This section describes methods that could be used for detecting and quantifying biological contaminants of emerging concern (BioCEC) in various environmental matrices (e.g., air, water, and soil) and vectors (Figure 1, see also Conceptual Exposure Model subsection 1.3 Defining the Environment). Reliable detection of a pathogen in environmental matrices and vectors requires a high degree of confidence in its identity and quantitation. Specific matrix subtypes are listed in Table 1. In addition to environmental matrices and their subtypes, Table 1 includes selected references and resources detailing sampling methods for each matrix. Different environmental matrices require specific sampling techniques to ensure representativeness and data quality and to avoid bias or error. Environmental sampling typically involves the development of a sampling plan (e.g., how, when, and where samples will be collected and how many), physical collection of samples, transport and storage prior to analysis, and then analysis.

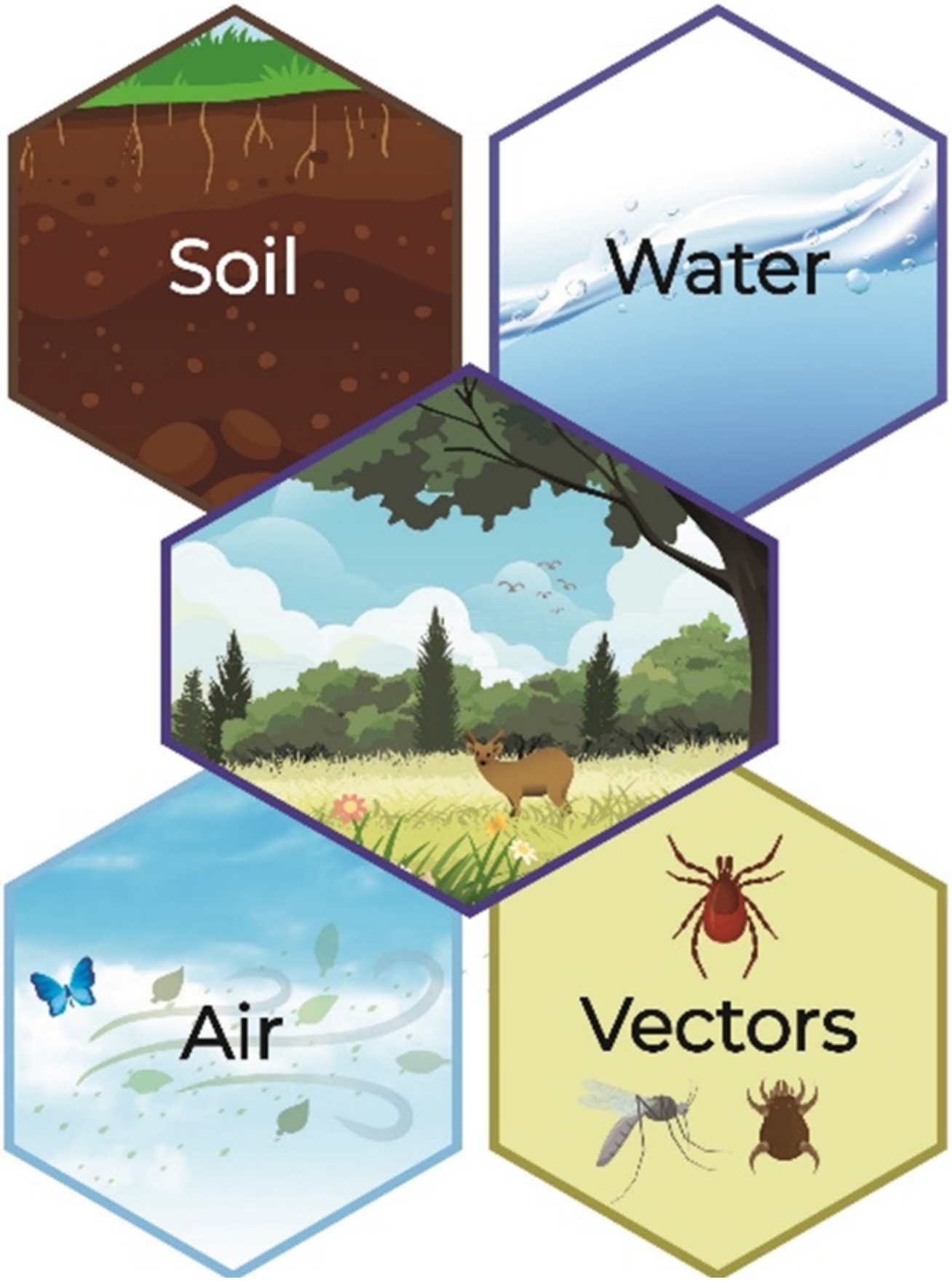



**Figure 1. Graphical depiction of environmental matrices.**

Designing and choosing an appropriate sampling method is complex and depends on many factors including study objectives, environmental matrix type, BioCEC characteristics, analytical method used, and available resources, to name a

few. Thus, specific and comprehensive sampling methods and design plans for BioCEC are challenging to prescribe. Numerous resources cited here are publicly available and are continuously being updated and improved in recognition of the important step of sampling in BioCEC identification (see USEPA ORD 2024 [6LB8D98M] USEPA ORD. 2024. “Sample Collection.” Data and Tools. https://www.epa.gov/esam/sample-collection., Table 1; USEPA OW 2025 [PYUKNXLB] USEPA OW. 2025. “National Aquatic Resource Surveys.” Collections and Lists. https://www.epa.gov/national-aquatic-resource-surveys.; Zhang 2007 [QCC2HV9P] Zhang, Chunlong. 2007. Fundamentals of Environmental Sampling and Analysis. Wiley-Interscience. https://doi.org/10.1002/0470120681.). Designing and selecting an appropriate sampling method is critical prior to applying any analytical method used for BioCEC detection. This will most likely require engagement among decision-makers, public health officials, laboratory personnel, subject matter experts, and other relevant stakeholders to ensure analytical recovery, accuracy, and reliability.

**Table 1. Sampling guidance and resources by environmental matrices type and subtype**

| Environmental Matrix Type | Matrix Subtypes | Relevant References and Resources |
|---|---|---|
| Air/aerosol | Various sizes of airborne particles or aerosols | Outdoor bioaerosol sampling onto agar plates ( Kobziar et al. 2018 [I4RY5ZXV] Kobziar, Leda N., Melissa R. A. Pingree, Heather Larson, Tyler J. Dreaden, Shelby Green, and Jason A. Smith. 2018. “Pyroaerobiology: The Aerosolization and Transport of Viable Microbial Life by Wildland Fire.” Ecosphere 9 (11): e02507. https://doi.org/10.1002/ecs2.2507.)<br>Indoor aerosol sampling ( Kumar et al. 2021 [STE4L77L] Kumar, Pradeep, Mohd. Adnan Kausar, A. B. Singh, and Rajeev Singh. 2021. “Biological Contaminants in the Indoor Air Environment and Their Impacts on Human Health.” Air Quality, Atmosphere & Health 14 (11): 1723–36. https://doi.org/10.1007/s11869-021-00978-z.)<br>Aerosol sampling for disease surveillance ( Santarpia et al. 2023 [XX73BZCQ] Santarpia, Joshua L., Elizabeth Klug, Ashley Ravnholdt, and Sean M. Kinahan. 2023. “Environmental Sampling for Disease Surveillance: Recent Advances and Recommendations for Best Practice.” Journal of the Air & Waste Management Association 73 (6): 434–61. https://doi.org/10.1080/10962247.2023.2197825.) |
| Water | Drinking water<br>Freshwater<br>Surface water<br>Brackish water<br>Saltwater<br>Stormwater<br>Wastewater | Drinking water: Standard Methods for the Examination of Water and Wastewater ( APHA et al. 2023 [945V9BC7] APHA, AWWA, and WEF. 2023. Standard Methods for the Examination of Water and Wastewater. 24th ed. Edited by E. B. Braun-Howland and T. E. Baxter. American Public Health Association.)<br>Freshwater and surface water monitoring and sampling guidance ( Queensland Government 2018 [FW5ECGEX] Queensland Government. 2018. “Water Monitoring and Sampling Manual.” Environment \| Department of the Environment, Tourism, Science and Innovation, Queensland. https://environment.qld.gov.au/management/water/quality-guidelines/sampling-manual. BC Ministry of Environment, Lands and Parks. Water Management Branch. [8JZWTFVS] BC Ministry of Environment, Lands and Parks. Water Management Branch. n.d. “Freshwater Biological Sampling Manual.” Accessed August 13, 2025. https://www2.gov.bc.ca/assets/gov/environment/natural-resource-stewardship/nr-laws-policy/risc/freshwaterbio.pdf.); US Environmental Protection Agency (USEPA) national aquatic resources for surface water sampling methods ( USEPA OW 2025 [PYUKNXLB] USEPA OW. 2025. “National Aquatic Resource Surveys.” Collections and Lists. https://www.epa.gov/national-aquatic-resource-surveys.)<br>Brackish water and saltwater: guidance on water quality criteria, monitoring, and sampling ( USEPA 2014 [RELKTDQY] USEPA. 2014. “Passive Samplers for Investigations of Air Quality: Method Description, Implementation, and Comparison to Alternative Sampling Methods.” CAEPA 2019 [4NMXNVKR] CAEPA. 2019. “Water Quality Control Plan for Ocean Waters of California.” https://www.waterboards.ca.gov/water_issues/programs/ocean/docs/oceanplan2019.pdf.)<br>Stormwater: Saifur and Gardner 2021 [P353XVP2] Saifur, Sumaiya, and Courtney M. Gardner. 2021. “Loading, Transport, and Treatment of Emerging Chemical and Biological Contaminants of Concern in Stormwater.” Water Science and Technology 83 (12): 2863–85. https://doi.org/10.2166/wst.2021.187.discuss the significance of stormwater microbial contamination and detection of antibiotic-resistant genes in recreational and receiving water bodies<br>Wastewater: ( Alygizakis et al. 2020 [SRZ85H2Z] Alygizakis, Nikiforos A., Jakub Urík, Vasiliki G. Beretsou, et al. 2020. “Evaluation of Chemical and Biological Contaminants of Emerging Concern in Treated Wastewater Intended for Agricultural Reuse.” Environment International 138 (May): 105597. https://doi.org/10.1016/j.envint.2020.105597.); New York state COVID Resources doc |
| Soil/Sediments | Sand<br>Silt<br>Clay | Soil sampling ( Kobziar et al. 2018 [I4RY5ZXV] Kobziar, Leda N., Melissa R. A. Pingree, Heather Larson, Tyler J. Dreaden, Shelby Green, and Jason A. Smith. 2018. “Pyroaerobiology: The Aerosolization and Transport of Viable Microbial Life by Wildland Fire.” Ecosphere 9 (11): e02507. https://doi.org/10.1002/ecs2.2507.)<br>Soil remediation techniques and technologies ( Wang et al. 2024 [GREHZTLU] Wang, Fang, Leilei Xiang, Kelvin Sze-Yin Leung, et al. 2024. “Emerging Contaminants: A One Health Perspective.” The Innovation 5 (4). https://doi.org/10.1016/j.xinn.2024.100612.) |

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|---|---|---|
| Vectors | Ectoparasites (ticks, fleas, mites, flies, etc.)<br>Mosquitoes | CDC Mosquito Control Resources<br>CDC Tick Data and Resources<br>Tick surveillance programs and related health tools ( Eisen and Paddock 2021 [5BCLYYLX] Eisen, Rebecca J., and Christopher D. Paddock. 2021. "Tick and Tickborne Pathogen Surveillance as a Public Health Tool in the United States." Journal of Medical Entomology 58 (4): 1490–502. https://doi.org/10.1093/jme/tjaa087.) |

Microbiological detection and quantification methods are typically developed and tested across many laboratories and groups with well-defined method limitations and appropriate quality control practices (see Interstate Technology and Regulatory Council (ITRC) Environmental Molecular Diagnostics (EMD) Section 10 for quality control considerations). These standardized microbial methods usually represent the best technique currently available for the detection and/or quantification of a specific known pathogen (i.e., targeted analysis, which can identify organisms at genera, species, and/or even strain level classifications). To some degree, these standardized methods can be used or modified in some way to capture new groups or subtypes of known pathogens (i.e., suspect screening).

For a BioCEC, reliable and standardized analytical methods may not be readily available, especially for new pathogens that have not been encountered previously (i.e., non-target analysis). Figure 2 depicts the transition of analytical methods from targeted screening approaches that are designed to detect a particular organism, to less-specific suspect screening that can help identify the general identity of a new BioCEC based on evidence-developed hypotheses, to non-targeted screenings that can help identify BioCECs that do not fit a known description. These analytical methods and their applications are discussed in detail below in Description of Analytical Methods. Table 2 identifies whether the various methods described in Description of Analytical Methods can be used for targeted analysis, suspect screening, and non-targeted analysis, and provides examples for how these methods have been used. Table 2 provides a partial list of possible analytical methods and serves as a summary of those methods, which have been highlighted within this document.

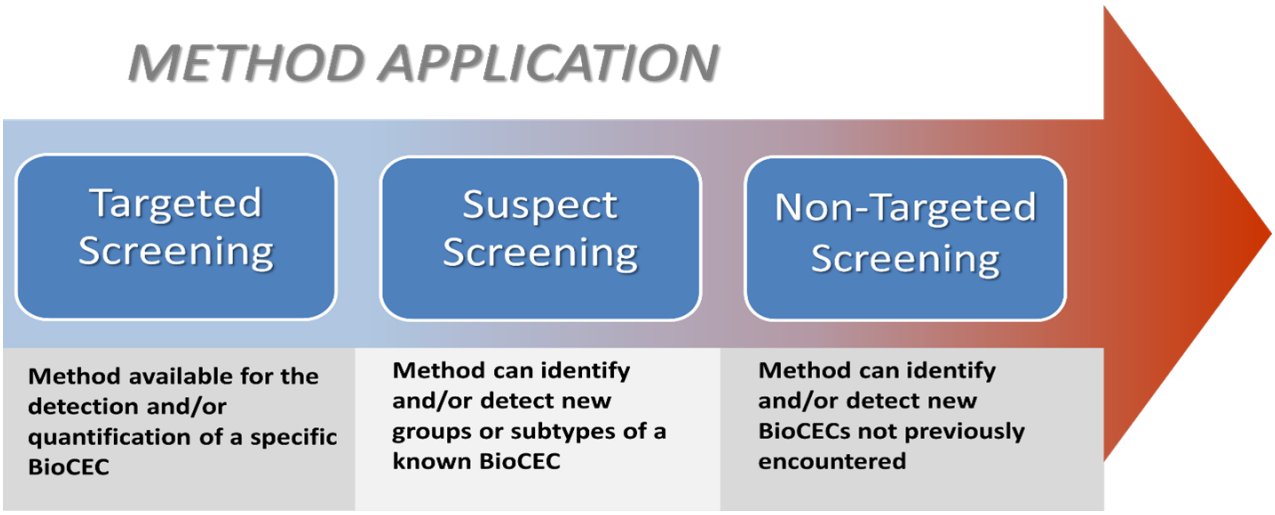



**Figure 2. Flowchart of the transition from targeted screening to non-target screening of biological contaminants of emerging concern.**

**Table 2. Overview of analytical methods and their applications for the detection of biological contaminants of emerging concern**

| Method | Targeted Usage[1] | Suspect Screening[2] | Non-Targeted Usage[3] |
|---|---|---|---|
| Microscopy | Yes. Commonly used in clinical settings for disease diagnosis. | Yes. Fluorescence microscopy is used for viral and bacterial monitoring in diverse marine samples (Noble and Fuhrman 1998).<br>Scanning electron microscopy coupled with energy dispersive x-ray analysis enabled identification and distinction between individual cells of pathogenic microbes (e.g., *Escherichia coli*, *Salmonella typhimurium*, *Listeria monocytogenes*) ( Khan et al. 2020 [CSCHYQW7] Khan, Muhammad Saiful Islam, Se-Wook Oh, and Yun-Ji Kim. 2020. "Power of Scanning Electron Microscopy and Energy Dispersive X-Ray Analysis in Rapid Microbial Detection and Identification at the Single Cell Level." Scientific Reports 10 (1): 2368. https://doi.org/https://dx.doi.org/10.1038/s41598-020-59448-8.). | Yes. Air quality monitoring using microscopy and machine learning can be used to detect particles ≤2.5 µm ( Wu et al. 2017 [LB5PZ67F] Wu, Yi-Chen, Ashutosh Shiledar, Yi-Cheng Li, et al. 2017. "Air Quality Monitoring Using Mobile Microscopy and Machine Learning." Light: Science & Applications 6 (9): e17046–e17046. https://doi.org/10.1038/lsa.2017.46.). |

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|---|---|---|---|
| Culture-Based Methods | Yes ( Bonnet et al. 2020 [DY8TQG8H] Bonnet, M., J. C. Lagier, D. Raoult, and S. Khelaifia. 2020. “Bacterial Culture through Selective and Non-Selective Conditions: The Evolution of Culture Media in Clinical Microbiology.” New Microbes and New Infections 34 (March): 100622. https://doi.org/10.1016/j.nmni.2019.100622.). | Yes. Used to detect filamentous fungi in water ( Babič et al. 2017 [RWZSDWQF] Babič, Monika Novak, Nina Gunde-Cimerman, Márta Vargha, et al. 2017. “Fungal Contaminants in Drinking Water Regulation? A Tale of Ecology, Exposure, Purification and Clinical Relevance.” International Journal of Environmental Research and Public Health 14 (6): 636. https://doi.org/10.3390/ijerph14060636.), yeasts and fungi from bioaerosols ( Kobziar et al. 2018 [I4RYSZXV] Kobziar, Leda N., Melissa R. A. Pingree, Heather Larson, Tyler J. Dreaden, Shelby Green, and Jason A. Smith. 2018. “Pyroaerobiology: The Aerosolization and Transport of Viable Microbial Life by Wildland Fire.” Ecosphere 9 (11): e02507. https://doi.org/10.1002/ecs2.2507.), and selection for tetracycline resistance in soil ( Wang et al. 2024 [GREHZTLU] Wang, Fang, Leilei Xiang, Kelvin Sze-Yin Leung, et al. 2024. “Emerging Contaminants: A One Health Perspective.” The Innovation 5 (4). https://doi.org/10.1016/j.xinn.2024.100612.). | Maybe. Culture conditions and medium are generally designed to select for specific organisms; however, non-selective agar plates have been used for general capture of culturable organisms in air and water matrices ( WHO 2003 [XM85BN38] WHO. 2003. “Assessing Microbial Safety of Drinking-Water: Improving Approaches and Methods.” https://www.who.int/publications/i/item/9241546301. Viani et al. 2020 [2XMITA3Q] Viani, Isabella, Maria Eugenia Colucci, Massimiliano Pergreffi, et al. 2020. “Passive Air Sampling: The Use of the Index of Microbial Air Contamination.” Acta Biomedica Atenei Parmensis 91 (3-S): 92–105. https://doi.org/10.23750/abm.v91i3-S.9434.). |
| Flow Cytometry (FC) | Yes (antibodies, fluorescent probes). For targeted analysis, it is often considered appropriate to use FC if fluorescently labeled antibodies against known epitopes for the BioCEC exist or can be prepared, allowing for positive identification of the agent.<br>Example: LITMUS RAPID-B system for detection of bacterial epitopes in food products 16S in situ hybridization with fluorescent probes to identify specific species of bacteria in a sample. | Yes. For suspect screening, FC can assist in identifying key characteristics of organisms. For bacterial screening, gram-staining using fluorophores can be conducted to identify populations within a mixed sample. Additionally, antibiotic resistance of a bacterial BioCEC can be assessed using live/dead fluorescent dye combinations following treatment. Examples: Flow Cytometry Antibiotic Susceptibility Testing ( Marutescu 2023 [LDKZJ43M] Marutescu, Luminita Gabriela. 2023. “Current and Future Flow Cytometry Applications Contributing to Antimicrobial Resistance Control.” Microorganisms 11 (5): 1300. https://doi.org/10.3390/microorganisms11051300.); gram-staining and viability staining ( Duquenoy et al. 2020 [AA8NLUJ2] Duquenoy, Aurore, Samuel Bellais, Cyrielle Gasc, Carole Schwintner, Joël Dore, and Vincent Thomas. 2020. “Assessment of Gram- and Viability-Staining Methods for Quantifying Bacterial Community Dynamics Using Flow Cytometry.” Frontiers in Microbiology 11 (June). https://doi.org/10.3389/fmicb.2020.01469.). | Yes (general capture/characterization of BioCEC based on size, complexity, etc.).<br>When the BioCEC is completely unknown, FC can be used to identify physical characteristics, notably size, of unknown agents. This can help delineate whether the BioCEC is of fungal, bacterial, or viral origins. Although viruses will often be too small to identify via FC, the absence of larger organisms may lead to the assumption of a viral pathogen. |
| Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry (MS) | Yes. Clinical samples or culture isolates can be rapidly identified or confirmed ( Hou et al. 2019 [3UJ3PZTL] Hou, Tsung-Yun, Chuan Chiang-Ni, and Shih-Hua Teng. 2019. “Current Status of MALDI-TOF Mass Spectrometry in Clinical Microbiology.” Journal of Food and Drug Analysis, Mass Spectrometry for Clinical Diagnosis, vol. 27 (2): 404–14. https://doi.org/10.1016/j.jfda.2019.01.001. Elbehiry et al. 2022 [QXYWZ429] Elbehiry, Ayman, Musaad Aldubaib, Adil Abalkhail, et al. 2022. “How MALDI-TOF Mass Spectrometry Technology Contributes to Microbial Infection Control in Healthcare Settings.” Vaccines 10 (11): 1881. https://doi.org/10.3390/vaccines10111881.). Can also be used as a rapid diagnostic technique ( Feucherolles et al. 2019 [V4V2J36P] Feucherolles, Maureen, Sven Poppert, Jürg Utzinger, and Sören L. Becker. 2019. “MALDI-TOF Mass Spectrometry as a Diagnostic Tool in Human and Veterinary Helminthology: A Systematic Review.” Parasites & Vectors 12 (1): 245. https://doi.org/10.1186/s13071-019-3493-9.). Can be used to identify protein toxins (e.g., enterotoxin B), botulinum neurotoxins, Shiga toxin, etc., ( Singhal et al. 2015 [4LM73W7L] Singhal, Neelja, Manish Kumar, Pawan K. Kanaujia, and Jugsharan S. Virdi. 2015. “MALDI-TOF Mass Spectrometry: An Emerging Technology for Microbial Identification and Diagnosis.” Frontiers in Microbiology 6 (August). https://doi.org/10.3389/fmicb.2015.00791.). | Yes. Used for detection of antibiotic-resistance in bacteria ( Florio et al. 2020 [GTATMNY3] Florio, Walter, Lelio Baldeschi, Cosmeri Rizzato, Arianna Tavanti, Emilia Ghelardi, and Antonella Lupetti. 2020. “Detection of Antibiotic-Resistance by MALDI-TOF Mass Spectrometry: An Expanding Area.” Frontiers in Cellular and Infection Microbiology 10 (November). https://doi.org/10.3389/fcimb.2020.572909.). | Maybe. In development, but heavily dependent on reliability and quality of spectral databases ( Ashfaq et al. 2022 [8CJQXV8G] Ashfaq, Mohammad Y., Dana A. Da’na, and Mohammad A. Al-Ghouti. 2022. “Application of MALDI-TOF MS for Identification of Environmental Bacteria: A Review.” Journal of Environmental Management 305 (March): 114359. https://doi.org/10.1016/j.jenvman.2021.114359.). Pinar-Méndez et al. 2021 [5NKJMCGH] Pinar-Méndez, Anna, Sonia Fernández, David Baquero, et al. 2021. “Rapid and Improved Identification of Drinking Water Bacteria Using the Drinking Water Library, a Dedicated MALDI-TOF MS Database.” Water Research 203 (September): 117543. https://doi.org/10.1016/j.watres.2021.117543.developed a drinking water library of more than 300 bacteria profiles representing 44 new genera and improved identification of water isolates). |

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| Polymerase Chain Reaction (PCR) and Quantitative Polymerase Chain Reaction (qPCR) | Yes. Targeted probes are used for diverse enteric pathogens in human and animal stool, potable water, river water, freshwater, stormwater, and soil ( Lappan et al. 2021 [P67CLPPB] Lappan, Rachael, Rebekah Henry, Steven L. Chown, et al. 2021. "Monitoring of Diverse Enteric Pathogens across Environmental and Host Reservoirs with TaqMan Array Cards and Standard qPCR: A Methodological Comparison Study." The Lancet Planetary Health 5 (5): e297–308. https://doi.org/10.1016/S2542-5196(21)00051-6.). Also used for monitoring antibiotic-resistant genes ( Zhang et al. 2022 [ASPYPPR6] Zhang, Yue, Yajie Guo, Tianlei Qiu, Min Gao, and Xuming Wang. 2022. "Bacteriophages: Underestimated Vehicles of Antibiotic Resistance Genes in the Soil." Frontiers in Microbiology 13 (August). https://doi.org/10.3389/fmicb.2022.936267. Franklin et al. 2021 [8GNQ66YM] Franklin, A. M., N. E. Brinkman, M. A. Jahne, and S. P. Keely. 2021. "Twenty-First Century Molecular Methods for Analyzing Antimicrobial Resistance in Surface Waters to Support One Health Assessments." Journal of Microbiological Methods 184 (May): 106174. https://doi.org/10.1016/j.mimet.2021.106174.) and virulence genes (e.g., toxin, adherence, secretion system, iron uptake, etc.) in environmental samples ( Xie et al. 2023 [FNW46ZJC] Xie, Shu-Ting, Long-Jun Ding, Fu-Yi Huang, et al. 2023. "VFG-Chip: A High-Throughput qPCR Microarray for Profiling Virulence Factor Genes from the Environment." Environment International 172 (February): 107761. https://doi.org/10.1016/j.envint.2023.107761.). | Yes. Used for broadly targeted probes (e.g., 16S, 18S, ITS genomic region).<br>Environmental DNA and RNA can be analyzed by PCR for bacteria, viruses, parasites, fungus, algae, eukaryotes, micro and macroinvertebrates, and vertebrates ( Bass et al. 2023 [XC2MDZE7] Bass, David, Kevin W. Christison, Grant D. Stentiford, Lauren S. J. Cook, and Hanna Hartikainen. 2023. "Environmental DNA/RNA for Pathogen and Parasite Detection, Surveillance, and Ecology." Trends in Parasitology 39 (4): 285–304. https://doi.org/10.1016/j.pt.2022.12.010.). | Yes, general, broad capture probes.<br>Degenerative probes can be used for tracking nitrogen metabolism in diverse microorganisms ( Keeley et al. 2020 [UPVIWY4S] Keeley, Ryan F., Laura Rodriguez-Gonzalez, U. S. F. Genomics Class, et al. 2020. "Degenerate PCR Primers for Assays to Track Steps of Nitrogen Metabolism by Taxonomically Diverse Microorganisms in a Variety of Environments." Journal of Microbiological Methods 175 (August): 105990. https://doi.org/10.1016/j.mimet.2020.105990.), identifying and differentiating between different SARS-CoV-2 variants ( Jessen et al. 2022 [XPZHNH56] Jessen, Randi, Line Nielsen, Nicolai Balle Larsen, et al. 2022. "A RT-qPCR System Using a Degenerate Probe for Specific Identification and Differentiation of SARS-CoV-2 Omicron (B.1.1.529) Variants of Concern." PLOS ONE 17 (10): e0274889. https://doi.org/10.1371/journal.pone.0274889.), and monitoring soil health by targeting bacterial biosynthesis gene domains ( Lemetre et al. 2017 [N6RSCXPB] Lemetre, Christophe, Jeffrey Maniko, Zachary Charlop-Powers, Ben Sparrow, Andrew J. Lowe, and Sean F. Brady. 2017. "Bacterial Natural Product Biosynthetic Domain Composition in Soil Correlates with Changes in Latitude on a Continent-Wide Scale." Proceedings of the National Academy of Sciences 114 (44): 11615–20. https://doi.org/10.1073/pnas.1710262114.). |
| Genomics or Metagenomics | Yes. Provides rapid and precise identification of microbial pathogen species and strains from cultured organisms and environmental samples ( Li et al. 2021 [5CXERHTS] Li, Na, Qingqing Cai, Qing Miao, Zeshi Song, Yuan Fang, and Bijie Hu. 2021. "High-Throughput Metagenomics for Identification of Pathogens in the Clinical Settings." Small Methods 5 (1): 2000792. https://doi.org/10.1002/smtd.202000792.). | Yes. Culture-independent metagenomic sequencing can be used to identify bacterial strains from an outbreak ( Loman et al. 2013 [QKQB928G] Loman, Nicholas J., Chrystala Constantinidou, Martin Christner, et al. 2013. "A Culture-Independent Sequence-Based Metagenomics Approach to the Investigation of an Outbreak of Shiga-Toxigenic Escherichia coli O104:H4." JAMA 309 (14): 1502–10. https://doi.org/10.1001/jama.2013.3231.). | Yes. Metagenomic sequencing is particularly useful for detecting emerging threats. It provides unbiased detection of all microorganisms present in an environmental sample ( Pérez-Cobas et al. 2020 [UNAUYTF6] Pérez-Cobas, Ana Elena, Laura Gomez-Valero, and Carmen Buchrieser. 2020. "Metagenomic Approaches in Microbial Ecology: An Update on Whole-Genome and Marker Gene Sequencing Analyses." Microbial Genomics 6 (8): e000409. https://doi.org/10.1099/mgen.0.000409.). It also provides functional information about the capabilities of microorganisms ( Davis et al. 2023 [HAZAQWSC] Davis, Benjamin C., Connor Brown, Suraj Gupta, et al. 2023. "Recommendations for the Use of Metagenomics for Routine Monitoring of Antibiotic Resistance in Wastewater and Impacted Aquatic Environments." Critical Reviews in Environmental Science and Technology 53 (19): 1731–56. https://doi.org/10.1080/10643389.2023.2181620.). |
| Fluorescence In Situ Hybridization (FISH) | Yes. Specific RNA and DNA-based probes targeting select pathogens are used.<br>Used for clinical diagnosis of known intracellular pathogens ( Prudent and Raoult 2019 [BUZ99TQR] Prudent, Elsa, and Didier Raoult. 2019. "Fluorescence in Situ Hybridization, a Complementary Molecular Tool for the Clinical Diagnosis of Infectious Diseases by Intracellular and Fastidious Bacteria." FEMS Microbiology Reviews 43 (1): 88–107. https://doi.org/10.1093/femsre/fuy040.).<br>Used for rapid and simultaneous identification of respiratory viruses in clinical samples ( Hepp et al. 2021 [AUR9L6NK] Hepp, Christof, Nicolas Shiaelis, Nicole C. Robb, et al. 2021. "Viral Detection and Identification in 20 Min by Rapid Single-Particle Fluorescence In Situ Hybridization of Viral RNA." Scientific Reports 11 (1): 19579. https://doi.org/10.1038/s41598-021-98972-z.). | Yes. Used for broadly targeted probes (e.g., 16S rRNA gene sequences, metabolic enzymes).<br>A modified FISH technique to target and preserve live cells is followed by fluorescence-activated cell sorting to isolate and culture subsets of live bacteria from environmental samples ( Batani et al. 2019 [CZGML94A] Batani, Giampiero, Kristina Bayer, Julia Böge, Ute Hentschel, and Torsten Thomas. 2019. "Fluorescence In Situ Hybridization (FISH) and Cell Sorting of Living Bacteria." Scientific Reports 9 (1): 18618. https://doi.org/10.1038/s41598-019-55049-2.).<br>Used in the identification of aerobic methane oxidizing bacteria in seawater and sediment samples ( Pernthaler and Amann 2004 [E3AA7U7S] Pernthaler, Annelie, and Rudolf Amann. 2004. "Simultaneous Fluorescence In Situ Hybridization of mRNA and rRNA in Environmental Bacteria." Applied and Environmental Microbiology 70 (9): 5426–33. https://doi.org/10.1128/AEM.70.9.5426-5433.2004.).<br>Used in the monitoring of the *Pseudomonad* genus for soil health ( Gougoulias and Shaw 2012 [HMAKQNJ7] Gougoulias, Christos, and Liz J. Shaw. 2012. "Evaluation of the Environmental Specificity of Fluorescence In Situ Hybridization (FISH) Using Fluorescence-Activated Cell Sorting (FACS) of Probe (PSE1284)-Positive Cells Extracted from Rhizosphere Soil." Systematic and Applied Microbiology, Special Issue: Fluorescence In Situ Hybridization (FISH), vol. 35 (8): 533–40. https://doi.org/10.1016/j.syapm.2011.11.009.). | Yes. Used for broadly targeted probes (e.g., eukaryotic and prokaryotic probes).<br>Used to monitor changes in microbial communities in different sample types such as clinical ( Gu et al. 2022 [4PQL2NU9] Gu, Junjie, Huayu Wang, Mengye Zhang, et al. 2022. "Application of Fluorescence In Situ Hybridization (FISH) in Oral Microbial Detection." Pathogens 11 (12): 1450. https://doi.org/10.3390/pathogens11121450.) and environmental ( Saccà et al. 2019 [WA8BB6NL] Saccà, Maria Ludovica, Valentina Elisabetta Viviana Ferrero, Robert Loos, et al. 2019. "Chemical Mixtures and Fluorescence in Situ Hybridization Analysis of Natural Microbial Community in the Tiber River." Science of the Total Environment 673 (July): 7–19. https://doi.org/10.1016/j.scitotenv.2019.04.011.).<br>Saccà et al. 2019 [WA8BB6NL] Saccà, Maria Ludovica, Valentina Elisabetta Viviana Ferrero, Robert Loos, et al. 2019. "Chemical Mixtures and Fluorescence in Situ Hybridization Analysis of Natural Microbial Community in the Tiber River." Science of the Total Environment 673 (July): 7–19. https://doi.org/10.1016/j.scitotenv.2019.04.011.used FISH to monitor bacterioplankton composition in river water and the impacts of industrial chemical exposure. |

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|---|---|---|---|
| Microbial Fingerprinting Methods | Yes. Fingerprinting can be used to help identify and confirm a suspected organism, down to strain variation. | Yes. Fingerprinting can be used to help narrow down suspects. Comparing the fingerprint developed to a standard database of microbial fingerprints should allow for the elimination of certain suspects in a pool. | No. Fingerprinting is not advised for non-targeted analysis. Many fingerprinting assays are based around the amplification of particular genes before applying restriction cutting. If the PCR targets are unknown, it is extremely difficult to produce a relevant fingerprint. |
| Advanced Isothermal Approaches | Yes. Isothermal methods are field deployable and can be used to identify pathogens in environmental samples ( Nieuwkerk et al. 2020 [6G98P5F9] Nieuwkerk, Dana M., Asja Korajkic, Erika L. Valdespino, Michael P. Herrmann, and Valerie J. Harwood. 2020. “Critical Review of Methods for Isothermal Amplification of Nucleic Acids for Environmental Analysis.” Journal of Microbiological Methods 179 (December): 106099. https://doi.org/10.1016/j.mimet.2020.106099.). | No. | No. |

[1] Targeted usage: The best technique currently available for the detection and/or quantification of a specific pathogen. “Yes” indicates that the method is appropriate, or applicable, for targeted usage.

[2] Suspect screening: Standardized methods that can be used or modified in some way to capture new groups or subtypes of known pathogens. “Yes” or “No” indicates whether the method is appropriate, or applicable, for suspect screening.

[3] Non-targeted usage: Application of a method to identify new pathogens that have not been encountered previously. “Yes,” “No,” or “Maybe” indicates whether the method is appropriate, or applicable, for non-targeted usage.

This section does not discuss the increasing use of data analytics (e.g., machine-learning approaches) for monitoring and forecasting contamination in the environment. For example, Mahmood et al. 2024 [GSW4SAFP] Mahmood, Mahnoor, Eric Minwei Liu, Amy L. Shergold, et al. 2024. “Mitochondrial DNA Mutations Drive Aerobic Glycolysis to Enhance Checkpoint Blockade Response in Melanoma.” Nature Cancer 5 (4): 659–72. https://doi.org/10.1038/s43018-023-00721-w.address the known data gaps (i.e., missing data) in groundwater quality databases by using two advanced data imputation algorithms. Results suggested that these machine learning–based algorithms can help identify sampling locations, provide geospatial information about contaminants, and prioritize analytes for testing to maximize sampling efforts and efficiently use available, but often limited, sampling resources. Nevertheless, prior to the application of more advanced data analytics, there is a need for reliable detection and quantification of BioCEC through direct analytical methods, which is the focus of this section.

# 1. Description of Analytical Methods

A previous ITRC effort generated detailed descriptions of EMDs, which is a collective term for advanced and emerging techniques for the analysis of biological and chemical characteristics of environmental samples (ITRC 2013). That ITRC EMD webtool and resource provides definitions of various terms also used in this section; they can be accessed by clicking on the word under the ITRC EMD Glossary tab. That ITRC resource also contains a detailed appendix of microbiology FAQs providing additional background information if needed (ITRC EMD Appendix D). The methods described below can be used for detection and/or quantification of BioCEC in the environmental matrices and subtypes listed in Table 1. As discussed in this section, selection of an analytical method will depend on numerous factors such as environmental matrix sample type; collection method used; BioCEC characteristics; potential recovery from and concentration in the original sample; and targeted analytical method accuracy, precision, and reliability. Thus, the potential challenges, advantages, disadvantages, and limitations of an analytical method are multifactorial and should be considered throughout the process of designing a sampling and collection plan and choosing an appropriate analytical method.

# 2. Microscopy

Microscopy is a general term used to describe the use of microscopes to view objects at a resolution that cannot be observed with the unaided eye. This method allows for the analysis of shape, size, and other characteristics that allow for the identification and classification of biological samples.

## 2.1 Direct or Light Microscopy

In this method, light is transmitted from a source (e.g., lamp) through a condenser, either below or above the sample. The light then passes through the sample to a magnifying lens (or objective), then to the oculars, where the enlarged sample image can be viewed.

### 2.2 Fluorescent Microscopy

This method relies on the underlying process of fluorescence, which occurs when a substance absorbs light of specific wavelengths and emits light at longer wavelengths. Thus, samples are the source of the visible light, in contrast to light microscopy. Most commonly, samples are stained with fluorescently labeled antibodies, nucleic acids, or fluorescent dyes, such as 4′,6-diamido-2-phenylindole, propidium iodide, and SYBR green. Samples that autofluorescence can also be detected. In fluorescent microscopy, samples are illuminated with a single or multiple wavelengths of light (excitation wavelength), and the emitted fluorescence (emission wavelengths) reaches the eye or detector ( WHO 2005 [DQ3463SM] WHO. 2005. Fluorescence Microscopy for Disease Diagnosis and Environmental Monitoring. https://applications.emro.who.int/dsaf/dsa281.pdf.).

### 2.3 Electron Microscopy

This method uses a beam of electrons as the source of illumination to magnify an object, allowing for the visualization of biological structures and composition. Types of electron microscopy include scanning electron microscopy and transmission electron microscopy.

## 3. Culture-Based Methods

Microorganisms can be cultured in either selective or non-selective media under various conditions (e.g., temperatures, atmospheric conditions, incubation times, etc.). The combination of media type and incubation conditions can be used to select for growth of specific microbial groups, subgroups, or species ( Lagier et al. 2015 [YAJ7NZ8Y] Lagier, Jean-Christophe, Sophie Edouard, Isabelle Pagnier, Oleg Mediannikov, Michel Drancourt, and Didier Raoult. 2015. “Current and Past Strategies for Bacterial Culture in Clinical Microbiology.” Clinical Microbiology Reviews 28 (1): 208–36. https://doi.org/10.1128/CMR.00110-14. Bonnet et al. 2020 [DY8TQG8H] Bonnet, M., J. C. Lagier, D. Raoult, and S. Khelaifia. 2020. “Bacterial Culture through Selective and Non-Selective Conditions: The Evolution of Culture Media in Clinical Microbiology.” New Microbes and New Infections 34 (March): 100622. https://doi.org/10.1016/j.nmni.2019.100622.). Growth of organisms under these specific conditions may be considered “presumptive,” meaning that both the organisms of interest and off-target organisms can grow. In these cases, further testing (using other methods) may be necessary to confirm the identity of the organism. Further sample processing may be required (e.g., filtration, heat, and/or acid treatment) to culture targeted microorganisms. Cell culture systems have also been used to detect, identify, and propagate pathogens (e.g., animal models, embryonated eggs, mammalian cell lines, amoebae) ( Vouga and Greub 2016 [UL4EE9BG] Vouga, M., and G. Greub. 2016. “Emerging Bacterial Pathogens: The Past and Beyond.” Clinical Microbiology and Infection 22 (1): 12–21. https://doi.org/10.1016/j.cmi.2015.10.010.).

## 4. Flow Cytometry

Flow cytometry (FC) is a method that analyzes individual cells or particles in suspension using a flow cytometer (see recent review by Robinson et al. 2023 [GGP7ZVSE] Robinson, J. Paul, Raluca Ostafe, Sharath Narayana Iyengar, Bartek Rajwa, and Rainer Fischer. 2023. “Flow Cytometry: The Next Revolution.” Cells 12 (14): 1875. https://doi.org/10.3390/cells12141875.). The flow rate and tubing that draw samples into the cytometer are optimized to allow for a single cell to be analyzed at a time. Analyzers within the flow cytometer use multiple lasers with a variety of wavelengths and angles to investigate an individual cell. From this, the cytometer will collect data on the scatter of visible light by the cell; certain wavelengths can excite fluorescent particles associated with the cell, and the emission from this can be collected by fluorescent detectors. The patterns of the visible light scatter and excitation/emission spectrums of the fluorescence signal can provide important information about the characteristics of the cells or particles (e.g., relative size, internal complexity, surface properties, identity, etc.). To maximize the discriminatory power of this method, samples can also be labeled with fluorescently tagged molecules targeting specific suspects (i.e., fluorescently labeled antibodies) that will allow for specific identification of the biological contaminants.

Method detection limits for FC include size and concentration of the biological agent. If the particles are too small, as is the case with the average virion, the cytometer will likely not be able to detect it. If the agent is too large (i.e., some parasites, or clumps of cells that were not dispersed properly), the detector will not properly categorize the particle. Similarly, the concentration of a target in a sample may also be a limiting factor for detection. If only a few representatives are present in a sample, it may be that any positives could be dismissed as erroneous detections.

A flow cytometer can be equipped with a cell sorter, which will allow for the collection and concentration of cells that meet an investigator's criterion. This can then allow for further investigations using the concentrated sample.

## 5. Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry (MS)

MALDI-TOF MS is a method that can rapidly identify pathogens or biological molecules by generating a spectral profile that is then compared against a library of reference spectral profiles of known biologicals ( Ashfaq et al. 2022 [8CJQXV8G] Ashfaq, Mohammad Y., Dana A. Da'na, and Mohammad A. Al-Ghouti. 2022. "Application of MALDI-TOF MS for Identification of Environmental Bacteria: A Review." Journal of Environmental Management 305 (March): 114359. https://doi.org/10.1016/j.jenvman.2021.114359.). Profiles are generated by ionizing biological particles (e.g., cellular proteins such as ribosomes), which move through a flight tube driven by an electric field separating the particles according to their mass and charge. The time-of-flight is measured by instrument detectors at the end of the flight tube. The x-axis of the spectra indicates the mass-to-charge values, and the y-axis shows the intensity of the signal.

## 6. Polymerase Chain Reaction (PCR)

PCR is a method used to amplify specific, targeted DNA sequences. The technique uses a pair of short synthetic DNA segments called primers that recognizes the start (5′) and end (3′) of the targeted DNA sequence. These primers help guide the enzyme, DNA polymerase, to copy the target DNA sequence through repeated cycles of heating and cooling. This enables the DNA strands to separate and for primers to anneal and then be extended by DNA polymerase to exponentially generate detectable copies of the target DNA sequence ( NIH: National Human Genome Research Institute 2025 [2CMPPAC2] NIH: National Human Genome Research Institute. 2025. "Polymerase Chain Reaction (PCR)." https://www.genome.gov/genetics-glossary/Polymerase-Chain-Reaction-PCR.).

### 6.1 Quantitative Polymerase Chain Reaction (qPCR)

This is a very sensitive technique used to amplify short gene sequences (e.g., 80–150 base pairs). It provides real-time monitoring of the exponential amplification process via fluorescence probes or dyes. The detected fluorescence is proportional to the amount of DNA in the reactions, facilitating precise quantification of DNA by interpolation from standard curves. Primers are designed via primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?GROUP_TARGET=on) or can be derived from published methods or other peer-reviewed literature.

### 6.2 Digital Polymerase Chain Reaction (dPCR)

This is an advanced nucleic acid quantification method that has no need for standard curves to determine quantities of target DNA. Digital polymerase chain reaction involves partitioning a sample into thousands of small volume reactions, each containing zero or at least one DNA molecule. The number of positive partitions is counted to absolutely determine the exact number of target molecules by using the Poisson mass probability distribution.

### 6.3 High Throughput Polymerase Chain Reaction

High throughput PCR enables the simultaneous amplification and detection of multiple target DNA sequences using a single integrated microfluidic circuit or similar platform ( Franklin et al. 2021 [8GNQ66YM] Franklin, A. M., N. E. Brinkman, M. A. Jahne, and S. P. Keely. 2021. "Twenty-First Century Molecular Methods for Analyzing Antimicrobial Resistance in Surface Waters to Support One Health Assessments." Journal of Microbiological Methods 184 (May): 106174. https://doi.org/10.1016/j.mimet.2021.106174.).

### 6.4 Multiplex Polymerase Chain Reaction

This enables simultaneous detection of multiple DNA targets in a single PCR using distinct primers (ITRC 2013; Ramírez et al. 2015 [6MIIBHWJ] Ramírez, Juan Carlos, Carolina Inés Cura, Otacilio da Cruz Moreira, et al. 2015. "Analytical Validation of Quantitative Real-Time PCR Methods for Quantification of Trypanosoma cruzi DNA in Blood Samples from Chagas Disease Patients." The Journal of Molecular Diagnostics 17 (5): 605–15. https://doi.org/10.1016/j.jmoldx.2015.04.010.). It provides more information when working with scarce samples.

## 7. Genomics

Genomics is a field of biology focused on studying all the DNA of an organism (i.e., its genome). Sequencing technologies have evolved over the last few decades ( Rolando et al. 2024 [9XQI444Y] Rolando, Justin C., Arek V. Melkonian, and David R. Walt. 2024. “The Present and Future Landscapes of Molecular Diagnostics.” Annual Review of Analytical Chemistry 17 (Volume 17, 2024): 459–74. https://doi.org/10.1146/annurev-anchem-061622-015112.). The first widely used method was developed by Walter Gilbert and Allan Maxam and involved radiolabeled adenosine triphosphate–modified DNA resolved by gel electrophoresis. Frederick Sanger developed first-generation sequencing that used dideoxynucleotides for chain-termination and DNA sequences by gel electrophoresis. Newer sequencing technologies are frequently referred to as next-generation sequencing (NGS). These NGS technologies enable parallel analysis of clinical and environmental samples and are classified into short- and long-read sequencers. The short-read sequencers include Illumina sequencing-by-synthesis via reversible terminator chemistry, Thermo Fisher Scientific Ion Torrent semiconductor chips, and Roche 454 pyrosequencing (no longer available but mentioned here for historical purposes). The long-read sequencing technologies include PacBio single-molecule real-time and Oxford nanopore electrical current density sequencing.

These NGS technologies are used for genomics, metagenomics, and metatranscriptomics of microbes in environmental samples. Microbial genomics entails bioinformatic assembly of short and/or long reads to generate complete pathogen genomes in pure cultures, which aids their identification ( Knight et al. 2018 [R8IH4A22] Knight, Rob, Alison Vrbanac, Bryn C. Taylor, et al. 2018. “Best Practices for Analysing Microbiomes.” Nature Reviews Microbiology 16 (7): 410–22. https://doi.org/10.1038/s41579-018-0029-9.). Metagenomics involves sequencing the entire microbiome in an environmental sample, which yields detailed genomic and taxonomic information for pathogens. Metatranscriptome analysis uses RNA sequences to profile active genes (e.g., virulence and antimicrobial resistance genes) to help evaluate microbial activity and discriminate live from dormant or dead pathogens. NGS technologies generate vast amounts of sequence data, necessitating the curation of databases organized for querying and retrieval of information, and phylogenetic and phylogenomic analysis ( Vidanagamachchi and Waidyarathna 2024 [7BB38CA6] Vidanagamachchi, S. M., and K. M. G. T. R. Waidyarathna. 2024. “Opportunities, Challenges and Future Perspectives of Using Bioinformatics and Artificial Intelligence Techniques on Tropical Disease Identification Using Omics Data.” Frontiers in Digital Health 6 (November): 1471200. https://doi.org/10.3389/fdgth.2024.1471200.).

## 8. Fluorescence In Situ Hybridization (FISH)

FISH is a method used to visualize and enumerate specific types of microorganisms or groups of microorganisms in an environmental sample (ITRC EMD). FISH can provide information regarding the abundance of microorganisms or genes of interest in a sample, cell morphology and growth characteristics, spatial distributions and associations with other microorganisms, and microbial community structure. The FISH method involves (1) the fixation and permeabilization of microorganisms to make their cellular membranes permeable to fluorescently labeled oligonucleotide probes, (2) hybridization of these probes to nucleic acid targets in the microorganisms, (3) washing to remove excess probe, and (4) visualization and enumeration via microscopy or another method, such as FC (Flow Cytometry) for high-speed counting.

## 9. Microbial Fingerprinting Methods

### 9.1 Phospholipid Fatty Acid (PLFA)

This method analyzes the key component of microbial cellular membranes and involves several steps, including (1) lipid extraction from the sample, (2) column separation/fractionation, (3) phospholipid modifications, (4) separated and modified components detected by a flame ionization detector, and (5) the generation of a chromatogram profile. PLFA fingerprinting methods provide a measure of total viable biomass and a broad-based profile of the microbial community composition and is best suited for assessing microbial responses as a result of a treatment (e.g., decontamination) or natural or anthropogenic induced environmental changes (ITRC 2013; Quideau et al. 2016 [B8EI5MGG] Quideau, Sylvie A., Anne C. S. McIntosh, Charlotte E. Norris, Emily Lloret, Mathew J. B. Swallow, and Kirsten Hannam. 2016. “Extraction and Analysis of Microbial Phospholipid Fatty Acids in Soils.” Journal of Visualized Experiments (JoVE), no. 114 (August): e54360. https://doi.org/10.3791/54360.

Lewe et al. 2021 [34NNPP5C] Lewe, Natascha, Syrie Hermans, Gavin Lear, et al. 2021. “Phospholipid Fatty Acid (PLFA) Analysis as a Tool to Estimate Absolute Abundances from Compositional 16S rRNA Bacterial Metabarcoding Data.” Journal of Microbiological Methods 188 (September): 106271. https://doi.org/10.1016/j.mimet.2021.106271.).

## 9.2 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE is a non-quantitative technique that provides a DNA-based profile of the microbial community and allows identification of the predominant organisms, generally to the family or genus level. This is achieved by separating PCR-amplified fragments of a targeted gene (e.g., 16S rRNA gene) to allow visualization of patterns of distinguishable bands representing different microorganisms within a sample. DGGE analysis cannot, however, quantify specific organisms or microbial functions present within a sample. This method can also be used to identify and compare the presence/absence of specific organisms among samples. (ITRC 2013).

## 9.3 Pulsed Field Gel Electrophoresis (PFGE)

PFGE is a non-quantitative analysis that allows for the production of a DNA-based profile on genetic material up to 10 megabase pairs in length. This contrasts with traditional gel electrophoresis, which only reliably resolves up to 20 kilobase pairs ( Sharma-Kuinkel et al. 2016 [JJQNX9FX] Sharma-Kuinkel, Batu K., Thomas H. Rude, and Vance G. Fowler. 2016. “Pulse Field Gel Electrophoresis.” In The Genetic Manipulation of Staphylococci: Methods and Protocols, edited by Jeffrey L. Bose. Springer. https://doi.org/10.1007/7651_2014_191.). By using only specific restriction enzymes to cut the genomic material, specific lengths of genomic material will be generated based on the host. These can then be separated using a specialized electrophoresis device that has pairs of electrodes placed at different angles. By changing the angle of the electric field during the run, it effectively gives more distance to resolve the DNA fragments, allowing for the observance of larger bands of DNA. This is ideal for suspect screening methods involving well-characterized suspects, as the DNA band profile that is developed needs to be compared to existing profiles for identification. Additionally, its ability to resolve large genomes lends the technique credence in the identification of bacteria or eukaryotic contaminants.

## 9.4 Multilocus Sequence Typing (MLST)

MLST is a non-quantitative analysis that uses DNA sequencing to determine the identity of an unknown. PCR (Polymerase Chain Reaction (PCR)) is performed with primers directed at variable regions within several key genes. These PCR products are then sequenced and compared to existing databases of genomes (or subsets specifically curated for MLST) to determine the identity of the unknown ( Larsen et al. 2012 [9U8D5WLG] Larsen, Mette V., Salvatore Cosentino, Simon Rasmussen, et al. 2012. “Multilocus Sequence Typing of Total-Genome-Sequenced Bacteria.” Journal of Clinical Microbiology 50 (4): 1355–61. https://doi.org/10.1128/jcm.06094-11.). Originally, this was performed using seven key genes, but as the technique has been expanded to include a wider variety of organisms, the exact set of genes to analyze has some variability. This technique has applications for targeted analyses and suspect screening. Although it can be used for non-targeted analysis, it may be more productive to perform whole genome sequencing of an unknown contaminant.

## 9.5 Restriction Fragment Length Polymorphism (RFLP)

RFLP is a non-quantitative analysis that relies on restriction enzymes’ ability to cut at specific sites ( Hashim and Al-Shuhaib 2019 [JJ94PM72] Hashim, Hayder O., and Mohammed Baqur S. Al-Shuhaib. 2019. “Exploring the Potential and Limitations of PCR-RFLP and PCR-SSCP for SNP Detection: A Review.” Journal of Applied Biotechnology Reports 6 (4): 137–44. https://doi.org/10.29252/JABR.06.04.02.). This method is based on the differences in DNA sequences between BioCEC (e.g., between different strains or subtypes of the same microbial species). For example, DNA sequences for a certain gene may be different between similar BioCEC, but the gene product performs the same function between the similar BioCEC. In this case, these nucleotide differences, or polymorphisms, can be detected when they change where restriction enzymes cut. This is often used in tandem with PCR amplification of particular genes to reduce the background noise of genetic material in a sample. The PCR fragments are then subjected to restriction enzyme cuts, and the size of the resulting fragments is resolved through a technique such as gel electrophoresis. This helps inform the fingerprint of a given sample. This is best used in suspect screening or targeted analysis, as the produced fingerprint needs to be compared to existing fingerprints for identification.

## 9.6 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

T-RFLP is a modification of RFLP that uses PCR primers with fluorescent probes. Rather than analyzing the entirety of the fragments produced by a restriction enzyme cutting a PCR product, only the fluorescently labeled ends of the PCR product (the terminal ends) are analyzed for their change in size ( De Vrieze et al. 2018 [KINBEPP4] De Vrieze, Jo, Umer Z. Ijaz, Aaron M. Saunders, and Susanne Theuerl. 2018. “Terminal Restriction Fragment Length Polymorphism Is an ‘Old School’ Reliable Technique for Swift Microbial Community Screening in Anaerobic Digestion.” Scientific Reports 8 (1): 16818.

https://doi.org/10.1038/s41598-018-34921-7.). It effectively reduces the amount of analysis needed to determine differences in the fingerprint, while potentially missing polymorphisms occurring in the middle of the PCR product. The same limitations that apply to RFLP apply here as well.

## 10. Isothermal Amplification Approaches

Isothermal amplification techniques use constant temperature and DNA strand–displacing enzymes that facilitate the extension of gene-specific primers on double-stranded DNA. Exponential amplification of the target sequence is due to isothermal cyclic repetition of these processes and, unlike PCR, does not require thermal denaturation for the primers to bind to template DNA. There are several kinds of isothermal amplification systems, and three are listed below:

- Recombinase polymerase amplification ( Piepenburg et al. 2006 [UVSEXV95] Piepenburg, Olaf, Colin H. Williams, Derek L. Stemple, and Niall A. Armes. 2006. “DNA Detection Using Recombination Proteins.” PLOS Biology 4 (7): e204. https://doi.org/10.1371/journal.pbio.0040204.) uses recombinase protein to mediate the invasion of primers into the double-stranded DNA and uses single-stranded binding proteins to stabilize the complex for elongation by an isothermal polymerase such as Phi-29.
- Helicase-dependent amplification ( Vincent et al. 2004 [4Y4TDBSY] Vincent, Myriam, Yan Xu, and Huimin Kong. 2004. “Helicase-dependent Isothermal DNA Amplification.” EMBO Reports 5 (8): 795–800. https://doi.org/10.1038/sj.embor.7400200.) uses a DNA helicase to enzymatically unwind double-stranded DNA to generate single-stranded complementary strand templates for primer extension by DNA polymerase.
- Loop-mediated isothermal amplification ( Notomi et al. 2000 [CGILLTXK] Notomi, Tsugunori, Hiroto Okayama, Harumi Masubuchi, et al. 2000. “Loop-Mediated Isothermal Amplification of DNA.” Nucleic Acids Research 28 (12): e63. https://doi.org/10.1093/nar/28.12.e63.) involves inner and outer primers that generate a self-priming dumbbell structure with two stem loops that, after multiple rounds of amplification, generate large self-amplifying concatemers (which is a term for a long, continuous DNA molecule that contains multiple copies of the same DNA sequence linked in series).

The advantages of these isothermal amplification approaches are simplicity (constant temperature eliminates the need for thermal cyclers), speed (rapid detection in 30 minutes), and suitability to be deployed in the field (e.g., lateral flow assays). The disadvantage of these approaches is a higher limit of detection relative to PCR.