
# Biotechnology and the environment

UNIT 16

*European Initiative for Biotechnology Education*

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***The European Initiative for Biotechnology Education (EIBE) seeks to promote skills, enhance understanding and facilitate informed public debate through improved biotechnology education in schools and colleges throughout the European Union (EU).***

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# Biotechnology and the environment

UNIT 16

*European Initiative for Biotechnology Education*

MATERIALS

## Contents



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These pages (and the other EIBE Units) are available throughout Europe and the rest of the world on the World Wide Web. They can be found at:

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All of the EIBE Units on the World Wide Web are Portable Document Format (PDF) files. This means that the high-quality illustrations, colour, typefaces and layout of these documents will be maintained, whatever computer you have (Macintosh - including Power PC, Windows, DOS or Unix platforms).

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# About this Unit

These materials have been devised by practising teachers and educationalists from several European countries, brought together with financial support and encouragement from DGXII of the European Commission, under the auspices of EIBE, the European Initiative for Biotechnology Education.

The EIBE materials have been extensively tested in workshops involving teachers from across Europe.

The views expressed in this Unit and the activities suggested herein are those of the authors and not of the European Commission.

# Safety

In all of the EIBE Units, we have tried to check that all recognised hazards have been identified and that suitable precautions are suggested.

Where possible, the proposed procedures are in accordance with commonly-adopted general risk assessments. If a special risk assessment may be necessary, this has been indicated.

However, users should be aware that errors and omissions can be made, and that different employers and educational authorities adopt different standards. Therefore, before doing any activity, users should always carry out their own risk assessment. In particular, any local rules issued by employers or educational authorities MUST be obeyed, whatever is suggested in the EIBE Unit.

Unless the context dictates otherwise, it is assumed that:

- practical work is carried out in a properly equipped and maintained science laboratory;
- any mains-operated equipment is properly maintained;
- care is taken with normal laboratory operations such as heating substances;
- good laboratory practice is observed when chemicals or living organisms are used;
- eye protection is worn whenever there is any recognised risk to the eyes;
- pupils and/or students are taught safe techniques for activities such as handling chemicals and microorganisms.

INTRODUCTION

# Biotechnology and the environment

## Introduction

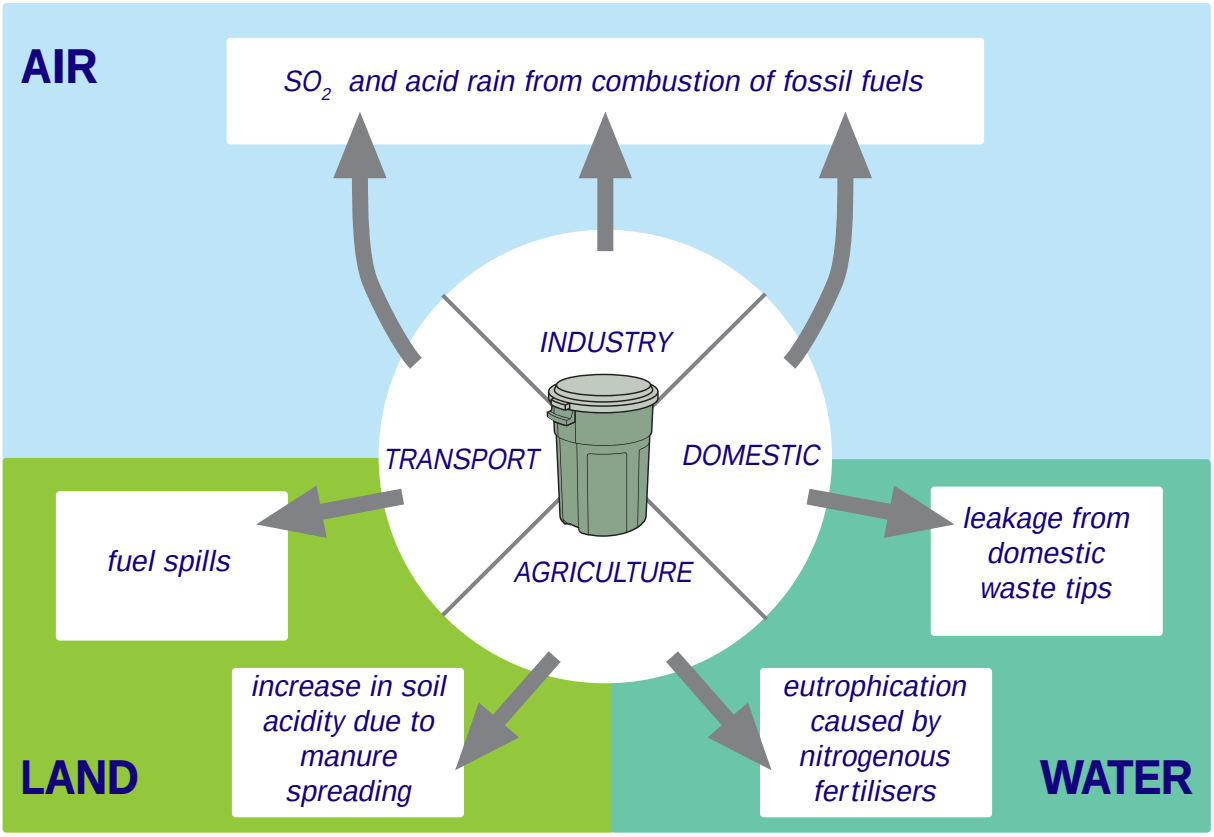
*Biotechnology is the integration of natural sciences and technical engineering in order to achieve the application of organisms, cells, parts thereof and molecular analogues for products and services used by mankind.* (EFB General Assembly, 1989)

Environmental biotechnology uses a combination of biological processes and technology to protect, maintain and restore the quality of the environment. For example, biodegradation processes are used for the treatment and safe disposal of sewage, industrial effluents and solid domestic and municipal refuse and provide opportunities for the recycling of valuable resources including water and energy. Understanding of biodegradation processes and associated technologies has progressed so much over the past 15-20 years that there is now a specialised bioremediation industry that can restore terrestrial and aquatic sites that have become polluted in various ways.

Environmental issues also encompass other aspects of biotechnology:

- agricultural biotechnology: plant varieties are being developed that either require lower levels of artificial fertilisers or have increased resistance to micro-organisms and insects;
- animal biotechnology: higher levels of production can be achieved from fewer animals so that less waste is produced and water and soil pollution can be reduced;
- microbial biotechnology: the activities of naturally occurring organisms can be enhanced, sometimes by genetic modification, to increase the yields of useful products and make new ones.

***Figure 1. Aspects of environmental disturbance caused by mankind***



## Environmental issues

Western society produces more and more waste. Contributing factors to this are:

- an increased standard of living and higher consumer demand;
- the use of disposable goods;
- the use of non biodegradable materials;
- the lack of proper recycling facilities for waste.

Four distinct sources of waste can be identified: domestic waste, municipal / industrial waste, bulk waste and radioactive waste. Each of these have the potential to alter or damage the natural environment i.e. soil, water and air.

A few examples illustrate this:

- Burning fossil fuels (by industry, domestic heating, and traffic) produces sulphur dioxide. This gas reacts with water to form acid rain which can damage the environment, including both trees and buildings. In a gaseous form sulphuric acid irritates the respiratory system and affects human health.
- Landfill sites used for solid waste disposal can lead to local complaints about a bad smells. More significantly, they produce methane, which if allowed to escape is a destructive greenhouse gas, but may be collected and used as an energy source. There is also the potential for volatile fatty acids to leak out and pollute the ground water.
- Farmers alter the acidity of the soil by spreading large amounts of manure on their arable land. Run off of nitrogenous compounds, both natural and artificial, can cause eutrophication of lakes, rivers and streams.
- Leakage of fuel hydrocarbons into the soil destroys fertility and pollutes water courses. The persistence of toxic residues in derelict land from industries that have closed down, e.g. coal tar from sites of disused gas works, interfere with re-use of land (known as 'brownfield sites') for building purposes. Even more distressing is the effect of large amounts of fuel or oil on aquatic ecosystems (salt or fresh water). Fish mortality and dead birds are the major visible impacts but are only two of the many harmful impacts of oil spills.
- In most large stores both food and non-food products are pre-packed. This increases the quantity of domestic waste and adds pressure on waste disposal. The alternative of increasing the proportion of waste that is re-cycled and incinerating the remainder is part of EU policy.
- Ever increasing demands for clean water can be met only by treating domestic and industrial waste water to make them suitable to re-use (see EIBE Unit 17 Case study B: *Providing clean water).*

These are just a few examples of the environmental impact of waste; daily examples can be found in the news.

In theory, the best way to prevent disturbance by waste is not to produce waste! This is difficult, so that we have to find ways to reduce the waste problem: traditionally this has be done by re-using, recycling and composting as much as possible. Such, more traditional, solutions are very important. However, biotechnology can also help by detecting, preventing and remediating environmentally damaging processes.

The following pages gives examples of the use of biotechnological processes to protect, maintain and restore the quality of the environment.

# Guidelines for the teacher

## Aims and objectives

That the students should understand:

- that society's activities should take into account the need to maintain a 'safe' environment;
- the consequences of disturbing the natural cycle of elements;
- that biotechnology can be used to detect, prevent and remediate environmental problems;
- that biotechnology itself can cause environmental problems;
- that in considering environmental issues the scientific facts have to be considered along with ethical, societal, legal and economical aspects.

## Content and method

Three chosen topics are approached through a variety of activities, including practical work, discussions and fieldwork.

### Detection of environmental damage

When the environment has been damaged, it is most likely that some abiotic factors have been changed within the ecosystem. In order to detect the damage one can use either physical or chemical measuring equipment or biosensors. Biosensors depend on living organisms that are particularly sensitive to a change in a specific abiotic factor.

### Prevention of environmental damage

Some energy demanding industrial processes whose products may damage the environment can be replaced by using enzyme systems, isolated either from natural living organisms or potentially even from genetically modified organisms (GMOs). Enzymes are biological catalysts; they are highly efficient and have many advantages over non-biological catalysts.

The development of GMOs in agriculture is also of increasing importance, for example the development of 'new' plants that are more resistant to diseases or pests can lead to less pollution from the sprays used to overcome these.

### Remediation of environmental damage

The degradation of pollutants by micro-organisms is the basis of bioremediation. The special activities needed are provided either by organisms naturally present or by adding relevant ones.

## The concept of cycles

All biological systems require materials for their structure and energy for their activities. This is true not only for individual organisms but also for the communities that they form in nature. This flux of energy and matter through communities implies a interdependency of organisms that is commonly represented in schemes as the cycling of nutrients, e.g. C, N, S compounds through plants via animals, people and microbes and back again into plants.

Students often represent cycles in nature in an incomplete manner, e.g. leaving out the decomposition and mineralisation stages that occur in the soil. Also they do not consider the ways in which the cycles link together.

Another world-wide persistent problem is the misconception that plants must be given organic nutrients such as carbohydrates and proteins to grow. This idea ignores reality where the cycling of nutrients involves the unique photosynthetic capacity of green plants and their nitrogen metabolism that enable them to use exclusively inorganic matter.

The traditional approach to teaching the concept of cycles only partly remedies this misconception. While teaching the cycle concepts in this unit it is important not to deny the relevance of the students' ideas but to identify them and to discuss them in relation to the scientific evidence.

### Organisation

The three topics in this unit focus on different applications of environmental biotechnology. In the 'detection' section, the accent is on health issues, the 'prevention' chapter looks at genetic modification and 'remediation' goes into biological aspects of cleaning polluted sites.

Depending on the actual classroom situation, the level of the students and the time available one, two or all three of the different approaches can be used, i.e. detection, prevention and remediation. If only one or two are studied thoroughly, it is recommended that the other(s) should also be discussed briefly.

Clearly, however, it is also important to stress that the best way to maintain a 'safe' environment is NOT to pollute, damage or alter it in the first place.

## Detection of environmental damage

This part of the unit deals with the biological importance of sulphur compounds: the effects of air pollution caused by sulphur compounds is considered first, fieldwork is then outlined which shows how to detect this form of air pollution using lichens as biological indicators.

### Objectives

Students should:

- understand the relationships between fossil fuels, $SO_2$, acid rain and respiratory problems;
- understand that the sensitivity of lichens to high levels of $SO_2$ can be used as indicators of its level;
- be able to make clear observations and records of the lichens in the research area and from them deduce the 'quality' of the air in relation to the level of $SO_2$.

### Field work

The procedures outlined are intended to standardise lichen coverage measurements so that results from different sites can be compared.

An extension of this investigation could be to include other abiotic factors in the results, i.e. the most frequently occurring direction of the wind, the presence of industry in the nearby and/or far away neighbourhood.

Ideally several different sites would be studied and compared. Searching for explanations of the differences between different sites has a greater interest than collecting data for a single site.

Identification of lichens is rather difficult for the inexperienced student. It is therefore advisable to use photographs or pictures in addition to a local key. It should be stressed that water content can alter the colour of a lichen. This investigation can also be carried out using only a limited (more easily identified) number of locally common species.

## Prevention of environmental damage

This part of the unit deals exclusively with nitrogen: nitrogen over-fertilization, nitrogen fixation by *Rhizobium* and, with the increasing understanding of the molecular mechanisms involved in nitrogen fixation, the possible role of biotechnology in preventing environmental damage.

### Objectives

Students should understand that;

- artificial fertilisers are made by chemical fixation of molecular nitrogen;
- optimization of biological processes of the nitrogen cycle has a role in reducing pollution;
- environmental problems, such as

nitrogen over fertilisation, are not easily overcome by biotechnology alone;

The students should know that:

- the availability of nitrogen in a fixed form is a limiting factor for plant growth and crop yield;
- the constant use of nitrogen-containing artificial fertilisers in agriculture influences the natural ecosystem of the soil and water courses;
- nitrogen fixation is carried out by specialised prokaryotes (bacteria), using a nitrogenase enzyme complex that reduces molecular nitrogen to ammonia;
- *Rhizobium*, forms nodules in the roots of leguminous plants that, through a symbiotic interaction with the host plant, can fix nitrogen; other free living bacteria make a smaller contribution;
- one aim of biotechnology research is to develop genetically modified *Rhizobium*, that could improve crop yield without fertilisers;

### What about the risks?

This unit provides an excellent opportunity to discuss risk assessment as regards release of GMOs to the environment. Therefore, this part of the unit should be concluded with the introduction of two ways of risk assessment that are commonly used in public discussion: the additive model and the synergistic model.

The additive model takes its arguments from experimental evidence of the risks involved. By analogy, conclusions are reached about the risks of releasing particular gene-modified organisms.

The synergistic model doubts the possibility of transferring knowledge gained by experience from closed systems (like the laboratory) to complex systems (like the environment). For risk assessment here all related aspects must be considered.

In the course of instruction the students should be asked if they see any risks connected to the release of genetically modified nitrogen fixing bacteria. The arguments can then be listed and decisions are categorised by the two models of risk assessment.

See also: *The development of risk analysis for the deliberate release of genetically modified organisms: an outline of the international discussion.* Reproduced as Annex 6 (pages 27-29) in EIBE Unit 10. *Transgenic plants: economy, environment and ethics.*

## Remediation of environmental damage

This part of the unit explores the role of biotechnology in removing or alleviating damage to the environment.

### Objectives

The student should understand:

- the purpose of remediation for society and the benefits of bioremediation;
- that bioremediation may be used in conjunction with more traditional physical and chemical approaches and is not appropriate or acceptable for all cases of pollution;
- the ways in which bioremediation technologies are designed to optimise the microbiological processes involved;
- the ways in which pollution of land and sea may occur and be dealt with by reference to the example of pollution by hydrocarbons;
- the role of biological processes in the cycling of carbon, nitrogen and sulphur and the relevance of the cycles to detection, prevention and remediation of environmental damage.

# Detection of environmental damage

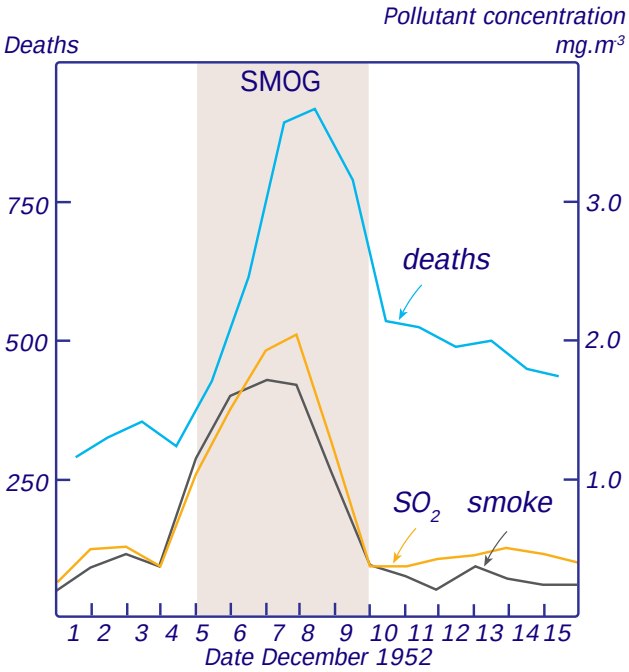
## London smogs

Not that long ago the city of London was a place where smog regularly occurred. Smog is a contraction of the words 'smoke' and 'fog'. During London smogs high levels of smoke from coal-burning industries mixed with fog creating very limited visibility. In such smog your hand would disappear before you when you stretched your arm and breathing it caused coughing and wheezing.

For five days in the autumn of 1952 London was enveloped in a dense smog that caused an increased death rate. About 1000 people more than the average died in those five days. Causes of death were bronchitis, pneumonia, asthma and other chronic respiratory diseases.

Figure 2 shows that both the sulphur dioxide and the soot content of the air was high during the period of increased deaths. As the burning of coal and wood generates both soot particles and sulphur dioxide, sulphur dioxide levels have been used since then as indicators of a healthy environment. This monitoring of the air is possible due to the fact that lichens are very sensitive to sulphur dioxide in the atmosphere (see page 13).

***Figure 2. Deaths and air pollutant levels during the London smog of 1952***




## Air pollution and health

What does polluted air do to the body? In cities with polluted air the following similar health problems are common: irritation of the respiratory system, wheezing, coughing, shortness of breath, nausea and headache. The severity of the symptoms depends on how long a person is exposed to the source of pollution, the concentration, the polluting agent and the age and health of the person. Many of the health problems associated with sulphur dioxide are also associated with high levels of particles or other pollutants in the air.

On a world-wide basis sulphur compounds are considered to be one of the major air pollution problems. Sulphur dioxide is a colourless gas, highly soluble in water which results in the formation of sulphurous acid. In the atmosphere, sulphurous acid is easily converted to sulphuric acid, which is the major acidic component of 'acid rain'.

Sulphuric acid aerosols and particulate sulphate compounds have been shown to be corrosive and potentially carcinogenic. Exposure to 400 parts per million of $SO_2$, causes lung oedema and bronchial inflammation; at lower concentrations the symptoms of throat irritation, bronchi-spasm and increased airway resistance are found. Sulphur dioxide may also play an important role in the aggravation of chronic illnesses such as asthma. The incidence and intensity of asthma attacks have been shown to be increased when asthmatics are exposed to higher levels of sulphates which are products of atmospheric sulphur dioxide reactions.

## Sulphur in the environment

Figure 3 shows the relative contribution of different processes to the global sulphur balance. An outline of the microbial contribution to the sulphur cycle is given in Figure 4.

Artificial (man-made) contributions include burning fossil fuels (coal, oil) in stationary sources such as power plants, refineries and to a small extent homes; extraction of materials from sulphur bearing ores, such as copper smelting; diesel fuels, and to a lesser extent petrol, contain sulphur and also contribute to sulphur dioxide in the air.

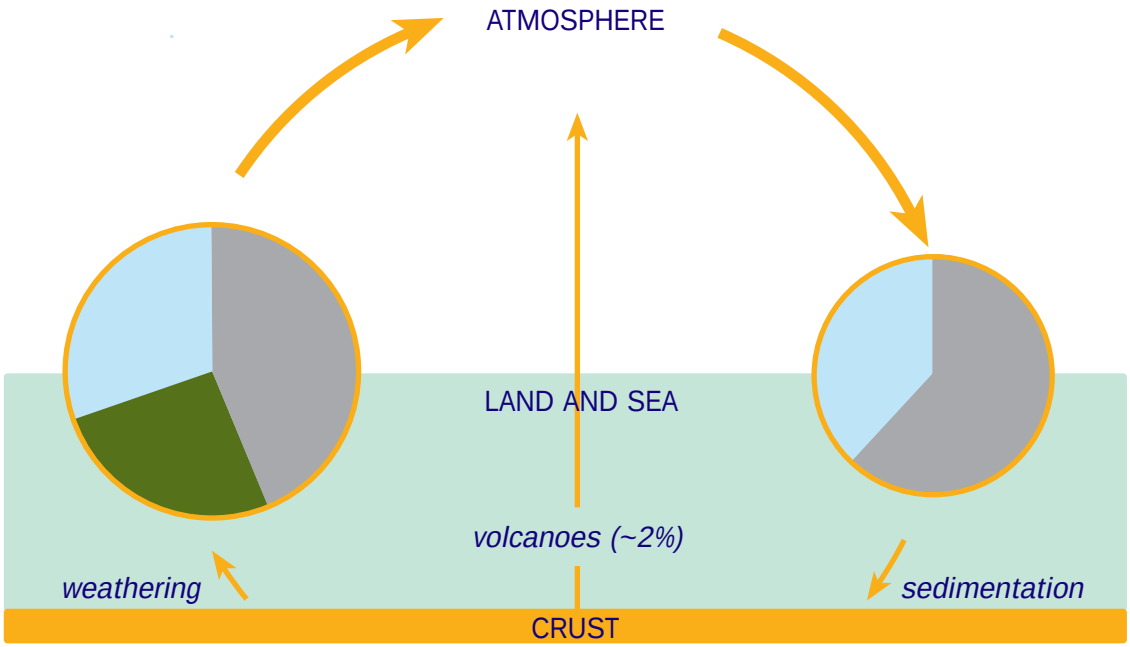
Sulphur dioxide is also emitted in significant amounts from natural sources such as sea spray and volcanic eruptions. However, natural sources rarely play an important role in the urban sulphur dioxide problem.

## Essential for life

The element sulphur is present in two amino acids, cysteine and methionine, and is therefore part of many proteins. Sulphur atoms can form cross-links between amino acids containing them. The links may be between two amino acids in the same chain, as in oxytocin, the hormone that causes contractions of the uterus during child birth, or vasopressin, the hormone that reduces urine production, often in combination with emotions or exercise. The links may also join two separate amino acid chains, as in antibodies and in the blood sugar regulating hormone, insulin.

Two vitamins, thiamine and biotin also contain sulphur. In humans, both are essential nutrients, just as is methionine. All these nutrients are widely available in food.

***Figure 3. The global balance of sulphur***




***EMISSIONS** from land/sea to atmosphere*

- *artificial emissions from human activities ($SO_2$)*
- *sea spray*
- *emissions from mud, bogs and biological decay. ($H_2S$ and dimethyl sulphide)*

***DEPOSITIONS** from atmosphere to land/sea*

- *artificial wet and dry deposition, from human activities ($SO_4^{2-}$)*
- *natural wet and dry deposition ($SO_4^{2-}$)*

## Air pollution monitoring

The amount of sulphur dioxide in the air can be monitored by chemical analysis. However, since lichens largely derive their water and essential nutrients from the atmosphere, they are affected by air and can provide useful bio-indicators of air pollution and $SO_2$ levels. A decline in numbers of lichens around factories was first observed in the 19th century.

## Lichens

Lichens are small, coloured but nondescript, and easily overlooked. A lichen is actually two organisms, either a green alga or a cyanobacterium and a fungus, existing in a symbiotic relationship known as mutualism. The alga photosynthesises and provides itself and the fungus with carbohydrates and some vitamins. In turn, the fungus provides the alga with physical protection; it obtains water vapour from the air, providing moisture for the alga.

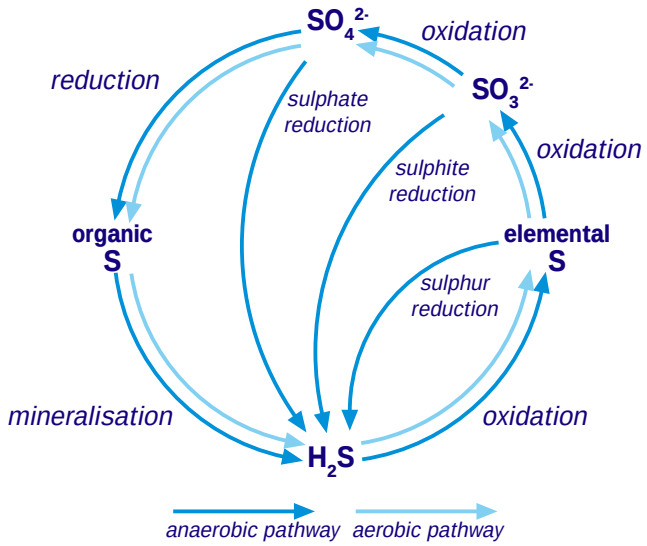
Lichens also have special adaptations permitting them to withstand extremes of moisture and temperature. When moisture is available, it is taken up by the fungus leading to a physical change, which allows more light to get through, triggering photosynthesis. When the atmosphere is dry, the lichen is dormant and does not grow.

## Sensitivity to $SO_2$

Lichens appear in a wide variety of habitats because they can tolerate, and in fact thrive, under difficult environmental conditions: they are often pioneer vegetation on bare sand, but more visible as dry-looking green or yellow patches on rocks, concrete, stone work and tree trunks.

Lichens are extremely sensitive to air pollution, particularly sulphur dioxide. Lichens absorb $SO_2$ from the air but have no means to excrete it, so concentrations are reached which disturb the symbiotic relationship. Some lichens are much more tolerant of $SO_2$ than others.

***Figure 4. Microbial transformations in the sulphur cycle***




The sensitivity of lichens to pollution varies with species and the pH of the surface on which they grows. At extreme low pH-values, i.e. an acid environment, lichens are not found. The chlorophyll molecules of the algae are damaged by the low pH; the lichen turns from its usual grey or green hues to the more unusual brown, yellow, pink or white as its chlorophyll is lost. The organism then loses its photosynthetic capability and dies.

It has been found that, with increasing $SO_2$ pollution in an area, loss of lichens occurs in stages:

- the first loss of lichens occurs on birches and conifers (acid bark and low buffering capacity)
- the next loss on oaks and sycamore (intermediate acidity and some buffering capacity)
- the last on trees like elm (alkaline bark and high buffering capacity)

Alkaline substrates such as basic bark or limestone to some extent counteract the acidity of $SO_2$ pollution but as $SO_2$ pollution increases only the more tolerant species will be found. Maps can be drawn up depicting air pollution (in terms of $SO_2$ concentration) that are based on studies of lichen diversity.

INVESTIGATION 1

# Measuring air pollution

## Materials

map
metre rule or cord (minimum length: 1 meter; subdivisions: 0.1 m)
sampling grid*
lichen identification key (local)
pictures or photographs of lichen
magnifying glass, tweezers
data collection sheet (page 15)
paper, pencil and clipboard
small plastic bags

** sampling grid: 0.5 m x 0.5 m, the width divided in two sections (0.25 m), the height divided in five sections (0.1 m).*

## Procedure

*Working in groups of 3 or 4:*

1. Read the sample selection notes (below), collect the materials listed and select 10 sample locations within one kilometre of the school.

*At each site:*

2. Mark the location on a map or sketch plan with an 'X' and an identification number.
5. Select the side of the sample site where there are the most lichens (probably SW to SE). Hold the grid against the sample site (for trees its lower edge should be 1 m above the ground).
6. Record (by species name, if possible) each lichen type found and the number of sections on the grid where it is present (the maximum for each lichen species will be 10 per grid).
7. For species that you cannot identify collect samples labelled with their location and position on the grid.

## Data processing

8. Compare and confirm identification of lichen samples with reference to books and other groups.
9. Calculate the TOTAL and MEAN (total / no of sites) data for each species recorded.
10. Taking into account the species present, their frequency and the nature of the substratum, compare your results with published data relating these to particular $SO_2$ concentrations.

### Sample selection notes

Choose similar sample sites at 10 locations, i.e. all trees, all rock / stone or all concrete. Each must be large enough for the sample grid to be laid against a face.

*For tree samples:*
If possible each group should use trees of the same species at each of their sites. Choose trees with alkaline bark if possible (ash, elm or sycamore); if this is not possible use oak, beech or birch (in that order of preference).

Choose large trees (circumference between 0.9 m and 2.80 m) with trunks that are clear of low branches and undergrowth.

# Data collection table

Name ..... Date .....

Group ..... Study area .....

**Location type** *(all 10 should be similar)*

- ❒ garden
- ❒ park
- ❒ alongside a road
- ❒ other give details .....

**Sample type** *(all 10 should be similar)*

- ❒ tree if so, what species .....
- ❒ timber
- ❒ stone
- ❒ concrete
- ❒ rock

**Record of results**

*List each lichen species found and record the number of grid units in which each lichen is present at each location.*

| ***LICHEN SPECIES*** | ***Number of GRID UNITS in which each lichen is present*** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Location identification number* | | | | | | | | | | *TOTAL* | *MEAN* |
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# Prevention of environmental damage

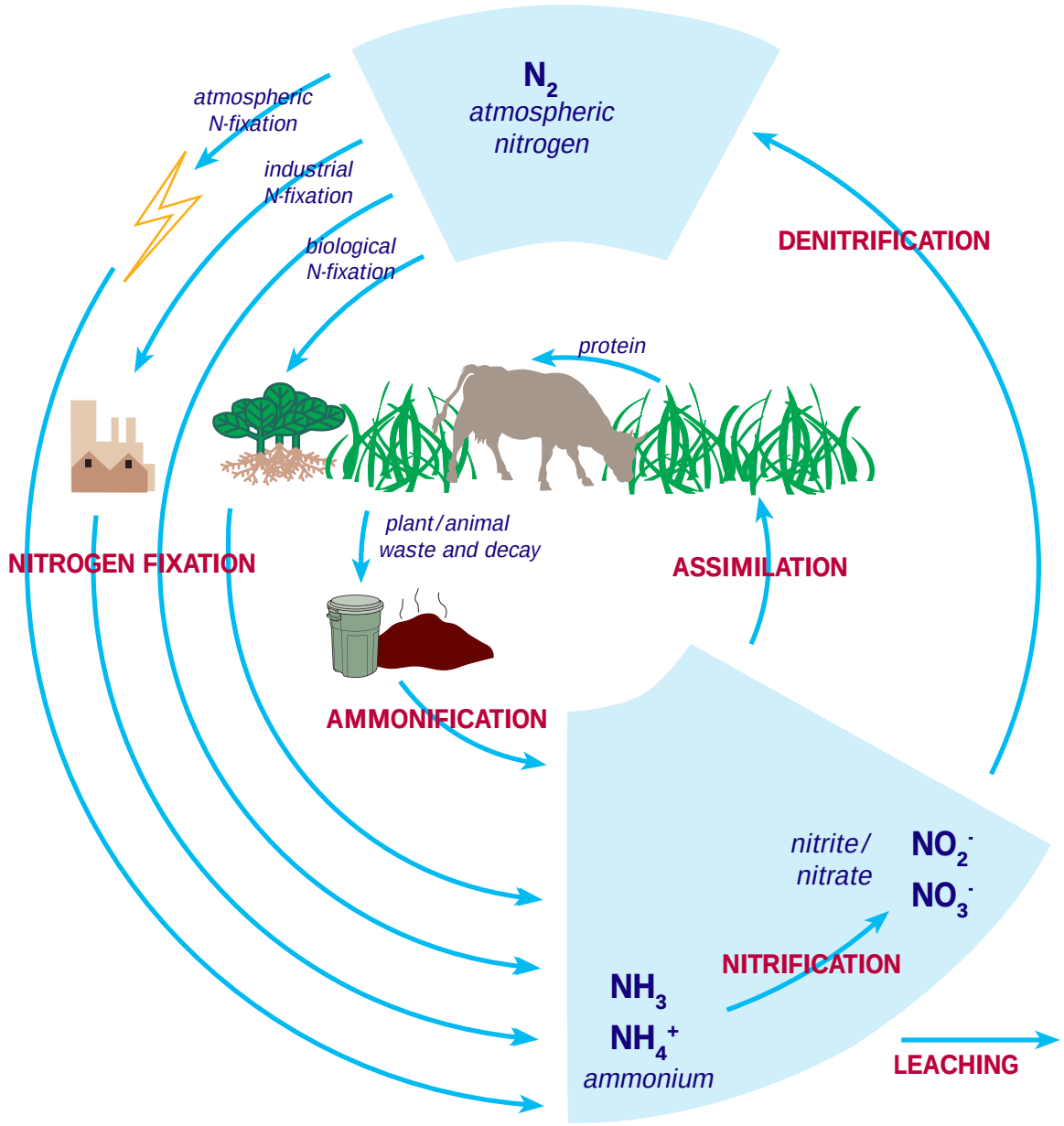
## The Nitrogen Cycle

- About 80% of the atmosphere is $N_2$.
- Every living organism needs nitrogen. It is used to synthesise proteins, nucleic acids and other important organic molecules.
- Animals cannot use atmospheric $N_2$ and depend on animal or plant food sources for their supply of nitrogen.
- Plants cannot use atmospheric $N_2$.

Plants and animals therefore depend on nitrogen compounds which arise in four main ways:

- biological nitrogen fixation (by bacteria);
- chemical / industrial processes for the manufacture of 'artificial' fertilisers;
- naturally in the atmosphere;
- decomposition of organic matter by micro-organisms.

***Figure 5. The nitrogen cycle***



### *Table 1. World population and food production*

| Year | World population ($x10^9$) | Number of people that have to be fed by an area of 1 ha |
|---|---|---|
| *1960* | *2.6* | *1.9* |
| *1970* | *3.7* | *2.6* |
| *1980* | *4.6* | *3.3* |
| *1990* | *5.5* | *3.9* |
| *2000* | *6.4** | *4.6** |
| *2010* | *7.6** | *5.4** |
| *2020* | *8.8** | *6.3** |
| *2030* | *10.5** | *7.5** |

** predicted figures*

## Man's dependence on the nitrogen cycle

The growth of the world's population (Table 1) and the advance of technology has meant that Man's impact on most ecosystems is increasing.

Agricultural practices have changed so that the effects of intensive cultivation may be both local and far reaching. For example, consider a fertile field in Italy. A good crop of broccoli takes some of the nutrients from the soil. The broccoli is flown to London and eaten, after digestion some of the nutrients are excreted as waste and, via sewage treatment, find their way to the North Sea. To maintain the fertility of the field in Italy for another crop, fertilisers will be necessary, while an excess of nutrients in rivers and seas can lead to excessive growth of algae, which in turn influences other ecosystems.

From Table 1 it can be seen that with the expected increase in the world population, more productive methods of cultivation will be needed.

## Biological nitrogen fixation

Nitrogen, one of the key elements in soil that are needed to make crops grow, is obtained from the nitrogen gas which makes up about 80% of the atmosphere. As plants cannot use nitrogen in the gaseous form, it has to be changed ('fixed') into ammonia, a nitrogen-containing compound that can be utilised. Although a chemical process is used commercially for manufacturing 'artificial' fertilizers, on a global scale about 85% of nitrogen fixation occurs through a biological process. About 60% of biological nitrogen fixation is on land; the other 40% occurs in seas and oceans. Plants directly use the ammonia produced by nitrogen fixation and also

### *Table 2. Nitrogen in the soil*

| Type of nitrogen fixation | Estimated contribution to soil nitrogen | |
|---|---|---|
| | t $year^{-1}$ (global) | kg $ha^{-1}$ $year^{-1}$ |
| *Natural (in the atmosphere)* | *45 x $10^6$* | |
| *Industrial (Haber-Bosch process)* | *40 x $10^6$* | |
| *Biological (nitrogenase enzyme)* | *175 x $10^5$* | |
| *free-living cyanobacteria living in rice fields* | | *30 - 50* |
| *other free living bacteria, e.g.* Azotobacter | | *0.4 - 0.8* |
| Rhizobium *symbiosis with legumes* | | *100 - 300* |
| *cyanobacteria symbiosis with* Azolla | | *300* |

other fixed forms that are produced from the decomposition of plants and other organic matter. These processes are two of the stages in the 'nitrogen cycle'.

## The biochemical and molecular basis of nitrogen fixation

Biological nitrogen fixation where $N_2$ is reduced to ammonia and immediately converted to an organic form, is a highly energy demanding process due to the N-N triple bond. The reduction process is catalysed by the nitrogenase enzyme complex and is subject to many regulatory controls. The genes for the production and control of this enzyme complex are part of a group of genes known as the *nif* regulon (see fig. 6).

## Nitrogen-fixing organisms

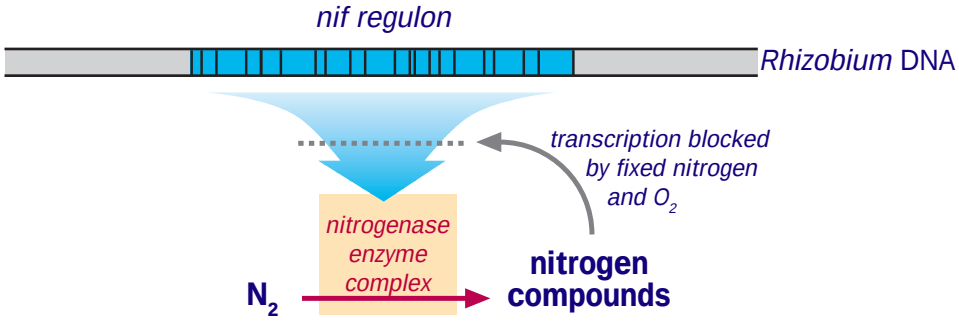
The process of biological nitrogen fixation on which our very existence depends is found only among micro-organisms and is limited to a small number of special types, generally known as nitrogen-fixing bacteria.

Nitrogenase enzyme complexes from all nitrogen-fixing organisms are remarkably similar, requiring a complex iron-molybdenum (or sometimes, -vanadium) cofactor. Nitrogenase activity is very sensitive to the presence of oxygen (see fig. 6). In aerobic bacteria inactivation by oxygen is prevented in several ways: the rapid removal of oxygen by respiration (*Azotobacter*, has the highest respiratory rate of any organism); production of protective slime layers; physical separation of the process in heterocysts of cyanobacteria; or, *in Rhizobium*, by controlling oxygen levels with an 'oxygen buffer', leghemoglobin. *Rhizobium* forms nitrogen-fixing root nodules in leguminous plants and can fix nitrogen only through a symbiotic interaction with its host plant. The critical leghemoglobin protein is genetically encoded partly by the plant and partly by the bacterium.

There are also anaerobic nitrogen fixing bacteria (e.g. certain species of *Clostridia*) which grow in oxygen-free environments and, therefore, whose nitrogenase is not exposed to the inhibiting effects of oxygen.

When comparing strains of *Rhizobium*, some are found to be good in their nitrogen fixing ability (*nif* regulon) and others in their ability to compete for nodule sites (*nod* regulon). Some of the strains used in commercial inocula have good nitrogen fixing capability but do not compete well with naturally occurring strains already in the soil in their ability to form nodules. The genetics of the *nod* regulon is being studied in the hope that it will be possible to transfer genes that will increase the competitiveness of the good nitrogen fixing strains.

***Figure 6. Regulation of nitrogen fixation***



## *Fig. 7. Isolation of the genes responsible for nodulation (nod⁺) from* Sinorhizobium meliloti

A cloning vector (in this case an artificial phage-plasmid hybrid known as a cosmid) is digested with the restriction enzyme *Eco*RI.

*Eco*RI restriction fragments (30 - 40 kilobase pairs) of DNA from *Sinorhizobium meliloti (nod⁺)* are prepared.

The *Sinorhizobium* DNA fragments and the vector DNA are incorporated into long lengths of DNA using DNA ligase.

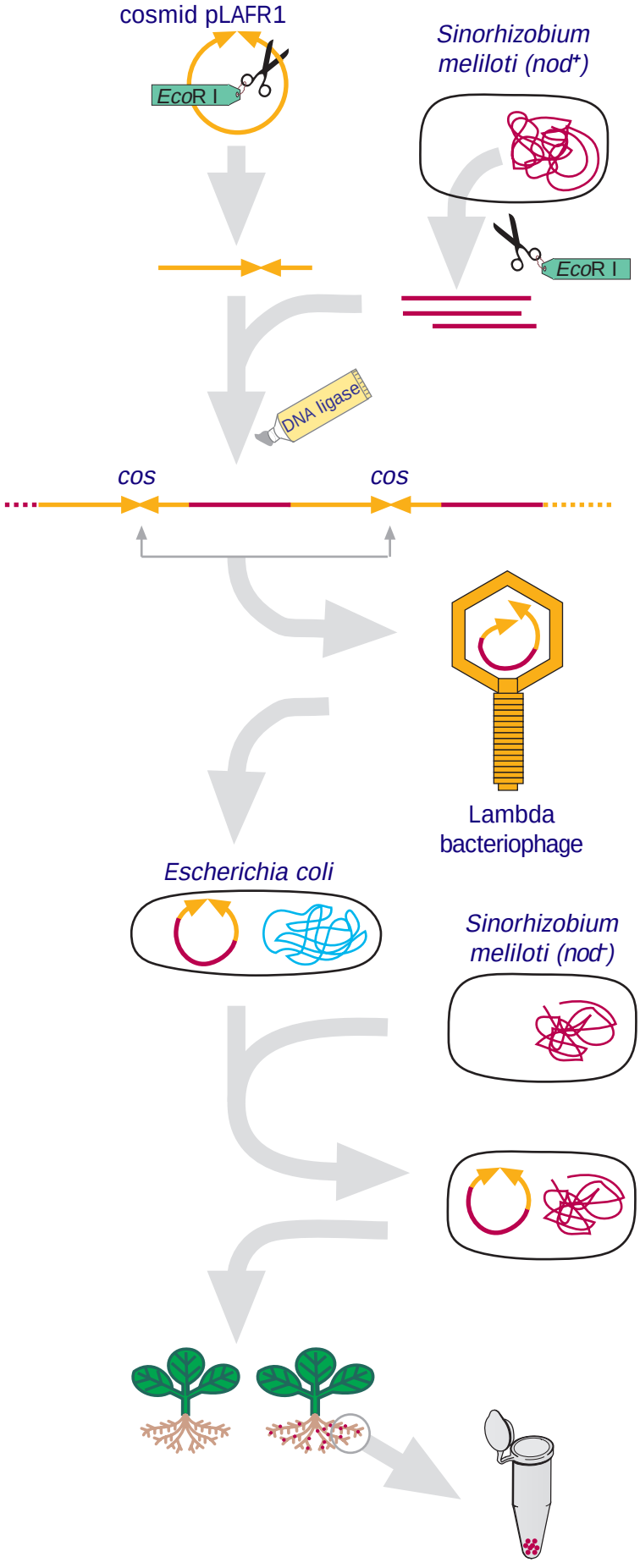
Lambda bacteriophage head and tail proteins are added, which recognise the *cos* sites on the long lengths of DNA and, if the DNA between these sites is of suitable length, cut and package it into viable phage particles.

The phage particles are used to transduce *E. coli* bacteria (the phage injects its DNA into the bacterial cell where it forms a new cosmid).

The cosmids are transferred by conjugation to cells of a mutant strain of *Sinorhizobium melitoli* that cannot form root nodules *(nod⁻).*

Leguminous plants are inoculated with the transformed *Sinorhizobium*; if the cosmid contains *nod⁺* genes root nodules will form on the plant.

Nodules are isolated from selected plants and the DNA extracted for identification of the genes involved in nodulation.



The purpose of the research is to reduce the reliance of agriculture on 'artificial' fertilisers and therefore the consequent pollution problems. But first it is necessary to isolate the genes responsible for root nodulation from the bacterium. Fig.7 (page 19) shows one way of doing this.

Much research has already been carried out to elucidate the biochemical and genetic basis of nodulation, but, because of the complexity of the symbiosis between higher plants and *Rhizobium*, it has still a long way to go.

## Practical activities 2 and 3

The following two investigations (pages 21 and 22) are designed to grow two types of nitrogen-fixing bacteria from the soil environment, i.e. one that is free living in soil, *Azotobacter*, and another that lives in a symbiotic association inside nodules on plant roots, *Rhizobium*. Despite their importance, they occur in only small numbers compared with other soil bacteria. Therefore, special selective methods which prevent or discourage the growth of other organisms have to be used to grow them from natural sources.

2. *Azotobacter*

The presence of *Azotobacter* in soil can be shown by the use of a culture medium to which no nitrogen is added, i.e. it is 'nitrogen-free'. This is an example of a selective medium.

3. *Rhizobium*

*Rhizobium* occurs in two forms; a free-living form in the soil and a symbiotic form in the nodules of leguminous plants such as peas and clover. However, *Rhizobium* is able to fix nitrogen only when growing within a nodule and therefore, it cannot be grown using a selective nitrogen-free medium. Their presence in nodules have to be shown by chemically sterilising the outside of the nodule, crushing it and inoculating onto a medium that contains nitrogen in a form that has already been 'fixed'.

INVESTIGATION 2

# A free-living bacterium that fixes nitrogen: *Azotobacter*

## Materials

soil sample
marker pen
adhesive tape
incubator at 25 ˚C

*Sterile:*

nutrient agar plate
nitrogen-free mineral salts* agar plate

* nitrogen-free mineral salts agar medium

To make 500 $cm^3$:

0.05 g $FeCl_3.6H_2O$
2 g $K_2HPO_4$
0.25 g $MgSO_4.7H_2O$
10 g glucose

Dissolve the above (start by dissolving $FeCl_3$ separately) in 500 $cm^3$ distilled water. Check the pH and adjust to 8.3 if necessary. Transfer to a bottle containing:

1.0 g $CaCO_3$
7.5 g agar

Autoclave (121 ˚C for 20 minutes). When dispensing, mix to disperse the $CaCO_3$ before pouring (allow 20 $cm^3$ of medium per Petri dish).

## Procedure

### WEEK 1

1. Take a Petri dish containing a nitrogen-free mineral salts agar medium and place 10 - 20 small crumbs of soil evenly over the surface of the medium. Cover and label the plate.
2. Repeat step 1 using a Petri dish containing nutrient agar medium, a non-selective medium.
3. Without inverting the plates (otherwise the soil will fall off) incubate at 25 ˚C for up to a week.

### WEEK 2

4. Examine the plates for microbial growth. Colonies of *Azotobacter* look mucoid (slimy) and are often colourless. They will be around the soil particles.
5. Compare the growth on the nitrogen-free medium and the nutrient agar and explain any differences.
6. Examine the growth microscopically.

## Questions

1. What is the source of nitrogen for *Azotobacter* in this investigation?
2. Why do other micro-organisms not grow, or grow poorly, in this medium?
3. How would you investigate whether *Azotobacter* is also able to grow when given a source of fixed nitrogen?

INVESTIGATION 3

# Nitrogen fixation in root nodules: isolation of *Rhizobium*

## Materials

plant with root nodules (e.g. clover)
scissors
small beaker or dish containing industrial methylated spirits (IMS)
Bunsen burner and mat
marker pen
adhesive tape
incubator at 25 ˚C
forceps
glass rod
wire loop
industrial methylated spirits (IMS) in a small beaker covered in foil
*(CAUTION: flammable, should be kept covered, away from flames)*

*Sterile:*

water, 100 $cm^3$
beakers or dishes for washing, 3
Petri dish or cavity microscope slide, 1
dropper pipette, 1
mannitol yeast extract agar plate*, 1
nitrogen-free mineral salts agar plate (see page 24), 1

* mannitol yeast extract agar medium

To make 500 $cm^3$:

0.25 g $K_2HPO_4$
0.1 g $MgSO_4.7H_2O$
0.05 g NaCl
0.5 g yeast extract
5 g mannitol

Dissolve the above in 500 $cm^3$ distilled water. Check the pH and adjust to 7.0 if necessary. Transfer to a bottle containing:

0.15 g $CaCO_3$
7.5 g agar

Autoclave (121 ˚C for 20 minutes). When dispensing, mix to disperse the $CaCO_3$ before pouring (allow 20 $cm^3$ of medium per Petri dish).

## Procedure

### WEEK 1

1. Choose a length of root from a clover plant that has root nodules, cut a small piece (about 1 cm).
2. Wash the piece of root thoroughly free of soil under the tap.
3. Immerse in IMS for 1 minute.

*Aseptic techniques should now be used.*

4. Using sterile metal forceps (flamed with alcohol and cooled) transfer the sample through three changes of sterile water to remove all the alcohol.
5. Using sterile equipment, place a drop of sterile water a Petri dish or in the well of a cavity slide, add the nodule bearing root and crush it with a glass rod or forceps.
6. Flame a wire loop, take a loopful of the milky liquid from the crushed nodule and make a streak plate on mannitol-yeast extract agar medium. Label the plate.
7. Re-sterilise the loop and make a second streak plate on nitrogen-free mineral salts agar medium. Label the plate.
8. Incubate the plates at 25 ˚C for up to a week.

### WEEK 2

9. Examine the plates for microbial growth. Colonies of *Rhizobium* are shiny, mucoid (slimy) and off-white; other colonies are contaminants from soil.
10. Compare the growth on the two media and explain the differences.

## Questions

1. What is the purpose of the IMS?
2. Why are you using two different media?
3. What are the sources of nitrogen in the two media?
4. The bacteria also require a source of carbon and energy. Which ingredients of the media provide it?
5. How successful were you in sterilising the outside of the nodule?

# Remediation of environmental damage

Remediation is a process whereby environmental hazards that arise from accumulated toxic chemicals and other hazardous wastes are reduced or eliminated. Physical and chemical processes are available for effecting remediation but biological systems are also being developed for *bioremediation* which depends on the activities of living organisms and their enzymes. It is common for remediation to be approached through combinations of physical, chemical and biological means.

Bioremediation is relevant where there are problems with, for example, contaminated land, groundwater, seas and oceans and gaseous emissions from industrial and commercial operations. The processes involved have many features in common with the *biotreatment* of wastes such as sewage and there is much overlap between the two approaches for dealing with pollution. Important examples of the problems for which solutions are sought through bioremediation include:

- renewal of derelict inner city and suburban industrial sites ('brown field sites') for improving the quality of life through redevelopment to provide alternative uses such as building houses and commercial and recreational activities instead of expansion into 'green field sites';
- removal of contamination by heavy metals from land on which piles of metal-containing ash remain after the closure of metal works, gas works and power stations; also coal tar and heavy fuel oil on gas works sites;
- restoration of completed landfill sites for domestic, municipal and industrial uses;
- decontamination of ground water and land containing hydrocarbons that have leaked from corroded fuel storage tanks and railway trains and run-off from motorways; also chlorinated hydrocarbons from industrial cleaning fluids;
- treatment ground water and surface water courses contaminated by acid drainage from coal and spoil heaps and disused mines;
- cleaning oil pollution of seas and oceans that arises following accidental damage to oil tankers or acts of war;
- changing the emphasis in approach from the philosophy of managing the waste that society produces to one of preventing or minimizing the occurrence of pollution;

The living systems involved in bioremediation are commonly micro-organisms but consideration is also being given to the use of plants in extracting metals from contaminated land, i.e. *bioaccumulation*, and also in volatilising them to the atmosphere. The attraction of using micro-organisms is their ability to metabolise an extremely wide range of natural and synthetic substances because of the great diversity of their metabolic activities.

Bioremediation technologies have many attractions but they have to be balanced against their disadvantages and attractions of competing physical and chemical technologies (see Table 3, page 24). As a relatively new technology, improvements in several areas are needed for bioremediation to reach its full potential, e.g. integration with other disciplines and technologies, site characterisation technologies, methods and criteria for assessing treatability, modelling for scale-up from laboratory to field and predicting performance in the field, provision of databases from documentation of field experiments.

## The market for bioremediation

As industries increasingly believe that savings in the costs of energy and raw

## Table 3. Benefits and disadvantages of bioremediation technologies

| BENEFITS | DISADVANTAGES |
|---|---|
| *operational cost savings* vs *other technologies* | *applicability depends on type of pollutant and local conditions* |
| *minimal site disruption* | *may have long time-scale* |
| *low capital cost* | *proving feasibility is time consuming and expensive* |
| *destruction of pollutants, i.e. not transferring the problem elsewhere* | *public concern for safety of large-scale on-site treatment* |
| *exploitation of interactions with other technologies* | *needs input from other technologies* |

materials are to be gained from using cleaner production methods, the demand for bioremediation processes increases. Bodies such as the Organisation for Economic Co-operation Development (OECD) based in Brussels report on these developments and future trends and needs. Accurate estimates for the world market value of environmental technologies are difficult to make but they range up to more than $500 billion for the beginning of the 21st century, representing an annual growth rate of 5-10% since 1990. This scale of increase points to these activities reaching a global market of about half the size of the established chemicals industry.

For Europe, where predictions for market figures approach $100 billion, the leading sectors are control of air pollution, treatment of waste management, remediation of contaminated land and waste treatment. The last two mentioned are the fastest growing sectors because, respectively, there are tens of thousands of contaminated sites to be cleaned up and landfill sites are becoming scarce as stricter controls for their use are introduced to reduce emissions of methane, a greenhouse gas.

Needs vary in different countries depending on existing levels environmental infrastructure and markets. For example, much growth, particularly in waste water treatment, is occurring in countries such as Greece, southern Italy, Portugal and parts of Spain with financial support from the EU whereas slower growth is occurring where strictly enforced environmental standards already apply, e.g. Denmark, Finland, Germany and the Netherlands. Countries in which there are some sectors that need further improvement to reach EU standards lie somewhere in between; these include Belgium, Ireland, northern Italy and the UK.

### Bioremediation technologies

Bioremediation processes can be applied directly to the polluted area, i.e. *in situ* treatment, and to polluted material that has been removed from the site for treatment in another locality, i.e. *ex situ* treatment. There are two approaches to *in situ* treatment: in *bioenhancement* the conditions of the polluted area, such as nutrient provision, irrigation and aeration, are enhanced in order to improve the abilities of relevant organisms that are naturally present to transform the pollutants into non-dangerous substances; in *bioaugmentation* cultures of micro-organisms that are known to perform the required transformations are added to the polluted area. Polluted soil or water that is removed

from the site for *ex situ* treatment undergoes conventional processes such as composting in heaps and waste water treatment in bioreactor tanks or may be subjected to *land farming* whereby it is applied to contained areas of soil where pollutants are removed by natural biological processes, possibly aided by bioenhancement or bioaugmentation. The treated material is then available for re-use elsewhere.

Although bioaugmentation may seem to be the most obvious approach to choose and, indeed, it is in commercial use, it is important to realise that this strategy is not an easy one to apply successfully. When making a suitable microbial culture to add to a polluted site, thereby speeding up the natural microbial processes, it is vital to realise that in nature one type of micro-organism is rarely able to function alone. Therefore, it is usually necessary to use as the inoculum a mixture (consortium) of a variety of micro-organisms because in nature they are part of a complex ecological community within which many kinds of interactions take place and affect growth, metabolism and survival. The interactions may be beneficial through commensalism, synergism and mutualism but the outcome may be negative through competition, amensalism, parasitism and predation which work against success of added "foreign" cultures.

Successful inoculation of a polluted site with a suitable mixture of a variety of types depends on there being appropriate conditions of pH, oxygen, nutrients and water. However, these conditions alone are unlikely to be enough to ensure that they function successfully. To increase the chances of success, it is necessary to ensure that, for example, a sufficiently large amount of the inoculum is applied, it is delivered in a suitable formulation (e.g. as a liquid or on solid particles to prevent it being washed away) and a suitable ecological niche is created by including special nutrients.

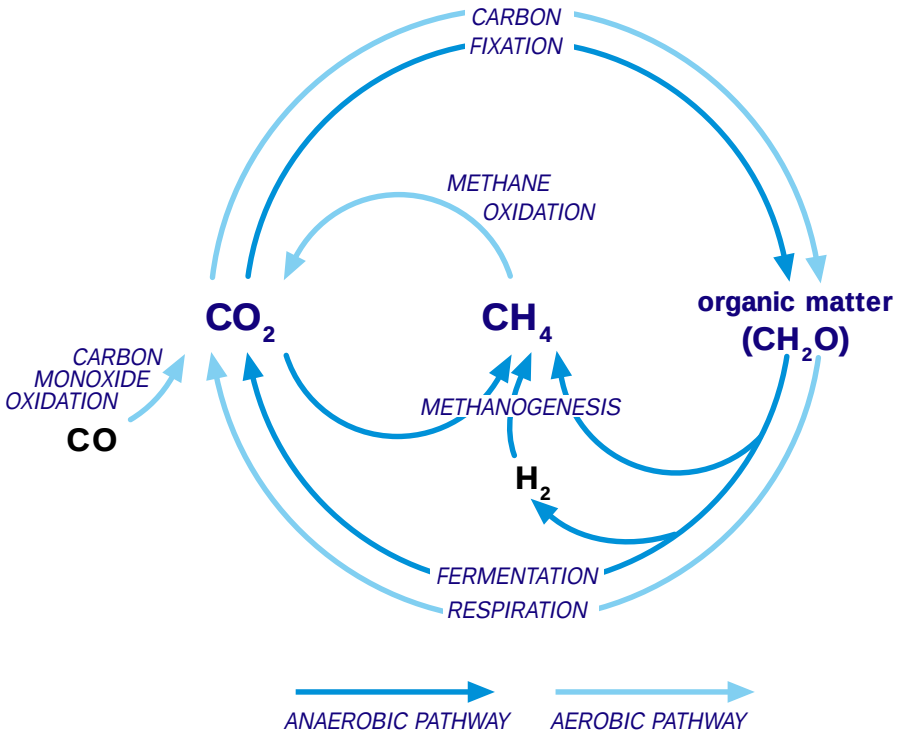
It is frequently suggested that genetically modified organisms (GMOs) have major potential for cleaning up the environment but, again, caution must be expressed. There are two main biological limitations of GMOs which have to be overcome for their use in *in situ* and *ex situ* bioremediation, i.e. compared with natural microbial consortia, their abilities have to be as good and they have to be as physiologically robust. Additionally, legislation and public opinion provides another hurdle.

## The carbon cycle and global warming

An understanding of the carbon cycle is central to the international agreement in Kyoto in 1997 to combat global warming by reducing carbon dioxide emissions by 5% by 2012 and the continuing attempts to achieve or improve on this target. Figure 8 (page 26) illustrates that biotreatment and bioremediation have some relevance to these discussions. For example, methane gas in the atmosphere functions as a greenhouse gas and is 27 times more potent than carbon dioxide in this respect. Anaerobic production of methane by micro-organisms contributes to the amount of methane in the atmosphere which is reason for the introduction of restrictions in the EU on the use of landfill sites from which methane is generated. Carbon dioxide is a product of the action of micro-organisms involved in bioremediation and in other natural processes such as decomposition and respiration by plants and animals. Its production by the burning of wood and fossil fuels can be considered as returning to the atmosphere carbon dioxide that was removed by photosynthesis long ago.

On the other hand, the high demand that plants have for this gas (removing about 15% from the atmosphere annually) has led to speculation about the possible value of enlarging forests to remove harmful carbon dioxide from the atmosphere - a process which might be considered to be a form of

**Figure 8. The carbon cycle**




bioremediation. However, the function of forests as 'carbon sinks' introduces other uncertainties and its validity must await a better understanding of its scientific and quantitative basis, for example, possible consequences of greater areas of forestation causing increased absorption of sunlight, of carbon dioxide-induced changes in species distribution and in plant physiology whereby trees (and phytoplankton in the oceans) begin to release more carbon dioxide than they take in, and of the emissions that will inevitably occur when the trees are ultimately used.

## Hydrocarbons as pollutants

Hydrocarbons provide a valuable example of pollution because they are among the most common contaminants of groundwater and are in oil that when spilled at sea generate much publicity and outrage. Hydrocarbons contain carbon and hydrogen only. They are components of fuels including gasoline, diesel, jet fuel and heating oil, and of solvents and wood preservatives. There is a variety of different hydrocarbons components in crude and refined oils. Examples of some of the types that are found in oils are shown in Figure 9. There may also be sulphur compounds present.

Different types of hydrocarbons also possess different physical and chemical properties that influence their persistence and biodegradability. For example, they may be in non-aqueous phase or soluble in water and also differ in their boiling point which influences the amount of volatilisation that may occur: gasoline contains principally lighter, low boiling point compounds such as pentanes and benzene but creosote and coal tar contain high boiling point compounds.

## Biodegradation of hydrocarbons

Although virtually all petroleum hydrocarbons can be oxidised to mainly carbon dioxide and water, the rate at which the process takes place is variable and depends on such factors as the nature and amount of the hydrocarbons, the prevailing environmental factors and the types, numbers and capabilities of the

micro-organisms present. This, in turn, is influenced by the quantity and types of other substances, either naturally present or intentionally added as supplements, that provide nutrients and oxygen as electron acceptor for the biodegradation reactions. Other physical limitations that are characteristic of oils are their hydrophobicity (water repelling properties) and low solubility. Therefore, physical contact between micro-organisms and oil must be enhanced. This is achieved by special properties of the organisms and the addition of supplements to improve the oleophilic (oil loving) properties of the environment.

## Hydrocarbon pollution on land

The schematic representation of a hydrocarbon 'plume' that would occur in the soil and groundwater beneath a leaking underground gasoline storage is given in Figure 10. Most hydrocarbon-utilising micro-organisms are aerobic and, therefore, the significance of the presence of aerobic and anaerobic zones is obvious. The activities of aerobic micro-organisms will be reduced in large spills because the limited amount of available oxygen at the edges of the plume is quickly consumed.

There are some well-documented examples of the degradation of plumes of dissolved hydrocarbons, including creosote and petroleum, by naturally occurring micro-organisms without human intervention. Indirect evidence is provided from observations such are those in the US that although 90% of all underground tanks are used to store gasoline, the most frequently identified contaminants of groundwater were chlorinated solvents, suggesting that natural biodegradation had reduced petroleum contamination to below detectable levels. However, knowledge is not yet sufficiently advanced to predict with certainty the effectiveness of biodegradation in a variety of circumstances nor the extent to which a plume may migrate before it is biodegraded.

In addition to the use of natural degradation, success has been reported for the removal of hydrocarbon pollution from

**Figure 9. Some classes of hydrocarbons present in oil**

***Aliphatics and cycloaliphatics***

*alcanes, e.g. hexadecane, $CH_3(CH_2)_{14}CH_3$*

*alkenes, e.g. 1-octene, $CH_2=CH(CH_2)_5CH_3$*

*cycloalkanes, e.g. cyclopentane, $C_5H_{10}$*

***Aromatics***

*arenes, e.g. benzene, $C_6H_6$*

*e.g. 1,2-dimethylnaphthalene*

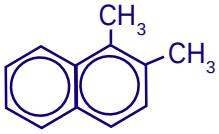



***Fig 10. Profile of a groundwater 'plume' undergoing natural bioremediation***

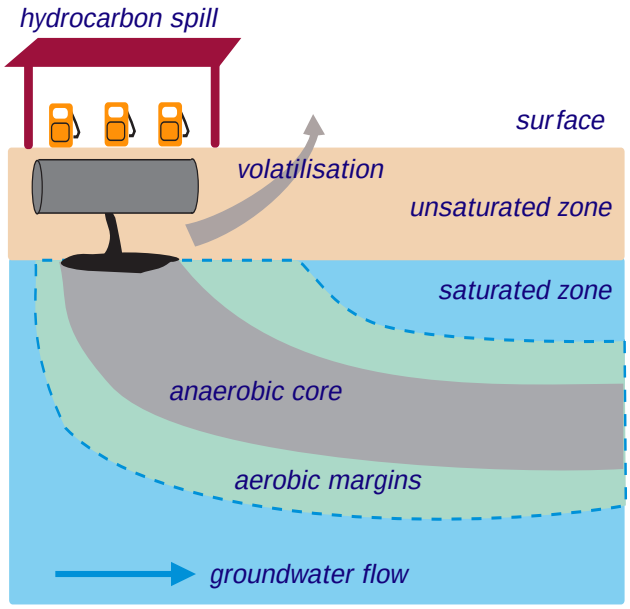
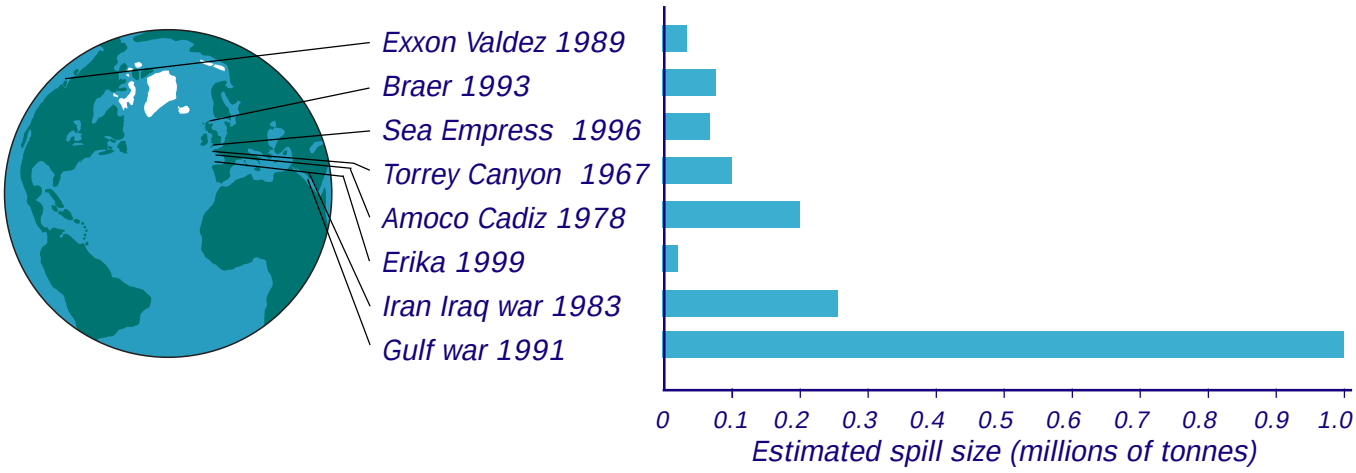


***Figure 11. Major marine oil pollution incidents***




soil and groundwater by bioaugmentation. An example is the treatment of soil and groundwater contaminated with hydrocarbons such as benzene, toluene, ethylbenzene and xylenes that have leaked from underground tanks by injecting of mixtures of micro-organisms. It is necessary to construct wells and trenches to ensure good distribution of the micro-organisms to the contaminated plume which may be 10 metres below the surface and to inject large volumes, e.g. 3 litres of culture/cubic metre. Reductions of about 50% in the amounts of hydrocarbons from an initial level of e.g. 2500 ppm have been recorded after one month and about 95% after four months to meet local regulatory limits.

However, in addition to the need for a successful technology, it must be emphasised that a major factor that influences the cost of bioremediation is acceptance by regulators, pressure groups and the general public. Indeed, when bioremediation meets strong is opposition, the increase in the costs of implementation might be greater than the conventional approach removing contaminated material by excavation and pumping.

## Hydrocarbon pollution at sea

Probably more than 2 million tonnes of oil enter the sea each year of which less than 20% comes from the oil industry - refineries, offshore operations and tankers. Of the remainder, about 50% enters the sea from industrial effluent, sewage and rivers and some 25% from non-tanker shipping and natural seepage. Although oil spills from tankers occur only occasionally, they are very visible and emotive because of the widespread problems that can be caused to wildlife, beaches and the fishing and tourist industries. Several incidents have affected seas and coastlines around Europe.

Oil degradation occurs by photo-oxidation and other chemical processes, aided at sea by the action of wind and waves in breaking up oil slicks, as well by biological action. At sea the usual environmental factors control the biodegradability of oil - oxygen supply, inorganic nutrients, temperature, water availability, pH vale, sources of carbon other than oil - with the added factor of salinity.

In the normal course of events, it appears that these various abiotic and biotic degradation processes are effective, otherwise greater consequences of natural seepage and minor spillages would be apparent. Evaporation and the other abiotic processes referred to above can remove 20-40% of an oil spillage incident. Even in oil

spills such as those illustrated in Figure 11, the polluting material eventually disappears through slow, natural processes, aided when possible by initial emergency work to contain and remove oil by physical means and aid its dispersal by spraying with detergents. An example of a situation where such emergency work was not possible is the Iran/Iraq conflict in 1983 when an estimated 300,000 litres of oil per day gushed into the Persian Gulf for almost a month from damaged oil wells. However, the expected catastrophic effects of, for example, elimination of the phytoplankton that form the basis of the marine food chain, were much less than anticipated because of the combined natural action of evaporation, wind and waves and biodegradation by marine bacteria on the oil slicks.

## Case study: the Exxon Valdez incident

In the Exxon Valdez incident in 1989, some 40 million litres of crude oil was released and contaminated 1750 km of the coastline of Prince William Sound, Alaska. This provide the opportunity for the first major bioremediation exercise was undertaken. Traditional methods were used to wash the oil from the coastline but oil that had penetrated into the beaches was not affected by this approach. Therefore the USA Environmental Protection Agency (EPA) conducted laboratory and field studies to explore the possibilities for integrating bioremediation with the other clean-up measures.

It was already known that bacteria capable of oxidising hydrocarbons are widespread and that, although their activities were limited by inadequate supplies of N and P, they could be improved by supplementation with these nutrients. This scenario was confirmed at the site of the Exxon Valdez spill. There was an abundance of hydrocarbon-utilising bacteria in the beach environment and in laboratory studies almost all of the alkanes in the Alaskan oil and much of the polycyclic aromatic hydrocarbons were degraded in 6 weeks with an inorganic supplement containing N and P.

The method of application of the supplement needed careful consideration in order that it remained associated with the oil and not washed away by tides and storms. Therefore, the N and P was added to the beaches in the form of oleophilic 'fertilisers', initially as a liquid formulation The source of N was urea in oleic acid; P was provided as tri(laureth-4)-phosphate. However, the oleophilic liquid formulation did not penetrate well beneath the beach surface because it remained with the oil and incorporation into briquettes held in nets was also ineffective. This difficulty was overcome by incorporating the N and P supplement in a slow-release granule formulation. The result was a visible improvement in 2 weeks in treated sections of the beach as compared with the controls; within 16 months, 60-70% of the oil had been degraded. No deleterious effects on the marine ecosystem were reported.

Not unexpectedly there has been debate about the relative influence of abiotic and biotic factors on the outcome. Regarding costs, more than $10 million per day was spent on the partially successful washing of the rocky shores of Prince William Sound whereas bioremediation of several hundred metres of coastline is estimated to have cost less than $1 million in total. However, this does not include the cost of efficacy and safety testing which was probably ten times that amount.

Much was learned about the principles of microbial growth, microbial diversity and microbial ecology, organic chemistry, the value of joint field and laboratory studies, and the importance of interdisciplinary interactions (the basis of biotechnology) from what is still the largest *in situ* bioremediation exercise ever undertaken.