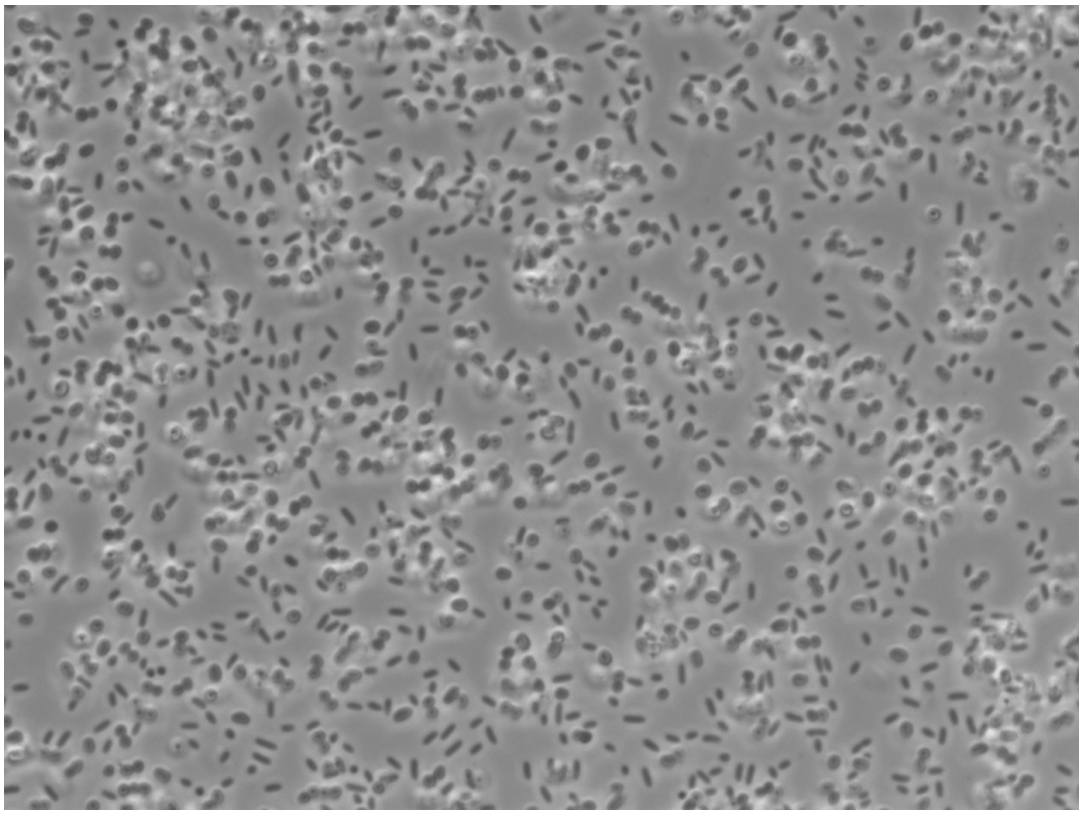
Who does what: how do we figure out what microbes do in the environment?

Miss: there are thousands of different microbes in the soil, so how do we know who does what in the environment?



A mixture of bacteria from the environment as seen under the microscope at x1,000 magnification (courtesy of Dr Nasmille Larke-Mejia, University of East Anglia, UK)

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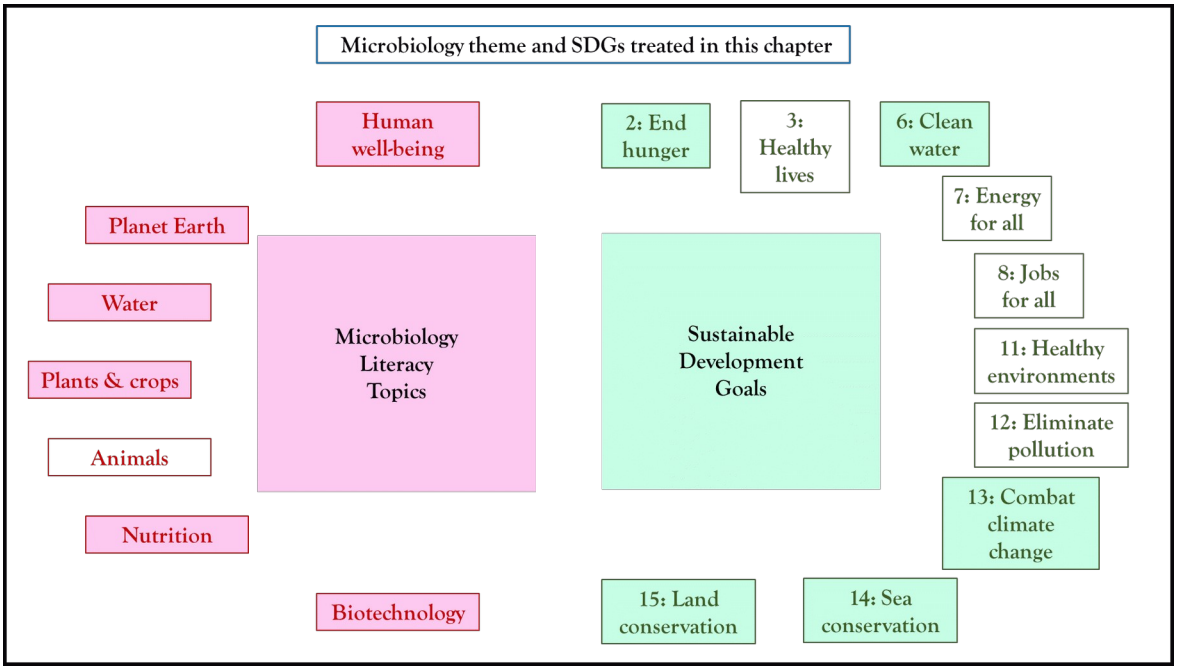
Who does what?

Storyline

Soil contains thousands of different types of microbes, all of which use many different types of foods (substrates) in order to grow. When you take a few grams of soil and wash out the microbes and view them under the microscope, they all look pretty much the same (see picture above) and so it is difficult to tell who is who and indeed what they eat in order to grow. We can try to isolate and cultivate the different microbes in the laboratory and, indeed, this approach has served microbiologists very well over the past 150 years. Once we have them growing in the lab, we can offer them different types of food to see what they prefer, and find out how they grow by studying their metabolism. And, we can also look at their DNA, the genetic material that makes up their genome (chromosomes), and find out what genes they contain. This will give us clues as to what they are, what they eat, and maybe even what their role (function) may be in the environment. However, many microbes can't be grown in the laboratory at present, because we have not yet found the food/conditions they need. Therefore, microbiologists who study the ecology of microbes in their natural habitats have developed a whole series of new techniques – a "toolkit" – that gives information about key molecules in the cell, in order to find out which microbes live in the studied environment and what they grow on. You may wonder: Why do we want to know this? In fact, microbes are incredibly important in driving all of the nutrient cycles on our planet and by understanding their function, we can help preserve the health of the Earth.



The Microbiology and Societal Context

Microbes in our biosphere are vital for the health of our Earth. They make major contributions to all of the nutrient cycles on land and in the oceans and play key roles in food webs, agriculture, waste treatment, water purification and human health. They can also be used to make useful industrial chemicals, foodstuffs and antibiotics. Of the millions of different

microbes in the environment, microbiologists have only "captured" a fraction and grown and studied them in the laboratory. A major focus of many microbiologists in the last 20 years or so has been the field of microbial ecology, finding out where different microbes occur in the environment, how they grow and how they contribute to the function of the Earth's biosphere. The rapid advances in DNA sequencing techniques, which allow scientists to sequence the complete genomes of organisms (from microbes to humans), has enabled microbiologists to discover the wealth of microbial diversity present in the environment. This knowledge can be used to find out what exactly their function is, how human activity is affecting their activity, and how for example, climate change will influence the microbes that sustain life on Earth.

Who does what? – The microbiology

1. ***The environment contains thousands of different types of microbes***. Virtually all environments on Earth, for example, soil, mud, lakes and oceans, contain thousands of different types of microorganisms, all of which use a multitude of different types of foods (in the microbial world we call these substrates). However, if you wash microbes off the surface of soil particles and look at them under the microscope, most look very similar in size and shape, especially bacteria (see above). Microbiologists like to look at different kinds of bacteria (and all other types of microbes, but we will use the example of bacteria here to illustrate certain principles of microbial ecology) and try to find out what they are and what substrates they are using. This enables scientists to determine what different bacteria are doing in the environment, and investigate how important they are in the health and wellbeing of the planet, because they play critical roles in nutrient cycling and are a major part of the Earth's food chain.

There are two major ways in which microbiologists investigate bacteria. Firstly, they can take a sample from the environment (let's use soil as an example), mix a small amount of this soil with a liquid growth medium containing all of the nutrient sources required for growth and a specific carbon source which will enable the growth of specific types of bacteria, such as a sugar like glucose. They then dilute out this solution many times and spread it out onto Petri dishes containing the same medium solidified with agar. The aim of this exercise is to isolate/purify individual bacteria by diluting them so that single cells become separated from one another on the agar surface and can multiply and form "pure" colonies that do not touch one another and mix together as they grow. For example, our initial solution may contain 10 million cells per millilitre, or 1 million in 0.1ml, which is what we might spread on the dish. A dish with 100 colonies is okay, so we need to dilute the initial solution 10,000x! When we incubate the petri dish, the individual cells grow and multiply, one cell becoming 2, 2 cells becoming 4, and so on. After incubating these Petri dishes for one or several days, each cell may have produced 1 million cells and more, which can be seen by eye as a colony on agar surface. The colonies of different bacteria can be individually propagated on new petri dishes, and the pure cultures can be examined in detail to study their metabolism (what they eat and how they do it). Furthermore, the DNA from these bacteria can be extracted and sequenced to provide insights into the genes of these microbes. These genes provide important information about potential metabolic functions, as well as allow to determine the identity of the organisms (their taxonomy) and their relatedness with other microbes (their phylogeny).

2. ***Not all bacteria can be cultivated in the laboratory.*** Many bacteria in the environment are impossible or very difficult to culture in the laboratory at present, so microbiologists have devised new ways of studying them. Taking our example of soil, a few grams of soil can be taken

back to the laboratory and the DNA containing the genomes of all of the bacteria present in the soil can be released by gently breaking open the bacteria with chemicals. This DNA can then be purified, again using chemicals, and analysed. The big difficulty at this stage is that there are millions of genes from different bacteria that need to be sorted out. Fortunately, there are a few tricks (now routine methods for microbial ecologists) that can be used to search for certain genes which enable microbiologists to find out which bacteria are in this particular soil sample. When bacterial DNA from a community of soil bacteria is isolated in the laboratory, a technique known as the polymerase chain reaction (PCR) can be used to capture specific gene sequences. The gene that microbiologists study to determine which bacteria are in an environmental sample is called the 16S rRNA gene. Every bacterium has at least one of these genes and their sequences differ from bacterium to bacterium: microbial ecologists use them a bit like a barcode. Interestingly, differences in the sequence of the 16S rRNA increase as bacteria diverge from one another in evolutionary time, so the barcodes serve also as molecular clocks. When these barcodes are retrieved from this microbial community DNA, they can be compared with the barcodes from the thousands of bacteria which have been cultivated and characterised in the laboratory over many years to see how similar they are to known ones. Of the thousands of different bacteria present in that soil sample (often termed the microbiome), some will be similar to those microbiologists have grown in the laboratory before but many others will be completely new.

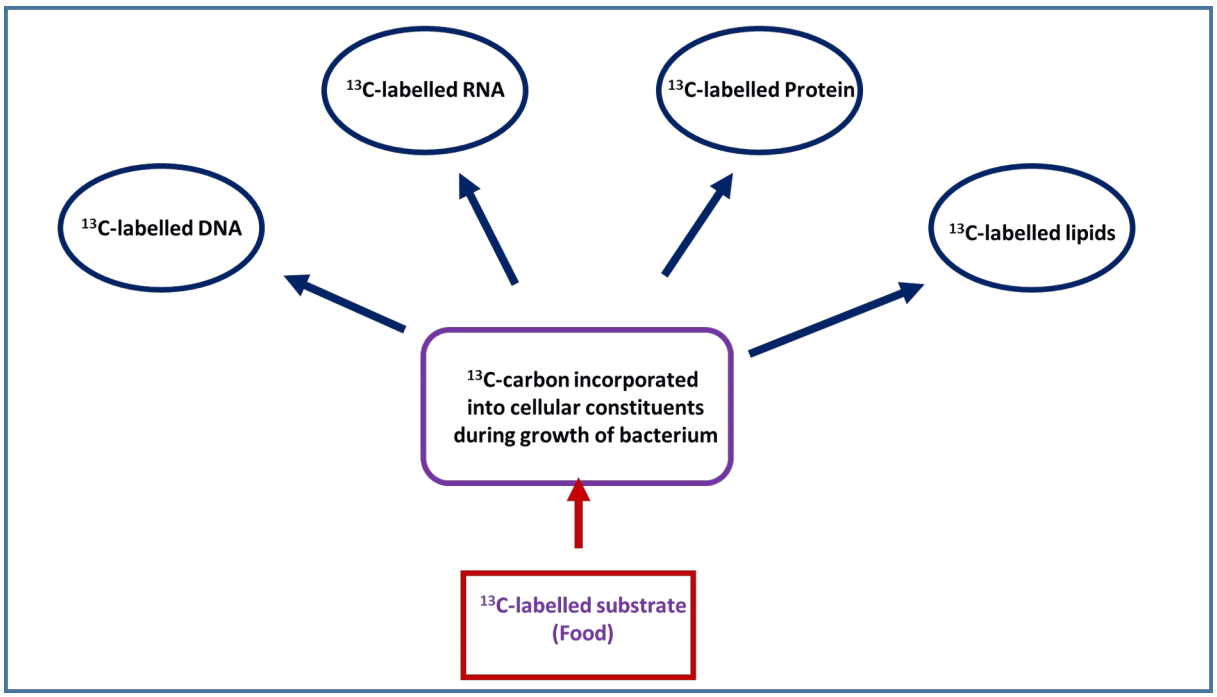
3. ***The genes that bacteria have in their genomes tell us what their function might be.*** By studying the growth and substrates of bacteria in the laboratory and determining which genes and in turn, which enzymes and metabolic pathways they use to grow on these various specific substrates, we can make predictions about the function of these microbes in the environment. If we use an example here: there are bacteria in the environment that can grow on methane, a very strong greenhouse gas. These bacteria are called methanotrophs and they can be found anywhere where methane is produced, for example in soil and mud near stagnant ponds, soil in paddy fields where rice is grown, and in soil above a landfill where methane is released from the degradation of organic waste. Studying the metabolism of these bacteria in the lab has allowed the identification of methane-specific genes which are present in the genomes of all of these methanotrophs. One of these methane-specific genes is used as a functional gene marker, again a specialised sort of barcode that only these types of bacteria have in their DNA. So, if we have DNA isolated from soil samples, we can PCR amplify and sequence these methane genes and compare them with previously known methane genes (stored in a methane gene database) that have been obtained from cultures of methanotrophs in the laboratory. In this way we can find new methanotrophs, which are eating the climate gas methane before it escapes into the atmosphere.

4. ***You are what you eat!*** By extracting DNA from environmental samples and sequencing certain genes, we can get a good idea of the different bacteria present and look at certain key functions. However, this does not always tell us "who is active and who is doing what". One way to determine which bacteria are actively degrading specific substrates in an environmental sample is to feed them a "labelled" substrate and follow where the label goes and what happens to it afterwards.

Elements are defined by the number of protons in the atom. For example, carbon, C, has 6 protons and ordinarily 6 neutrons, to give an atomic mass of 12 (https://www.youtube.com/watch?v=wMx1l86XFLU). However, it may also have 7 or 8 neutrons, giving atomic masses of 13 and 14, respectively, collectively making the ^{12}C, ^{13}C and

^{14}C carbon isotopes. Because ^{13}C is heavier than ^{12}C, and ^{14}C is radioactive, these isotopes can be distinguished from ^{12}C, and hence can be used in biology as labels to investigate biochemical processes, for example tracing the fate of a particular substrate through a metabolic network or a microbial consortium. ^{14}C is an unstable isotope, which is why it is radioactive, whereas ^{13}C is a stable isotope, so not radioactive, which makes it easier to work with. Isotope-labelled substrates are produced by specialised companies for use by microbial ecologists (and others).

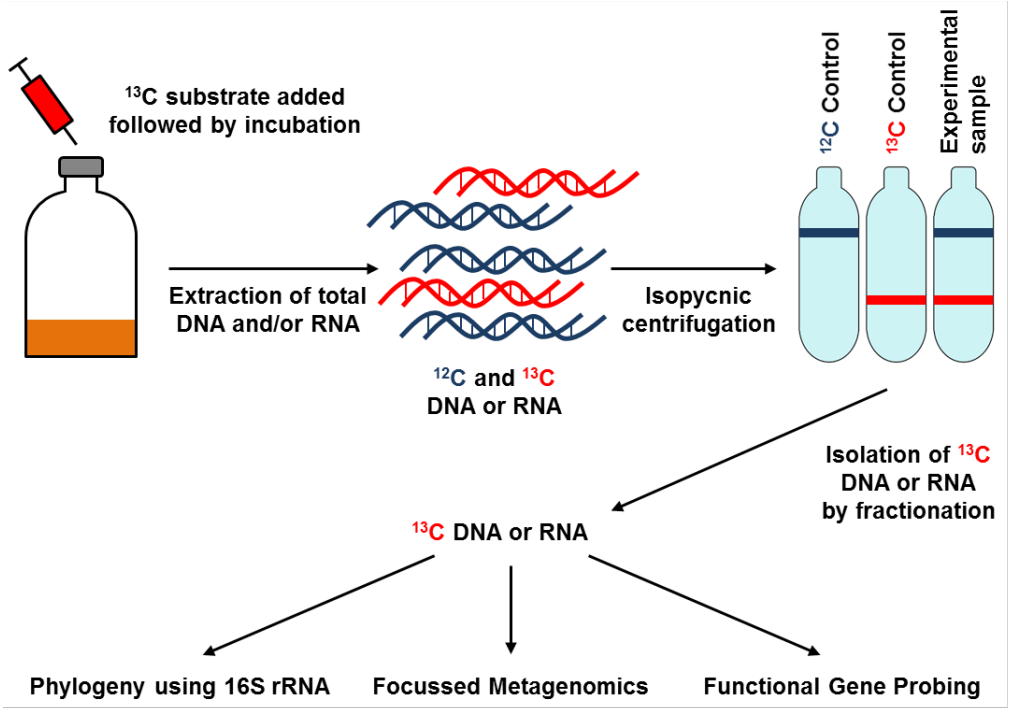
Any carbon substrate (foodstuff) for bacteria which is labelled with ^{13}C will get incorporated into all of its cellular constituents, including DNA, RNA, proteins and lipids (see Figure 2) because they all contain carbon. This makes these constituents heavier (isotopically-labelled) in the bacteria which have eaten the offered substrate, and enables scientists to distinguish them from cell components in other bacteria which that have not been labelled with ^{13}C.



Incorporation of ^{13}C-labelled substrate into a bacterial cell and cellular components that are subsequently labelled.

5. Bacterial cells in soil can be labelled with ^{13}C substrates. If we consider the example of methane-eating bacteria (methanotrophs) as an illustration, then these bacteria will be in a few grams of soil alongside thousands of other types of bacteria that can't use methane. (i) When a few grams of soil are incubated in a sealed flask along with air and a few millilitres of ^{13}C-labelled methane gas, then only the methanotrophs present in the soil will grow on the methane and incorporate the ^{13}C into their cellular constituents (DNA, RNA, protein, lipids) which become heavier. None of the thousands of other bacteria will incorporate ^{13}C into the cell and so will remain "light" (= normal). (ii) The task now is to isolate the ^{13}C-labelled cell components of the methanotrophs. In the case of DNA, we can use a technical trick called isopycnic density gradient centrifugation. Total DNA (containing heavy ^{13}C-labelled DNA from methanotrophs along with the lighter ^{12}C-DNA from the thousands of other bacteria) can be purified from soil using different chemicals to yield DNA from the total community of bacteria in that soil. (iii) The DNA is then loaded into a tube containing a concentrated solution of the heavy metal caesium (in fact the salt caesium chloride) and spun very fast in a centrifuge for 48 hours. This creates an enormous gravitational force in the tube which forces the caesium

towards the bottom of the tube, thereby creating a stable density gradient. During the centrifugation, all of the DNA molecules migrate to the positions in the tube corresponding to their specific densities, with the heavy ^{13}C-DNA separating from the light ^{12}C-DNA (see Figure below). The ^{13}C-DNA, which is exclusively from methanotrophs because they are the only bacteria that can grow on methane and incorporate the ^{13}C-carbon into their cellular materials, can then be extracted from the centrifuge tube with a needle and syringe to separate it from the ^{12}C-DNA that came from all of the other non-active, non-methane bacteria. The purified ^{13}C-DNA can then be analysed: by PCR amplification and sequencing their 16S rRNA genes and allows to identify the methanotrophs (who), as well as the functional genes involved (what). This technique is known as DNA-Stable Isotope Probing (DNA-SIP) and is summarised below.



DNA-stable isotope probing. The blue and red bands in the tubes represent unlabelled and the heavier ^{13}C-labelled DNA, respectively

6. Variations on a theme. Other components of bacterial cells which have been labelled with ^{13}C-carbon, such as RNA, proteins and lipids, can also be analysed to reveal which bacteria are active in the environment. In the case of RNA, a different type of solution is used in the density gradient centrifugation separation method depicted above and the technique is known as RNA-SIP. ^{13}C-labelled proteins and lipids can also be examined using mass spectrometry techniques to identify labelled proteins and lipids. Protein-SIP is a particularly powerful technique to look at key enzymes of target bacteria, but it requires a lot of computing power, extensive analyses and very large databases to identify key marker proteins out of the millions of proteins present in soil.

The examples given above used ^{13}C-labelled methane, but virtually any substrate for which a fully isotopically-labelled compound is available, such as sugars, complex organic molecules like starch or cellulose, and pollutants such as polyaromatic hydrocarbons, chlorinated solvents, could be used in SIP experiments. Using more sophisticated scientific instruments, it is even possible to visualise individual cells that have been labelled with ^{13}C-

compounds. For example, a Raman spectrophotometer linked to a microscope can be used to detect cellular components that have incorporated ^{13}C atoms. Multi-isotope imaging mass spectrometry (nano-SIMS) is another very sensitive way of finding out if a bacterial cell has taken up and used a particular isotope, not only carbon, but for example nitrogen, oxygen or metal atoms. Food substrates labelled with radioactive isotopes can also be used to label bacteria in environmental samples. For example, low doses of ^{14}C-labelled compounds which are incorporated into individual cells can be visualised under a microscope. The radioactivity of bacteria that take up the ^{14}C label is detected by fixing them onto a microscope slide and exposing them to an emulsion which causes silver grains to form adjacent to the radioactive cells (autoradiography). This blackened area can then be seen under the microscope. The method is termed microautoradiography (MAR). If the same slide is analysed by a technique known as fluorescent *in situ* hybridisation (FISH), which involves labelling the same cells with a 16S rRNA probe (see section 2), the taxonomic identity of the active bacterium can be determined at the same time. This combination technique is known as MAR-FISH and, although technically quite difficult, it provides a powerful way to determine what the various microbe in the environment are doing.

Relevance for Sustainable Development Goals and Grand Challenges

- **Goal 2. End hunger, achieve food security and improved nutrition and promote sustainable agriculture**. Identifying which microbes are abundant and play a key role in promoting the nutrition and health of plants is vital if we are to improve soil productivity. The way in which microbes in soils respond to changes in agricultural practices, such as reduced nitrogen fertilisation and land management are also key to sustainable agriculture.
- **Goal 6. Ensure availability and sustainable management of water and sanitation.** Microbes play a pivotal role in waste-water treatment plants. For example, it is important to know which microbes are responsible for clean-up of pollutants and the removal of nitrogen compounds, and how the microbial communities change in response to perturbations. Management of water resources using microbes is playing a vital role in improving water quality.
- **Goal 13. Take urgent action to combat climate change and its impacts**. Microbes are key players in all of Earth's biogeochemical cycles and it is important that we know who the key players are, where they occur in the environment, and how they impact the production and consumption of climate-active gases, such as methane, carbon dioxide, dimethylsulfide, isoprene and nitrous oxide. The abundance and activity of key carbon, nitrogen and sulfur cycling microbes in the environment, and how they respond to human-made changes in the environment, is essential if we are to provide solutions to global warming and air quality in our biosphere.
- **Goal 14. Conserve and sustainably use the oceans, seas and marine resources for sustainable development.** Nutrient cycles within the world's oceans depend on the activity of microbes, and a sound knowledge of who are the major players in these cycles, particularly the carbon cycle which determines the primary productivity of the oceans, enables a better understanding of how to manage marine resources. The response of microbes in the oceans to the effects of our changing climate, such as ocean acidification, also requires a sound knowledge of the changes in distribution, diversity and activity of marine microbes.

- **Goal 15. Protect, restore and promote sustainable use of terrestrial ecosystems, sustainably manage forests, combat desertification, and halt and reverse land degradation and halt biodiversity loss.** Microbial diversity in the terrestrial environment is arguably more important than the diversity of animals and plants. The "unseen majority" on Earth, i.e. the microbes, play vital roles in ecosystem services and changes in land-use, loss of forests, desertification, soil degradation, all of which impact the diversity and activity of microbes. A detailed mechanistic understanding of the function of microbes in a changing landscape are essential to be able to predict the effects of a changing terrestrial environment. We often focus on macro-biodiversity loss but microbiologists would argue that we must not lose microbial diversity. If we don't study microbial ecology, how will we know if this is already happening?

Pupil Participation

1. ***Class discussion***
    a. What is a microbiome?
    b. How many different microbiomes can you think of?
    c. What microbes might you find there?
    d. What might they grow on?
    e. Are there any environments on Earth in which you wouldn't find microbes?

2. ***Pupil stakeholder awareness***

How do microbes affect your everyday life? Some hints and subtopics include food production and agriculture, foodstuffs made using microbes, human health and disease, defence against disease, antibiotics, probiotics, climate change.

3. ***Class experiments***
    a. Collect barcodes from various food items and sort them as if they were marker genes for different bacteria. Can you group them into similar patterns?
    b. Each group pick a different environment and think what different groups of microbes might be present, what they might eat and how many of each there might be.
    c. Think about some environments on Earth in which only microbes might survive and suggest what they might grow on (clues: hot springs, volcanic mudpots, alkaline soda lakes)
    d. Could there be microbial life on Mars? What would microbes need to grow there?
    e. The name game: think up an imaginary bacterium, suggest where it lives, what it eats and then make up a name for it based upon it's habitat and food.
    f. Think of some polluted environments and find out if microbes can help clean up these environments.

The evidence base, further reading and teaching aids

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Glossary

Biosphere: the regions of the Earth containing all living organisms.
Fluorescent *in situ* hybridisation (FISH): a technique used to visualise cells by staining them with a specific fluorescent dye linked to a nucleic acid probe.
Isopycnic gradient centrifugation: the separation of substances according to their buoyant density, usually in a salt or sugar gradient generated by centrifugation.
Metagenomics: the study of genetic material recovered directly from environmental samples
Methanotroph: a bacterium that grows on methane
Microbiome: a characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties
Nano-SIMS: Nanoscale secondary ion mass spectrometry is a method used to visualise the elemental and isotopic composition of cells.
Phylogeny: a systematic way to look at the evolutionary history and relationships between organisms
Polymerase chain reaction (PCR): a technique in molecular biology to rapidly make millions of copies of small amounts of a specific DNA molecule, by amplifying it to make large enough amounts to study in detail.
Raman microspectroscopy: a spectroscopic technique which analyses the vibration of molecules and provide a structural fingerprint of the components of cells. It is based on the interaction of light with chemical bonds in cellular material.
Stable isotope probing (SIP): A microbial ecology method to identify specific functional groups of microorganisms that incorporate stable-isotope-labelled carbon (^{13}C) or nitrogen (^{15}N) sources are assimilated into microbial biomass of environmental samples, revealing phylogenetic and functional information about the microorganisms responsible for the metabolism of a particular substrate.
Taxonomy: the study of naming, defining and classifying groups of biological organisms.
Taxonomic marker gene: a conserved gene used to classify organisms, for example the 16S ribosomal RNA gene is widely used in the classification of bacteria.