OPINION

Identifying microorganisms responsible for ecologically significant biogeochemical processes

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Abstract | Throughout evolutionary time, and each day in every habitat throughout the globe, microorganisms have been responsible for maintaining the biosphere. Despite the crucial part that they play in the cycling of nutrients in habitats such as soils, sediments and waters, only rarely have the microorganisms actually responsible for key processes been identified. Obstacles that have traditionally impeded fundamental microbial ecology inquiries are now yielding to technical advancements that have important parallels in medical microbiology. The pace of new discoveries that document ecological processes and their causative agents will no doubt accelerate in the near future, and might assist in ecosystem management.

Since its nineteenth-century foundations in the work of Beijerinck[1] and Winogradsky[2], environmental microbiology has been concerned with how microorganisms in terrestrial and aquatic environments change our world. Conceptually, environmental microbiology resides at the interface between two vigorously expanding disciplines: environmental science[3] and microbial ecology[4] (FIG. 1). Each discipline has recently undergone major developments, with expanding areas of research and the generation of considerable amounts of new data. However, it seems likely that the information still awaiting discovery greatly exceeds our current knowledge, given that nearly all current information about prokaryotes is based on measurements performed on <5,000 isolated species, which represent ~0.1% of the total estimated diversity of prokaryotes in the biosphere[5–7].

The Earth's habitats present complex gradients of environmental conditions that include extreme variations in temperature, light, pH, pressure, salinity and both inorganic and organic compounds (materials ranging from elemental sulphur to ammonia, hydrogen gas, and methane; and from cellulose and lignin to fats, proteins, lipids, nucleic acid and humic substances). Each geochemical setting (for example, anaerobic peatlands, oceanic hydrothermal vents, soil humus and deep subsurface sediments) features its own set of resources that can be physiologically exploited by microorganisms. The free-energy-governed interactions between these resources, their settings, the microorganisms themselves and ~3.5 billion years of evolution are probably the source of the metabolic diversity of the microbial world[8]. Microorganisms are the primary agents of geochemical change, and the global biomass of prokaryotes is approximately equal to that of all other (eukaryotic) life forms[9].

Their small size, ubiquitous distribution, high specific surface area, potentially high rate of metabolic activity, physiological responsiveness, genetic malleability, potentially rapid growth rate and unrivalled enzymatic and nutritional diversity cast microorganisms in the role of recycling agents for the biosphere. Enzymes accelerate reaction rates between thermodynamically unstable substances. Perhaps the most ecologically important types of enzymatic reactions are those that catalyse oxidation/reduction reactions between electron donors and electron acceptors. These reactions allow microorganisms to generate metabolic energy, and to survive and grow. Microorganisms procreate by carrying out complex, genetically regulated sequences of biosynthetic and assimilative intracellular processes. Each daughter cell has essentially the same macromolecular and elemental composition as its parent. Therefore, the integrated metabolism of all nutrients is implicit in microbial growth. The growth and survival of microorganisms drives the geochemical cycling of the elements, detoxifies many organic and inorganic contaminants, makes essential nutrients present in the biomass of one generation available to the next, and maintains the conditions required by other inhabitants of the biosphere[10–12] (TABLE 1).

This article presents a perspective on past and current attempts to discover the identity of microorganisms that are responsible for catalysing key biogeochemical reactions in *in situ* soils, sediments and waters. The traditional challenges to reaching this goal are discussed, as are recent innovations to overcome these challenges. Insights are sought by contrasting ways of documenting causality in medical microbiology — Koch's postulates — with those of environmental microbiology.

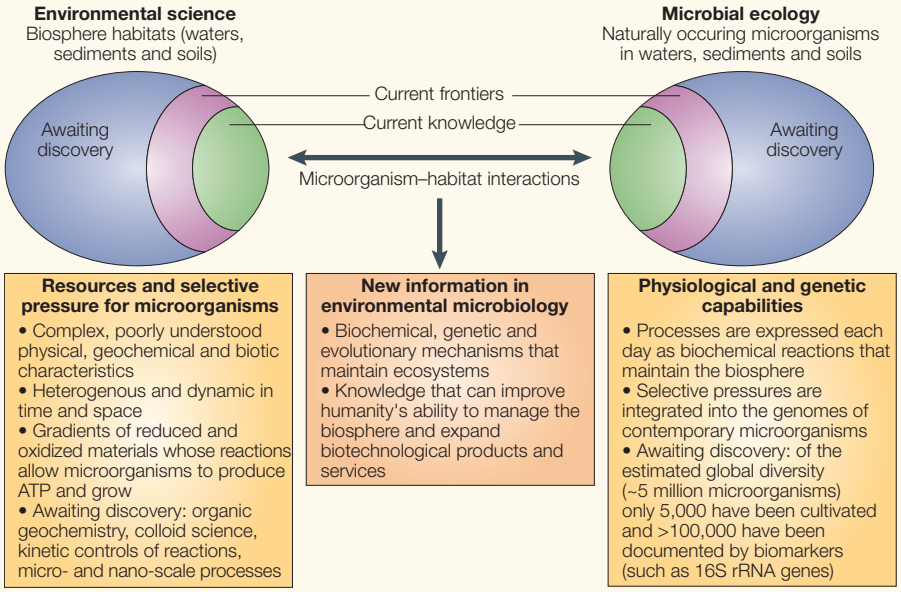

Figure 1 | **Conceptual representation of the interactions between environmental science and microbial ecology.** Interactions between environmental science (left sphere) and microbial ecology (right sphere) allow new discoveries to be made at the interface between microorganisms and their habitats. Microbial ecology and environmental microbiology overlap considerably; nonetheless, advancements in the latter are represented by the central arrow.

Enrichment culturing from nature

Some of the earliest and most influential investigations in the history of environmental microbiology relied on enrichment culturing strategies[1,13] to identify and isolate individual microbial cultures capable of carrying out novel metabolic processes, such as growth on ammonia as an energy source, fixation of atmospheric nitrogen into cell protein, and the use of unusual (perhaps pollutant) organic compounds as carbon and energy sources or final electron acceptors. FIGURE 2 provides an integrated overview of the procedures used in environmental microbiology to conduct such inquiries, and how to interpret the resultant data.

Enrichment culturing uses a sample of a naturally occurring microbial community as an inoculum for laboratory-prepared growth medium that is designed to select a small subset of the initial community. The logic behind enrichment culturing involves devising growth conditions that allow particular members of the community to grow and eventually dominate within the mixed population that was initially present. For instance, if one is interested in finding aerobic microorganisms that can grow on benzene (oxidizing it to CO_2 and incorporating the substrate carbon into new cells), then the enrichment medium would contain benzene as the sole carbon and energy source, and oxygen as the electron acceptor. A 1 g soil inoculum can contain 40,000 species[7,14], although only a small percentage of these would be expected to grow on benzene. After a 1–2 week incubation, benzene degraders would become dominant. Then, by plating small volumes of the enriched populations onto benzene growth medium solidified with agar, individual colonies of benzene degraders can be picked, further purified, isolated and characterized using appropriate physiological, biochemical and/or genetic procedures.

It is important to note that naturally occurring microbial communities used as inocula typically consist of uncharacterized, highly diverse populations (see above), which usually are morphologically non-distinct rods and cocci. Each cell has the genetic potential to carry out a multitude of metabolic processes, although conditional regulation can severely limit gene expression in the natural environment. Furthermore, dormancy (or very slow growth) is the norm for most cells in nature, because all habitats are nutrient limited[15]. Therefore, the presence in

Table 1 | **Examples of physiological processes catalysed by microorganisms in biosphere habitats**

Process	Nature of process	Typical habitat	References
Carbon cycle			
Photosynthesis	Light-driven CO_2 fixation into biomass	Ow, Fw, FwS, Os	42–44
C respiration	Oxidation of organic C to CO_2	All	45
Cellulose decomposition	Depolymerization, respiration	Sl	46
Methanogenesis	CH_4 production	Sw, FwS, Os	47,48
Aerobic CH_4 oxidation	CH_4 becomes CO_2	All	49,50
Anaerobic CH_4 oxidation	CH_4 becomes CO_2	Os	51
Biodegradation			
Synthetic organic compounds	Decomposition, CO_2 formation	All	52,53
Petroleum hydrocarbons	Decomposition, CO_2 formation	All	54
Fuel additives (MTBE)	Decomposition, CO_2 formation	Sl, Sw, Gw	55
Nitroaromatics	Decomposition, CO_2 formation	Sl, Sw, Gw	56,57
Pharmaceuticals, personal care products	Decomposition	Sl, Sw, Gw	53,58
Chlorinated solvents	Compounds are dechlorinated through respiration in anaerobic habitats	Sl, Sw, Gw	59,60
Nitrogen cycle			
N_2 fixation	N_2 gas becomes NH_3	Sl, Ow	61
NH_4^+ oxidation	NH_3 becomes NO_2^-, NO_3^-	Sl, Sw	62,63
Anaerobic NH_4^+ oxidation	NO_2^- and NH_3 become N_2 gas	Sw, Os	64,65
Denitrification	NO_3^- is used as an electron acceptor and converted to N_2 gas	Sl, Sw	66,67
Sulphur cycle			
S_2 oxidation	S^{2-} and S^0 become SO_4^{2-}	Os, FwS	68
SO_4^{2-} reduction	SO_4^{2-} is used as an electron acceptor and converted to S^0 and S^{2-}	Os, Sw, Gw	69
Other elements			
H_2 oxidation	H_2 is oxidized to H^+, electrons reduce other substances	Sw, Sl, Os, FwS	48
Hg methylation and reduction	Organic Hg is formed and Hg^{2+} is converted to Hg	FwS, Os	70,71
(per)chlorate reduction	Oxidants in rocket fuel and other sources are converted to chloride	Gw	72
U reduction	U oxyanion is used as an electron acceptor; therefore immobilized	Gw	73
As reduction	As oxyanion is used as an electron acceptor; therefore toxicity is diminished	FwS, Gw	74
Fe oxidation, acid mine drainage	FeS ores are oxidized, strong acidity is generated	FwS, Gw	75

As, arsenic; C, carbon; CH_4, methane; CO_2, carbon dioxide; Fe, iron; FeS, iron sulphide; Fw, freshwater; FwS, freshwater sediment; Gw, groundwater; H_2, hydrogen; Hg, mercury; Hg^{2+}, mercuric ion; MTBE, methyl tertiary butyl ether; N_2, nitrogen; NH_3, ammonia; NH_4^+, ammonium; NO_2^-, nitrite; NO_3^-, nitrate; Os, ocean sediments; Ow, ocean waters; S^0, elemental sulphur; S^{2-}, sulphide; Sl, soil; SO_4^{2-}, sulphate; Sw, sewage; U, uranium.

an environmental sample of a particular organism that is capable of a particular process cannot be taken as evidence that the process is occurring *in situ*[8,16].

Striving for ecological significance

Implicit in enrichment procedures is the ability of microorganisms to respond and change when subjected to environmental perturbations. The nature of microbial responsiveness during enrichment culturing is clear: resuscitation from dormancy and growth of (often) minor populations during incubation periods lasting days–years. But even if relatively brief incubations preclude shifts in population dynamics owing to growth and death, microorganisms still respond to environmental change. For instance, intricate biochemical signalling pathways allow cells to sense and respond to key nutrients (for example, light, oxygen, other electron acceptors and carbon sources[17]), stress (such as acid, oxidative damage or inhibitory substances[18]), and cell-to-cell signalling molecules (quorum sensing pheromones[19]). The time frames for these responses range from nanoseconds (light) to milliseconds (oxygen, toxicity) to minutes (enzyme synthesis) or hours (sporulation).

This remarkable propensity of populations within naturally occurring microbial communities to change is a blessing for microbiologists practising enrichment culture. However, it is a major impediment for those seeking to interpret physiological and ecological measurements performed on laboratory-incubated environmental samples such as water, soils or sediments. The validity of measurements conducted on microbial communities removed from their original field setting is uncertain, because we cannot be sure that conditions imposed on the native microorganisms (post-sampling and incubation) have not quantitatively or qualitatively altered these populations and their physiological reactions. Potentially misleading 'bottle effects' are implicit in all measurements performed on sampled microbial communities[20,21]. This situation has been likened to the Heisenberg Uncertainty Principle in quantum chemistry, which formally recognizes the mutual exclusivity of simultaneous determination of the position and momentum of an electron[8]. When one begins in a field site and strives to dissect site-derived samples, the closer microorganisms in the community are examined, the more likely the resultant information is to suffer from artefacts imposed by the sampling and/or measurement procedures. The investigator's inability to obtain disturbance-free field samples and to fully characterize, understand and duplicate field conditions in the laboratory undermines the acceptance of laboratory measurements performed on field samples as valid surrogates for true, *in situ* field processes (FIG. 2).

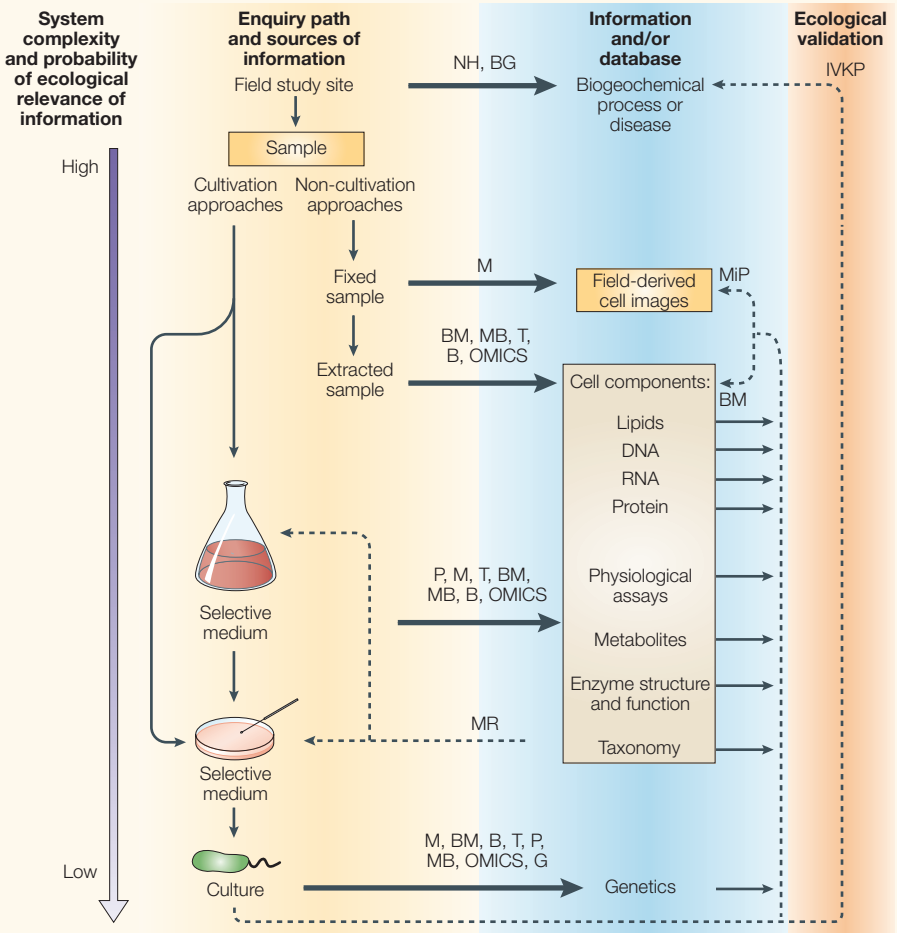

Figure 2 | **Model for the generation and interpretation of environmental microbiological information, with emphasis on field relevance and ecological validation of data.** Column 1 provides a scale for evaluating the likely ecological relevance of information in the other three columns. With each successive methodological step away (down) from direct field measurements, the risk of artefacts (ecologically misleading data) increases. Column 2 provides an outline of microbiological procedures (cultivation-based or non-cultivation-based) that are used as sources of information about microorganisms in nature. Column 3 shows the types of information created by various methodological procedures (arrows extending from column 2 to 3). The dashed arrows in column 4 show the main feedback pathways that can be used to validate the ecological relevance of microbiological data. Dashed arrows connecting column 3 to 2 show a means for improving growth media, as guided by field-derived 'omics' information. B, biochemical characterization; BG, biogeochemistry; BM, biomarkers (for example, 16S rRNA genes, lipids); G, genetic characterization (for example, operons, regulation); IVKP, inoculation to verify Koch's postulates; M, microscopy; MB, molecular biological characterization (for example, cloning and sequencing); MiP, microscopic probing (immuno- and omics-based visualization in field-fixed cells); MR, medium refinement based on expressed genes and other biomarkers discovered in field samples; NH, natural history; OMICS, genomics, proteomics, metabolomics, transcriptomics, and so on; P, physiological characterization; T, taxonomic characterization.

When measurements are performed *in situ* within a field study site (such as soil, a lake or an ocean), the complexity of the system is high, and the information obtained (such as geochemical parameters and field observations) is directly applicable to the system under study. However, as the sources of microbiological information become simplified (that is, farther removed from the field study site), the risk of obtaining ecologically questionable information increases. If investigators take the path of cultivation-based inquiry, not only might ecologically insignificant organisms be isolated, but the laboratory conditions selected for subsequent testing might also fail to activate ecologically relevant genes (FIG. 2). During the past two decades, great efforts have been made to develop methods for non-cultivation-based inquiry

Table 2 | **Contrasts between information on causality in medical and environmental microbiology**

Medical microbiology	Environmental microbiology
Traits of habitats studied	
Humans are globally distributed and evolutionarily stable	Soils, sediments and waters are globally distributed, but show high physical and geochemical variability in time and space
Consistent uniform resources for microbial colonization	Highly variable resources; severe but unpredictable nutritional limitation is the rule
Reliably simulated in laboratory media or animal models	Unreliably simulated in the laboratory because geochemical complexity defies characterization
Low-diversity microbial community offers few background organisms that confound isolation of causative agent	High community diversity; thousands of background organisms that can be mistaken for causative agents
Characteristics of microbial processes	
Diseases are reliably recognized in the field	Biogeochemical reactions are often difficult to document in the field; discerning geochemical footprints of processes is a challenge in open field sites
Immense negative impact on host; intervention is essential	Robust, reliable processes often have positive impacts, regardless of human understanding or intervention
Huge impetus for scientific study (disease prevention)	Historically, little impetus for scientific study relative to human disease
Steps to identify potential causative agents	
Pathogens are often culturable because the habitat (host) can be simulated in laboratory media or animal models	Biogeochemical agents have often not yet been cultured because habitats are poorly understood and difficult to simulate
A single agent is often the cause	Large- and small-scale habitat diversity might select for many different agents within flexible ecological guilds that carry out processes
Relatively high chance of isolating the correct organism because it comes from a low-diversity community	Relatively low chance of isolating ecologically significant agents because community diversity is immense. Process might stem from many cooperating populations
Ways of linking identity to field processes	
Koch's postulates are well established	Koch's postulates rarely apply
	Topic of ongoing multidisciplinary research involving microscopy, biomarker probes, stable isotopic signatures autoradiography and stable isotopic probing

(for example, the use of 16S rRNA in investigations of microbial diversity). The resultant molecular, biomarker and genomic information has been revolutionary in terms of the insights that have been attained[5,22–24]. The non-cultivation-based procedures have succeeded in generating ecologically significant information. However, both cultivation-based and non-cultivation-based enquiries are imperfect and biased[25]. It is for this reason that ecological validation is necessary (FIG. 2).

Environmental Koch's postulates?

In 1884, Robert Koch[26] developed fundamental criteria for proving that a particular microorganism (*Bacillus anthracis*) was responsible for a particular process (anthrax disease) in a particular habitat (sheep). This generalized four-step guideline, known as Koch's postulates, is as follows: (i) the microorganism should be found in all cases of the disease in question, and the microorganism's distribution in the body should be in accordance with the lesions observed; (ii) the microorganism should be grown in pure culture *in vitro* (or outside the body of the host) for several generations; (iii) when such a pure culture is inoculated into susceptible animal species, the typical disease must result; and (iv) the microorganism must again be isolated from the lesions of such experimentally produced disease.

Koch's postulates have been the gold standard in medical microbiology for establishing causality, and have survived intact to the present, with minor modifications that accommodate recent molecular biological techniques[27,28]. However, for microbiologists concerned with ecological processes, linking a microorganism's identity to its activity in its habitat has, with several exceptions, proven difficult. Below, I suggest why medical microbiologists have so far been more successful than environmental microbiologists in identifying causative agents.

TABLE 2 compares and contrasts, for medical and environmental microbiology, four key factors that influence the determination of causality: the complexity of the habitat and its inhabitants, the process of interest, identifying a potential causative agent, and linking this agent to the process of interest in the field. As stated in TABLE 2, human disease is readily recognized in the field and has an enormous detrimental impact. Therefore, the impetus for understanding and intervening is also enormous. By contrast, the impetus for discovery and management of ecologically important biogeochemical reactions has been less pressing — perhaps because biogeochemical processes in field habitats are not easy to discern, and because such processes generally proceed regardless of intervention.

Culturability is probably the other main factor that has allowed medical microbiologists to flourish while environmental microbiologists have perhaps fallen out of step. Culturability is a direct reflection of two interacting issues: the relative ratio of target to non-target organisms in the initial inoculum; and an ability to accurately simulate the native habitat in media. When Robert Koch embarked down the cultivation-based path (FIG. 2), his initial field sample (blood from a diseased sheep) was essentially a monoculture containing a "large number of regular, rod-shaped, colorless, immotile structures"[26] that were microscopically discernible. Compare this to the vast, confusing zoo of candidates (for example, 40,000 species and 10^9 cells per gram of soil) that confronts a soil microbiologist. Furthermore, Koch found that the blood-borne bacilli readily reproduced on solid media containing "nutrient gelatin or boiled potato"[26]. Easy culturability is not a given in medical microbiology (for example, *Treponema pallidum* (syphilis) and *Mycobacterium leprae* (leprosy) cannot yet be grown *in vitro*[28]). However, the uniform, stable, globally distributed nutrient conditions of the human body are undeniably easy to mimic in growth

Table 3a | **Selected examples of efforts to identify microorganisms responsible for field biogeochemical processes**

Process	Microorganism	Setting	Strategy	Commentary	Refs
Nitrogen fixation	*Rhizobium* spp.	Nodule on root of legume plant in field	Inoculate soil lacking native rhizobia	Koch's postulates succeed. Root infection selects for bacterial symbiont. Nitrogen fixation results from inoculation and nodulation	76
Biodegradation of TCE	*Dehalococcoides* spp.	Groundwater beneath air force base	Inoculate subsurface habitat where TCE persists	A version of Koch's postulates succeeds. Metabolism only occurred after inoculation	77
Glutamate uptake and DNA synthesis	Unknown	Water samples from Long Island Sound and Narragansett Bay	Microscopy plus microautoradiography; incubated in laboratory	First attempt to capture microscopic image of cells incorporating radio-labelled compounds; contrived laboratory setting required	78
Nitrification (and $^{14}CO_2$ fixation)	*Nitrobacter* spp. (autotroph)	Sediment samples from Mammoth Cave, Kentucky, enriched on nitrate incubated in laboratory	Microscopy plus micro-autoradiography and immunofluorescent detection of cells	First attempt to apply both fluorescent antibodies and autoradiography to soil microorganisms; contrived laboratory setting; $^{14}CO_2$ assimilation did not measure nitrification directly	79
Nitrification (and $^{14}CO_2$ fixation)	*Nitrosococcus* spp., *Nitrosomonas* spp. (autotrophs)	Seawater in bottles incubated aboard ship	Microscopy plus micro-autoradiography and immunofluorescent detection of cells	Brief incubation in bottles under 'field-like conditions'; two known nitrifiers probed with fluorescent antibodies; $^{14}CO_2$ assimilation did not measure nitrification directly	80
Amino-acid assimilation, DNA synthesis	α-Proteobacteria, *Cytophaga-Flavobacterium* group	Coastal California seawater samples	Microscopy plus micro-autoradiography and 16S rRNA-based FISH	40 ml samples incubated in laboratory for 3 hours. Uptake of tritiated glucose and amino acids was measured by autoradiography. 16S rRNA-based FISH identified active cells	81
Organic and inorganic nutrient assimilation	β-Proteobacteria	Activated sewage sludge samples	Microscopy plus micro-autoradiography and 16S rRNA-based FISH	2 ml samples incubated in laboratory for 2–3 hours. Uptake of ^{14}C-acetate, -butyrate, -bicarbonate and ^{32}P-phosphate measured and imaged via autoradiography. 16S rRNA-based FISH identified active cells	82
Glucose and acetate assimilation	Candidatus *Meganema perideroedes*	Activated sewage sludge samples	Microscopy plus quantitative micro-autoradiography and 16S rRNA FISH	2 ml samples harvested the day before and kept at 4°C then incubated in laboratory for 1 hour at 21°C. Uptake of ^{14}C-acetate and ^{14}C-glucose measured and imaged using autoradiography. 16S rRNA-based FISH identified active cells	83
Aerobic methane oxidation	*Methylomonas* spp.	Baltic Sea sediment sample	Simultaneous FISH probing of identity (rRNA) and activity (methane monoxygenase mRNA)	Demonstrated principle of using fixed samples for determining both identity and activity. Probed community enriched on methane and incubated in laboratory for 4 weeks. Methane oxidation not confirmed geochemically	84

FISH, fluorescence *in situ* hybridization; TCE, trichloroethene.

media compared with the uncharacterized, site-specific, heterogeneous complexity of soils, sediments and waters (FIG. 1). Many biogeochemical processes are not catalysed by individual microorganisms, but instead by cooperating populations (consortia). Moreover, it seems likely that guilds of physiologically equivalent microorganisms in different habitats can be compositionally distinctive (TABLE 2). So, identifying ecologically significant microorganisms using Koch's postulates has been evasive because of a combination of lack of impetus, community complexity and the limitations of cultivation techniques. Fortunately, there are other methods of ecological validation that do not require cultivation. These often rely on microscopic probing of field-fixed cell images for DNA, RNA or other biomarkers indicative of cell identity and/or activity (FIG. 2; TABLE 2).

Linking field processes to agents

Progress has been made in many areas that will contribute to the successful identification of ecologically significant microorganisms. These areas include the impetus for inquiry, deciphering community complexity, improving cultivation procedures, as well as the development of new strategies and techniques that can largely substitute for Koch's postulates (during the interim period that microorganisms in biosphere habitats remain uncultivated). The substitutes for Koch's postulates are outlined in TABLE 2, and detailed examples are discussed below and presented in TABLES 3a,b.

The increasing impetus for understanding microbially mediated environmental processes probably reflects the growing public and governmental awareness of the frailty of our planet[29] under the combined stresses of population growth, climate change, pollution and disease transmission. Understanding the complexity of both habitats and naturally occurring microbial communities is an important focus of current research in environmental microbiology (FIG. 1). Examples include the geochemical characterization of the ocean floor[30–32] and Lake Vostok[33], and recent whole-community genome-sequencing efforts[24,34,35]. Cultivation strategies have already taken a significant leap forward through efforts in which minimally altered environmental samples are used to meet the complex and subtle nutritional needs of naturally occurring microorganisms[36–38].

Using FIG. 2 as a map to visualize the steps towards progress in environmental microbiology, there are three obvious avenues for increasing the ecological validity of information. First, if the culture media improve in

Table 3b | **Selected examples of efforts to identify microorganisms responsible for field biogeochemical processes**

Process	Microorganism	Setting	Strategy	Commentary	Refs
Anaerobic methane oxidation	Archaea and sulphate reducers	Ocean sediments adjacent to methane sources (Pacific and Black Sea)	Follow stable isotopic signature of ^{13}C-methane into community biomarkers, cells and site carbonate deposits	All biomarker, microscopy and geochemical assays performed on field-fixed samples. Resultant data support a single explanation: methane is oxidized anaerobically by a consortium of bacteria related to methanogens and sulphate reducers	51, 85–87
Assimilation of acetate and methane	*Desulfotomaculum acetoxidans*, type I methanotrophs	Samples of sediments from Tamar mud flat and Lake Loosdrecht	SIP, following ^{13}C-labelled substrates into lipid biomarkers	Small sediment cores incubated in laboratory for 8 hours (acetate) and 14 days (methane). Polar lipid-derived fatty acids extracted and analysed by gas chromatography/isotope ratio mass spectrometry	88
Assimilation of methanol	α-Proteobacteria *Acidobacterium* spp.	Sample of oak forest soil	SIP, following ^{13}C-labelled substrate into DNA	10 g sieved, air-dried samples fed ^{13}C-methanol at 0.5% concentration for 44 days. ^{13}C DNA fraction analysed for16S rDNA sequences	89
Phenol biodegradation	*Thauera* spp.	Sample from laboratory bioreactor	RNA SIP, ^{13}C-labelled RNA extracted, reverse transcribed and sequenced	First demonstration that RNA pool can be rapidly labelled. ^{13}C atoms traced into ribosome fraction of community within 24–72 hours. Sequencing of reverse-transcribed RNA revealed identity of active microorganisms	90
Methanol oxidation by denitrifying microorganisms	*Methylobacillus* spp., *Methylophilus* spp.	Sample from laboratory bioreactor	DNA SIP, confirmed by FISH and micro-autoradiography	^{13}C atoms traced into DNA during 24-hour incubation. Cloned 16S rDNA sequences confirmed by FISH; these confirmed by microautoradiography using radioactive methanol	91
Naphthalene biodegradation	*Polaromonas naphthalenivorans*	Contaminated field sediment in South Glens Falls, New York	Field-based DNA SIP	Addition of ^{13}C-naphthalene to field site sediment; respiration assay confirmed *in situ* biodegradation; extraction and sequencing of 16S rRNA genes in ^{13}C-DNA identified responsible population. A representative of this population cultivated	92
Nitrogen fixation	*Trichodesmium*	Ocean water	Recognizable filamentous colonies collected by filtration and assayed, physiologically, in bottles aboard ship	*Trichodesmium* fixes nitrogen in ocean waters; nitrogen fixation assays used closed-bottle incubations; field activity confirmed by immunodetection of nitrogenase enzyme in field samples	93–95
Photosynthesis	*Prochlorococcus* spp.	Ocean water	Rates of cell division and photosynthesis inferred from circadian cell cycles at many depths	Field-fixed cells analysed by flow cytometry; specific growth rate; CO_2 fixation estimated by analysing circadian cell-cycle patterns;	21

FISH, fluorescence *in situ* hybridization; SIP, stable isotope probing.

ecological relevance, then the microorganisms that are eventually isolated are more likely to be those that are active in nature. Second, as analyses of field-fixed samples deliver increasingly sophisticated information about expressed genes and proteins used by microorganisms in their native habitats, inferences can be made about *in situ* physiological conditions, carbon substrates and nutritional needs. Such information can, in turn, guide the design of media so that new microorganisms can be cultivated. Last, the several paths of information flow for validating data shown in FIG. 2 need to be more widely used. These validation paths are: following Koch's postulates by inoculating field sites, use of pure-culture-derived 'omics' biomarkers to guide analyses performed on extracted samples, and the use of microscopy and biomarker probes to confirm the field relevance of information from both pure cultures and extracted samples.

Selected examples of past and current investigations aimed at linking identity of microorganisms to their field activity are shown in TABLES 3a,b. The entries were chosen to be representative of the types of strategies, techniques, challenges and breakthroughs that have occurred in environmental microbiology over the past several decades. The emphasis is on identifying microorganisms and being sure that they catalyse biogeochemical reactions *in situ* — in real-world field sites containing soil, sediment or water. The first two entries (symbiotic nitrogen fixation and biodegradation of trichloroethene in contaminated groundwater) reveal that Koch's postulates can be powerful and insightful when used in environmental microbiology. However, Koch's postulates are only applicable in limited contexts because the active microorganisms must be cultivated, and must initially be present in low numbers or be absent from the inoculated habitat.

The next six entries in TABLE 3a illustrate the foundations and later developments in microscopy-based attempts to link identity to activity without using Koch's postulates. Microscopy and microautoradiography were initially used to see which cells in mixed microbial communities incorporated radiolabelled substrates. Later, microautoradiography was combined with cell-specific probing: fluorescent antibodies targeting cell-surface antigens of cultivated bacteria or fluorescent oligonucleotides targeting sequences of taxonomically revealing rRNA, often derived from uncultivated microorganisms. Recent efforts

have developed another strategy that has the potential of avoiding all laboratory incubations by using fluorescence *in situ* hybridization (FISH) to probe naturally occurring microorganisms for both identity (rRNA sequence) and activity (indirectly, through hybridization with the mRNA of expressed functional genes).

Another promising methodological development is stable isotope probing (SIP) (TABLE 3b). This strategy follows the stable isotopic signature of an assimilated substrate (for example, a carbon source) into the populations responsible for substrate metabolism in complex microbial communities. Because the assimilated substrate has a distinctive signature mass (for example, density or $^{13}C/^{12}C$ ratio), labelled cells or biomarkers can be separated and/or analysed in ways that reveal the identity of active cells. Without question, the most elegant example of SIP to date is from a series of investigations documenting anaerobic methane oxidation in deep waters adjacent to methane sources in the Black Sea and in coastal California and Oregon. These investigations were successful because the field study sites contained a substrate (methane) that was fortuitously labelled with a unique stable isotopic signature. Such situations are rare.

To implement SIP in other contexts, a stable isotope-labelled substrate (for example, ^{13}C label) is dosed to a community and later retrieved in biomarkers. Such biomarkers include phospholipid fatty acids (whose molecular structures are taxonomically informative), and DNA and rRNA, both of which are sources of 16S rRNA gene sequences. Early SIP studies established 'proof of principle' for the dosing approach. However, these investigations were carried out on enrichment cultures (laboratory-based model soils exposed to high concentrations of ^{13}C-labelled substrates for many weeks). More recently, refinements in the SIP approach have included analysing the labelled RNA fraction (RNA is rapidly turned over in cells and labelling does not require that the populations undergo growth) and verification of rRNA sequences discovered through SIP with both FISH and microauto-radiography. SIP has also been applied in a field situation (naphthalene-contaminated sediment), leading to the discovery and later cultivation of an ecologically significant bacterium, *Polaromonas naphthalenivorans*.

The final two entries in TABLE 3b focus on long-studied nitrogen-fixing and photosynthetic microorganisms found in ocean waters. *Trichodesmium* is a relatively large, filamentous, morphologically recognizable cyanobacterium whose global presence and potential for nitrogen fixation are undeniable. By the strict criteria developed in this article, nitrogen fixation by *Trichodesmium* has not yet been directly demonstrated because the nitrogen fixation assay relies on ship-incubated water samples. Nonetheless, biomarker studies carried out on field-fixed samples have shown that nitrogen-fixation genes are transcribed and translated *in situ*. Members of the genus *Prochlorococcus* are other widely distributed ocean inhabitants (recognizable by flow cytometry). Representatives of *Prochlorococcus* spp. have been cultivated and their genomes have been sequenced[39]. *In situ* photosynthesis by *Prochlorococcus* spp. was demonstrated without laboratory-based incubation.

Outlook

The ultimate goals of environmental microbiology are to understand the mechanistic relationships between habitat characteristics, evolutionary pressures, microbial diversity, and biochemical processes and their genetic controls (FIG. 1). Processes carried out by microorganisms in soils, sediments, oceans, lakes and groundwaters have an important impact on environmental quality, agriculture and global climate change. Identifying ecologically significant microorganisms is like finding a needle in an unusual haystack — a haystack with individual pieces that can, during the search, change into misleading needles. For more than a century, environmental microbiologists have been confronted by vast, unknown microbial diversity (the haystack), by population responsiveness (the misleading needles), by an enormous size differential between humans (~1 m) and microorganisms (~1 μm), and by the task of documenting the geochemical impact of microorganisms in open, heterogeneous field sites. The complexity of natural systems has, almost without exception, made it impossible to directly observe the identity of microorganisms and their activities in waters, sediments and soils. Instead, indirect approaches have emerged.

As the frontiers of environmental science and microbial ecology advance (FIG. 1), we are assured of a vast supply of new hypotheses relating microbial diversity to mechanisms of ecologically significant physiological adaptation. Current challenges include discovering the role of uncultivated ocean[40] and soil[41] microorganisms that are widely dispersed, but whose metabolic functions are a complete mystery. The new bioinformatic tools and feedback-based investigative strategies available to environmental microbiologists (FIG. 2) guarantee complementation and convergence of information generated by cultivation- and non-cultivation-based procedures. By strengthening and extending the model built on Koch's postulates, future inquiries will surely accelerate the progress in linking ecologically important microorganisms to their activity in real-world habitats.

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Competing interests statement

The author declares no competing financial interests.

Online links

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