to identify pathogens and AMR. To obtain comparable results, it is important to set standardization for both phenotypic and genotypic methods for pathogens and AMR monitoring. For this to become possible, methods and database should be better curated to improve the consistence, quality and availability of surveillance data. This should need international organization such as WHO to coordinate.

4) **Extension of surveillance to a broad range of pathogens and AMR**

The current surveillance of pathogens is usually confined to a very limited number of microbial indicators. It is not enough to cope with the rising health risks. It is suggested to extend the range to more medically or health relevant species, such as the antibiotic-resistant priority pathogens listed by WHO, and infectious diseases-causing virus, especially those without efficient vaccine. For AMR monitoring, it is suggested to extend from commonly consumed antibiotics to last resort or newly introduced antibiotics to determine the spread speed of these less abundant resistance.

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.

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References

[1] M. May, Tomorrow's biggest microbial threats, Nat. Med. 27 (2021) 358–359. https://doi.org/10.1038/s41591-021-01341-6.

[2] N. Sims, B. Kasprzyk-Hordern, Future perspectives of wastewater-based epidemiology: monitoring infectious disease spread and resistance to the community level, Environ. Int. 139 (2020) 105689. https://doi.org/10.1016/j.envint.2020.105689.

[3] J. O'Neill, Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations, Welcome Trust; HM Government, London, 2014.

[4] World Bank, Global Economic Prospects, 2021. https://openknowledge.worldbank.org/handle/10986/34710.

[5] World Health Organization, Ten Threats to Global Health in 2019, 2019. https://www.who.int/news-room/spotlight/ten-threats-to-global-health-in-2019.

[6] A. Karkman, T.T. Do, F. Walsh, M.P.J. Virta, Antibiotic-resistance genes in waste water, Trends Microbiol. 26 (2018) 220–228. https://doi.org/10.1016/j.tim.2017.09.005.

[7] D.A. Larsen, K.R. Wigginton, Tracking COVID-19 with wastewater, Nat. Biotechnol. 38 (2020) 1151–1153. https://doi.org/10.1038/s41587-020-0690-1.

[8] A. Zahedi, P. Monis, D. Deere, U. Ryan, Wastewater-based epidemiology-surveillance and early detection of waterborne pathogens with a focus on SARS-CoV-2, Cryptosporidium and Giardia, Parasitol. Res. (2021). https://doi.org/10.1007/s00436-020-07023-5.

[9] P.M. Choi, B.J. Tscharke, E. Donner, J.W. O'Brien, S.C. Grant, S.L. Kaserzon, R. Mackie, E. O'Malley, N.D. Crosbie, K.V. Thomas, J.F. Mueller, Wastewater-based epidemiology biomarkers: past, present and future, TrAC Trends Anal. Chem. (Reference Ed.) 105 (2018) 453–469. https://doi.org/10.1016/j.trac.2018.06.004.

[10] L.W. Wu, D.L. Ning, B. Zhang, Y. Li, P. Zhang, X.Y. Shan, Q.T. Zhang, M. Brown, Z.X. Li, J.D. Van Nostrand, F.Q. Ling, N.J. Xiao, Y. Zhang, J. Vierheilig, G.F. Wells, Y.F. Yang, Y. Deng, Q.C. Tu, A.J. Wang, T. Zhang, Z.L. He, J. Keller, P.H. Nielsen, P.J.J. Alvarez, C.S. Criddle, M. Wagner, J.M. Tiedje, Q. He, T.P. Curtis, D.A. Stahl, L. Alvarez-Cohen, B.E. Rittmann, X.H. Wen, J.Z. Zhou, D. Acevedo, M. Agullo-Barcelo, G.L. Andersen, J.C. de Araujo, K. Boehnke, P. Bond, C.B. Bott, P. Bovio, R.K. Brewster, F. Bux, A. Cabezas, L. Cabrol, S. Chen, C. Etchebehere, A. Ford, D. Frigon, J.S. Gomez, J.S. Griffin, A.Z. Gu, M. Habagil, L. Hale, S.D. Hardeman, M. Harmon, H. Horn, Z.Q. Hu, S. Jauffur, D.R. Johnson, A. Keucken, S. Kumari, C.D. Leal, L.A. Lebrun, J. Lee, M. Lee, Z.M.P. Lee, M.Y. Li, X. Li, Y. Liu, R.G. Luthy, L.C. Mendonca-Hagler, F.G.R. de Menezes, A.J. Meyers, A. Mohebbi, A. Oehmen, A. Palmer, P. Parameswaran, J. Park, D. Patsch, V. Reginatto, F.L. de los Reyes, A.N. Robles, S. Rossetti, J. Sidhu, W.T. Sloan, K. Smith, O.V. de Sousa, K. Stephens, R.M. Tian, N.B. Tooker, D.D. Vasconcelos, S. Wakelin, B. Wang, J.E. Weaver, S. West, P. Wilmes, S.G. Woo, J.H. Wu, L.Y. Wu, C.W. Xi, M.Y. Xu, T. Yan, M. Yang, M. Young, H.W. Yue, Q. Zhang, W. Zhang, Y. Zhang, H.D. Zhou, M. Brown, G.W.M. Consortium, Global diversity and biogeography of bacterial communities in wastewater treatment plants, Nat. Microbiol. 4 (2019) 1183–1195. https://doi.org/10.1038/s41564-019-0426-5.

[11] World Health Organisation, Disease Outbreaks by Year, 2020. https://www.who.int/emergencies/disease-outbreak-news.

[12] World Health Organisation, Antimicrobial Resistance: No Action Today, No Cure Tomorrow, 2011. https://www.who.int/director-general/speeches/detail/antimicrobial-resistance-no-action-today-no-cure-tomorrow.

[13] World Health Organization, Global Priority List of Antibiotic Resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics, 2017. https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed.

[14] K.K. Kumarasamy, M.A. Toleman, T.R. Walsh, J. Bagaria, F. Butt, R. Balakrishnan, U. Chaudhary, M. Doumith, C.G. Giske, S. Irfan, P. Krishnan, A.V. Kumar, S. Maharjan, S. Mushtaq, T. Noorie, D.L. Paterson, A. Pearson, C. Perry, R. Pike, B. Rao, U. Ray, J.B. Sarma, M. Sharma, E. Sheridan, M.A. Thirunarayan, J. Turton, S. Upadhyay, M. Warner, W. Welfare, D.M. Livermore, N. Woodford, Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study, Lancet Infect. Dis. 10 (2010) 597–602. https://doi.org/10.1016/S1473-3099(10)70143-2.

[15] Y.Y. Liu, Y. Wang, T.R. Walsh, L.X. Yi, R. Zhang, J. Spencer, Y. Doi, G. Tian, B. Dong, X. Huang, L.F. Yu, D. Gu, H. Ren, X. Chen, L. Lv, D. He, H. Zhou, Z. Liang, J.H. Liu, J. Shen, Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study, Lancet Infect. Dis. 16 (2015) 161–168. https://doi.org/10.1016/S1473-3099(15)00424-7.

[16] World Health Organisation, Global Action Plan on Antimicrobial Resistance, 2015. https://www.who.int/antimicrobial-resistance/global-action-plan/en/.

[17] World Health Organisation, Global Antimicrobial Resistance Surveillance System (GLASS), 2015. https://www.who.int/glass/en/.

[18] J.Q. Su, X.L. An, B. Li, Q.L. Chen, M.R. Gillings, H. Chen, T. Zhang, Y.G. Zhu, Metagenomics of urban sewage identifies an extensively shared antibiotic resistome in China, Microbiome 5 (2017) 84. https://doi.org/10.1186/s40168-017-0298-y.

[19] X.L. An, J.Q. Su, B. Li, W.Y. Ouyang, Y. Zhao, Q.L. Chen, L. Cui, H. Chen, M.R. Gillings, T. Zhang, Y.G. Zhu, Tracking antibiotic resistome during wastewater treatment using high throughput quantitative PCR, Environ. Int. 117 (2018) 146–153. https://doi.org/10.1016/j.envint.2018.05.011.

[20] Y. Che, Y. Xia, L. Liu, A.D. Li, Y. Yang, T. Zhang, Mobile antibiotic resistome in wastewater treatment plants revealed by Nanopore metagenomic sequencing, Microbiome 7 (2019) 44. https://doi.org/10.1186/s40168-019-0663-0.

[21] M. Munir, K. Wong, I. Xagoraraki, Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan, Water Res. 45 (2011) 681–693. https://doi.org/10.1016/j.watres.2010.08.033.

[22] E. Wistrand-Yuen, M. Knopp, K. Hjort, S. Koskiniemi, O.G. Berg, D.I. Andersson, Evolution of high-level resistance during low-level antibiotic exposure, Nat. Commun. 9 (2018) 1599. https://doi.org/10.1038/s41467-018-04059-1.

[23] L. Cui, Y.-J. Zhang, W.E. Huang, B.-F. Zhang, F.L. Martin, J.Y. Li, K.-S. Zhang, Y.-G. Zhu, Surface-enhanced Raman spectroscopy for identification of heavy metal arsenic(V)-mediated enhancing effect on antibiotic resistance, Anal. Chem. 88 (2016) 3164–3170. https://doi.org/10.1021/acs.analchem.5b04490.

[24] Y. Xing, S. Wu, Y. Men, Exposure to environmental levels of pesticides stimulates and diversifies evolution in Escherichia coli toward higher antibiotic resistance, Environ. Sci. Technol. 14 (2020) 8770–8778. https://doi.org/10.1021/acs.est.0c01155.

[25] A. Di Cesare, E.M. Eckert, S. D'Urso, R. Bertoni, D.C. Gillan, R. Wattiez, G. Corno, Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants, Water Res. 94 (2016) 208–214. https://doi.org/10.1016/j.watres.2016.02.049.

[26] J. Botelho, H. Schulenburg, The role of integrative and conjugative elements in antibiotic resistance evolution, Trends Microbiol. 29 (2021) 8–18. https://doi.org/10.1016/j.tim.2020.05.011.

[27] J. Li, T. Wang, B. Shao, J.Z. Shen, S.C. Wang, Y.N. Wu, Plasmid-mediated quinolone resistance genes and antibiotic residues in wastewater and soil adjacent to swine feedlots: potential transfer to agricultural lands, Environ. Health Perspect. 120 (2012) 1144–1149. https://doi.org/10.1289/ehp.1104776.

[28] Y.G. Zhu, M. Gillings, P. Simonet, D. Stekel, S. Banwart, J. Penuelas, Human dissemination of genes and microorganisms in Earth's Critical Zone, Global Change Biol. 24 (2018) 1488–1499. https://doi.org/10.1111/gcb.14003.

[29] K. Lehmann, T. Bell, M.J. Bowes, G.C.A. Amos, W.H. Gaze, E.M.H. Wellington, A.C. Singer, Trace levels of sewage effluent are sufficient to increase class 1 integron prevalence in freshwater biofilms without changing the core community, Water Res. 106 (2016) 163–170. https://doi.org/10.1016/j.watres.2016.09.035.

[30] F.H. Wang, M. Qiao, J.Q. Su, Z. Chen, X. Zhou, Y.G. Zhu, High throughput profiling of antibiotic resistance genes in urban park soils with reclaimed water irrigation, Environ. Sci. Technol. 48 (2014) 9079–9085. https://doi.org/10.1021/es502615e.

[31] United Nations, 2017 UN World Water Development Report, Wastewater: the Untapped Resource, 2017. https://www.unep.org/resources/publication/2017-un-world-water-development-report-wastewater-untapped-resource.

[32] J.Q. Su, B. Wei, W.Y. Ou-Yang, F.Y. Huang, Y. Zhao, H.J. Xu, Y.G. Zhu, Antibiotic resistome and its association with bacterial communities during sewage sludge composting, Environ. Sci. Technol. 49 (2015) 7356–7363. https://doi.org/10.1021/acs.est.5b01012.

[33] P. Gerner-Smidt, J. Besser, J. Concepcion-Acevedo, J.P. Folster, J. Huffman, L.A. Joseph, Z. Kucerova, M.C. Nichols, C.A. Schwensohn, B. Tolar, Whole genome sequencing: bridging one-health surveillance of foodborne diseases, Front. Public. Health. 7 (2019) 172. https://doi.org/10.3389/Fpubh.2019.00172.

[34] One Health Global, One Health Global Network Webportal, 2015. http://www.onehealthglobal.net/what-is-one-health/.

[35] World Health Organization, GLASS Whole-Genome Sequencing for Surveillance of Antimicrobial Resistance, 2020. https://apps.who.int/iris/handle/10665/334354.

[36] M. Yang, X. Liu, Y. Luo, A.J. Pearlstein, S. Wang, H. Dillow, K. Reed, Z. Jia, A. Sharma, B. Zhou, D. Pearlstein, H. Yu, B. Zhang, Machine learning-enabled non-destructive paper chromogenic array detection of multiplexed viable pathogens on food, Nature Food 2 (2021) 110–117. https://doi.org/10.1038/s43016-021-00229-5.

[37] K. Yang, Q.-L. Chen, M.-L. Chen, H.-Z. Li, H. Liao, Q. Pu, Y.-G. Zhu, L. Cui, Temporal dynamics of antibiotic resistome in the plastisphere during microbial colonization, Environ. Sci. Technol. 54 (2020) 11322–11332. https://doi.org/10.1021/acs.est.0c04292.

[38] K.E. Wardenburg, R.F. Potter, A.W. D'Souza, T. Hussain, M.A. Wallace, S. Andleeb, C.A.D. Burnham, G. Dantas, Phenotypic and genotypic characterization of linezolid-resistant Enterococcus faecium from the USA and Pakistan, J. Antimicrob. Chemother. 74 (2019) 3445. https://doi.org/10.1093/jac/dkz367.

[39] C.V. Raman, K.S. Krishnan, A new type of secondary radiation, Nature 121 (1928) 501. https://doi.org/10.1038/121501c0.

[40] C.V. Raman, A change of wave-length in light scattering, Nature 121 (1928) 619. https://doi.org/10.1038/121619b0.

[41] M.Q. Li, D.P. Canniffe, P.J. Jackson, P.A. Davison, S. FitzGerald, M.J. Dickman, J.G. Burgess, C.N. Hunter, W.E. Huang, Rapid resonance Raman microspectroscopy to probe carbon dioxide fixation by single cells in microbial communities, ISME J. 6 (2012) 875–885. https://doi.org/10.1038/ismej.2011.150.

[42] L. Cui, K. Yang, H.Z. Li, H. Zhang, J.Q. Su, M. Paraskevaidi, F.L. Martin, B. Ren, Y.G. Zhu, Functional single-cell approach to probing nitrogen-fixing bacteria in

soil communities by resonance Raman spectroscopy with $^{15}N_2$ labeling, Anal. Chem. 90 (2018) 5082–5089. https://doi.org/10.1021/acs.analchem.7b05080.
[43] L. Cui, D. Zhang, K. Yang, X. Zhang, Y.-G. Zhu, Perspective on surface-enhanced Raman spectroscopic investigation of microbial world, Anal. Chem. 91 (2019) 15345–15354. https://doi.org/10.1021/acs.analchem.9b03996.
[44] Y. Wang, W.E. Huang, L. Cui, M. Wagner, Single cell stable isotope probing in microbiology using Raman microspectroscopy, Curr. Opin. Biotechnol. 41 (2016) 34–42. https://doi.org/10.1016/j.copbio.2016.04.018.
[45] B. Lorenz, C. Wichmann, S. Stockel, P. Rosch, J. Popp, Cultivation-free Raman spectroscopic investigations of bacteria, Trends Microbiol. 25 (2017) 413–424. https://doi.org/10.1016/j.tim.2017.01.002.
[46] H.-Z. Li, D. Zhang, K. Yang, X.-L. An, Q. Pu, S.-M. Lin, J.-Q. Su, L. Cui, Phenotypic tracking of antibiotic resistance spread via transformation from environment to clinic by reverse D_2O single-cell Raman probing, Anal. Chem. 92 (2020) 15472–15479. https://doi.org/10.1021/acs.analchem.0c03218.
[47] K. Yang, H.-Z. Li, X. Zhu, J.-Q. Su, B. Ren, Y.-G. Zhu, L. Cui, Rapid antibiotic susceptibility testing of pathogenic bacteria using heavy-water-labeled single-cell Raman spectroscopy in clinical samples, Anal. Chem. 91 (2019) 6296–6303. https://doi.org/10.1021/acs.analchem.9b01064.
[48] H.-Z. Li, Q.-f. Bi, K. Yang, B.-X. Zheng, Q. Pu, L. Cui, D_2O-Isotope-Labeling approach to probing phosphate-solubilizing bacteria in complex soil communities by single-cell Raman spectroscopy, Anal. Chem. 91 (2019) 2239–2246. https://doi.org/10.1021/acs.analchem.8b04820.
[49] L. Guo, C. Ye, L. Cui, K. Wan, S. Chen, S. Zhang, X. Yu, Population and single cell metabolic activity of UV-induced VBNC bacteria determined by CTC-FCM and D_2O-labeled Raman spectroscopy, Environ. Int. 130 (2019) 104883. https://doi.org/10.1016/j.envint.2019.05.077.
[50] L. Cui, K. Yang, Y.G. Zhu, in: Marc G. Dumont, Marcela Herna ndez García (Editors), Stable Isotope-Labeled Single-Cell Raman Spectroscopy Revealing Function and Activity of Environmental Microbes, Springer Nature, New York, 2019, pp. 95–107.
[51] O.O. Olaniyi, K. Yang, Y.-G. Zhu, L. Cui, Heavy water-labeled Raman spectroscopy reveals carboxymethylcellulose-degrading bacteria and degradation activity at the single-cell level, Appl. Microbiol. Biotechnol. 103 (2018) 1455–1464. https://doi.org/10.1007/s00253-018-9459-6.
[52] L. Cui, K. Yang, G. Zhou, W.E. Huang, Y.-G. Zhu, Surface-enhanced Raman spectroscopy combined with stable isotope probing to monitor nitrogen assimilation at both bulk and single-cell level, Anal. Chem. 89 (2017) 5793–5800. https://doi.org/10.1021/acs.analchem.6b04913.
[53] Y. Wang, J. Xu, L. Kong, B. Li, H. Li, W.E. Huang, C. Zheng, Raman-activated sorting of antibiotic-resistant bacteria in human gut microbiota, Environ. Microbiol. 22 (2020) 2613–2624. https://doi.org/10.1111/1462-2920.14962.
[54] X. Jing, H. Gou, Y. Gong, X. Su, L. Xu, Y. Ji, Y. Song, I.P. Thompson, J. Xu, W.E. Huang, Raman-activated cell sorting and metagenomic sequencing revealing carbon-fixing bacteria in the ocean, Environ. Microbiol. 20 (2018) 14268. https://doi.org/10.1111/1462-2920.14268.
[55] W.E. Huang, K. Stoecker, R. Griffiths, L. Newbold, H. Daims, A.S. Whiteley, M. Wagner, Raman-FISH: combining stable-isotope Raman spectroscopy and fluorescence in situ hybridization for the single cell analysis of identity and function, Environ. Microbiol. 9 (2007) 1878–1889. https://doi.org/10.1111/j.1462-2920.2007.01352.x.
[56] G. Azemtsop Matanfack, A. Pistiki, P. Rosch, J. Popp, Raman (18) O-labeling of bacteria in visible and deep UV-ranges, J. Biophot. 13 (2021), e202100013. https://doi.org/10.1002/jbio.202100013.
[57] J. Kelly, R. Patrick, S. Patrick, S.E.J. Bell, Surface-enhanced Raman spectroscopy for the detection of a metabolic product in the headspace above live bacterial cultures, Angew. Chem. Int. Ed. 57 (2018) 15686–15690. https://doi.org/10.1002/anie.201808185.
[58] W.R. Premasiri, J.C. Lee, A. Sauer-Budge, R. Theberge, C.E. Costello, L.D. Ziegler, The biochemical origins of the surface-enhanced Raman spectra of bacteria: a metabolomics profiling by SERS, Anal. Bioanal. Chem. 408 (2016) 4631–4647. https://doi.org/10.1007/s00216-016-9540-x.
[59] G. Bodelón, V. Montes-García, V. López-Puente, E.H. Hill, C. Hamon, M.N. Sanz-Ortiz, S. Rodal-Cedeira, C. Costas, S. Celiksoy, I. Pérez-Juste, L. Scarabelli, A. La Porta, J. Pérez-Juste, I. Pastoriza-Santos, L.M. Liz-Marzán, Detection and imaging of quorum sensing in Pseudomonas aeruginosa biofilm communities by surface-enhanced resonance Raman scattering, Nat. Mater. 15 (2016) 1203–1211. https://doi.org/10.1038/nmat4720.
[60] J. Milucka, T.G. Ferdelman, L. Polerecky, D. Franzke, G. Wegener, M. Schmid, I. Lieberwirth, M. Wagner, F. Widdel, M.M.M. Kuypers, Zero-valent sulphur is a key intermediate in marine methane oxidation, Nature 491 (2012) 541. https://doi.org/10.1038/nature11656.
[61] W.E. Huang, R.I. Griffiths, I.P. Thompson, M.J. Bailey, A.S. Whiteley, Raman microscopic analysis of single microbial cells, Anal. Chem. 76 (2004) 4452–4458. https://doi.org/10.1021/ac049753k.
[62] N. Majed, C. Matthäus, M. Diem, A.Z. Gu, Evaluation of intracellular polyphosphate dynamics in enhanced biological phosphorus removal process using Raman microscopy, Environ. Sci. Technol. 43 (2009) 5436–5442. https://doi.org/10.1021/es900251n.
[63] C.-S. Ho, N. Jean, C.A. Hogan, L. Blackmon, S.S. Jeffrey, M. Holodniy, N. Banaei, A.A.E. Saleh, S. Ermon, J. Dionne, Rapid identification of pathogenic bacteria using Raman spectroscopy and deep learning, Nat. Commun. 10 (2019) 4927. https://doi.org/10.1038/s41467-019-12898-9.
[64] G.J. Puppels, F.F.M. de Mul, C. Otto, J. Greve, M. Robert-Nicoud, D.J. Arndt-Jovin, T.M. Jovin, Studying single living cells and chromosomes by confocal Raman microspectroscopy, Nature 347 (1990) 301–303. https://doi.org/10.1038/347301a0.
[65] K.S. Lee, M. Palatinszky, F.C. Pereira, J. Nguyen, V.I. Fernandez, A.J. Mueller, F. Menolascina, H. Daims, D. Berry, M. Wagner, R. Stocker, An automated Raman-based platform for the sorting of live cells by functional properties, Nat. Microbiol. 4 (2019) 1035–1048. https://doi.org/10.1038/s41564-019-0394-9.
[66] N. Majed, A.Z. Gu, Application of Raman microscopy for simultaneous and quantitative evaluation of multiple intracellular polymers dynamics functionally relevant to enhanced biological phosphorus removal processes, Environ. Sci. Technol. 44 (2010) 8601–8608. https://doi.org/10.1021/es1016526.
[67] K.C. Schuster, E. Urlaub, J.R. Gapes, Single-cell analysis of bacteria by Raman microscopy: spectral information on the chemical composition of cells and on the heterogeneity in a culture, J. Microbiol. Methods 42 (2000) 29–38. https://doi.org/10.1016/S0167-7012(00)00169-X.
[68] K.C. Schuster, I. Reese, E. Urlaub, J.R. Gapes, B. Lendl, Multidimensional information on the chemical composition of single bacterial cells by confocal Raman microspectroscopy, Anal. Chem. 72 (2000) 5529–5534. https://doi.org/10.1021/ac000718x.
[69] D. Kusić, B. Kampe, P. Rösch, J. Popp, Identification of water pathogens by Raman microspectroscopy, Water Res. 48 (2014) 179–189. https://doi.org/10.1016/j.watres.2013.09.030.
[70] S. Stockel, S. Meisel, M. Elschner, P. Rosch, J. Popp, Identification of Bacillus anthracis via Raman spectroscopy and chemometric approaches, Anal. Chem. 84 (2012) 9873–9880. https://doi.org/10.1021/Ac302250t.
[71] A. Silge, W. Schumacher, P. Rösch, P.A. Da Costa Filho, C. Gérard, J. Popp, Identification of water-conditioned Pseudomonas aeruginosa by Raman microspectroscopy on a single cell level, Syst. Appl. Microbiol. 37 (2014) 360–367. https://doi.org/10.1016/j.syapm.2014.05.007.
[72] S. Stöckel, S. Meisel, M. Elschner, F. Melzer, P. Rösch, J. Popp, Raman spectroscopic detection and identification of Burkholderia mallei and Burkholderia pseudomallei in feedstuff, Anal. Bioanal. Chem. 407 (2015) 787–794. https://doi.org/10.1007/s00216-014-7906-5.
[73] A. Ashkin, J.M. Dziedzic, Optical trapping and manipulation of viruses and bacteria, Science 235 (1987) 1517–1520. https://doi.org/10.1126/science.3547653.
[74] H.A. Pohl, The motion and precipitation of suspensoids in divergent electric fields, J. Appl. Phys. 22 (1951) 869–871. https://doi.org/10.1063/1.1700065.
[75] C. Xie, J. Mace, M.A. Dinno, Y.Q. Li, W. Tang, R.J. Newton, P.J. Gemperline, Identification of single bacterial cells in aqueous solution using confocal laser tweezers Raman spectroscopy, Anal. Chem. 77 (2005) 4390–4397. https://doi.org/10.1021/ac0504971.
[76] U.-C. Schröder, A. Ramoji, U. Glaser, S. Sachse, C. Leiterer, A. Csaki, U. Hübner, W. Fritzsche, W. Pfister, M. Bauer, J. Popp, U. Neugebauer, Combined dielectrophoresis–Raman setup for the classification of pathogens recovered from the urinary tract, Anal. Chem. 85 (2013) 10717–10724. https://doi.org/10.1021/ac4021616.
[77] W.E. Huang, M.J. Bailey, I.P. Thompson, A.S. Whiteley, A.J. Spiers, Single-cell Raman spectral profiles of *Pseudomonas fluorescens* SBW25 reflects *in vitro* and *in planta* metabolic history, Microb. Ecol. 53 (2007) 414–425. https://doi.org/10.1007/s00248-006-9138-5.
[78] M.F. Escoriza, J.M. Vanbriesen, S. Stewart, J. Maier, Studying bacterial metabolic states using Raman spectroscopy, Appl. Spectrosc. 60 (2006) 971–976. https://doi.org/10.1366/000370206778397290.
[79] L. He, S. Wang, M. Cortesão, M. Wu, R. Moeller, P. Setlow, Y.-q. Li, Single-cell analysis reveals individual spore responses to simulated space vacuum, npj Microgravity 4 (2018) 26. https://doi.org/10.1038/s41526-018-0059-7.
[80] S. Pahlow, S. Meisel, D. Cialla-May, K. Weber, P. Rosch, J. Popp, Isolation and identification of bacteria by means of Raman spectroscopy, Adv. Drug Deliv. Rev. 89 (2015) 105–120. https://doi.org/10.1016/j.addr.2015.04.006.
[81] Q.H. Gan, X.T. Wang, Y. Wang, Z.Y. Xie, Y. Tian, Y.D. Lu, Culture-free detection of crop pathogens at the single-cell level by micro-Raman spectroscopy, Adv. Sci. 4 (2017) 1700127. https://doi.org/10.1002/Advs.201700127.
[82] K. Maquelin, L.-P.i. Choo-Smith, T. van Vreeswijk, H.P. Endtz, B. Smith, R. Bennett, H.A. Bruining, G.J. Puppels, Raman spectroscopic method for identification of clinically relevant microorganisms growing on solid culture medium, Anal. Chem. 72 (2000) 12–19. https://doi.org/10.1021/ac991011h.
[83] K. Maquelin, L.-P. Choo-Smith, H.P. Endtz, H.A. Bruining, G.J. Puppels, Rapid identification of *Candida* species by confocal Raman microspectroscopy, J. Clin. Microbiol. 40 (2002) 594–600. https://doi.org/10.1128/jcm.40.2.594-600.2002.
[84] R.M. Jarvis, R. Goodacre, Discrimination of bacteria using surface-enhanced Raman spectroscopy, Anal. Chem. 76 (2004) 40–47. https://doi.org/10.1021/ac034689c.
[85] P.C.A.M. Buijtels, H.F.M. Willemse-Erix, P.L.C. Petit, H.P. Endtz, G.J. Puppels, H.A. Verbrugh, A. van Belkum, D. van Soolingen, K. Maquelin, Rapid identification of mycobacteria by Raman spectroscopy, J. Clin. Microbiol. 46 (2008) 961–965. https://doi.org/10.1128/jcm.01763-07.
[86] R. Goodacre, é.M. Timmins, R. Burton, N. Kaderbhai, A.M. Woodward, D.B. Kell, P.J. Rooney, Rapid identification of urinary tract infection bacteria using hyperspectral whole-organism fingerprinting and artificial neural networks, Microbiology 144 (1998) 1157–1170. https://doi.org/10.1099/00221287-144-5-1157.
[87] C. Farber, D. Kurouski, Detection and identification of plant pathogens on maize kernels with a hand-held Raman spectrometer, Anal. Chem. 90 (2018) 3009–3012. https://doi.org/10.1021/acs.analchem.8b00222.

[88] S. Kloß, B. Kampe, S. Sachse, P. Rösch, E. Straube, W. Pfister, M. Kiehntopf, J. Popp, Culture independent Raman spectroscopic identification of urinary tract infection pathogens: a proof of principle study, Anal. Chem. 85 (2013) 9610–9616. https://doi.org/10.1021/ac401806f.
[89] S. Kloß, P. Rösch, W. Pfister, M. Kiehntopf, J. Popp, Toward culture-free Raman spectroscopic identification of pathogens in ascitic fluid, Anal. Chem. 87 (2015) 937–943. https://doi.org/10.1021/ac503373r.
[90] Y. Maeda, Y. Sugiyama, A. Kogiso, T.-K. Lim, M. Harada, T. Yoshino, T. Matsunaga, T. Tanaka, Colony fingerprint-based discrimination of Staphylococcus species with machine learning approaches, Sensors 18 (2018) 2789. https://doi.org/10.3390/s18092789.
[91] N. Arend, A. Pittner, A. Ramoji, A.S. Mondol, M. Dahms, J. Rüger, O. Kurzai, I.W. Schie, M. Bauer, J. Popp, U. Neugebauer, Detection and differentiation of bacterial and fungal infection of neutrophils from peripheral blood using Raman spectroscopy, Anal. Chem. 92 (2020) 10560–10568. https://doi.org/10.1021/acs.analchem.0c01384.
[92] X. Fan, W. Ming, H. Zeng, Z. Zhang, H. Lu, Deep learning-based component identification for the Raman spectra of mixtures, Analyst 144 (2019) 1789–1798. https://doi.org/10.1039/C8AN02212G.
[93] W. Lu, X. Chen, L. Wang, H. Li, Y.V. Fu, Combination of an artificial intelligence approach and laser tweezers Raman spectroscopy for microbial identification, Anal. Chem. 92 (2020) 6288–6296. https://doi.org/10.1021/acs.analchem.9b04946.
[94] S. Yu, H. Li, X. Li, Y.V. Fu, F. Liu, Classification of pathogens by Raman spectroscopy combined with generative adversarial networks, Sci. Total Environ. 726 (2020) 138477. https://doi.org/10.1016/j.scitotenv.2020.138477.
[95] S. Meisel, S. Stoeckel, P. Roesch, J. Popp, Identification of meat-associated pathogens via Raman microspectroscopy, Food Microbiol. 38 (2014) 36–43. https://doi.org/10.1016/j.fm.2013.08.007.
[96] Y. Liu, J. Xu, Y. Tao, T. Fang, W. Du, A. Ye, Rapid and accurate identification of marine microbes with single-cell Raman spectroscopy, Analyst 145 (2020) 3297–3305. https://doi.org/10.1039/C9AN02069A.
[97] S. Shanmukh, L. Jones, Y.P. Zhao, J.D. Driskell, R.A. Tripp, R.A. Dluhy, Identification and classification of respiratory syncytial virus (RSV) strains by surface-enhanced Raman spectroscopy and multivariate statistical techniques, Anal. Bioanal. Chem. 390 (2008) 1551. https://doi.org/10.1007/s00216-008-1851-0.
[98] C. Fan, Z. Hu, L.K. Riley, G.A. Purdy, A. Mustapha, M. Lin, Detecting food- and waterborne viruses by surface-enhanced Raman spectroscopy, J. Food Sci. 75 (2010) M302–M307. https://doi.org/10.1111/j.1750-3841.2010.01619.x.
[99] Y.-T. Yeh, K. Gulino, Y. Zhang, A. Sabestien, T.-W. Chou, B. Zhou, Z. Lin, I. Albert, H. Lu, V. Swaminathan, E. Ghedin, M. Terrones, A rapid and label-free platform for virus capture and identification from clinical samples, Proc. Natl. Acad. Sci. Unit. States Am. 117 (2020) 895–901. https://doi.org/10.1073/pnas.1910113117.
[100] D. Zhang, X. Zhang, R. Ma, S. Deng, X. Wang, X. Wang, X. Zhang, X. Huang, Y. Liu, G. Li, J. Qu, Y. Zhu, J. Li, Ultra-fast and onsite interrogation of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) in waters via surface enhanced Raman scattering (SERS), Water Res. 200 (2021) 117243. https://doi.org/10.1016/j.watres.2021.117243.
[101] O. Žukovskaja, S. Kloß, M.G. Blango, O. Ryabchykov, O. Kniemeyer, A.A. Brakhage, T.W. Bocklitz, D. Cialla-May, K. Weber, J. Popp, UV-Raman spectroscopic identification of fungal spores important for respiratory diseases, Anal. Chem. 90 (2018) 8912–8918. https://doi.org/10.1021/acs.analchem.8b01038.
[102] E. Witkowska, T. Jagielski, A. Kamińska, A. Kowalska, A. Hryncewicz-Gwóźdź, J. Waluk, Detection and identification of human fungal pathogens using surface-enhanced Raman spectroscopy and principal component analysis, Anal. Methods 8 (2016) 8427–8434. https://doi.org/10.1039/C6AY02957D.
[103] N.E. Dina, A.M.R. Gherman, V. Chiş, C. Sârbu, A. Wieser, D. Bauer, C. Haisch, Characterization of clinically relevant fungi via SERS fingerprinting assisted by novel chemometric models, Anal. Chem. 90 (2018) 2484–2492. https://doi.org/10.1021/acs.analchem.7b03124.
[104] B.S. Luo, M. Lin, A portable Raman system for the identification of foodborne pathogenic bacteria, J. Rapid Methods Autom. Microbiol. 16 (2008) 238–255. https://doi.org/10.1111/j.1745-4581.2008.00131.x.
[105] Y. Liu, H. Zhou, Z. Hu, G. Yu, D. Yang, J. Zhao, Label and label-free based surface-enhanced Raman scattering for pathogen bacteria detection: a review, Biosens. Bioelectron. 94 (2017) 131–140. https://doi.org/10.1016/j.bios.2017.02.032.
[106] Y. Wang, S. Ravindranath, J. Irudayaraj, Separation and detection of multiple pathogens in a food matrix by magnetic SERS nanoprobes, Anal. Bioanal. Chem. 399 (2011) 1271–1278. https://doi.org/10.1007/s00216-010-4453-6.
[107] N. Xiao, C. Wang, C. Yu, A self-referencing detection of microorganisms using surface enhanced Raman scattering nanoprobes in a test-in-a-tube platform, Biosensors 3 (2013) 312–326. https://doi.org/10.3390/bios3030312.
[108] H. Zhang, X. Ma, Y. Liu, N. Duan, S. Wu, Z. Wang, B. Xu, Gold nanoparticles enhanced SERS aptasensor for the simultaneous detection of Salmonella typhimurium and Staphylococcus aureus, Biosens. Bioelectron. 74 (2015) 872–877. https://doi.org/10.1016/j.bios.2015.07.033.
[109] T.-Y. Liu, K.-T. Tsai, H.-H. Wang, Y. Chen, Y.-H. Chen, Y.-C. Chao, H.-H. Chang, C.-H. Lin, J.-K. Wang, Y.-L. Wang, Functionalized arrays of Raman-enhancing nanoparticles for capture and culture-free analysis of bacteria in human blood, Nat. Commun. 2 (2011) 538. https://doi.org/10.1038/ncomms1546.
[110] H. Wang, Y. Zhou, X. Jiang, B. Sun, Y. Zhu, H. Wang, Y. Su, Y. He, Simultaneous capture, detection, and inactivation of bacteria as enabled by a surface-enhanced Raman scattering multifunctional chip, Angew. Chem. Int. Ed. 54 (2015) 5132–5136. https://doi.org/10.1002/anie.201412294.
[111] P.-J. Huang, L.-K. Chau, T.-S. Yang, L.-L. Tay, T.-T. Lin, Nanoaggregate-embedded beads as novel Raman labels for biodetection, Adv. Funct. Mater. 19 (2009) 242–248. https://doi.org/10.1002/adfm.200800961.
[112] S. Uluok, B. Guven, H. Eksi, Z. Ustundag, U. Tamer, I.H. Boyaci, Designing multilayered nanoplatforms for SERS-based detection of genetically modified organisms, J. Nano Res. 17 (2015) 43. https://doi.org/10.1007/s11051-014-2849-5.
[113] V. Deckert, T. Deckert-Gaudig, D. Cialla-May, J. Popp, R. Zell, S. Deinhard-Emmer, A.V. Sokolov, Z. Yi, M.O. Scully, Laser spectroscopic technique for direct identification of a single virus I: faster cars, Proc. Natl. Acad. Sci. Unit. States Am. 117 (2020) 27820–27824. https://doi.org/10.1073/pnas.2013169117.
[114] A. van Belkum, C.-A.D. Burnham, J.W.A. Rossen, F. Mallard, O. Rochas, W.M. Dunne, Innovative and rapid antimicrobial susceptibility testing systems, Nat. Rev. Microbiol. 18 (2020) 299–311. https://doi.org/10.1038/s41579-020-0327-x.
[115] D. Berry, E. Mader, T.K. Lee, D. Woebken, Y. Wang, D. Zhu, M. Palatinszky, A. Schintlmeister, M.C. Schmid, B.T. Hanson, N. Shterzer, I. Mizrahi, I. Rauch, T. Decker, T. Bocklitz, J. Popp, C.M. Gibson, P.W. Fowler, W.E. Huang, M. Wagner, Tracking heavy water (D2O) incorporation for identifying and sorting active microbial cells, Proc. Natl. Acad. Sci. U.S.A. 112 (2015) E194. https://doi.org/10.1073/pnas.1420406112.
[116] X. Yi, Y. Song, X. Xu, D. Peng, J. Wang, X. Qie, K. Lin, M. Yu, M. Ge, Y. Wang, D. Zhang, Q. Yang, M. Wang, W.E. Huang, Development of a fast Raman-assisted antibiotic susceptibility test (FRAST) for the antibiotic resistance analysis of clinical urine and blood samples, Anal. Chem. 93 (2021) 5098–5106. https://doi.org/10.1021/acs.analchem.0c04709.
[117] M. Zhang, W. Hong, N.S. Abutaleb, J. Li, P.-T. Dong, C. Zong, P. Wang, M.N. Seleem, J.-X. Cheng, Rapid determination of antimicrobial susceptibility by stimulated Raman scattering imaging of D_2O metabolic incorporation in a single bacterium, Adv. Sci. 7 (2020) 2001452. https://doi.org/10.1002/advs.202001452.
[118] W. Hong, C.W. Karanja, N.S. Abutaleb, W. Younis, X. Zhang, M.N. Seleem, J.-X. Cheng, Antibiotic susceptibility determination within one cell cycle at single-bacterium level by stimulated Raman metabolic imaging, Anal. Chem. 90 (2018) 3737–3743. https://doi.org/10.1021/acs.analchem.7b03382.
[119] Y. Song, L. Cui, J.Á.S. López, J. Xu, Y.-G. Zhu, I.P. Thompson, W.E. Huang, Raman-Deuterium Isotope Probing for in-situ identification of antimicrobial resistant bacteria in Thames River,, Sci. Rep. 7 (2017) 16648. https://doi.org/10.1038/s41598-017-16898-x.
[120] H.K. Allen, J. Donato, H.H. Wang, K.A. Cloud-Hansen, J. Davies, J. Handelsman, Call of the wild: antibiotic resistance genes in natural environments, Nat. Rev. Microbiol. 8 (2010) 251–259. https://doi.org/10.1038/nrmicro2312.
[121] H. Ochman, J.G. Lawrence, E.A. Groisman, Lateral gene transfer and the nature of bacterial innovation, Nature 405 (2000) 299–304. https://doi.org/10.1038/35012500.
[122] A.A. Adegoke, A.C. Faleye, G. Singh, T.A. Stenström, Antibiotic resistant superbugs: assessment of the interrelationship of occurrence in clinical settings and environmental niches, Molecules 22 (2017) 29. https://doi.org/10.3390/molecules22010029.
[123] R. Girones, M. Antonia Ferrús, J. Luis Alonso, J. Rodriguez-Manzano, B. Calgua, A. de Abreu Corrêa, A. Hundesa, A. Carratala, S. Bofill-Mas, Molecular detection of pathogens in water - the pros and cons of molecular techniques, Water Res. 44 (2010) 4325–4339. https://doi.org/10.1016/j.watres.2010.06.030.
[124] C. Cenciarini-Borde, S. Courtois, B. La Scola, Nucleic acids as viability markers for bacteria detection using molecular tools, Future Microbiol. 4 (2009) 45–64. https://doi.org/10.2217/17460913.4.1.45.
[125] S. Guajardo-Leiva, J. Chnaiderman, A. Gaggero, B. Díez, Metagenomic insights into the sewage RNA virosphere of a large city, Viruses-Basel 12 (2020) 1050. https://doi.org/10.3390/v12091050.
[126] M.A. Jahne, N.E. Brinkman, S.P. Keely, B.D. Zimmerman, E.A. Wheaton, J.L. Garland, Droplet digital PCR quantification of norovirus and adenovirus in decentralized wastewater and graywater collections: implications for onsite reuse, Water Res. 169 (2020) 115213. https://doi.org/10.1016/j.watres.2019.115213.
[127] Q. Liu, Y. Zhang, W. Jing, S. Liu, D. Zhang, G. Sui, First airborne pathogen direct analysis system, Analyst 141 (2016) 1637–1640. https://doi.org/10.1039/c5an02367j.
[128] D. Calderón-Franco, M.C.M. van Loosdrecht, T. Abeel, D.G. Weissbrodt, Free-floating extracellular DNA: systematic profiling of mobile genetic elements and antibiotic resistance from wastewater, Water Res. 189 (2021) 116592. https://doi.org/10.1016/j.watres.2020.116592.
[129] S.P. Rudko, N.J. Ruecker, N.J. Ashbolt, N.F. Neumann, P.C. Hanington, Enterobius vermicularis as a novel surrogate for the presence of helminth ova in tertiary wastewater treatment plants, Appl. Environ. Microbiol. 83 (2017). https://doi.org/10.1128/AEM.00547-17. e00547-00517.
[130] S.A. Bustin, Why the need for qPCR publication guidelines?-The case for MIQE, Methods 50 (2009) 217–226. https://doi.org/10.1016/j.ymeth.2009.12.006.
[131] K.E. Shannon, D.Y. Lee, J.T. Trevors, L.A. Beaudette, Application of real-time quantitative PCR for the detection of selected bacterial pathogens during

municipal wastewater treatment, Sci. Total Environ. 382 (2007) 121–129. https://doi.org/10.1016/j.scitotenv.2007.02.039.
[132] J.L. Alonso, I. Amorós, I. Cañigral, Development and evaluation of a real-time PCR assay for quantification of Giardia and Cryptosporidium in sewage samples, Appl. Microbiol. Biotechnol. 89 (2011) 1203–1211. https://doi.org/10.1007/s00253-010-2984-6.
[133] M. Achak, S.A. Bakri, Y. Chhiti, F.E.M.h. Alaoui, N. Barka, W. Boumya, SARS-CoV-2 in hospital wastewater during outbreak of COVID-19: a review on detection, survival and disinfection technologies, Sci. Total Environ. 761 (2021) 143192. https://doi.org/10.1016/j.scitotenv.2020.143192.
[134] J. Flannery, S. Keaveney, P. Rajko-Nenow, V. O'Flaherty, W. Dóre, Norovirus and FRNA bacteriophage determined by RT-qPCR and infectious FRNA bacteriophage in wastewater and oysters, Water Res. 47 (2013) 5222–5231. https://doi.org/10.1016/j.watres.2013.06.008.
[135] G. Medema, L. Heijnen, G. Elsinga, R. Italiaander, A. Brouwer, Presence of SARS-coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in The Netherlands, Environ. Sci. Technol. Lett. 7 (2020) 511–516. https://doi.org/10.1021/acs.estlett.0c00357.
[136] W. Randazzo, P. Truchado, E. Cuevas-Ferrando, P. Simón, A. Allende, G. Sánchez, SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a low prevalence area, Water Res. 181 (2020) 115942. https://doi.org/10.1016/j.watres.2020.115942.
[137] R.T. Hayden, Z. Gu, J. Ingersoll, D. Abdul-Ali, L. Shi, S. Pounds, A.M. Caliendo, Comparison of droplet digital PCR to real-time PCR for quantitative detection of cytomegalovirus, J. Clin. Microbiol. 51 (2013) 540–546. https://doi.org/10.1128/JCM.02620-12.
[138] M.A. Ibekwe, S.E. Murinda, S. Park, A. Obayiuwana, M.A. Murry, G. Schwartz, T. Lundquist, Comparative use of quantitative PCR (qPCR), droplet digital PCR (ddPCR), and recombinase polymerase amplification (RPA) in the detection of Shiga toxin-producing E. coli (STEC) in environmental samples, Water 12 (2020) 3507. https://doi.org/10.3390/w12123507.
[139] E. Stachler, K. Crank, K. Bibby, Co-occurrence of crAssphage with antibiotic resistance genes in an impacted urban watershed, Environ. Sci. Technol. Lett. 4 (2019) 216–221. https://doi.org/10.1021/acs.estlett.9b00130.
[140] N. Tomita, Y. Mori, H. Kanda, T. Notomi, Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products, Nat. Protoc. 3 (2008) 877–882. https://doi.org/10.1038/nprot.2008.57.
[141] K. Mao, H. Zhang, Z. Yang, Can a paper-based device trace COVID-19 sources with wastewater-based epidemiology? Environ. Sci. Technol. 54 (2020) 3733–3735. https://doi.org/10.1021/acs.est.0c01174.
[142] H. Yuan, Y. Chao, S. Li, M.Y.H. Tang, Y. Huang, Y. Che, A.S.T. Wong, T. Zhang, H.C. Shum, Picoinjection-enabled multitarget loop-mediated isothermal amplification for detection of foodborne pathogens, Anal. Chem. 90 (2018) 13173–13177. https://doi.org/10.1021/acs.analchem.8b03673.
[143] M. Xu, H. Fu, D. Chen, Z. Shao, J. Zhu, W.Q. Alali, L. Chen, Simple visualized detection method of virulence-associated genes of Vibrio cholerae by loop-mediated isothermal amplification, Front. Microbiol. 10 (2019) 2899. https://doi.org/10.3389/fmicb.2019.02899.
[144] M. Golabi, M. Flodrops, B. Grasland, A.C. Vinayaka, T.L. Quyen, T. Nguyen, D.D. Bang, A. Wolff, Development of reverse transcription loop-mediated isothermal amplification assay for rapid and on-site detection of avian influenza virus, Front. Cell. Infect. Mi. 11 (2021) 652048. https://doi.org/10.3389/fcimb.2021.652048.
[145] C. Takano, M. Seki, D.W. Kim, H. Gardner, R.E. Mclaughlin, P.E. Kilgore, K. Kumasaka, S. Hayakawa, Development of a novel loop-mediated isothermal amplification method to detect guiana extended-spectrum (GES) β-lactamase genes in Pseudomonas aeruginosa, Front. Microbiol. 10 (2019) 25. https://doi.org/10.3389/fmicb.2019.00025.
[146] T. Kostić, M. Ellis, M.R. Williams, T.M. Stedtfeld, J.B. Kaneene, R.D. Stedtfeld, S.A. Hashsham, Thirty-minute screening of antibiotic resistance genes in bacterial isolates with minimal sample preparation in static self-dispensing 64 and 384 assay cards, Appl. Microbiol. Biotechnol. 99 (2015) 7711–7722. https://doi.org/10.1007/s00253-015-6774-z.
[147] V. Kumar, P. Guleria, Application of DNA-nanosensor for environmental monitoring: recent advances and perspectives, Curr. Pollut. Rep. (2020). https://doi.org/10.1007/s40726-020-00165-1.
[148] M.E.E. Alahi, S.C. Mukhopadhyay, Detection methodologies for pathogen and toxins: a review, Sensors 17 (2017) 1885. https://doi.org/10.3390/s17081885.
[149] World Health Organization, Molecular Methods for Antimicrobial Resistance (AMR) Diagnostics to Enhance the Global Antimicrobial Resistance Surveillance System. Geneva, 2019. https://www.who.int/glass/resources/publications/molecular-methods-for-amr-diagnostics/en/.
[150] P. Santiago, A. Jiménez-Belenguer, J. García-Hernández, R. MontesEstellés, M. Hernández Pérez, M.A. Castillo López, M. Antonia Ferrús, Y. Moreno, High prevalence of Salmonella spp. in wastewater reused for irrigation assessed by molecular methods, Int. J. Hyg Environ. Health 221 (2018) 95–101. https://doi.org/10.1016/j.ijheh.2017.10.007.
[151] X.L. An, J.Y. Wang, Q. Pu, H. Li, J.Q. Su, High-throughput diagnosis of human pathogens and fecal contamination in marine recreational water, Environ. Res. 190 (2020) 109982. https://doi.org/10.1016/j.envres.2020.109982.
[152] H. Waseem, S. Jameel, J. Ali, H.S.U. Rehman, I. Tauseef, U. Farooq, A. Jamal, M.I. Ali, Contributions and challenges of high throughput qPCR for determining antimicrobial resistance in the environment: a critical review, Molecules 24 (2019) 163. https://doi.org/10.3390/molecules24010163.
[153] Q.X. Wen, L. Yang, R. Duan, Z.Q. Chen, Monitoring and evaluation of antibiotic resistance genes in four municipal wastewater treatment plants in Harbin, Northeast China, Environ. Pollut. 212 (2016) 34–40. https://doi.org/10.1016/j.envpol.2016.01.043.
[154] W. Calero-Cáceres, M. Muniesa, Persistence of naturally occurring antibiotic resistance genes in the bacteria and bacteriophage fractions of wastewater, Water Res. 95 (2016) 11–18. https://doi.org/10.1016/j.watres.2016.03.006.
[155] F.Y. Ramírez-Castillo, A. Loera-Muro, M. Jacques, P. Garneau, F.J. Avelar-González, J. Harel, A.L. Guerrero-Barrera, Waterborne pathogens: detection methods and challenges, Pathogens 4 (2015) 307–334. https://doi.org/10.3390/pathogens4020307.
[156] Q.L. Chen, X.T. Fan, D. Zhu, X.L. An, J.Q. Su, L. Cui, Effect of biochar amendment on the alleviation of antibiotic resistance in soil and phyllosphere of Brassica chinensis L, Soil Biol. Biochem. 119 (2018) 74–82. https://doi.org/10.1016/j.soilbio.2018.01.015.
[157] G.B. Zhu, X.M. Wang, T. Yang, J.Q. Su, Y. Qin, S.Y. Wang, M. Gillings, C. Wang, F. Ju, B.R. Lan, C.L. Liu, H. Li, X.E. Long, X.M. Wang, M.S.M. Jetten, Z.F. Wang, Y.G. Zhu, Air pollution could drive global dissemination of antibiotic resistance genes, ISME J. 15 (2021) 270–281. https://doi.org/10.1038/s41396-020-00780-2.
[158] H.J. Wang, L.Y. Hou, Y.Q. Liu, K.S. Liu, L.P. Zhang, F.Y. Huang, L. Wang, A.Z.R. Rashid, A.Y. Hu, C.P. Yu, Horizontal and vertical gene transfer drive sediment antibiotic resistome in an urban lagoon system, J. Environ. Sci. 102 (2021) 11–23. https://doi.org/10.1016/j.jes.2020.09.004.
[159] Z.M. Han, Y. Zhang, W. An, J.Y. Lu, J.Y. Hu, M. Yang, Antibiotic resistomes in drinking water sources across a large geographical scale: multiple drivers and co-occurrence with opportunistic bacterial pathogens, Water Res. 183 (2020) 116088. https://doi.org/10.1016/j.watres.2020.116088.
[160] X. Liu, P. Xiao, Y.Y. Guo, L.M. Liu, J. Yang, The impacts of different high-throughput profiling approaches on the understanding of bacterial antibiotic resistance genes in a freshwater reservoir, Sci. Total Environ. 693 (2019) 133585. https://doi.org/10.1016/j.scitotenv.2019.133585.
[161] D.Q. Mao, S. Yu, M. Rysz, Y. Luo, F.X. Yang, F.X. Li, J. Hou, Q.H. Mu, P.J.J. Alvarez, Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants, Water Res. 85 (2015) 458–466. https://doi.org/10.1016/j.watres.2015.09.010.
[162] A. Novo, S. André, P. Viana, O.C. Nunes, C.M. Manaia, Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater, Water Res. 47 (2013) 1875–1887. https://doi.org/10.1016/j.watres.2013.01.010.
[163] J. Lee, J.H. Jeon, J. Shin, H.M. Jang, S. Kim, M.S. Song, Y.M. Kim, Quantitative and qualitative changes in antibiotic resistance genes after passing through treatment processes in municipal wastewater treatment plants, Sci. Total Environ. 605 (2017) 906–914. https://doi.org/10.1016/j.scitotenv.2017.06.250.
[164] K.F. Yu, P. Li, Y.L. He, B. Zhang, Y.H. Chen, J.H. Yang, Unveiling dynamics of size-dependent antibiotic resistome associated with microbial communities in full-scale wastewater treatment plants, Water Res. 187 (2020) 116450. https://doi.org/10.1016/j.watres.2020.116450.
[165] N. Release, WHO publishes list of bacteria for which new antibiotics are urgently needed, Neurosciences 38 (2017) 444–445.
[166] S. Ishii, T. Segawa, S. Okabe, Simultaneous quantification of multiple food- and waterborne pathogens by use of microfluidic quantitative PCR, Appl. Environ. Microbiol. 79 (2013) 2891–2898. https://doi.org/10.1128/aem.00205-13.
[167] H.K. Allen, Antibiotic resistance gene discovery in food-producing animals, Curr. Opin. Microbiol. 19 (2014) 25–29. https://doi.org/10.1016/j.mib.2014.06.001.
[168] E. Luby, A.M. Ibekwe, J. Zilles, A. Pruden, Molecular methods for assessment of antibiotic resistance in agricultural ecosystems: prospects and challenges, J. Environ. Qual. 45 (2016) 441–453. https://doi.org/10.2134/jeq2015.07.0367.
[169] S.G. Tringe, E.M. Rubin, Metagenomics: DNA sequencing of environmental samples, Nat. Rev. Genet. 6 (2005) 805–814. https://doi.org/10.1038/nrg1709.
[170] P. Hugenholtz, G.W. Tyson, Metagenomics, Nature 455 (2008) 481–483. https://doi.org/10.1038/455481a.
[171] F.M. Aarestrup, M.E. Woolhouse, Using sewage for surveillance of antimicrobial resistance, Science 367 (2020) 630–632. https://doi.org/10.1126/science.aba3432.
[172] P. Fresia, V. Antelo, C. Salazar, M. Gimenez, B. D'Alessandro, E. Afshinnekoo, C. Mason, G.H. Gonnet, G. Iraola, Urban metagenomics uncover antibiotic resistance reservoirs in coastal beach and sewage waters, Microbiome 7 (2019) 35. https://doi.org/10.1186/s40168-019-0648-z.
[173] R. Baum, J. Luh, J. Bartram, Sanitation: a global estimate of sewerage connections without treatment and the resulting impact on MDG progress, Environ. Sci. Technol. 47 (2013) 1994–2000. https://doi.org/10.1021/es304284f.
[174] R.S. Hendriksen, P. Munk, P. Njage, B. Van Bunnik, L. McNally, O. Lukjancenko, T. Röder, D. Nieuwenhuijse, S.K. Pedersen, J. Kjeldgaard, Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage, Nat. Commun. 10 (2019) 1–12. https://doi.org/10.1038/s41467-019-08853-3.
[175] M. Lamba, D.W. Graham, S. Ahammad, Hospital wastewater releases of carbapenem-resistance pathogens and genes in urban India, Environ. Sci. Technol. 51 (2017) 13906–13912. https://doi.org/10.1021/acs.est.7b03380.

[176] M.L. Petrovich, A. Zilberman, A. Kaplan, G.R. Eliraz, Y. Wang, K. Langenfeld, M. Duhaime, K. Wigginton, R. Poretsky, D. Avisar, Microbial and viral communities and their antibiotic resistance genes throughout a hospital wastewater treatment system, Front. Microbiol. 11 (2020) 153. https://doi.org/10.3389/fmicb.2020.00153.
[177] N.P. Marathe, F. Berglund, M. Razavi, C. Pal, J. Dröge, S. Samant, E. Kristiansson, D.J. Larsson, Sewage effluent from an Indian hospital harbors novel carbapenemases and integron-borne antibiotic resistance genes, Microbiome 7 (2019) 1–11. https://doi.org/10.1186/s40168-019-0710-x.
[178] J. Subirats, A. Sànchez-Melsió, C.M. Borrego, J.L. Balcázar, P. Simonet, Metagenomic analysis reveals that bacteriophages are reservoirs of antibiotic resistance genes, Int. J. Antimicrob. Agents 48 (2016) 163–167. https://doi.org/10.1016/j.ijantimicag.2016.04.028.
[179] E.C. Pehrsson, P. Tsukayama, S. Patel, M. Mejía-Bautista, G. Sosa-Soto, K.M. Navarrete, M. Calderon, L. Cabrera, W. Hoyos-Arango, M.T. Bertoli, Interconnected microbiomes and resistomes in low-income human habitats, Nature 533 (2016) 212–216. https://doi.org/10.1038/nature17672.
[180] B. Berglund, J. Fick, P.E. Lindgren, Urban wastewater effluent increases antibiotic resistance gene concentrations in a receiving northern European river, Environ. Toxicol. Chem. 34 (2015) 192–196. https://doi.org/10.1002/etc.2784.
[181] B. Christgen, Y. Yang, S. Ahammad, B. Li, D.C. Rodriquez, T. Zhang, D.W. Graham, Metagenomics shows that low-energy anaerobic– aerobic treatment reactors reduce antibiotic resistance gene levels from domestic wastewater, Environ. Sci. Technol. 49 (2015) 2577–2584. https://doi.org/10.1021/es505521w.
[182] F. Ju, B. Li, L. Ma, Y. Wang, D. Huang, T. Zhang, Antibiotic resistance genes and human bacterial pathogens: co-occurrence, removal, and enrichment in municipal sewage sludge digesters, Water Res. 91 (2016) 1–10. https://doi.org/10.1016/j.watres.2015.11.071.
[183] S. Conlan, P.J. Thomas, C. Deming, M. Park, A.F. Lau, J.P. Dekker, E.S. Snitkin, T.A. Clark, K. Luong, Y. Song, Single-molecule sequencing to track plasmid diversity of hospital-associated carbapenemase-producing Enterobacteriaceae, Sci. Transl. Med. 6 (2014). https://doi.org/10.1126/scitranslmed.3009845, 254ra126.
[184] P.G. Cantalupo, B. Calgua, G. Zhao, A. Hundesa, A.D. Wier, J.P. Katz, M. Grabe, R.W. Hendrix, R. Girones, D. Wang, Raw sewage harbors diverse viral populations, mBio 2 (2011), e00180. https://doi.org/10.1128/mBio.00180-11.
[185] H.W. Young, D.W. York, Reclaimed water reuse in Florida and the South gulf coast, Florida Water Resour. J (1996) 32–36.
[186] K. Rosario, C. Nilsson, Y.W. Lim, Y. Ruan, M. Breitbart, Metagenomic analysis of viruses in reclaimed water, Environ. Microbiol. 11 (2009) 2806–2820. https://doi.org/10.1111/j.1462-2920.2009.01964.x.
[187] K. Bibby, J. Peccia, Identification of viral pathogen diversity in sewage sludge by metagenome analysis, Environ. Sci. Technol. 47 (2013) 1945–1951. https://doi.org/10.1021/es305181x.
[188] Y.A. Wang, Y.F. Hu, F. Liu, J. Cao, N. Lv, B.L. Zhu, G.P. Zhang, G.F. Gao, Integrated metagenomic and metatranscriptomic profiling reveals differentially expressed resistomes in human, chicken, and pig gut microbiomes, Environ. Int. 138 (2020) 105649. https://doi.org/Artn.105649.
[189] Z. Liu, U. Klümper, Y. Liu, Y. Yang, Q. Wei, J.-G. Lin, J.-D. Gu, M. Li, Metagenomic and metatranscriptomic analyses reveal activity and hosts of antibiotic resistance genes in activated sludge, Environ. Int. 129 (2019) 208–220. https://doi.org/10.1016/j.envint.2019.05.036.
[190] P. Mullany, Functional metagenomics for the investigation of antibiotic resistance, Virulence 5 (2014) 443–447. https://doi.org/10.1021/acs.est.0c01155.
[191] K.A. Kazimierczak, K.P. Scott, D. Kelly, R.I. Aminov, Tetracycline resistome of the organic pig gut, Appl. Environ. Microbiol. 75 (2009) 1717–1722. https://doi.org/10.1128/AEM.02206-08.
[192] F. Wichmann, N. Udikovic-Kolic, S. Andrew, J. Handelsman, Diverse antibiotic resistance genes in dairy cow manure, mBio 5 (2014), e01017. https://doi.org/10.1128/mBio.01017-13.
[193] P.C. Blainey, The future is now: single-cell genomics of bacteria and archaea, FEMS Microbiol. Rev. 37 (2013) 407–427. https://doi.org/10.1111/1574-6976.12015.
[194] N. Ye, J. Qin, W. Shi, X. Liu, B. Lin, Cell-based high content screening using an integrated microfluidic device, Lab Chip 7 (2007) 1696–1704. https://doi.org/10.1039/b711513j.
[195] P.Y. Chiou, A.T. Ohta, M.C. Wu, Massively parallel manipulation of single cells and microparticles using optical images, Nature 436 (2005) 370–372. https://doi.org/10.1038/nature03831.
[196] Y. Wang, Y. Ji, E.S. Wharfe, R.S. Meadows, P. March, R. Goodacre, J. Xu, W.E. Huang, Raman activated cell ejection for isolation of single cells, Anal. Chem. 85 (2013) 10697–10701. https://doi.org/10.1021/ac403107p.
[197] J.K. Jarett, M. Džunková, F. Schulz, S. Roux, D. Paez-Espino, E. Eloe-Fadrosh, S.P. Jungbluth, N. Ivanova, J.R. Spear, S.A. Carr, Insights into the dynamics between viruses and their hosts in a hot spring microbial mat, ISME J. 14 (2020) 2527–2541. https://doi.org/10.1038/s41396-020-0705-4.
[198] Z. Zhu, W. Zhang, X. Leng, M. Zhang, Z. Guan, J. Lu, C.J. Yang, Highly sensitive and quantitative detection of rare pathogens through agarose droplet microfluidic emulsion PCR at the single-cell level, Lab Chip 12 (2012) 3907–3913. https://doi.org/10.1039/c2lc40461c.
[199] F. Lan, B. Demaree, N. Ahmed, A.R. Abate, Single-cell genome sequencing at ultra-high-throughput with microfluidic droplet barcoding, Nat. Biotechnol. 35 (2017) 640. https://doi.org/10.1038/nbt.3880.
[200] F.B. Dean, S. Hosono, L. Fang, X. Wu, A.F. Faruqi, P. Bray-Ward, Z. Sun, Q. Zong, Y. Du, J. Du, Comprehensive human genome amplification using multiple displacement amplification, Proc. Natl. Acad. Sci. Unit. States Am. 99 (2002) 5261–5266. https://doi.org/10.1073/pnas.082089499.
[201] F.B. Dean, J.R. Nelson, T.L. Giesler, R.S. Lasken, Rapid amplification of plasmid and phage DNA using phi29 DNA polymerase and multiply-primed rolling circle amplification, Genome Res. 11 (2001) 1095–1099. https://doi.org/10.1101/gr.180501.
[202] M. Podar, C.B. Abulencia, M. Walcher, D. Hutchison, K. Zengler, J.A. Garcia, T. Holland, D. Cotton, L. Hauser, M. Keller, Targeted access to the genomes of low-abundance organisms in complex microbial communities, Appl. Environ. Microbiol. 73 (2007) 3205–3214. https://doi.org/10.1128/AEM.02985-06.
[203] X. Liu, R. Painter, K. Enesa, D. Holmes, G. Whyte, C. Garlisi, F. Monsma, M. Rehak, F. Craig, C.A. Smith, High-throughput screening of antibiotic-resistant bacteria in picodroplets, Lab Chip 16 (2016) 1636–1643. https://doi.org/10.1039/c6lc00180g.
[204] M.E. Sieracki, N.J. Poulton, O. Jaillon, P. Wincker, C. de Vargas, L. Rubinat-Ripoll, R. Stepanauskas, R. Logares, R. Massana, Single cell genomics yields a wide diversity of small planktonic protists across major ocean ecosystems, Sci. Rep. 9 (2019). https://doi.org/10.1038/S41598-019-42487-1.
[205] L.Z. Allen, T. Ishoey, M.A. Novotny, J.S. McLean, R.S. Lasken, S.J. Williamson, Single virus genomics: a new tool for virus discovery, PloS One 6 (2011), e17722. https://doi.org/10.1371/journal.pone.0017722.
[206] S. Picelli, Single-cell RNA-sequencing: the future of genome biology is now, RNA Biol. 14 (2017) 637–650. https://doi.org/10.1080/15476286.2016.1201618.
[207] D.D. Licatalosi, R.B. Darnell, RNA processing and its regulation: global insights into biological networks, Nat. Rev. Genet. 11 (2010) 75–87. https://doi.org/10.1038/nrg2673.
[208] T.-Q. Zhang, Z.-G. Xu, G.-D. Shang, J.-W. Wang, A single-cell RNA sequencing profiles the developmental landscape of Arabidopsis root, Mol. Plant 12 (2019) 648–660. https://doi.org/10.1016/j.molp.2019.04.004.
[209] S.B. Blattman, W. Jiang, P. Oikonomou, S. Tavazoie, Prokaryotic single-cell RNA sequencing by in situ combinatorial indexing, Nat. Microbiol 5 (2020) 1192–1201. https://doi.org/10.1038/s41564-020-0729-6.
[210] A. Kuchina, L.M. Brettner, L. Paleologu, C.M. Roco, A.B. Rosenberg, A. Carignano, R. Kibler, M. Hirano, R.W. DePaolo, G. Seelig, Microbial single-cell RNA sequencing by split-pool barcoding, Science 371 (2021) 798. https://doi.org/10.1126/science.aba5257.
[211] S. Rodrigue, R.R. Malmstrom, A.M. Berlin, B.W. Birren, M.R. Henn, S.W. Chisholm, Whole genome amplification and de novo assembly of single bacterial cells, PloS One 4 (2009), e6864. https://doi.org/10.1371/journal.pone.0006864.
[212] T. Woyke, A. Sczyrba, J. Lee, C. Rinke, D. Tighe, S. Clingenpeel, R. Malmstrom, R. Stepanauskas, J.-F. Cheng, Decontamination of MDA reagents for single cell whole genome amplification, PloS One 6 (2011), e26161. https://doi.org/10.1371/journal.pone.0026161.
[213] A. Raghunathan, H.R. Ferguson, C.J. Bornarth, W. Song, M. Driscoll, R.S. Lasken, Genomic DNA amplification from a single bacterium, Appl. Environ. Microbiol. 71 (2005) 3342–3347. https://doi.org/10.1128/AEM.71.6.3342-3347.2005.
[214] C.A. Hutchison, H.O. Smith, C. Pfannkoch, J.C. Venter, Cell-free cloning using φ29 DNA polymerase, Proc. Natl. Acad. Sci. Unit. States Am. 102 (2005) 17332–17336. https://doi.org/10.1073/pnas.0508809102.
[215] A. Heintz-Buschart, P. Wilmes, Human gut microbiome: function matters, Trends Microbiol. 26 (2018) 563–574. https://doi.org/10.1016/j.tim.2017.11.002.
[216] J.D. Neufeld, M. Wagner, J.C. Murrell, Who eats what, where and when? Isotope-labelling experiments are coming of age, ISME J. 1 (2007) 103–110. https://doi.org/10.1038/ismej.2007.30.
[217] D. Berry, A. Loy, Stable-isotope probing of human and animal microbiome function, Trends Microbiol. 26 (2018) 999–1007. https://doi.org/10.1016/j.tim.2018.06.004.
[218] M. Egert, A.A. De Graaf, H. Smidt, W.M. De Vos, K. Venema, Beyond diversity: functional microbiomics of the human colon, Trends Microbiol. 14 (2006) 86–91. https://doi.org/10.1016/j.tim.2005.12.007.
[219] W.-Y. Ouyang, J.-Q. Su, H.H. Richnow, L. Adrian, Identification of dominant sulfamethoxazole-degraders in pig farm-impacted soil by DNA and protein stable isotope probing, Environ. Int. 126 (2019) 118–126. https://doi.org/10.1016/j.envint.2019.02.001.
[220] D.K. Wissenbach, K. Oliphant, U. Rolle-Kampczyk, S. Yen, H. Hoeke, S. Baumann, S.B. Haange, E.F. Verdu, E. Allen-Vercoe, M. von Bergen, Optimization of metabolomics of defined in vitro gut microbial ecosystems, Int. J. Med. Microbiol. 306 (2016) 280–289. https://doi.org/10.1016/j.ijmm.2016.03.007.
[221] F.C. Pereira, K. Wasmund, I. Cobankovic, N. Jehmlich, C.W. Herbold, K.S. Lee, B. Sziranyi, C. Vesely, T. Decker, R. Stocker, B. Warth, M. von Bergen, M. Wagner, D. Berry, Rational design of a microbial consortium of mucosal sugar utilizers reduces Clostridiodes difficile colonization, Nat. Commun. 11 (2020) 5104. https://doi.org/10.1038/s41467-020-18928-1.
[222] T. Xu, Y. Gong, X. Su, P. Zhu, J. Dai, J. Xu, B. Ma, Phenome–genome profiling of single bacterial cell by Raman-activated gravity-driven encapsulation and sequencing, Small 16 (2020) 2001172. https://doi.org/10.1002/smll.202001172.